Improved characterization of protein sources and implications on evaluation of rations for dairy cows

by

Louis Johannes Venter

Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Agriculture (Animal Science)

at

University of Stellenbosch

Department of Animal Sciences

Faculty of AgriSciences

Supervisor: Dr. Emiliano Raffrenato

Co-supervisor: Prof. Antonio Faciola

Date: March 2017

DECLARATION

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Date: March 2017

ABSTRACT

This study looked at a recent development in a new laboratory assay used to determine the unavailable nitrogen (uN) fraction in non-forage feeds. The need for this new assay was brought about as the current methods used to determine the uN fraction provide inconsistent results with varying amounts of AA being liberated. Furthermore, the need to improve nitrogen utilization efficiency (NUE) in dairy cows is necessary to reduce harmful waste to the environment and decrease feed costs. For that reason an effective approach to improve NUE, as well as improve protein characterization, is by improving the estimation of protein fractions such as the unavailable one. However, due to the assay being a novel procedure the implementation of the procedure in our laboratory required slight modifications in order to replicate the procedure. Nonetheless, successful results were obtained on 19 protein sources that are commonly used in South Africa with comparable values being achieved in different laboratories. Although the procedure was developed to determine the uN fraction, it became apparent that the rumen degradable (RDP) and undegradable protein (RUP) fractions need to be determined in order to quantify uN. Consequently, the new assay has provided a drastic improvement in past procedure to determine the uN fraction with comparable values for both RDP and RUP in the literature. As for the uN fraction, the new assay reports significantly higher values than previously presumed ranging from 34.53g kg^{-1} CP in sunflower meal to 447.70 g kg^{-1} CP in feather meal. These values indicate that there has been a drastic underestimation in the uN fraction, which has resulted in high levels of N excretion as well as an unnecessary expense for the farmer.

The uN fraction, and relative assay, has also been recently implemented in the Cornell Net Carbohydrate and Protein System (CNCPS) which in addition to the former acid detergent insoluble nitrogen (ADIN) method, allows for a comparison to be made between the different procedures. Using this nutritional model to make this comparison in 10 rations that were supplied to us, it was evident that NUE could be improved in addition to income over feed costs (IOFC). When implementing the uN fraction, as opposed to the traditional detergent system, the rations resulted in a reduction of IOFC from R0.10 to R39.50 per cow/day. In addition, the nutritional model showed a range of milk loss from 0.31 l to 7.90 l per cow/day as a result of the true protein unavailable to the animal. Moreover, by optimizing the rations for both IOFC and productive N, an improved composition was noted which resulted in an improved IOFC ranging from R0.16 to R7.16 per cow/day. However, owing to the new assay being a novel and an *in vitro* procedure, we would recommend further studies on both *in vitro* and *in vivo* trials to confirm our findings.

ACKNOWLEDGMENTS

I would like to take this opportunity to acknowledge my gratitude to the people and institutions for their contributions toward the study that made the successful completion of this study possible:

Dr. E. Raffrenato from the Department of Animal Sciences, University of Stellenbosch (US), who made up the study committee, for his guidance, criticism, advice and support.

The Protein Research Foundation of South Africa for their financial aid.

My friends, fellow postgraduate students and staff at the Department of animal science (SU) for their support and help.

My parents for their continued guidance and support throughout my studies.

NOTES

The language and style used in this thesis are in accordance with the requirements of the *Journal of Dairy Science*. This thesis represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

ABBREVIATIONS

AA	Amino acids
ADF	Acid detergent fibre
ADIC	Acid detergent insoluble crude protein
ADIN	Acid detergent insoluble nitrogen
ADIP	Acid detergent insoluble protein
AF	Adjustment factor
BAEE	Benzoylarginine ethyl ester
BCAA	Branched chain amino acid
BN	Basal nitrogen
BT	Blood meal Tisca
BTEE	Benzoyltyrosine ethyl ester
BUN	Blood urea nitrogen
CC	Canold Coldpress
CHO	Carbohydrates
CNCPS	Cornell net carbohydrate and protein system
CP	Crude protein
CS	Canola Solvent
CSNDF	Maize silage neutral detergent fibre
DM	Dry matter
DMI	Dry matter intake
EAA	Essential amino acids
EE	Ether extract
E	Extrublend
EMPS	Efficiency of microbial protein synthesis
EP	Expeller

FC	Feather meal cooked
FC	Fibrous carbohydrates
FG	Fishmeal Ganslobaai
FNF	Fishmeal Namibia flame dried
FNS	Fish meal Namibia steam dried
FOM	Fermentable organic matter
FS	Feather meal spray dried
FS	Fullfat soya
FS	Soya Freestate
GIT	Gastrointestinal tract
HS	Soya hulls
id	Intestinal digest
ID	Intestinal digestibility
IOFC	Income over feed costs
IUN	Intestinal undigestible nitrogen
LS	Lignosulfonate
LSM	Least squares means
MBT	Mobile bag technique
MCP	Microbial crude protein
MCP	Microbial protein
MCPS	Microbial protein synthesis
MNE	Milk nitrogen efficiency
MN	Manure nitrogen
MP	Metabolizable protein
mTSP	Modified three step procedure
MUN	Milk urea nitrogen

MY	Milk yield
NAN	Non ammonia nitrogen
NDF	Neutral detergent fibre
NDICP	Neutral detergent insoluble crude protein
NDIN	Neutral detergent insoluble nitrogen
NDIP	Neutral detergent insoluble protein
NFC	Non-fibrous carbohydrates
N	Nitrogen
NPN	Non protein nitrogen
NRC	National research council
NUE	Nitrogen utilization efficiency
OM	Organic matter
PC	Positive control
peNDF	Physical effective neutral detergent fibre
RDP	Rumen degradable protein
rd	Rumen digest
RF	Rumen fluid
RUP	Rumen undegradable protein
SBM	Soya bean meal
SC	Soya Continental
SEM	Standard errors
SEN	Specific endogenous nitrogen
SE	Solvent extracted
SI	Small Intestines
SoA	Soya Aminomax
SP	Soya Profile Feeds

SuA	Sunflower Aminomax
SuC	Sunflower Continental
SuG	Sunflower Gautenq Oil
SuM	Sunflower Majesty
TDN	Total digestible nutrients
TMP	Total milk protein
TPI	Total protein intake
TP	True protein
TSP	Three step procedure
uCP	Unavailable crude protein
uN	Unavailable nitrogen
UPLC	Ultra performance liquid chromatography
UT	Urea transporters
VFA	Volatile fatty acids
WSC	Water soluble carbohydrates

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Chapter 1

GENERAL INTRODUCTION

The need to improve nitrogen utilization efficiency (NUE) in the dairy industry has been exacerbated by the high cost of protein sources and the growing environmental concerns. In an attempt to rectify this, focus on reducing N excretion has been accomplished by reducing N intake thanks to improved knowledge of feed fractions and feed formulation systems. However, the success of N reduction relies on the ability to satisfy animal requirements and therefore reduce N waste. To accomplish this, nutritionists need to know the N availability in feeds in order to not affect milk production. While there are several nutritional models to assist with reducing N in feeds, they all rely on empirical formulas which require constant updates. Consequently, the accuracy of nutritional models rely on the accuracy of categorizing N in feeds.

The unavailable nitrogen (uN) fraction in feeds has been associated with acid detergent insoluble nitrogen (ADIN) and classified as undegradable in both the rumen as well as the intestines (Goering *et al*., 1972). However, it was found that when a feed was exposed to heat and moisture in order to stimulate a Maillard reaction, a higher ADIN value was found (Klopfenstein and Britton, 1987; Weiss *et al*., 1989; Van Soest and Mason, 1991). This has resulted in varying degrees of digestibility being associated to this fraction (NRC, 2001). As a result attempts have been made to improve the quantification of the uN fraction by reviewing past procedures from Tilley and Terry (1963) to the modified three step procedure (mTSP) by Gargallo (2006) (Ross *et al*., 2013). Consequently, a new laboratory assay was developed that estimates the uN fraction in non-forage feeds. The new assay has reported interesting results so far with reports of a 20 g difference in N digestibility and 2 kg in milk yield for a lactation study (Gutierrez-Botero *et al*., 2014). Furthermore, this new assay has been implemented in the Cornell net Carbohydrate and Protein System (CNCPS; v.6.55) which allows for a comparison to be made between the ADIN method and the new assay in terms of predicting uN (Tylutki, 2015).

Using this new lab assay, tests were performed on a variety of protein sources used in the dairy industry in South Africa. This was done in order to quantify the impact this new assay would have on NUE as well as income over feed costs (IOFC) in commercial South African dairy farms.

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Chapter 2. Digestion, efficiency and characterization of protein sources in dairy cows: a review.

2.1. INTRODUCTION

The rising consumer demand for animal products has led to an increase in animal production, which has placed focus on both the quality and safety of production (Tamminga, 2003). However, due to the large contribution of nonpoint nitrogen (N) pollution to water from animal production (Thomann *et al*., 1994), more focus has been placed on environmental concerns. Improvements in nitrogen utilization efficiency (NUE) would lead to a reduction in N loss (Kohn *et al*., 1997), and therefore N pollution as either ammonia volatilization, nitrate leaching to the ground, and N surface run off could be reduced (Jonker *et al*., 1998).

The level of feed nitrogen (N) which is assimilated into animal products ranges from about 10% to 40% (Van der Hoek, 1998). Among animal species, feed N conversion efficiency fluctuates immensely with the highest being associated with poultry and pigs and the lowest with beef cattle species (Flachowsky, 2002; Huhtanen and Hristov, 2009). As for dairy cows, usually, less than 30% of N intake is retained in milk and therefore, large amounts of N are excreted approximately equally in urine and feces, with damaging impacts on the environment (Castillo *et al*., 2000; Calsamiglia *et al*., 2010). Data regarding NUE vary but ranges between 13% and 31% in grazing systems and 40% and 45% in confinement systems with balanced rations (Delagarde *et al*., 1997; Verité and Delaby, 2000). Over the years an effective approach in reducing N excretion has been achieved by feeding less N on the farm, thanks to an improved knowledge of both the biology of the cow, feed fractionation and characterization. However, this becomes cost effective only if we are able to satisfy animal requirements and thus reduce wasted N. This review looks at the N pathways in dairy cows and focuses on the latest developments with regard to protein fractionation and characterization in various systems and models and laboratory approaches that are being used.

2.2. Protein

2.2.1. Nitrogen metabolism

The gastrointestinal tract (GIT) plays an important role in both the energy (Reynolds, 1995) and nitrogen (N) metabolism in ruminants. However, the gut tissue is only held accountable for 25% to 40% of whole body protein synthesis (Lobley, 1993). This is due to the much larger exchanges and transactions of N metabolites occurring, which often results in catabolic fates of N because of fermentation processes, and

mechanisms essential to maintain a healthy rumen. However, losses are associated with these processes and are only exacerbated by factors such as the dietary composition, intake and the productive state of the animal.

Nitrogen is incorporated into dairy cattle via several pathways. Among these pathways is dietary N intake, recycled N or endogenous N (Van Soest, 1994). However, the rumen is only responsible for the output of ammonia-N, rumen undegradable nitrogen (RUP) (dietary or endogenous) and microbial protein (MCP). As a result, two diverse steps separate the N metabolism in the rumen, into either protein degradation which provides N for bacteria, or protein degradation which provides N for microbial protein synthesis (Bach *et al*., 2005).

The amount of ammonia N output from the rumen can be increased significantly when excess rumen degradable protein (RDP) in relation to the ruminal microorganisms is fed. When this occurs the excess RDP is degraded to ammonia N, which is absorbed, metabolized in the liver to urea and lost in the urine (Van Soest, 1994). However, as in most mammalian species, which have the ability to transfer blood urea nitrogen (BUN) back to the (GIT), ruminants are able to supply this N source to the ruminal microorganisms, thereby recycling N (Houpt, 1959). Alternatively, N can also be recycled within the rumen, as a result of proteolytic bacteria and protozoa, which digest the rumen bacteria, thereby decreasing outflow and increasing ammonia release in the rumen (Lapierre and Lobley, 2001). This recycling ability is of significant importance to the survival of ruminants in contrast to other mammals which render it unfavorable from a nutritional perspective (Marini and Van Amburgh, 2003). In fact, 19 to 96% of urea recycling back to the gut is as a result of endogenous production (Lapierre and Lobley, 2001). Even though numerous physiological changes have been reported (Leng *et al.*, 1985; Cirio and Boivin, 1990), the increased reabsorption levels of urea in animals consuming low N diets (Isozaki *et al.*, 1994), in an attempt to recoup urea, is still not clearly understood as the mechanism which is responsible for this is unclear. It was not until the discovery of urea transporters (UT) in the GIT that the assumption of a mechanism could be made (Ritzhaupt *et al.*, 1997; Ritzhaupt *et al.*, 1998). As a result a lot of focus has been placed on feeding strategies in order to prevent the loss of N as urea, since the manipulation of rumen protein degradation is an effective method for reducing N loss (Tamminga, 1996). Two methods of achieving this N efficiency are to either reduce the amount of feed N converted to urea, or to improve the conversion of urea-N into bacterial protein (Lapierre and Lobley, 2001). Table 2.1 below shows how much N transits to urea and the potential recouping benefits. Approximately 40% to 80% of urea-N produced by the liver is recycled to the gut of which 35% to 55% is further converted to anabolic use in cattle.

In a study by Marini and Van Amburgh (2003), a similar conclusion arose supporting previous studies which stated that, when low N diets are fed, urea is redirected to the GIT. This conclusion arose due to the increased plasma cleared by the GIT in relation to the kidneys on a lower N diet. However, they reported an increase in UT expression when high N diets were fed, which was contrary to their initial hypothesis. They believe the reason for this is due to the high ammonia level produced, as a result of high N diets, which suppresses urease secretion (Bunting *et al.*, 1989) and thereby transports urea back into the blood. As a result of this, N recycling, at high dietary N levels, is decreased, which adds little to the N economy of the rumen (Marini and Van Amburgh, 2003).

Table 2.1. Species comparison of (^{15}N ^{15}N) urea kinetics (g N/d).

Species	N Intake	Digestible N	Urea-N synthesis	Urea-N to gut	Gut return: synthesis
Dairy cows [1]	450.00	301.00	262.00	171.00	0.67
Steers [2]	64.00	33.10	35.40	28.10	0.79
Sheep [3]	17.10	11.50	16.30	9.90	0.61
Human [4]	10.30	…	11.30	4.40	0.39
Cats [5]	1.70	1.50	1.10	0.20	0.15

[1]Lapierre *et al*., unpublished; [2]Archibeque *et al*., 2000; [3]Lobley *et al*., 2000; [4]McClelland and Jackson, 1996; [5]Russell *et al*., 2000.

For instance, Holstein heifers have shown the ability to capture about 43% of the N recycled to the GIT when fed a low N ration compared to 6% when fed high N rations (Figure 2.1). The entry of digestible N can increase in lactating dairy cows from 50 to 60% depending on the inflow of recycled N to the rumen (Lapierre and Lobley, 2001). This emphasized the significant importance of endogenous N in ruminants, especially when a low dietary N ration is fed.

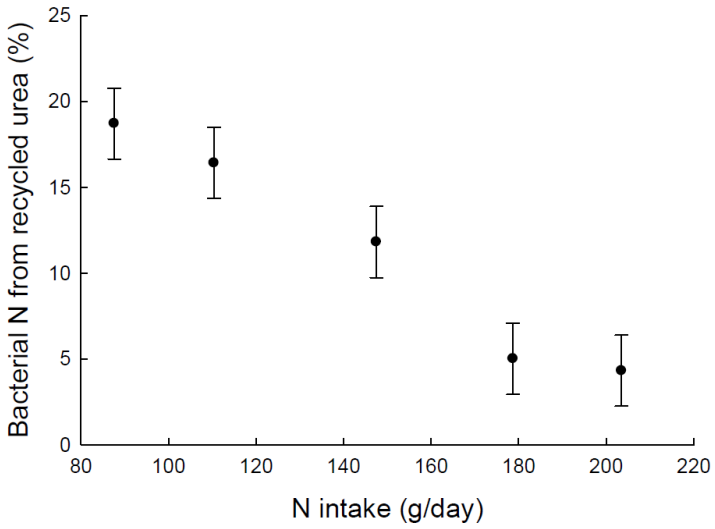

Figure 2.1. The proportion of bacteria flowing out of the rumen that has been synthesized using recycled N at different daily N intakes (Higgs, 2009; Marini and Van Amburgh, 2003).

2.3. Rumen degradation

As feed enters the rumen it is subjected to a wide variety of microorganisms, which attaches to the undigested feed (Brock *et al.*, 1982). According to Craig *et al.*, (1987) about 70% to 80% of the microorganisms attach to the undigested feed. Once the bacteria have attached, a second step of cell-bound microbial protease activity occurs (Brock *et al.*, 1982)**.** However, only 30% to 50% of the attached microbial bacteria have proteolytic activity (Prins *et al.*, 1983). It is as a result of the proteolytic activity of different microbes, that these large proteins can be broken down in a symbiotic relationship, into peptides and amino acids (AA) (Wallace *et al.*, 1997). The peptides can further be degraded into AA by peptidases, which can be incorporated into MCP or deaminated to volatile fatty acids (VFA), carbon dioxide and ammonia (Tamminga, 1979). The degree of protein degradation will depend on the proteolytic activity of the ruminal microflora, as well as the type of protein (Wallace *et al.*, 1997). Once the protein has been deaminated the byproducts, AA and peptides are transported, in microbial cells depending on the availability of energy. If there are enough carbohydrates (CHO) the AA are transaminated or used directly for MCP synthesis. However in the case of limiting CHO, the AA are deaminated and fermented into VFA (Tamming, 1979). This is unfortunately not the only time when AA are not utilized for MCP, as some microbes are unable to transport AA from the cytoplasm to the extracellular environment. These AA's as well as AA in excess in the cytoplasm, are excreted as ammonia (Tamminga, 1979).

2.4. Factors affecting ruminal degradation

The three main factors that affect MCP degradation are the type of protein, passage rate, pH and the interaction with other nutrients (Schwingel and Bates, 1996; Yang and Russell, 1992; Devant *et al.*, 2001; Lana *et al*., 1998; Cardozo *et al.*, 2000, 2002; Mould and Orskov, 1983). Nonetheless according to Cardozo *et al.*, (2004) the breakdown of protein can also be influenced by not only the level of proteolysis but also by the changes in peptidolysis and deamination.

2.4.1. Type of protein

Proteins vary in their degree of solubility, which affects their susceptibility to microbial protease (Romangnolo *et al*., 1994). Proteins that are soluble are broken down easier than insoluble proteins, however, this is not always the case, as the structure of protein itself also affects the degradation, i.e. tertiary structure, quaternary structure or specific peptide bonds. This implies that higher solubility does not always correspond to higher degradability (Schwingel and Bates, 1996; Yang and Russell, 1992).

2.4.2. Passage rate and pH

Several factors affect the microbial population which in turn affects rumen degradation. Among these factors is the ration fed (Devant *et al.*, 2001; Lana *et al*., 1998), the ruminal passage rate (Ørskov and McDonald, 1979) and the ruminal pH (Cardozo *et al.*, 2000, 2002). When looking at the ruminal passage rate, there is an inverse relationship with protein degradation. This implies that when there is an increase in the ruminal passage rate, there is a decrease in protein degradation or vice versa. Although these changes may be small, it will result in an increase in protein flow to the small intestines (SI) (Ørskov and McDonald, 1979). Referring to the ruminal pH, which is optimal for proteolytic enzyme activity at a range of 5.5 to 7.0, (Kopecny and Wallace, 1982) there tends to be a decrease in protein degradation at the lower end of that pH range. However, results from studies have shown that the pH is not the only factor which affects the degradation, but also the ration fed and therefore the microbial population (Cardozo *et al.*, 2000; Cardozo *et al.*, 2002 Devant *et al.*, 2001; Lana *et al*., 1998).

2.4.3. Interactions with other nutrients

Besides the ruminal pH, passage rate and microbial population, the importance of other enzymatic activity should not be neglected. The enzymatic activity of amylase increases protein degradation, as starch tends to interfere with this process. In fact with the addition of amylase an increase from 6 to 20 percent units was noted in cereal grains (Assoumani *et al.* 1992). Similar positive reports using amylase were found by others (Tománková and Kopečný, 1995). Apart from amylase, cellulase has also been reported to

increase protein degradation (Kohn and Allen, 1995; Abdelgadir *et al.*, 1996). The reason for this increase is due to the plant proteins being embedded in a fibre matrix, which needs to be broken down before the proteins can be exposed to proteases. Therefore maximum degradation of protein requires several proteolytic and non-proteolytic enzymes, as well as the combined effects of several microbial and enzymatic activities (Endres and Stern, 1993). This goes in agreement with the drop in protein degradation at the lower end of the pH range, following a reduction in cellulolytic bacteria activity, therefore a reduced fibre degradation which in turn reduces access of proteolytic bacteria, thereby diminishing protein degradation (Devant *et al.*, 2001; Lana *et al*., 1998; Cardozo *et al.*, 2000, 2002; Mould and Ørskov, 1983).

2.5. Intestinal digestion

2.5.1. Small and large intestines

The N containing compounds found in the small intestines include feed protein that has escaped rumen fermentation as well as microbial and endogenous protein (Hvelplund, 1984). The amount of N in the small intestines as a result of microbial protein as well as feed protein that escaped rumen fermentation depends on several factors such as ration composition, processing, enzyme supplementation and the level of feeding (McDonald *et al.*, 2011). As for the amount of endogenous N (saliva, bile/gastric/pancreatic secretions, and mucous membrane cells) it can be divided into two fractions. The first fraction is basal N loss (BN) which includes the N that is unrelated to the quality and quantity of protein but dependent on the DM passing through and the second fraction is specific endogenous N (SEN) which is related to the quality and quantity of protein and other ingredients, i.e fibre. These endogenous fractions are of particular importance as their digestibility determines the amount of AA absorbed for production.

In contrast to the small intestines, the contribution of N absorption in the large intestines is assumed to be very minimal if any at all (McDonald *et al.*, 2011). As a result, digestibility coefficients based on collection and analysis of ileum digesta are normally more accurate in terms of N absorbed than fecal collection analysis. This is believed to be due to the elimination of the error associated with the collection from the lower gut (feces) (McDonald *et al.*, 2011).

2.6. Nitrogen requirements and deficiencies

Providing a sufficient amount of essential amino acids (EAA) is vital in achieving maximum milk protein production (Schingoethe, 1996), as well as providing the option of reducing protein intake, by

increasing the efficiency of metabolizable protein (MP) utilization (Haque *et al.*, 2012). However, achieving an optimal inclusion level is complicated, as the CP in a ration is converted to rumen microbial protein. Therefore, in order to meet AA requirements of high producing lactating cattle, a significant amount of protein is needed to bypass rumen degradation, in order to be digested postruminally and absorbed (Schingoethe, 1996). It is, therefore, imperative that the balancing of AA's present for absorption from the GIT becomes of vital importance in lactating cows (Schingoethe, 1996). In fact, according to the limiting AA theory, in which a specific AA's supply can influence the incorporation of an AA profile, depending on the specific dietary and physiological conditions, it is thus essential to ensure that this limiting AA phenomenon is used for efficient manipulation (Weekes *et al.*, 2006). Numerous experiments have tested the responses of a single AA, which has led to a general agreement that AA deficiencies are common in lactating cattle (Weekes *et al.*, 2006). Regrettably, the quantitative assessment of AA nutrition of ruminants is bestowed with the difficulty of determining the transformation by ruminal microbes. As a result of this uncertainty, it is difficult to determine if a specific AA supplement has rectified the deficiency or caused an imbalance (Weekes *et al.*, 2006). The specific AA requirements for ruminants use to be estimated based on the AA requirements of non-ruminants (NRC, 1989) and according to the milk protein composition (Jacobson *et al.*, 1970). In fact, in a study by Chamberlain and Yeo, (2003) various protein sources infused postruminally resulted in various protein levels in milk. According to Schingoethe (1996), the AA requirements of ruminants for growth, maintenance, reproduction and metabolic processes at a tissue level are likely to relate to that of non-ruminants, however, there is limited data available to validate this.

For high producing lactating cows, giving approximately 45 kg of milk per day, it is thought that the AA composition of the milk should be closely related to the AA requirements. This is supposedly due to the use of essentially 90% of dietary AA's for milk production at such a high performance (Schingoethe, 1996). Although argued between nutritionists and researchers, studies have proposed that the most likely limiting AA's in milk production is lysine (Lys) and methionine (Met) (Burris *et al.*, 1976; Schwab *et al.*, 1982; Schwab *et al.*, 1976) trailed by phenylalanine (Phe), isoleucine (Ile), threonine (Thr) (Derrig *et al.*, 1974; Vik-Mo *et al.*, 1974; Nichols *et al.*, 1998; Piepenbrink and Schingoethe, 1998; Liu *et al.*, 2000), histidine (His) and arginine (Arg) (Vanhatalo *et al.*, 1999). Currently, the accuracy in determining the requirements and delivery of intestinally absorbable AA is low, thereby making it difficult to balance diets for metabolizable protein (MP) using metabolic models (Swanepoel *et al.*, 2010). The concern, with the current models, is that there is a lack of variability that can be held accountable for, from raw material to cow's environment or the interaction thereof. On the other hand a better understanding of the limiting AA, as well as the effect of supplementing them, is required, but due to the difficulty in performing a large dose-

response study on lactating cows in defined parameters, comparing models' predictions on limiting AA which would improve the understanding and estimates of nutrient supplies which will ultimately improve ration formulation on a AA level (Swanepoel *et al.*, 2010).

An ideal AA profile proposed by Rulquin *et al*, (2007), expressed as percentage of MP (or PDIE for the INRA feeding system, (Jarrige, 1989) is 7.3% Lys, 2.5% Met, 8.9% Leu, 3.0% His, and 4.6% Phe. As for the composition of Ile 5.2% and Val 5.8%, they were calculated relative to Lys in milk protein (Swaisgood, 1995) and the value of Trp 5.3%, was calculated by applying the Trp:Lys ratio in milk proteins (Swaisgood, 1995). Table 2.2 below compares the different profiles of AA for dairy cows in the literature. Using this proposed ideal AA profile by Rulquin *et al.* (2007) , Haque *et al.*, (2012) concluded that the EAA were supplied in sufficient quantities and that the addition of other EAA only increased milk and protein yield, and therefore the MP utilization as a result of the increased protein intake.

Table 2.2. Different recommended profiles of AA for dairy cows in the literature.

	Recommendations for dairy cows				**Profile in milk**
AA[1]	Fraser *et al.* (1991)	Rohr and Lebzien (1991)	Doepel *et al.* (2004)	Rulquin *et al.* (2007)	Swaisgood (1995)
Lys	100	100	100	100	100
Met	37	31	35	34	34
Leu	110	123	131	122	118
His	36	33	33	42	33
Ile	61	71	74	61	71
Phe	62	76	72	63	59
Thr	47	75	71	55	52
Val	73	81	85	73	80
Trp	15	-	-	-	18
Arg	43	63	67	43	41

[1]Requirements of AA other than Lys are calculated in proportion to the Lys concentration reported. (values are in % of Lys)

Similar results were obtained in a more recent study by Haque *et al.* (2015)**,** with the exception of a deficiency of Thr and an oversupply of His. Suggesting that the MP of His should be less than the proposed

3.0% and that of Lys 7.0% as well as Thr 4.5% were too low to maximize milk protein synthesis (Haque *et al.*, 2015).

In a study by Weekes *et al.* (2006) in which cows were fed a base diet low in protein, with infusions of saline a negative control (NC), complete AA mixture positive control (PC), PC without Lys (PC-Lys), PC without His (PC-His), PC without Met (PC-Met) and PC without Lys, His, and Met (PC-BCAA). Infusion of PC resulted in an increased plasma concentration of several AA's, insulin, urea, acetate, β-hydroxybuturate and glucagon. Whereas the infusion of PC-Lys, PC-His and PC-Met caused an increase in EAA, glucose, insulin and glucagon concentration in plasma as well as a decrease in growth hormone when compared to the NC infusion. A two-fold drop of the deficient AA in the plasma concentration was also recorded when PC-Met, PC-Lys, and PC-His were infused, however, the milk protein remained within 12% of its basal value. Further, a decreased in dry matter intake (DMI) by 35% in the first two days occurred but it was recuperated thereafter. As for the milk fat yields an increase of 258 and 320 g/d occurred for the infusions of PC-Lys and PC-His respectively, compared to 728 g/d on the NC ration. Once the infusions deficient in Met, Lys or His was corrected a protein milk yield increase of 27% occurred as a result of increasing a single AA in plasma. However, in these corrections, the hormone concentration was not affected. Furthermore, a low protein to fat ratio in milk was a characteristic of either an imbalance or a deficiency. But even though a similar plasma AA profile results from both a deficiency as well as an imbalanced diet. An imbalance diet in relation to a deficient diet is better able to override the imperfections and maintain a higher milk protein yield than expected from a deficient diet (Weekes *et al.*, 2015).

2.7. Microbial protein synthesis

There is a wide variety of microbial species in the rumen, which all differ in their nutrient requirements and their metabolism. Consequently, it is imperative to understand their requirements in order to understand the N metabolism in the rumen. Among the rumen microorganisms, protozoa make up roughly 40% of the microbial biomass (Russell and Rychlik, 2001) and they are able to digest both protein and CHO. In fact, protozoa are able to digest fibrous carbohydrates (FC) as well as non-fibrous carbohydrates (NFC) (Williams and Withers, 1991) and get their major supply of protein from bacteria. Although protozoa make up almost half of the microbial biomass, they are selectively retained in the rumen, thus they only supply approximately 11% of the CP that goes to the SI (Shabi *et al.*, 2000). Regardless, protozoa play a major role in the breakdown of protein and are not only responsible for the engulfing of large molecules, CHO, protein and ruminal bacteria (Van Soest, 1994), but they also have an important role

in regulating N turnover in the rumen (Van Soest, 1994). This is due to the inability of protozoa to utilize ammonia N (Onodera *et al.*, 1977), and therefore, a portion of formerly ingested insoluble protein is returned to the rumen fluid later in a soluble protein form (Dijkstra, 1994). In fact, besides the reduction in protein degradation, as well as that of peptides and AA from the defaunation of protozoa (Ivan *et al.*, 1991), there is also a reduction in ammonia N concentration in the rumen (Eugène *et al.*, 2004). However, there is a general agreement that the true value of protozoa to ruminants is not clear (Bach *et al.*, 2005).

Bacteria are able to utilize both protein and CHO as an energy source, with the latter being the main energy source. Carbohydrates can also be used in addition with ammonia, to synthesize protein (Stern and Hoover, 1979). However, the synthesis of microbial protein is dependent on the amount, as well as the type of CHO. If the source of CHO is readily fermentable, such as starch or sugar, the synthesis is more effective compared to a CHO source like cellulose (Stern and Hoover, 1979). In fact a number of studies, both *in vitro* (Stern *et al.*, 1978; Henning *et al.*, 1991) and *in vivo* (Cameron *et al.*, 1991; Casper and Schingoethe, 1989), have demonstrated lower ammonia N concentrations due to the infusion of readily fermentable CHO, as a result of an improved N uptake by ruminal microbes. This implies that when protein degradation surpasses that of CHO fermentation, there will be a loss of N as ammonia and alternatively if CHO fermentation surpasses that of protein degradation, MCP synthesis will possibly be reduced (Nocek and Russell, 1988). However studies do not show consistent results, with some studies reporting positive results (Casper and Schingoethe, 1989; Herrera-Saldana *et al.*, 1990; Matras *et al.*, 1991) while others show no results (Henning *et al.*, 1991; Newbold and Rust, 1992). This may be due to the variation of ingredients used, or possibly the confounded availability of protein and CHO availability (Bach *et al.*, 2005).

Ruminal bacteria can be classified as either cellulolytic or amylolytic, depending on their desired energy substrate. According to Russell *et al.*, (1992), microbes which degrade structural CHO are cellulolytic and microbes which degrade nonstructural CHO are amylolytic**.** Russel *et al*. (1992) also proposed that cellulolytic microbes have a low upkeep, grow slowly and their major source of N is from ammonia N and that amylolytic microbes have a high upkeep, grow quickly and their major N source is from ammonia, peptides, and AA. Nonetheless, in several studies, the supply of either AA and/or peptides, have shown positive results in relation to microbial growth regardless of the microbes being classified as cellulolytic or amylolytic (Maeng and Baldwin, 1976; Argyle and Baldwin, 1989; Kernick, 1991)**.** In another study done by Atasoglu *et al*, (2001) it was proven that there was an inverse relationship between ammonia N incorporation and AA incorporation in a pure culture of cellulolytic bacteria. This implied that cellulolytic bacteria would utilize AA if presented with it. Comparable results were obtained with peptides; although a greater preference for AA, N incorporation was noted by cellulolytic bacteria than peptides

(Atasoglu *et al.*, 2001). However, approximately 80% of cell N is obtained from ammonia N, regardless of a typical ruminal concentration of AA and peptides. Although an increase in fibre digestion, microbial protein synthesis, and microbial growth efficiency, have resulted from the inclusion of peptides and branched-chain AA (BCAA), which will ferment to branched chain VFA in the ruminal fluid (Russell and Sniffen, 1984; Thomsen, 1985). The utilization of these AA's and peptides is probably the result of direct incorporation into microbial protein and/or the utilization of the carbon skeleton, for new microbial AA or for energy production (Bryant, 1973).

According to Russel *et al.* (1983) rumen microbes which ferment NFC obtain about 66% of their protein from AA or peptides, implying that the rest comes from ammonia N. They further stated that this proportion was not subjective to the rate of microbial growth and that if no CHO were present all the peptide N would be turned to ammonia N. Initially a proposed maximum microbial protein synthesis (MCPS) of 1.2 g peptides N/kg, was stated from the assumption that NFC fermenting bacteria utilize 66% of available N from peptides and that bacteria convert peptides into MCP with an 80% efficiency (Russell *et al.*, 1983). However, in several studies (Atasoglu *et al.*, 1999; Siddons *et al.*, 1985; Firkins *et al.*, 1987) a negative relationship with ammonia N and microbial protein was established. This implies that the accumulation of ammonia N in the rumen is due to the preferred utilization of peptides and AA as an N or energy source. As a result, the proposed 1.2 g of peptides N/kg cannot apply because the amount of bacteria derived from ammonia N is not fixed. Therefore a maximum MCPS in relation to peptide concentration in the rumen is yet to be determined (NRC, 1996). In a more recent study by Atasoglu *et al.* (2001), results showed that rumen microbes had difficulty synthesizing certain AA i.e. Phe, Leu and Ile (Allison, 1969; Oltjen *et al.*, 1971; Amin and Onodera, 1997). It could, therefore, be proposed that microbial growth may be limited due to certain AA such as Lys. Therefore microbial growth can be increased by the addition of certain AA.

2.7.1. Factors affecting microbial protein synthesis

Factors that are important in MCPS namely, CHO, N sources, other nutritional (i.e. sulfur) and non-nutritional factors (i.e. ruminal pH or dilution rate) all play an important role. According to St-Pierre, (2001), who conducted a meta-analysis, there is no relationship between ruminal pH and the efficiency of microbial protein synthesis (EMCPS). This statement was justified by several *in vitro* studies (Hoover and Miller, 1991; De Veth and Kolver, 2001; Calsamiglia *et al.*, 2002). However, ruminal pH does have a negative relationship with total bacterial N flow (St-Pierre, 2001). So although a low pH arises from the fermentation of a high quantity of organic matter (OM), it simultaneously results in an increase in MCPS (Hoover and Stokes, 1991). Alternately fermentation and microbial growth are affected by a change in a

dilution rate of liquid and solid fractions in the rumen content (Isaacson *et al.*, 1975; Russell *et al.*, 1992). The dilution rate of liquid and solid fractions in the ruminal content relies on various factors such as intake, (Merchen *et al.*, 1986; Forbes and France, 1993), particle size (Uden, 1988; Woodford and Murphy, 1988) and percentage forage in a ration (Rode and Satter, 1988). Evidence of an increase in microbial synthesis and EMCPS were proven by *in vitro* studies when there was either an increase in liquid, (Isaacson *et al.*, 1975) solid, (Hoover *et al.*, 1984; Schadt *et al.*, 1999) or both dilution rates (Crawford *et al.*, 1980; Shriver *et al.*, 1986). As the dilution rate increased, the OM digestibility, as well as the energy for microbial growth, decreased, which lowered the expected bacterial N flow. The explanation for the increase in MCPS and EMCPS, as a result of a higher ruminal dilution rate, is credited by the selection of ruminal microbe species with a higher growth rate, a greater percentage of the microbial population in the exponential phase of growth as well as a dilution of maintenance requirements of microbes. High dilution rates are also responsible for a shorter ruminal retention time which reduces both bacterial lysis and bacterial attack by protozoa (Stern and Hoover, 1979; Firkins *et al.*, 1992; Hoover and Miller, 1991). The conflicting results obtained *in vivo* in relation to that of *in vitro* in terms of EMCPS is attributed to modification of passage rates *in vivo* without causing changes in other variables at the same time, therefore, resulting in confusion.

2.8. Efficiency of microbial protein synthesis

An ultimate achievement in ruminant nutrition would be to capitalize on both microbial growth, as well as the capture of RDP into ruminal microbial cells. Emphasis on the EMCPS is essential because maximizing the utilization of degradable N that enters the rumen would result in less N losses and improve the supply of AA to the SI (Bach *et al.*, 2005). Since it is assumed that energy is the most limiting factor in microbial growth, a common microbial growth test is to determine the grams of microbial N/unit of rumen accessible energy. This is usually stated as true OM or CHO fermented. Therefore in order to maximize microbial growth, it is imperative to maximize microbial protein per unit fermentable matter (Bach *et al.*, 2005). However even though EMCPS is a useful indicator in determining how much energy is used in N incorporation into microbes, EMCPS is unable to determine the quantity of N utilized by the microbes (Bach *et al.*, 2005). In fact, according to the same meta-analysis previously mentioned (St-Pierre, 2001), the EMCPS is not influenced by ammonia N concentration in the rumen, which implies that EMCPS is unable to determine the efficiency at which bacteria N is utilized in the rumen. The efficiency of microbial protein synthesis has been proposed to have a negative relationship with ruminal N balance, meaning that when there is a high N balance, the EMCPS is low compared to when N is limiting (NRC, 2001). This

statement is, however, difficult to rationalize and has limitations (Bach *et al.*, 2005). In the calculation of ruminal N balance (RDP supply minus RDP requirements), the RDP requirement is calculated as a function of total digestible nutrients (TDN) with the assumption of a constant efficiency adjustment for MCPS (0.131 x adjusted TDN). A possible reason for the negative relationship implied by NRC (2001) is that as TDN availability increases, the EMCPS decreases because TDN sustains microbial growth and relies on degradation of OM (Bach *et al.*, 2005). Actually, according to St-Pierre, (2001), there is a negative relationship between EMCPS and fermented OM, implying that the relationship stated by the NRC (2001) may be due to the negative relationship between EMCPS and TDN.

When it comes to measuring the NUE at a microbial level, numerous authors have proposed alternate methods. For example, Griswold *et al*, (2003) stated that the NUE should be determined by the proportion of N intake altered into microbial N, whereas (Bach *et al.*, 1999), suggested expressing, the division of grams of bacterial N by the grams of available N and multiplying it by 100. Where the available N consists of N that could ultimately be utilized by rumen bacteria, such as the RDP and endogenous protein. Regardless of the method used, the simplicity of determining NUE is higher in *in-vitro* situations compared to *in-vivo*. This is due to the complexity of determining the available N in-vivo, as the N that is recycled by the saliva and rumen wall, from urea and ammonia need to be estimated and is rarely reported in literature (Bach *et al.*, 2005).

Once again, according to the meta-analysis previously mentioned (St-Pierre, 2001), the NUE can be used to successfully explain the efficiency of N utilized by rumen microbes. In fact, NUE and EMCPS appear to be complementary because since they are reliable indicators of either N or energy use respectively. As a result, a quadratic relationship between EMCPS and NUE was established (St-Pierre, 2001). From this, it was possible to determine a level at which there was a maximum efficiency of both energy use and N captured. According to the NRC, (2001) this maximum level of efficiency occurs when there is an EMCPS between 25 to 36 g of bacterial N/kg of fermentable organic matter (FOM), and an NUE of 69 g microbial N/100 g of rumen available N. However it is worth mentioning the limitations associated with the measurements of grams of microbial N rather that grams of AA N in bacterial cells because although according to NRC 2001 80% of microbial N is in the AA form, the physiological state may vary.

2.9. Nitrogen efficiency

Nitrogen utilization efficiency can generally be defined as the percentage of N output in relation to the total percentage of N consumed (Calsamiglia *et al.*, 2010). The most simplistic approach to increase

NUE would therefore be to reduce protein intake. Furthermore, since protein is expensive a reduction in feed costs would occur. However, it is imperative that accurate prediction of N requirements, as well as the steady supply of carbohydrates and protein for lactating cows, can be made if a reduction in feed N is to be achieved (Recktenwald and Van Amburgh, 2006).

An increase in milk protein would result in an increase in N output as well as an improved N utilization. However, any N that does not accrete tissue or accumulate in milk is lost in faeces or urine (Lapierre and Lobley, 2001). As faecal N is fixed (Marini and Van Amburgh, 2003) the best approach to reduce excretion would therefore be by reducing urinary N excretion (Marini and Van Amburgh, 2003). As a result of dairy cows not being able to store N like energy, one method of measuring NUE is using the milk nitrogen efficiency (MNE) index. The MNE index is determined by the ratio of the amount of milk excreted to the milk divided by the amount of N ingested. Using this index, values ranging between 20 and 35% have been obtained in commercial dairy herds. This indicates that 65 to 80% of the ingested N is excreted in manure (Chase *et al.*, 2012). As the level of CP increases in a ration, the value of MNE seems to decrease. In Table 2.3 below results from a study in which a range of CP rations were fed can be seen (Colmenero and Broderick, 2006). Furthermore, Table 2.3 shows that a moderately constant level of N in milk can be seen throughout the range of CP rations fed. As for the excretion of manure nitrogen (MN) and urine-N, an increase in excretion is seen as the ration CP increases, with urine-N being the main route of excreting excess N.

On account of this low efficiency of N in feeding programs, ammonia emissions from dairy farms have raised concerns. In fact, for the USA, the dairy sector is accountable for 23.6% of total ammonia emissions in animal production (USEPA, 2004). Although ammonia is not emitted directly but indirectly via the conversion of urea N to ammonia by urease found in fecal matter, (Chase *et al.*, 2012) this should not be neglected as approximately 70% of ingested N is lost as ammonia volatilization and leaching (Klausner, 1993; Castillot *et al.*, 2000; Spears *et al.*, 2003). As a result, the oversupply of N in the dairy sector may have severe effects not only in terms of production performance but on a reproductive performance, profit, environment and public perception (Klopfenstein *et al.*, 2002; NRC, 2001). Therefore in order to establish optimum production and minimize environmental pollution, a better understanding for a need to progressing on protein requirements for high production cows is required (Klopfenstein *et al.*, 2002; NRC, 2001).

Furthermore, challenges are associated with measurement NUE, as a wide range of variation 15% to 40% exists among experiments. This variation is believed to be as a result of different feeding practices

and/or experimental conditions, which therefore implies that improvements are possible (Calsamiglia *et al.*, 2010)**.** In fact, according to (Tamminga, 1992) the most significant factor contributing to the inefficiency of N utilization is the rumen metabolism. Nonetheless, although the metabolic processes are responsible for the inefficiency, it is more feasible to alter rumen microbial fermentation instead, which has brought about a high level of research regarding the optimization of rumen fermentation and N flow to the SI (Calsamiglia *et al.*, 2010)**.** This resulted in either the recommendation for balancing RUP and RDP to regulate protein degradation and the supply of fermentable energy or the modification of AA supplied to the SI (NRC, 2001; Alderman and Cottrill, 1993). Even though this research has enriched our understanding of microbial fermentation as well as N utilization, it has only resulted in small improvements of NUE. For example, in the last 48 years, the average NUE reported in US dairy cattle was 23.7% and 24.0%, according to (Stone *et al.*, 1960) and (Hristov and Huhtanen, 2008), respectively. Astonishingly, the N fractions which have been commended in feeding strategies have not improved N utilization and the only factor which appears to be strongly associated with NUE is CP content (Huhtanen and Hristov, 2009)**.** After numerous years, the absence of an improved understanding has led to confusion (Calsamiglia *et al.*, 2010).

2.10. Protein level

Dietary protein plays an important part in both the nutrition of the cow, as well as the sustainability of the farm. This is because the inclusion level of protein may affect DMI, milk yield, milk composition, feed costs, the environment and reproduction (Hristov and Giallongo, 2014). Although there are several factors affected by the protein level in a ration, achieving a low level of protein must be considered with a potential risk in loss of milk production. On the other hand, the benefit of reducing protein level, from an economical perspective, is the reduction in feed costs, which can ultimately lead to an improved farm profitability. Other benefits include a reduction in N inputs and N lost in urine, as well as an improved NUE (Hristov and Giallongo, 2014). However, before considering to reduce protein levels in a ration, it is imperative that the ration meets the requirements of the animal for other nutrients, especially energy. Furthermore, a ration deficient in MP requirements can affect long term production (Hristov and Giallongo, 2014).

As for the level of protein inclusion that is classified as low or high, numerous studies have reported findings. For example, in a study by Colmenero and Broderick (2006) rations were fed with CP ranging from 13.5% to 19.4%, in which the RDP increased from 9.3% to 12.7% and the RUP increased from 4.2% to 6.7% of the DM. Even though the trial did not report any statistical difference between DMI and milk

yield (MY), the ration containing 13.5% CP, in relation to the ration containing 16.5% CP, resulted in approximately 0.68 kg/d reduction in DMI, ($P > 0.22$) as well as a 2.0 kg reduction in milk ($P = 0.10$). These results can be seen in Table 2.3 below. In a different study by Aschemann *et al*. (2012) in which production was 29.03 kg/d and feed was restricted (thereby not allowing DMI determination), a CP level of 12% did not affect milk production, but nutrient digestibility, as well as microbial protein synthesis, was depressed in the rumen. Furthermore, in several studies on higher producing cows, variable effects were noted on the DMI when the ration CP or MP was reduced. Generally, when the DMI is reduced, as a result of feeding a MP deficient ration, the milk production also declined (Lee *et al.,* 2011; Lee *et al* 2012a). On the other hand, if the DMI did not decline the milk production was not different from ration that provided adequate MP (Lee *et al*., 2012b; Histov and Giallongo *et al*., 2014). Moreover, the CP/MP deficient rations showed a general reduction in total tract apparent neutral detergent fibre (NDF) digestibility of 6 to 20%. Remarkably this did not seem to affect neither the milk production nor the milk fat content. However, the same production level was not achieved as the cows receiving an excess MP ration (Lee *et al*., 2012b). These production losses as a result of low protein diets appear to be due to a reduced DMI, causing impaired rumen function, and deficient RDP causing a reduction in fibre digestion, and inadequate supply of key AA's, thereby limiting milk protein synthesis (Hristov and Giallongo, 2014). Overall, the data indicates that diets with RDP of around 9 to 10% (of dietary DM) decrease fibre digestibility, but do not appear to have a consistent effect on ruminal microbial protein synthesis. (Hristov and Giallongo, 2014). According to Reynal and Broderick (2005), the optimal level of RDP on a DM basis is 11.7%, if a reasonable compromise between profitability and environmental waste is made and 12.3% if maximum milk true protein yield is the aim with an expense of uN excretion (237 to 293 g/d).

The reduced response in DMI in relation to the low protein ration is critical, and must be considered (Lee *et al.*, 2012). In fact, (Huhtanen and Hristov, 2009) reported an analysis of 31 studies that were published between 1995 and 2008 in the Journal of Dairy Science. This analysis revealed that in 5 of 7 experiments, the increase in milk protein yield was due to an increased DMI, whereas the other 2 were due to an increased CP as there was no effect on DMI. According to Hristov and Giallongo (2014), a high producing cow (providing about 39.91 kg/d) receiving a balanced ration with 16% or even 15% CP, will not be affected in terms of milk production and composition. Conversely, a ration containing less than 15% CP (MP deficiency of less than 12%) will probably lead to a reduction in milk yield, which to some extent is due to a reduction in DMI. However, this could be alleviated by providing protected AA limiting production, which could again be due to an effect on DMI (Histov and Giallongo, 2014). Several other studies have reported similar results. For instance, (Nadeau *et al.*, 2007) concluded that a ration balanced

with RUP and RDP containing 16% to 17% CP is adequate for early lactating cows. Leonardi *et al.* (2003) concluded that for high producing cows there is no difference in terms of milk or milk protein yield when fed a ration with 16.1% compared to 18.8% CP and, more recently, Hofherr and collaborators (2010) (Hofherr *et al.*, 2010) reported cows producing high yields (39.91 to 49.9 kg/d) when fed rations with 14% to 15% CP. Therefore, if the level of CP in a ration is too low it will compromise microbial protein production, ruminal digestion, energy and protein availability (Clark *et al.*, 1992)**,** and if it is too high it will result in higher unavailable nitrogen (uN), due to the production of ammonia from AA, that is not incorporated into microbial protein in the rumen (Broderick *et al.*, 1991). Thus an optimal level of RDP would allow for a reduction in ration CP with no effect on milk yield and allow for reduced feed costs as well as N losses (Reynal and Broderick, 2005)**.**

Although numerous studies have successfully proven that a ration providing a balanced CP of 16% is sufficient, there are always considerations that need to be taken into account, as there is always a risk associated with nutritional alterations (Chase *et al.*, 2012). Some areas of concern when implementing a ration low in CP according to a survey in the USA were the consistency and quality of day to day ration mixing on the farm, day-to-day variations in forage quality and DM, absence of on farm DM determination and the use of this to alter the amount of feed added to mixer wagon, TMR feeding or component fed, herd grouping and the accuracy of both sample collection as well as lab analysis (Chase *et al.*, 2012). Nonetheless, there is clear indication that many herds can lower their CP content without affecting milk production. This is especially true on farms that are feeding more than 16.5% CP or have a herd milk urea nitrogen (MUN) concentration greater than 12 mg/dL provided the management of the other nutrients is up to standard (Chase *et al.*, 2012).

Table 2.3. Nitrogen intake and excretion from cows fed rations varying in CP content

Item	Ration CP, %				
	13.5	**15.0**	**16.5**	**17.9**	**19.4**
N intake, g/day	483.0	531.0	605.0	641.0	711.0
Milk N, g/day	173.0	180.0	185.0	177.0	180.0
Total manure N, g/day	309.0	316.0	376.0	410.0	467.0
Fecal N, g/day	196.0	176.0	186.0	197.0	210.0
Urinary N, g/day	113.0	140.0	180.0	213.0	257.0
Urinary N, % of manure N	36.5	44.3.0	47.8	52.0	55.0
Milk N, % of N intake	36.5	34.0	30.8	27.5	25.4

Adapted from Colmenero and Broderick (2006).

As the level of protein, increases above optimal level (i.e. 16% CP) there is a decline in NUE (Colmenero and Broderick, 2006), and in order to reduce the protein level, rumen microbe requirements for RUP and glucogenic nutrients for the cow must be met to prevent decreasing feed intake as well as AA used for milk protein synthesis (Cabrita *et al.*, 2007)**.** In conclusion by looking at table 2.3 above, the benefits of reducing CP in a ration can be seen. As the % CP in the rations increased there was an increase in the total N excretion, with urinary N being the main excreting route of excess N, reduction in milk N efficiency and a constant production of both N excreted in the milk as well as the portion of total manure N found in the fecal portion (Chase *et al.*, 2012). From the studies mentioned above it can concluded, that if a cow producing up to 39.9 kg/d is fed a ration with more than 16% CP, its protein intake can be classified as high and if its CP% is less than 15%, its protein intake can be classified as too low. This results in classifying the optimal CP inclusion level to be between 15 and 16% for cows producing up to 39.9 kg of milk per day.

2.11. Characterization of protein

The value of CP in a nutrient source (%N x 6.25) comprises of true protein (TP) and NPN. So, although the contribution of TP and NPN make up the value of CP, the nutrition value of CP for ruminants is better defined by its rate and extent of break down in the rumen, as well as the fraction of RDP and RUP that occur (Schwab *et al.*, 2003). A great deal of effort has been done to determine the value of CP in feed sources, through the development of feed analysis and computer models predictions (Schwab *et al.*, 2003). The first step in characterizing CP is to determine RDP and RUP fractions with relative precision. Several different approaches have been taken, to determine these two separate and distinct fractions. Namely the *in situ*, *in vitro*-enzymatic, *in vitro*-chemical, and *in vitro*-multi-chemical method (Schwab *et al.*, 2003).The RDP fraction consists of a mixture of peptides, free amino-acids, and ammonia, which is essential for microbial growth, activity, as well as the synthesis of microbial protein. The RDP fraction is, therefore, necessary for ruminal fermentation. The RDP requirements on a dry matter basis (DM), for optimal digestion and synthesis, depend on the function of CHO digested in the rumen, efficiency of microbial growth, and the proportional relationship between ruminal supply and microbial requirements for the nutrients in this fraction (Schwab *et al.*, 2003). As for the RUP fraction, it supplies digestible AA as well as determine the amount of unavailable N (uN). The requirements of RUP on a DM basis is a function of the necessity of MP from RUP, as well as the quality of nutrients supplied by the RUP (i.e. AA composition)

(Schwab *et al.*, 2003). This is where the distinction between CP and MP can be made, as CP is a measurement of all N in a feed source, and MP is the amount of true protein that is delivered to the SI. This distinction leads to nutritional models balancing rations on a MP value instead of a CP value because if two rations have the same CP value, their MP value can vary greatly from one another (Block, 2006).

2.12. Determination of RDP and RUP

2.12.1. *In situ* method

This is the most widely used method, which has been adopted in several countries as well as in the latest dairy NRC model (NRC, 2001). Feed samples are placed into a nylon or Dacron polyester bag, with a pore size ranging between 40 to 60 µm, which is placed into the rumen of a ruminally cannulated cow. The bag is then removed at varying times and the amount of undigested CP determined. This procedure allows for a minimum of 3 fractions to be determined,

1. $RDP = a + b \times (kd/(kd + kp))$,
2. $RUP = b \times (kp/(kd + kp)) + c$, (Ørskov and McDonald, 1979; Eq. 1 and 2)

From these equations, a is soluble protein, b is potentially RDP (insoluble CP) and c is RUP (rumen indigestible protein or unavailable protein). As for the b fraction value however, it depends on the digestion rate (kd) as well as the passage rate (kp), which has either a fixed value depending on the system (Scandinavian, French and Italian systems; Schwab *et al*, (2003) or different fixed values for forages and concentrates (DVE/OEB system; Schwab *et al*, (2003) or variable values for feedstuff depending on intake, feedstuff and diet characteristics (Australian model, 2001 dairy NRC) (Schwab *et al.*, 2003). However, it is important to note that several factors affecting the kp, (particle size and density) are not included in these calculations, and may affect the potential outcomes (Schwab *et al.*, 2003).

In addition, there is a limitation is the disappearance of soluble proteins from the bag. Therefore as a result of soluble proteins varying in the rate of ruminal degradation, it cannot be presumed that soluble proteins are completely degraded in the rumen (NRC, 2001). In fact, the soluble protein fraction a consists of fine particles, intact protein and NPN which comprises of peptides which are not entirely broken down in the rumen (Gierus *et al.*, 2005) and approximately 7 to 13% non-ammonia N (NAN) escapes the rumen (Aufrere *et al.*, 2002), implying a likely over-prediction of RDP. However in a meta-study by Broderick *et al*. (2010), it was reported that a 22% under-prediction of RDP, may be due to the incorrect predictions of

passage rate (kp) or MCP which is presumed to be 130 g/kg total digestible nutrients (TDN) discounted for which is not broken down in the rumen (Tedeschi *et al.*, 2015).

2.12.2. *In vitro* enzymatic and fermentative methods

Numerous methods have been used with the aim of finding a procedure which performs as well as *in situ*. These methods used can be classified as ruminal or non-ruminal *in vitro* methods. The former involves the incubation with ruminal digest and the latter involves the incubation of free cell enzymes. Regardless of the method used, the rate of protein breakdown is determined by the rate of AA and ammonia build up, which are both products of protein breakdown (Schwab *et al.*, 2003) or by measuring the residual protein (Ross *et al.*, 2013). Nonetheless determining the release AA or ammonia is complicated by the uptake thereof by microbes causing an underestimation of degradability. This is further acerbated by the release of the same by-products as a result of the catabolism of microbes as well as feed residual in the inoculum, causing overestimation of degradability. Nevertheless, the latter underestimation is corrected by the inclusion of an inhibitor *in vitro* (Broderick, 1987) and the former is corrected by the inclusion of blanks (Schwab *et al.*, 2003). The measurement of residual protein can be instead biased by non-effective measurements of microbial contamination. As a result, much work has been done on non-ruminal *in vitro* method in order to get away from the interfering microbes. However no single *in vitro* method has been found to be acceptable across feedstuff.

2.12.3. *In vitro* chemical method

This procedure involves the use of a solvent to fractionate CP into RDP and RUP or to at least establish a relationship between N solubility and degradability of protein. Nonetheless, it appears that CP solubility is not the same as CP degradation in the rumen for any one given solvent (NRC, 2001). In addition, soluble proteins differ in ruminal degradation and insoluble proteins differ in ruminal degradation resistance (NRC, 2001). It, therefore, seems that no single solvent will be able to fractionate CP across diverse feed stuff (Schwab *et al.*, 2003).

2.12.4. *In vitro* multi-chemical methods

This method, implemented by the CNCPS (v.6.55), fractionates CP into 5 fractions, using 3 solvents, as well as a protein precipitating agent. The fractions include A (NPN) which is assumed to be 100% degraded in the rumen, B1 (rapidly degradable true protein), B2 (moderately degradable true protein and large peptides), B3 (slowly degradable true protein, which is calculated as the difference between neutral detergent insoluble CP, NDICP, and acid detergent insoluble CP, ADICP) and C (undegraded true

protein quantified as ADICP) which is assumed to be undegradable, i.e. unavailable protein (Schwab *et al.*, 2003). The CP that is related with the insoluble residue of ADF extract is referred to as ADICP and that which is related to the insoluble residue of NDF extract devoid of sodium sulfite is referred to as NDICP. In contrast with the *in situ* method, the A and C fractions do not need a degradation rate (kd), with the exception of specialized feeds assigned a kd to the A fraction. However, the three B fractions are assigned different kd values (Schwab *et al.*, 2003).The CNCPS model further identifies a relationship between kd and kp and that kp differs with feed intake, feed as well as diet characteristics. Thus two separate equations are used for predicting kp of forages and concentrates. In addition, the kp values are adjusted for individual feeds with the use of a multiplicative adjustment factor (AF), which are calculated differently for forages and concentrates (Russell *et al.*, 1992; Mertens, 1997; Mertens, 2002).

When determining the RDP fraction using either the CNCPS or NRC (2001), there is a lack of consideration for the passage lag and digestion lag in the equations, which need to be brought to attention as meal vary in size, feeding between meals, passage lag (Murphy *et al.*, 1993) and digestion lag (Varga and Hoover, 1983; Nocek and Grant, 1987; Coblentz *et al.*, 2000; Mustafa *et al.*, 2000).

2.13. Determining RUP digestibility

Accurate estimates of RUP fraction components across a range of feedstuff is critical for balancing rations for RUP. Therefore differences in RUP digestibility across various feedstuff can be held accountable for the increasing in the number of feeding standards. As for RUP digestibility, numerous methods have been used, namely, *in vivo* procedures, *in vitro* techniques, non-ruminal animal biomass, acid detergent insoluble CP (ADICP) and *in situ* method. Of these methods the *in situ* (bag technique) is the most commonly reported method (Schwab *et al.*, 2003). However a good alternative, at least for protein sources instead of the bag technique, is the 3 step *in situ/in vitro* process (Calsamiglia and Stern, 1995) or alternatively a more recent *in vitro* procedure announced in a conference paper by Ross *et al.*, (2013).

In the NRC (2001) model, the allocated approximations of RUP digestibility to feedstuff, are the result of the approximate mean reported in the literature according to the mobile bag technique and the 3-step procedure. For feeds with limited or no data, the French protein system (Jarrige, 1989) was used to assign values. In contrast, the CNCPS system assigns digestibility coefficients to protein fractions instead of assigning them to feedstuff. The values assigned to the protein fractions (A = 1.00, B1 =1.00, B2 = 1.00, B3 = 0.80, and C = 0.00) are not always the same for the B1, B2, B3, and C as specified, especially for that of fraction C (NRC, 2001; McNiven *et al.*, 2002). This mean that in the CNCPS the RUP digestibility

predicted values are not fixed as in the NRC (2001) system. Nonetheless, the benefit of this to the CNCPS system is not known (Schwab *et al.*, 2003).

2.14. Evolution of nutritional models

Scientific research involving protein requirements for growing cattle probably started in 1908 (Forbes, 1924). However, it was only until the late 1980's and 1990's when desktop computers and software became available to determine requirements with a series of complex equations. This brought about the development of nutritional models (Tedeschi *et al.*, 2015).

2.14.1. National Research Council (NRC)

The first revision of the dairy NRC was published in 1950 followed by its second revision in 1956 with a title of "Nutritional Requirements of Dairy Cattle" (NRC, 1956). At this stage the protein requirements for maintenance were calculated in terms of digestible protein and that 0.27 kg/453.6 kg of metabolic body weight ($BW^{0.75}$). In 1978 when the dairy NRC's fifth revision was made (NRC, 1978), immense alterations came about regarding the calculation of protein requirements, such as unavailable feed protein and protein solubility, as suggested by Swanson, (1977). It was only until the sixth revision that the concept of RUP and microbial CP (MCP) were proposed as being the key sources of MP (NRC, 1996). In the seventh and most recent revision of the dairy NRC (NRC, 2001), the theory of degradation kinetics for nutrient protein to compute readily, potentially and unwanted protein fractions was incorporated. This allowed the model to develop further in terms of predicting energy, protein, and AA requirements. In fact, in the seventh edition the recommended EAA changed from 2.2 to 2.4% for methionine and from 6.6 to 7.2% for lysine with an optimal lys:met ratio of 3 in order to adjust the formula for lactating cows (NRC, 2001). Additionally, this model uses the *in situ* fermentation method, explained earlier, to determine their RUP/RDP fraction. As for the MP for lactation, it is calculated from the net protein value in milk. This is done with an efficiency of 67% which, according to (Ruiz *et al.*, 2002), is probably not constant for ration deficient in ruminal N and may be as high as 75% (NRC, 1985).

2.14.2. Cornell Net Carbohydrate and Protein System (CNCPS)

As all nutritional models are gradually improved over the years, the CNCPS is no different. The first publication of CNCPS occurred in 1992 and 1993 in a series of papers (Fox *et al.*, 1992; O'Connor *et al.*, 1993; Russell *et al.*, 1992; Sniffen *et al.*, 1992), and has been improved over the last 15 years. In CNCPS version 4 protein was divided into 5 fractions with A representing rapidly available NPN, B1 rapidly

available true protein, B2 intermediate ruminal digestion rate, B3 slow degradation and C indigestible, bound to lignin (Tylutki and Fox, 2000). In CNCPS version 5 the protein fractions were still divided into 5 fractions but classified as A being the NPN, B1 the soluble true, B2 the non-cell wall, B3 available cell wall and C the unavailable cell wall (Tedeschi *et al.*, 2015). However, in the CNCPS version 6.1, the peptides were moved from the NPN fraction A to the soluble fraction B1 (Van Amburgh *et al.*, 2010). Furthermore, in a conference paper by Ross *et al*, (2013), a new lab assay was described, and compared with the two-step *in vitro* procedure (Tilley and Terry, 1963), the three step *in vitro* procedure (TSP) (Calsamiglia and Stern, 1995), which includes the use of the *in situ* bag technique plus an additional two *in vitro* steps, as well as a modified three step procedure (mTSP) (Gargallo *et al.*, 2006). These procedures are used to determine intestinal protein digestion in ruminants. The new Cornell lab assay differs from the procedures mentioned, as it makes use of an enzyme mixture consisting of trypsin, chymotrypsin, lipase and amylase at activity levels found in both sheep and cattle digesta in order to substitute pancreatin (Ross *et al.*, 2013). In addition, the new Cornell assay replaces the use of a bags, with Erlenmeyer flasks and, in doing so, reduces the loss of sample as well as variation between samples. This also allows for an improvement in the recovery of undegraded feed N, as a small pore size filter paper can be used (Ross *et al.*, 2013). As a result, the new Cornell assay allows for comparison with other published assays as well as acid detergent insoluble N (ADIN) and permits the recovery of residues for amino acid analysis (Ross *et al.*, 2013).

2.15. Processing of protein sources

Most of the protein sources used in dairy cattle nutrition comes from oilseeds such as canola, sunflower, and soya. The processing of these oilseeds is done in order to obtain residues of oil cake and meals, which improves the nutritional value of the raw material. The two key methods used for extracting the oil from the oilseeds are either mechanical applied pressure or solvent extraction. The mechanical applied pressure procedure, forcefully presses out the oil, while the solvent extraction, makes use of an organic solvent, usually hexane, to dissolve the oil from the seed (McDonald *et al*., 2011). The effect of the process allows for the complete, or partial removal of the thick coat, or husk around some seeds, which is generally high in fibre and low in digestibility. This removal of the husk is referred to as decortication, and generally has a positive effect on the nutritive value of the raw material, as it lowers the fibre content and has an effect in improving apparent digestibility of other constituents (McDonald *et al*., 2011). If one looks at animal protein sources, such as fishmeal, in relation to oilseed proteins, although the oilseed may approach that of fishmeal in terms of protein quality, they are not comparable. This is due to oilcakes having

poorly balanced AA ratios, with a large shortfall of at least one essential AA. Generally, oilseed proteins are low in both Cys and Met with a variable, but typically low Lys content. So although the protein quality in a specific oilseed is fairly constant, the same cannot be said for the protein quality of the derived oilcake or meal. This is as a result of the specific conditions used to extract the oil from the oilseed. For example, the high temperatures, in connection with the high pressure, associated with the expeller process, may denature the protein and thereby lower its digestibility with a chance of decreasing its nutritive value. However, this may be somewhat beneficial to ruminants, due to the reduction in rumen degradability (RDP) and control of deleterious substances, e.g. gossypol. Nonetheless the degree of heating is important if the raw seed is under-heated little effect is seen on ruminal degradation, but on the other hand, if over-heating occurs, a reduction in intestinal digestibility occurs (McDonald *et al.*, 2011). Therefore oilcakes are seen as a relatively good "bypass" protein source. It's worth noting that in solvent extractions such high temperatures are not achieved, so the protein value of the meals is just about the same as the raw seed (McDonald *et al*., 2011).

In a study by Castro *et al*, (2007), ruminal degradability and intestinal digestibility of protein and amino acids in treated soya meal products were determined. The treated soya bean meal (SBM) products that were compared were solvent extracted (SE) SBM, expeller (EP) SBM, lignosulfonate (LS) SBM and heat and soyahulls (HS) SBM. In this comparison, the results showed that the SE SBM increased degradability of CP and AA in relation to EP, LS, and HS SBM. This could be due to the SE SBM having a greater fraction of soluble protein, as well as a faster rate of rumen degradation. Furthermore, SE SBM increased the RUP from 42 to 68%. However, if EAA to the SI were to be enhanced EP, and LS SBM would be used. Table 2.4 below shows some of these mentioned results from (Castro *et al.*, 2007).

Table 2.4 Effects of different methods of treatment of soyabean meal on intestinal disappearance of CP and AA *in situ* and *in vitro*[1]. Adapted from Castro *et al.* (2007).

	Treatment				Contrasts				Treatment[2]				Contrasts			
Item	**SE**	**EP**	**LS**	**HS**	SEM, n = 4	SE vs. others	EP vs. LS, HS	LS vs. HS	**SE**	**EP**	**LS**	**HS**	SEM, n = 4	SE vs. others	EP vs. LS,HS	LS vs. HS
	(*In situ*[3] disappearance)								(*In vitro*[4] disappearance)							
CP%	98.500	98.400	99.100	98.900	0.120	0.010	0.001	0.078	87.500	76.700	74.300	79.400	3.210	0.003	0.968	0.147
Essential AA, %																
His	97.600	98.100	98.900	98.500	0.360	0.025	0.143	0.434	87.600	72.600	74.800	84.000	3.600	0.022	0.151	0.088
Ile	99.300	99.300	99.600	99.400	0.090	0.050	0.007	0.016	89.500	72.800	73.600	83.600	2.960	0.002	0.094	0.020
Leu	99.200	99.300	99.600	99.400	0.090	0.007	0.021	0.013	86.700	67.300	67.500	86.700	2.800	0.001	0.144	0.028
Lys	98.500	98.700	99.300	99.000	0.170	0.008	0.022	0.217	97.800	84.400	82.200	87.800	2.500	0.001	0.817	0.092
Met	100.000	100.000	100.000	100.000	—	—	—	—	85.700	76.600	71.200	76.500	3.870	0.036	0.568	0.354
Phe	99.000	99.300	99.600	99.200	0.110	0.015	0.510	0.027	87.700	69.900	69.300	87.700	2.230	0.001	0.158	0.013
Thr	98.800	99.100	99.500	99.200	0.140	0.017	0.154	0.170	90.100	73.900	73.700	82.700	3.100	0.003	0.241	0.059
Val	99.000	99.000	99.500	99.100	0.130	0.077	0.017	0.054	92.300	74.500	76.000	80.400	3.170	0.001	0.315	0.303
Nonessential AA, %																
Ala	99.000	99.100	99.500	99.200	0.150	0.031	0.036	0.125	89.800	71.700	72.600	80.300	2.480	0.001	0.112	0.036
Arg	99.700	99.800	99.800	99.800	0.137	0.287	0.793	0.955	80.100	78.100	60.500	67.200	8.310	0.102	0.053	0.141
Cys	98.500	100.000	99.500	100.000	0.370	0.009	0.533	0.290	90.500	83.900	75.500	81.600	4.180	0.065	0.323	0.326
Glx[5]	99.100	99.400	99.600	99.500	0.150	0.015	0.356	0.489	88.000	74.100	71.500	76.800	3.910	0.013	0.995	0.364
Gly	97.300	97.800	98.700	98.400	0.400	0.015	0.054	0.464	90.700	73.800	75.100	84.900	2.590	0.001	0.062	0.017
Pro	100.000	100.000	100.000	100.000	—	—	—	—	82.500	67.000	63.400	75.900	3.910	0.012	0.582	0.043
Ser	98.000	98.400	99.100	98.700	0.320	0.030	0.172	0.433	90.200	67.600	68.700	74.600	2.870	0.001	0.226	0.131
Tyr	98.200	98.000	99.000	98.400	0.180	0.070	0.004	0.041	94.800	80.000	73.400	85.300	5.010	0.007	0.887	0.064

[1]Performed on rumen degradation residues after 16 h incubation *in situ*.

[2]Treatments: SE = solvent-extracted; EP = expeller; LS = lignosulfonate; HS = heat and soyhulls.

[3]Intestinal disappearance (mobile bag technique), % of 16-h rumen residues.

[4]*In vitro* digestion (Calsamiglia and Stern, 1995), % of 16-h rumen residues.

[5]Glx = Glu plus Gln.

In Table 2.4 the effect of processing can be seen on non-animal protein source and in Table 2.5 below the effects of processing on animal protein sources can be seen. In Table 2.5 the importance of drying fishmeal is perceived as overdrying can make a significant difference (83 as to 69 g/kg CP). The two main approaches used to dry fishmeal are either indirect or direct drying withy the latter approach having less control of drying than the former (McDonald *et al*., 2011).

Table 2.5. Effect of various heat treatments on the available lysine contents of fishmeal (McDonald *et al*., 2011).

Treatment	**Available lysine, g/kg CP**
Freeze dried	86
Oven dried	
105°C for 6 hours	83
170°C for 6 hours	69

2.16 CONCLUSIONS

Nitrogen utilization efficiency is becoming an increasingly more important factor to consider. This is as a result of the increasing prices of protein sources and environmental concerns. The simplest approach to improve NUE is by reducing the level of protein fed in a ration. Currently, farms are still feeding CP levels above 16.5% which is the recommended inclusion level for optimal CP. Surpassing this level has shown no benefit in terms of milk yield and should thus be reduced. However, in order to effectively reduce the N level in a ration, the prediction of the protein fractions need to be accurate.

Numerous different approaches are constantly updated to determine different fractions within protein and different nutritional models adjust their methods to estimate requirements of the same fractions. This results in the lack of general agreement on a specific approach which will lead to the best prediction. This leaves us to decide which procedure is the most accurate. Further protein characterization research is therefore imperative to accurately quantify individual fractions, but only if accompanied by *in vivo* studies to better quantify and satisfy animal requirements, in relationship to the specific fractions. A particular area of concern is the uN fraction as it is associated with a high level of variation across protein sources. This has led to the development of a new laboratory procedure, which is believed to better estimate the uN fraction and therefore improve nitrogen utilization efficiency and farm profitability.

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Chapter 3. Implementing a new assay for protein digestibility on common protein sources used in the South African dairy industry.

ABSTRACT

Predicting the unavailable nitrogen (uN) fraction with the acid detergent insoluble nitrogen (ADIN) method for feeds has been done with discrepancy due to reports of varying amounts of AA being liberated. This led to the development of a new laboratory assay that not only estimates the uN fraction, but also determines the rumen degradable (RDP) and undegradable protein (RUP) fractions. In an attempt to implement the new assay complications arose which resulted in a modified new assay. Using this modified new assay we estimated the RDP, RUP and uN fractions of nineteen protein sources that are commonly fed in South Africa. The protein sources ranged from animal origin to plant origin with some differing in processing method. The results obtained in this study using the modified new assay for uN in g kg^{-1} CP are; blood meal 1 (39.25), blood meal 2 (38.61), fish meal 3 (15.49), fish meal 4 (17.18), fish meal 5 (25.81), feather meal 6 (20.65), feather meal 7 (44.77), canola meal 8 (14.80), canola meal 9 (22.14), extrublend 10 (7.43), soya meal 11 (8.58) , soya meal 12 (9.78), soya meal 13 (11.29), soya meal 14 (10.28), soya Aminomax 15 (9.25), sunflower meal 16 (3.45), sunflower meal 17 (5.72), sunflower meal 18 (11.07), sunflower Aminomax 19 (6.41). Alternatively, the ADIN estimated values for uN for the same samples are 0.061, 0.075, 0.324, 0.055, 0.288, 2.298, 10.861, 2.430, 3.147, 1.454, 0.769, 1.232, 1.354, 0.634, 1.019, 1.713, 1.719, 1.564, and 1.487. These results showed a drastic difference for uN predictions between the two methods. As for the RDP and RUP values obtained for these samples the values were comparable to other literature. We believe that the new assay provides a much better estimation for uN fraction and shows a high level of sensitivity. However results need to be confirmed by *in vivo* studies. Furthermore, we believe that by using the new assay a better prediction for animal requirements can be made which will improve nitrogen utilization efficiency (NUE) and income over feed costs (IOFC).

3.1. INTRODUCTION

Feed formulation systems distinguish between organic nitrogen (N) which is degraded in the rumen and rumen-undegradable protein N (also called bypass or escape protein), which is mostly digested post-rumen, when assessing proteins for ruminants (Alderman and Jarrige, 1987). When it comes to the digestibility of the rumen undegradable protein (RUP) fraction, the Agricultural Research Council (ARC - 1980, 1984) stipulated that the true digestibility of RUP may be constant at 0.85. However, the National Research Council assigned a constant value of 80% for RUP across all feedstuff (NRC, 2001). Nonetheless,

other systems encountered variations in digestibility ranging between 0.60 and 0.95 (Alderman, 1987). In fact, the UK Metabolisable Protein System (Webster, 1987) as well as Goering *et al*. (1972) assume that the N bound to acid detergent fibre (acid detergent insoluble N, ADIP) is both undegradable in the rumen as well as indigestible in the intestines and that the RUP digestibility can be calculated using an equation by Webster *et al*. (1984). Within the ADIP fraction, the indigestible N which is recovered may be lignin-bound N and part of Maillard reaction products and/or tannin-protein complexes (Van Soest *et al*., 1987). In relation to these presumed indigestible N products, several papers looked at distiller by-products as well as other materials which were subjected to sufficient heat and moisture in order to induce a Maillard reaction (Theander, 1980). The findings reported that these by-products which were exposed to heat and moisture, in order to stimulate a Maillard reaction (processing), resulted in a higher ADIN when compared to the original grain. This lead to the suggestion that ADIN may not be completely indigestible as the "extra" ADIN had a significant digestibility, however as to whether the digestion occurs in the rumen or in the abomasum is unclear in the literature (Klopfenstein and Britton, 1987; Weiss *et al.*, 1989; Van Soest and Mason, 1991).

The Cornell Net Carbohydrate and Protein System (CNCPS, v.6.5) has static library values for intestinal protein digestibility for numerous protein fractions and the unavailable nitrogen (uN) is defined by the acid detergent insoluble protein (ADIP) fraction (Tylutki *et al*., 2008; Higgs *et al*., 2012). This ADIP fraction is assigned a 5% digestibility due to data signifying that some amino acids (AA) could be liberated and absorbed (NRC, 2001). In an attempt to improve the accuracy of the uN fraction quantification, due to the variation in AA liberation from the ADIP fraction, a review on past methods used to determine *in-vitro* digestibility was done in order to identify the problems in the procedure (Ross *et al.*, 2013). By comparing the changes in the procedures over the years from Tilley and Terry (1963) to the modified three step procedure (mTSP) by Gargallo (2006), it became apparent that there were some problems (Ross *et al.*, 2013). Consequently, a new laboratory assay was developed by Ross *et al.* (2013). The assay was implemented into the CNCPS (v. 6.5) thereby allowing the model to make a comparison between the ADIP method and the new assay in terms of predicting intestinal indigestible nitrogen (IUN) (Tylutki, 2015). The sensitivity of this implementation reported a 20 g difference in N digestibility as well as a 2 kg difference in milk yield in a lactation study (Gutierrez-Botero *et al*., 2014).

The objective of our work was to implement the new assay for the most commonly used protein sources in South Africa and compare the IUN values obtained by the ADIP method and the new laboratory assay. Even if the assay can be implemented to various feedstuff, when collecting the samples we focused on feeds mainly used by the South African dairy industry.

3.2. MATERIALS AND METHODS

3.2.1. Procedure

A schematic representation of the laboratory assay used to determine intestinal digestibility (ID) can be seen in Figure 3.1 below (Ross *et al*., 2013). Although the assay was not developed to determine RUP, it became a necessity in order to calculate ID. Owing to the complications of understanding the procedure of the assay, a clearer explanation follows.

1. 0.5 g of unground sample is weighed into four 125-ml Erlenmeyer flasks.
2. 0.5 g of maize silage prepared as suggested by Ross *et al.*, (2013) is weighed out into 8 125-ml Erlenmeyer flasks.
3. All the flasks are placed in a warm water bath (39.5°C) under continuous CO_2 flow, with 40 ml buffer (Goering and Van Soest, 1970).
4. With the exception of 4 Erlenmeyer flasks containing the prepared maize silage, 10 ml of rumen fluid is added and a 16 h *in vitro* rumen fermentation commences.
5. After 16 h of fermentation 2 flasks containing the sample, 2 flasks containing the prepared maize silage with rumen fluid and 2 flasks containing the maize silage without rumen fluid are removed from the water bath. These flasks are used to determine the RUP.
6. The remaining flasks receive pepsin followed by the enzyme mixture as explained by Ross *et al*, (2013) and are removed 24 h later. These flasks are used to determine the unavailable N (uN) and therefore the ID.
7. The RUP flasks are filtered if the particles are not fine or soluble. Otherwise, the samples are freeze dried. Thereafter the N content is determined.
8. The ID flasks are filtered and N content determined as above.

Laboratory Assay Flow Chart

Into 125-ml Erlenmeyer flasks weigh 0.5 g sample into 4 flasks; reagent flasks for blanks- use most concentrate unground; 2-mm grind for forages
0.5 g CS ND for ND digestibility 0.5 g CS ND with (+) and without (-) rumen fluid to correct for microbial contamination.

↓

Fermentation. Add 40 ml Van Soest buffer, pH 6.8 10 ml rumen fluid under anaerobic CO2 16-h; 39°C

2 sample flasks/feed. 2 CS ND flasks + and 2 - rumen fluid

Soluble or fine particles

Transfer to tared pan; freeze.
Freeze dry.
Weigh dried residue
Mix well and quantify N
on aliquot.
(IV residue†)

Otherwise

Filter on tared filters[1]
with boiling H_2O. Dry
residue; hot weigh
for DM digestion
Quantify N Dry residue.
hot weigh for DM digestion (ID residue†)

2 sample flasks/feed 2 CS ND flask + and 2 without rumen fluid

Place in shaking water bath, 39°C Acidify; add ~2 ml 3 M HCl < pH 2 (~1.9)
shake ~ 1 min. Neutralize with ~2 ml 2 M NaOH; shake. Add 10ml
Enzyme mix[3] U/ml in incubation: Trypsin, 24; Chymotrypsin, 20 amylase, 50,
bile and lipase, 4 **OR** Pancreatin, 1.72 mg/ml.

↓

Incubate 39°C, 24-h for DM digestion. Filter on tared filters with boiling H_2O

*NOTE: Quantitatively transfer all residues †Corrections

- IV residue = 'RUP'
  - original - assay blank and microbial contamination [((cs nd + rumen fluid)/g, DM)-((cs nd + rumen fluid)/g, DM)]*wt, DM)
  - new - assay blank and microbial contamination using the above and adjusting for feed NDIN content by CS NDIN digested
- ID residue = undigested N
  - original – assay blank
  - new – assay blank and microbial contamination using cs nd +/- rumen fluid carried through entire procedure

[1]Filters: 90 mm; Whatman 934AH, 1.5µm [2]Pepsin in pH 2 HCl: 16.6 mg/ml [3]Enzyme mix and Pancreatin prepared daily in 1.8 M KH2PO4. Enzyme mix prepared to contain the following U in 10 ml: Trypsin, 1680; chymotrypsin, 1400; amylase, 7050, and lipase, 280. For bile add 0.01g per 10ml of enzyme mix. If using pancreatin, prepared to contain 120.4 mg in 10 ml. Enzyme activity definitions Pepsin ΔA280nm of 0.001 per min at pH 2.0,37°C measured as TCA-soluble products using hemoglobin. Trypsin ΔA253nm of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoylarginine ethyl ester (BAEE). Chymotrypsin ΔA256nm of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyltyrosine ethyl ester (BTEE). Amylase One unit will liberate 1.0 mg maltose in 3 min at pH 6.9, 37°C. Bile from Bovine. Product number B3883. Brand Sigma-Aldrich. CAS-No 8008-63-7. Lipase One unit releases 1 uEq of acid from olive oil per min.

Figure 3.1. Schematic representation of the new procedure for protein characterization. Adapted from Ross *et al*. (2013).

3.2.2. Feedstuff

The samples chosen to analyze using this new procedure were the most commonly used protein sources fed to dairy cattle in South Africa. Nineteen samples were kindly provided by Bester (Bester Feed & Grain, Stellenbosch, South Africa) and Afgri (AFGRI, Ltd, South Africa). Using these samples *4 in vitros* with two duplicates of each sample were run, giving a total of 8 replications per sample. Most South African feed companies obtain their protein sources from Bester, which is the largest agricultural trading company (specialized in domestic and international marketing of agricultural commodities) of the country. We therefore strongly believe that the samples obtained are the best representation of what products are daily used by commercial dairy farms. Our objective was also to obtain samples, of both plant and animal origins, which differed in processing method and/or processing plant. This was done in order to see if there were any changes in the quality of a protein source from either different processing plants that used the same treatment or from different treatments.

3.2.3. Chemical and Statistical Analyses

All feed samples were ground through a 1-mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Dry matter (DM; 105°C for 24h), ash, crude protein (CP; LECO Africa Pty Ltd, Kempton Park, GP, South Africa), acid detergent fibre (ADF; Robertson and Van Soest, 1981), and neutral detergent fibre (NDF; Mertens, 2002) were determined. The neutral detergent insoluble nitrogen (NDIP) and acid detergent insoluble nitrogen (ADIP) were determined according to Licitra *et al*. (1996) with the exception of using the Leco N Gas Analyser FP528 instead of the Kjeldahl (1883) for CP determination. Owing to the procedure being new, sub-samples of the intact samples, rumen- or intestine-(i.e. abomasum) undigested residues were composited and analysed for AA. The calculations used to determine the AA make compensation for the microorganisms attached to the feed (Wang *et al*., 2016; Krawielitzki *et al*., 2006; Storm and Ørskov, 1983). The amino acids of the intact samples, as well as the residues of RUP and ID, were determined using water acquity ultra performance liquid chromatography (UPLC; Plumb *et al*., 2004). All analyses were done in 3 runs.

Samples were grouped based on type (soya oilcake, sunflower oilcake, canola oilcake, fish meal, feather meal and blood meal), origins (plant vs. animal), and class of product (sold and used mainly as escape protein source or not). The MIXED procedure of SAS software (Version 9.4, 2013; SAS Institute Inc., Cary, NC, USA) was used. The mentioned groups were individually tested for significance differences in the resulting protein fractions and amino acids, with run added as random factor. Differences within groups were determined by least significant difference method with a Tukey

adjustment. Statistical differences were considered significant at $P \leq 0.05$ and those between $0.05 < P \leq 0.10$ were considered trends. Results reported in tables are, if not otherwise indicated, treatment least square means (LSM) and respective means standard errors (SEM).

3.3. RESULTS AND DISCUSSION

3.3.1. Feeds

When collecting the samples we tried to obtain samples that not only differed in type but also in processing method. This would allow for analysis of the effect of processing methods on the same sample type. However, from the samples collected that underwent the same processing, it was difficult to distinguish whether the same or different cultivar or batch of raw product was used. It was therefore not possible to determine if there was a difference within the same processing method. The samples obtained for analysis as well as their chemical composition can be seen in Tables 3.1 and 3.2, respectively. The numbers assigned to each sample will be used throughout the study, next to the sample's name (e.g. feather meal-3), to refer to the specific sample (e.g. Spray dried feather meal, from Rainbow, South Africa).

Table 3.1. Protein sources, processing methods, and origin of the samples obtained for the study.

Number	Protein source	Processing method	Origin
1	Blood meal	Unknown	Tisca, Netherlands
2	Blood meal	Unknown	Neulux, Netherlands
3	Feather meal	Spray dried	Rainbow, South Africa
4	Feather meal	Cooked	County Fair, South Africa
5	Fish meal	Steam dried	Walvis Bay, Namibia
6	Fish meal	Flame dried	Walvis Bay, Namibia
7	Fish meal	Flame dried	Ganslobaai, Namibia
8	Canola	Cold pressed	Soil-Bredasdorp, South Africa
9	Canola	Solvent extracted	Soil-Bredasdorp, South Africa
10	Extrublend	Extruded	Soil-Bredasdorp, South Africa
11	Fullfat Soya oilcake	Extruded	Majesty Oil, South Africa
12	Soya oilcake	Solvent extracted	Continental Oil, South Africa
13	Soya oilcake	Solvent extracted	Profile Feeds, South Africa
14	Soya oilcake	Solvent extracted	Freestate, South Africa
15	Soya oilcake	Aminomax	Afgri, South Africa
16	Sunflower oilcake	Solvent extracted	Continental Oil, South Africa
17	Sunflower oilcake	Solvent extracted	Gauteng Oil, South Africa
18	Sunflower oilcake	Solvent extracted	Majesty Oil, South Africa
19	Sunflower oilcake	Aminomax	Afgri, South Africa

Table 3.2. Chemical composition (g kg^{-1} DM) of the individual protein sources used.

Protein source	Processing method	Origin	CP	Ash	ADF	NDF	EE
Blood meal	Unknown	Tisca, Netherlands	950.00	22.80	4.16	11.77	22.21
Blood meal	Unknown	Neulux, Netherlands	956.30	29.30	4.29	9.96	31.56
Feather meal	Spray Dried	Rainbow, South Africa	771.90	60.80	72.32	247.11	48.23
Feather meal	Cooked	County Fair, South Africa	903.10	33.30	271.40	392.67	56.86
Fish meal	Steam Dried	Walvis Bay, Namibia	678.10	227.80	11.86	477.88	122.36
Fish meal	Flame Dried	Walvis Bay, Namibia	671.90	216.20	4.44	31.32	112.55
Fish meal	Flame Dried	Ganslobaai, Namibia	706.30	141.80	12.76	507.57	108.21
Canola oilcake	Cold pressed	Soil-Bredasdorp, South Africa	346.90	56.30	237.45	205.96	58.56
Canola oilcake	Solvent	Soil-Bredasdorp, South Africa	398.40	68.90	247.62	226.73	62.21
Extrublend	Extruded	Soil-Bredasdorp, South Africa	405.00	52.10	174.35	195.09	109.87
Fullfat Soya oilcake	Extruded	Majesty Oil, South Africa	385.30	47.10	121.79	232.40	179.23
Soya oilcake	Solvent	Continental Oil, South Africa	513.10	57.30	78.74	112.16	16.67
Soya oilcake	Solvent	Profile Feeds, South Africa	520.00	61.80	65.04	94.46	12.21
Soya oilcake	Solvent	Freestate, South Africa	513.10	56.90	78.23	105.84	18.41
Soya oilcake	Aminomax	Afgri, South Africa	486.30	62.50	91.38	235.62	12.65
Sunflower oilcake	Solvent	Continental Oil, South Africa	398.10	48.40	341.30	435.92	23.23
Sunflower oilcake	Solvent	Gauteng Oil, South Africa	335.00	55.30	252.99	347.25	20.59
Sunflower oilcake	Solvent	Majesty Oil, South Africa	377.80	49.40	341.23	431.23	19.78
Sunflower oilcake	Aminomax	Afgri, South Africa	373.40	52.90	330.71	417.34	26.46

The chemical analysis results show a large variation throughout the samples as well as within some of the specific protein sources. This is partly attributed to the wide range of samples used which, as mentioned earlier, can be further classified into type, origin, and class of product (bypass or not). The NDF, ADF, EE and ash values, for all the soya oilcakes, sunflower oilcakes and canola oilcakes values fall within range according to the NRC (2001). No ADF or NDF values are reported for blood meal, feather meal or fish meal in the NRC (2001). However, all the EE and ash values fall within range according to the NRC (2001). The CP varied from 335 g kg^{-1} DM for sunflower oilcake-17 to 956.30 g kg^{-1} DM for blood meal-

2. The CP values reported for blood and fish meals are the only values that fall within the range reported by the NRC (2001). Three of the CP values for sunflower oilcake are numerically higher, three for soybean oilcake and one for canola oilcake, are numerically lower than the reported values in the NRC (2001). Nonetheless, all the reported CP values are consistent with literature values (Boucher *et al*., 2009; Kamalak *et al*., 2005; Mondal *et al*., 2008; Piepenbrink and Schingoethe, 1998; Tiwari *et al*., 2006; Woods *et al*., 2003). However, the variation seen within the solvent extracted sunflower oilcake, with CP values ranging from 335 to 398 g kg^{-1} DM can be attributed to different source or species of sunflower used by the processing plant because solvent extraction does not involve pressing and temperatures are relatively low. This implies that the original seed value is similar to the solvent product (McDonald *et al.*, 2011). The reported CP values for the animal origin products (fish, feather and blood meals) do not vary much within the same product with the exception of feather meal. The large variation in CP between the spray dried (771 g kg^{-1} DM) and the cooked (903 g kg^{-1} DM) feather meal could, therefore, be attributed to the difference in processing method as well as the composition of the meal (i.e. heads, feet, skin). Furthermore, unlike plant origin samples, most of the animal products involve heat and high pressure during processing which has an influence on protein quality (McDonald *et al.*, 2011).

3.3.2. Modifications of the assay

In our attempt to follow the new laboratory assay procedure as explained above, several complications arose. Nonetheless, results were obtained with some modifications to the procedure by Ross *et al*. (2013). The implemented changes are described below.

1. 0.5 g of a sample was weighed out into four 125-ml Erlenmeyer flasks of which two are for RUP determination (RUP-flasks) and two are for ID (ID-flasks). The original protocol states that most concentrates can be analyzed unground and that forages should be milled through a 2-mm sieve. However, we decided to mill all samples through a 1-mm sieve, using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to allow the method resulting in the intrinsic characteristics of the protein fraction. Preliminary tests clearly showed how particle size can interact with the digestive steps, thus biasing the final values (Damiran *et al*., 2008). Furthermore, smaller and homogenous particles within sample allow for a more representative sub-sample.
2. 0.5 g of maize silage neutral detergent fibre (CSNDF) as explained by Ross *et al.* (2013) was weighed out into eight flasks. Four of the flasks are used for RUP determination of which only two receive rumen fluid (CS+RF and CS-RF). The remaining four are used for ID determination, and

again only two flasks receive rumen fluid (CS+RF and CS-RF). These are used for microbial contamination correction.

3. After the addition of 40 ml of medium, according to Goering and Van Soest (1970), the flasks were placed in a water bath at 39.5°C under constant flow of CO_2, in order to maintain anaerobiosis. Thereafter 10 ml of rumen fluid was added which was collected from two cannulated cows, fed a total mixed ration for lactating cows, composited and filtered through four layers of cheese cloth. An extra filtration step of the rumen fluid with a 100-µm porosity polyester cloth was added in order to reduce particles contamination within the flasks. Subsequently, the flasks were left to ferment for 16 h.
4. After the 16 h fermentation, according to the original procedure, the RUP-flasks are removed (2 duplicate samples, 2 CS+RF and 2 CS-RF). The content of the flasks are then filtered through Gooch crucibles fitted with a 1.5 µm glass microfibre filters (Whatman 934AH, GE Healthcare, Buckinghamshire, UK), to minimize loss of fine particles. Boiling distilled water is normally used to aid the filtration. After filtration, the crucibles are dried at 100°C for 24 h and the N content determined. However, due to clogging of the filters during filtration, when either the ground or unground sample was used, the 1.5 µm glass fibre filter was removed leaving the sintered filters in an attempt to rectify the clogging. Nonetheless, removing the 1.5 µm glass fibre filters did not rectify the clogging and therefore filtration with Gooch crucibles was not implemented. A possible explanation for the clogging may be due to fine particles from some feedstuff blocking the pores. Others have suggested (B. Steinlicht, Dairyland Laboratories, Inc., Arcadia, WI - USA, personal communication) that the viscosity of the rumen fluid may play an important role. However, the extra filtration step of the rumen fluid with a 100-µm cloth, as previously mentioned, did not improve the filtration step. Alternatively, the Erlenmeyer flask content can be transferred to a centrifuge bottle (250-ml centrifuge bottles in our case, Nalgene, model 2189-0008, Thermo Fisher Scientific, Waltham, USA) and centrifuged at 14,000 rpm (i.e. 30100 RCF) for 10 minutes at 4°C (Avanti j-e series, Beckman Coulter, USA). The supernatant was then disposed and the pellet dried at 60°C for 48 h and hot weighed. The supernatant was disposed of as the N content is very low and theoretically represents only the microbial N from the collected rumen fluid. Analyses of the supernatant for N highlighted that no significant residual samples were lost and that centrifuge at high speed would therefore reduce microbial contamination from the rumen fluid. For highly soluble protein sources, freeze drying is however suggested. Thereafter the bottles were left for 14±2 h at room temperature to stabilize moisture content before N determination

5. After the 16 h ruminal fermentation, the ID flasks were relieved of their anaerobiosis state and remained in the water bath. Thereafter, HCl was added to lower the pH to 1.9-2.0 before pepsin was added, to simulate abomasal conditions. The initial protocol (Ross *et al.*, 2013) stated that about 2 ml of 3 M HCl solution should be used to lower the pH. However, we needed to use 2±0.7 ml of a 6 M HCl solution to achieve a pH of 1.9-2.0. After 1 h 2±0.4 ml of a 5 M NaOH solution was added in order to increase the pH to 5.0-5.5. Thereafter 10 ml of the enzyme mixture as explained by Ross *et al.* (2013) was added for 24 h, to simulate intestinal conditions, assuming a retention of 24 h. Thereafter the samples are supposed to be filtered as stated in the initial protocol. However, the samples did not filter at this stage either and were therefore transferred into the same centrifuge bottles mentioned earlier, centrifuged and dried as above.

Table 3.3. Mean rumen degradable protein (RDP), rumen undegradable protein (RUP) and unavailable CP (uCP) for the selected protein sources using the modified procedure by Ross *et al.* (2013), and acid and neutral detergent insoluble protein fractions (ADIP, NDIP). All values are in g kg^{-1} of crude protein.

Protein Source	Processing Method	Origin	RDP	RUP	uCP	ADIP	NDIP
Blood meal	Unknown	Tisca, Netherlands	32.65	959.29	392.51	0.85	8.50
Blood meal	Unknown	Neulux, Netherlands	90.68	911.21	386.11	0.66	7.23
Feather meal	Spray dried	Afrgri, South Africa	380.45	602.19	206.48	23.99	228.65
Feather meal	Cooked	Medows, South Africa	233.83	782.85	447.66	103.70	379.50
Fish meal	Steam dried	Walis bay, Namibia	203.28	760.54	154.9	2.93	286.36
Fish meal	Flame dried	Walis bay, Namibia	478.68	515.24	171.82	0.38	19.54
Fish meal	Flame dried	Ganslobaai, Namibia	222.3	783.55	258.11	3.06	363.23
Canola oilcake	Coldpress	Soil-Bredasdorp, South Africa	770.93	226.47	147.99	60.86	32.83
Canola oilcake	Solvent	Soil-Bredasdorp, South Africa	662.25	359.79	221.43	62.74	37.84
Extrublend	Extruded	Soil-Bredasdorp, South Africa	526.43	418.72	74.30	28.65	26.52
Fullfat soya	Extruded	Majesty Oil, South Africa	641.95	362.10	102.85	15.61	25.71
Soya oilcake	Solvent	Continental Oil, South Africa	581.35	440.53	85.78	12.75	40.09
Soya oilcake	Solvent	Profile Feeds, South Africa	541.53	421.68	112.91	20.98	32.65
Soya oilcake	Solvent	Freestate, South Africa	675.88	331.27	97.77	19.57	38.07
Soya oilcake	Aminomax	Afgri, South Africa	337.7	662.25	92.48	15.89	122.67
Sunflower oilcake	Solvent	Continental Oil, South Africa	824.2	212.16	34.53	37.51	45.64
Sunflower oilcake	Solvent	Gautenq Oil, South Africa	713.88	272.62	110.63	39.09	28.21
Sunflower oilcake	Solvent	Majesty, South Africa	877.48	101.10	57.24	40.48	30.23
Sunflower oilcake	Aminomax	Afgri, South Africa	791.68	225.43	64.07	53.86	37.04

3.3.3. Rumen degradable and undegradable protein

Even though rumen degradable (RDP) and undegradable (RUP) protein fractions were not the objective of the new laboratory assay, they had to be determined to determine the uCP. The most commonly used approach to determine these fractions has been the mobile bag technique (MBT) (Schwab *et al.*, 2003). However, there are alternative procedures such as the *in vitro* enzymatic methods, *in vitro* chemical and multi-chemical methods (Schwab *et al.*, 2003). Furthermore, the NRC (2001) and CNCPS use different methods to determine RUP digestibility. The CNCPS RUP digestibility values are assigned to

each protein fraction, while the NRC assigns fixed RUP digestibility values to single feedstuffs obtained from the mobile bag technique and the 3-step procedure (Schwab *et al*., 2003). For both, however, the total RUP depends on the specific rates of digestion and passage assigned to the protein fractions that will determine in a competitive way the ruminally escaped protein (Higgs *et al.*, 2015). Nonetheless, the values for RUP digestibility are similar (Schwab *et al.,* 2003). The values we obtained for RDP (RUP) using the modified Ross assay range from 32.65 ± 23 (959.29 ± 31) g kg^{-1} CP in blood meal-1 to 877.48 ± 59 (101.1 ± 59) g kg^{-1} CP in sunflower-18. Furthermore, the results from this study show that the low amount of RUP for sunflower Aminomax (225.4 g kg^{-1} CP) is comparable with solvent extracted sunflower oilcake values we obtained (212.2 and 272.6 g kg^{-1} CP). On the other hand the RUP of soya Aminomax (662.25 g kg^{-1} CP) is higher than the solvent soya (331.23 and 440.53 g kg^{-1} CP). The soya Aminomax is consistent with other reports in literature which have shown a clear distinction of the Aminomax processed soya (i.e. 552 vs. 812 g kg-1 CP for unprocessed vs. processed, respectively; Evans, 2013) However, the sunflower Aminomax RUP shows that not all treatments intended to increase escape protein are as equally effective across samples and plants. Rumen degradable protein estimates from the literature are comparable to ours (Table 3.4). Past studies have reported RDP of 302 g kg^{-1} CP for fish meal, 723 g kg^{-1} CP for soya oilcake (Prestløkken and Rise, 2003), 683 g kg^{-1} CP for soya oilcake, 550 g kg^{-1} CP for sunflower oilcake (Wang *et al.*, 2016), 30 g kg^{-1} CP for blood meal, 559 g kg^{-1} CP for canola meal and 310 g kg^{-1} CP for fish meal (Piepenbrink and Schingoethe, 1997). These reported values are slightly lower for canola meal and sunflower meal but higher for soya oilcake and fish meal in relation to our values. Furthermore, comparing our RUP (Table 3.4) values to literature 955 g kg^{-1} CP for blood meal, 407 g kg^{-1} CP for soya meal (Maiga *et al*., 1996), 919 g kg^{-1} CP for blood meal, 563 g kg^{-1} CP for fish meal and 395 g kg^{-1} CP for canola meal (Piepenbrink and Schingoethe, 1997) the results are comparable.

Table 3.4. Mean values obtained using the modified Ross assay with standard deviation in g kg^{-1} CP.

Protein Source	RDP	RUP
Blood meal	61.67 ± 31.38	935.25 ± 31.38
Feather meal	307.14 ± 101.03	692.52 ± 101.03
Fish meal	301.42 ± 50.53	686.44 ± 50.53
Canola meal	716.59 ± 77.54	293.13 ± 77.54
Soya meal	599.58 ± 77.78	397.82 ± 77.78
Sunflower meal	805.19 ± 63.46	397.83 ± 63.46

3.3.4. Unavailable nitrogen

The values obtained implementing the new assay are shown in Table 3.3 along with the ADIP and NDIP values of the same feeds. When comparing the ADIP and NDIP values to other literature (Table 3.5) and to the feed library of the NRC (2001; Table 3.6) a wide range is found. The ADIP values for sunflower oilcake and canola oilcake (cold-pressed) fall within the range of the NRC (2001). However, none of the reported NDIP values fall within the range of the same source of data. Looking at the values in Tables 3.5 and 3.6, the range into which a specific value should fall is unclear as numerous values in literature fall outside the range suggested by the NRC (2001). An explanation as to why our values do not fall within the range according to the NRC (2001) may be related to the number of samples used to determine the range for a specific source. For some ranges very few samples were used which may have led to misrepresentative averages. Furthermore, in some cases samples may have not been grouped based on the specific process (mechanical, solvent, extruded) which may lead to fabricated ranges. Nonetheless, looking at the ADIP and NDIP values, we obtained large differences within and between types of feed, with the variation being higher in animal proteins compared to plant protein sources. Giving an explanation for the wide variation is difficult, also because ADIP/NDIP values are calculated from ADF/NDF which is particularly low, and theoretically absent, in animal products (Tylutki, 2015) and, in any case, an artefact. Perhaps the need to report the ADIN and NDIN values have been undermined as their contribution to value in feed fractions has been minimal. Moreover, the significance of the value has probably been diminished as a result of studies reporting it as a poor indicator of protein digestibility (Weiss *et al*., 1989; Nakamura *et al*., 1994). This has led to the development of several alternate methods to determine protein digestibility, i.e. two-step procedure (Tilley and Terry, 1963), 3-step procedure (Calsamiglia and Stern, 1995), modified 3-step procedure (Gargallo *et al*., 2006), precision fed cecetomized rooster assay (Boucher *et al*., 2009), Ross assay (Ross *et al*., 2013).

Table 3.5. ADIP and NDIP values obtained during our study and from the literature in g kg^{-1} DM.

Protein Source		Modified Ross assay	NRC (2001)	[3]Aminomax NDS library	Schwab *et al.* (2003)	Woods *et al.* (2003)	Boucher *et al.* (2009)	Wang *et al.* (2015)	Heendeniya *et al.* (2012)	Maiga *et al.* (1996)	CNCPS Library (2015)
Blood meal	ADIP	0.94 ± 0.10	-	-	-	-	-	-	-	9.38	13.30
	NDIP	7.86 ± 0.60	-	-	-	-	-	-	-	-	13.30
Fish meal	ADIP	2.86 ± 0.12	-	-	-	-	-	-	-	-	15.06
	NDIP		-	-	-	-	-	-	-	-	52.77
Sunflower meal	ADIP	16.66 ± 0.70	14.00 ± 4.00	-	-	31.00± 09.00	-	11.00 ± 1.70	-	-	15.33
	NDIP	34.69 ± 7.78	55.00	-	-	-	-	20.4 ± 1.70	-	-	33.69
Sunflower Aminomax	ADIP	14.87 ± 0.72	-	5.33	-	-	-	-	-	-	-
	NDIP	37.04	-	4.823	-	-	-	-	-	-	-
Soybean meal	ADIP	8.47 ± 2.38	4.00	-	17.00 ± 6.4	41.00± 8.00	12.00 & 24.00	46.0 ±0.9	7.00	21.25	19.62
	NDIP	27.7± 3.15	7.00 ± 2.00	-	59.00 ± 2.98	-	64.00 & 61.00	6.70 ± 0.90	4.30	-	24.06
Soybean Aminomax	ADIP	10.19	-	5.33	-	-	13.00 & 18.00[1]	-	-	-	5.99
	NDIP	122.67	-	4.823	-	-	97.00 & 29.00[1]	-	-	-	15.75

Protein Source		**Modified Ross assay**	**NRC (2001)**	**[3]Aminomax NDS library**	**Schwab *et al.* (2003)**	**Woods *et al.* (2003)**	**Boucher *et al.* (2009)**	**Wang *et al.* (2015)**	**Heendeniya *et al.* (2012)**	**Maiga *et al.* (1996)**	**CNCPS Library (2015)**
Fullfat Soybean	ADIP	06.34	-	-	-	-	-	-	-	-	-
	NDIP	25.71	-	-	-	-	-	-	-	-	-
Canola Cold pressed	ADIP	24.30	24.00 ± 7.00	-	-	-	-	-	32.00[2]	-	22.15
	NDIP	32.83	63.00 ± 25.00	-	-	-	-	-	0.800[2]	-	7.922
Canola Solvent	ADIP	31.47	-	-	-	-	-	-	32.00[2]	-	25.73
	NDIP	37.84	-		-	-	-	-	8.00[2]	-	57.26
Extrublend	ADIP	14.54	-		-	-	-	-	-	-	-
	NDIP	26.52	-		-	-	-	-	-	-	-
Feather meal	ADIP	104.90	-		-	-	-	-	-	-	17.00
	NDIP		-		-	-	-	-	-	-	17.00

[1]The ADIN and NDIN values compared with soybean Aminomax are Soy Plus values found in the respective literature.
[2]The ADIN and NDIN values reported by Heendeniya *et al.* (2012) for canola were not specific in terms of processing method.
[3]Values obtained from the NDS library for Aminomax Pro.

Table 3.6. Nutrient composition (g kg^{-1} DM) of some protein sources (and respective sample size) commonly fed to dairy cattle. Modified from NRC (2001).

Protein Source	Number of samples	CP	Number of samples	ADF	Number of samples	ADIP	Number of samples	NDF	Number of samples	NDIP	Number of samples
Blood meal, ring dried	97	955.0 ± 83.0	84	-	-	-	-	-	-	-	-
Fishmeal Menhaden, meal, mech.	135	685.0 ± 44.0	147	-	-	-	-	-	-	-	-
Sunflower meal, solvent	47	284.0 ± 50.0	48	300.0 ±64.0	16	14.0 ± 4.0	3	403.0 ± 66.0	16	55.0	3
Soya meal solvent 48% CP	561	538.0 ± 21.0	549	62.0 ± 30.0	248	4.0	-	98.0 ± 56.0	248	7.0 ± 2.0	21
Canola meal, mech, extracted	230	378.0 ± 11.0	230	205.0 ± 51.0	82	24.0 ± 7.0	19	298.0 ± 66.0	81	63.0 ± 25.0	16
Feather meal hydrolysed	19	920.0	-	-	-	-	-	-	-	-	-

The assay to estimate unavailable or indigestible N and protein has only recently been implemented by few commercial laboratories in the USA (e.g. Dairyland, Dairy One, CVAS - USA) and besides the procedure published by Ross *et al.* (2013) and a follow up *in vivo* study (Gutierrez-Botero *et al*., 2014) not much data is comparable to our values. However, both of these studies and personal communications from the mentioned laboratories, have given us strong confidence on the values we are now publishing in South Africa. Estimation of the unavailable protein "C" fraction for ration formulation has been done for many years using an adjusted ADIP (i.e. 5% digestible) for the NRC (2001) and a 0% digestible ADIP for the CNCPS. The undigested protein was then dependent on the fractions proportions or on the specific feedstuff, for the CNCPS and the NRC, respectively. Both systems have been however heavily dependent on the detergent system that for some high protein sources (e.g. animal protein sources) is clearly a stretch. It is clear from our data (Table 3.3) that a drastic difference between the new assay and the ADIP method is apparent, similarly to how Ross and collaborators (2013) have shown. Using the modified procedure, the uCP values range from 34.53 in sunflower oilcake-16 to 392.51 g kg^{-1} CP in blood meal-1. However, the ADIP values range only from 0.66 in blood meal-2 to 103.70 feather meal 4. Furthermore, the two methods show very contrasting results with blood meal having the highest uCP according to our modified assay and having the lowest according to ADIP and therefore the largest difference between the two procedures. Ross *et al*. (2013) had, as well, shown a very high unavailable N for heat-damaged blood meal, and therefore the high uCP for blood meal-1 is probably linked to excessive heat during the process. If this value will be confirmed by *in vivo* results, it may have a profound effect on the continued use of, possibly, heat-damaged blood meal as a protein source, since approximately 392.51 g kg^{-1} of the protein provided is unavailable to the digestive system of the cow. Blood meal is also among the most expensive protein sources (R14500 per tonne; November 2016). Furthermore, a vast difference can be seen within a specific sample that underwent the same processing. For instance, the uCP values for solvent extracted sunflower oilcake ranges from 34.53 to 110.63 g kg^{-1}, while the ADIP values range from 37.51 to 40.48 g kg^{-1}CP. Besides the sensitivity of the modified assay, a possible explanation for this variation, since it cannot be related to processing, (solvent extraction does not in fact have a big effect on the solvent product; McDonald *et al.*, 2011) might be due to the interaction between the process and the specific feed. In this case, the acid detergent probably dissolves most of the protein or nitrogen that is not actually available to the animal and leave only unavailable protein within the cell wall matrix. The high sensitivity of the assay could be attributed to the drastic reduction in sample loss or to the improved enzymatic profile or both. This could imply that the assay is not only accurate in determining the uCP fraction across different samples but also within a specific sample. This might have resulted in immense underestimation of the uCP fraction in most feeds, but this inference can be confirmed

only by *in vivo* studies using at least some the same products that have been characterized by our study. However, values from both Ross *et al*. (2013) and our study confirm that there has been an underestimation of the uCP fraction in feeds. Although the blood meal results reported by Ross *et al*. (2013) are lower than ours, with the exception of the heat damaged blood meal, it should be stated that different blood meal samples were used but the processes have always high temperatures in common. Nonetheless, it still shows that a variation in the uCP fraction exists within and among samples indicating that a fixed percentage should not be used as it has been for the NRC (2001). On the other hand, other uCP values (e.g. canola and soya) reported by Ross *et al*. (2013) are comparable to ours, considering that different samples were used. Despite of the needed follow up by in vivo studies, we strongly believe that the new assay was successfully implemented in our laboratory and that the results should thus be acceptable. Figures 3.2 and 3.3 appendix 2 show a graphical representation of the protein fraction we determined.

Furthermore, as a result of the NRC (2001) dividing the CP value into 3 fractions A, B and C, with C resembling uCP (Schwab *et al.*, 2003). Looking at these results (Table 3.8) the unavailable CP for NRC (2001) still provide a much lower estimation of uCP in comparison to the modified Ross assay. In fact our values are double the values reported by NRC with the exception of feather meal. This is as a result of higher uCP values. Figures 2.2 and 2.3 show a graphical representation of the protein fraction determined using the modified Ross assay.

Table 3.8 Comparison of our uCP values to NRC (2001) in g kg^{-1} RUP.

Protein Source	**Modified Ross Assay uCP[1]**	**NRC (2001) uCP[2]**
Blood meal	416.26	200.00
Feather meal	472.29	350.00
Fish meal	283.99	100.00
Canola meal	630.13	250.00
Soya meal	248.40	70.00
Sunflower meal	345.49	100.00

[1] Mean values of the reported in Table 3.3
[2]RUP determined by (100-RUP digestibility)

3.4. Amino acids composition and digestibility

The AA results obtained from the intact, rumen and intestine residues are in Tables 3.9, 3.10 and 3.11. Comparing our results to NRC (2001), most of our values do not fall within the range. For example comparing blood meal we found Arg 79.2, His 29.8, Ile 30.5, Leu 94.8, Lys 150 and Met 14.1 but NRC (2001) found Arg 43.8, His 63.6, Ile 12.6, Leu 128.2, Lys 89.8, and Met 11.7 in g kg^{-1}CP. Furthermore, for sunflower we found Arg 127.6, His 15.2, Ile 34.9, Leu 60.6, Lys 131.2 and Met 10.3 and NRC (2001) found Arg 81.8, His 26.0, Ile 40.9, Leu 64.1, Lys 35.6, and Met 22.9 in g kg^{-1}CP. This may be as a result of the calculations we used to compensate for microbial contamination which depend on the NDF and CP level (Wang *et al*., 2016; Krawielitzki *et al*., 2006; Storm and Ørskov, 1983). Nonetheless, the disappearance of EAA and NEAA can be seen between the rumen and intestines. Generally, the total EAA and NEAA decreased as the samples moved from the rumen to the intestines. Canola-1 showed the largest disappearance of total EAA from the intact sample to the intestinal residue and fishmeal-6 showed the smallest disappearance. For NEAA, sunflower-17 and Blood-meal-1 resulted in the highest and smallest disappearance, respectively. In terms of EAA, there is a large variation in digestion between the rumen and the intestines. For instance the digestion of His in fullfat soya oilcake was 24.8 g kg^{-1} CP in the rumen vs. only 2.7 g kg^{-1} CP in the intestines, whereas the digestion of Lys was 82.0 g kg^{-1} and 21.7 g kg^{-1} CP, respectively. Furthermore, looking at sunflower-16 there was no digestion of Lys in the rumen while 48.0 g kg^{-1} CP are intestinally digested, whereas for Met it is 7.7 g kg^{-1} CP and 0.8 g kg^{-1} CP respectively. These differences in AA digestibility can help feed formulation programs to better meet the supply of limiting AA.

Table 3.9. Amino acids composition of the feeds in g kg^{-1} CP.

	Feedstuff																		
AA	BT	BN	SC	CS	CC	E	SoA	FS	SF	SP	SuM	SuC	SuG	SuA	FNF	FNS	FG	FS	FC
Arg	79.2	82.3	97.3	105.3	112.1	100.2	105.1	77.8	102.0	92.0	127.6	117.5	109.0	108.4	169.3	74.7	122.5	191.6	128.9
His	29.8	29.0	17.0	27.1	22.3	18.8	23.9	29.6	17.5	16.0	15.2	14.0	32.4	19.2	25.9	21.5	17.4	16.2	10.8
Ile	30.5	31.9	39.2	32.3	35.8	37.5	37.8	29.8	40.1	39.4	34.9	33.3	40.4	31.6	36.9	44.5	39.6	38.6	38.2
Leu	94.8	98.5	76.4	65.0	70.0	72.8	68.3	76.2	74.1	75.4	60.6	58.1	76.3	56.0	69.7	83.0	73.0	69.4	77.3
Lys	150.0	225.7	115.4	113.3	112.1	126.4	36.0	37.7	208.5	162.3	131.2	52.1	23.7	111.9	170.5	82.7	116.8	116.5	92.1
Met	14.1	13.9	8.1	12.1	12.5	9.0	13.3	14.8	10.1	8.9	10.3	10.7	16.5	12.6	25.1	32.1	24.4	8.9	8.9
Phe	54.7	54.5	52.2	41.9	35.1	41.7	66.0	63.8	62.1	50.5	42.8	36.7	81.3	55.3	37.6	42.8	38.0	38.9	49.3
Thr	49.2	46.1	34.9	37.2	41.8	36.8	31.5	37.7	28.7	31.3	28.8	32.8	36.1	29.2	37.9	45.4	38.3	39.2	42.9
Val	51.9	52.5	40.6	40.9	45.2	40.9	39.3	43.3	39.2	39.4	39.7	39.0	52.1	37.5	44.2	49.2	44.9	55.2	62.0
Total EAA	554.3	634.4	481.0	475.0	486.7	484.3	421.2	410.8	582.2	515.2	491.2	394.2	467.6	461.6	617.1	475.9	514.9	574.5	510.4
Ala	67.1	67.1	39.3	38.7	43.3	39.1	36.7	40.1	36.5	37.9	36.6	37.6	43.4	33.8	59.1	61.6	57.5	47.2	42.7
Asp	89.0	83.5	97.3	62.1	69.7	88.2	95.4	108.2	79.4	90.3	75.4	86.8	96.0	77.3	70.9	78.7	81.5	60.9	65.0
Cys	5.8	6.2	3.4	5.1	5.9	3.9	2.1	1.8	1.7	2.6	3.5	4.3	3.2	3.3	2.2	3.3	2.5	6.0	15.0
Glu	99.5	96.2	158.8	152.5	172.9	172.9	150.2	169.5	126.2	143.4	166.2	190.3	214.8	168.7	98.9	117.7	111.6	102.8	100.1
Gly	34.7	35.8	41.9	48.8	48.8	43.9	46.0	57.6	44.7	40.3	55.6	50.9	81.9	59.5	73.7	66.6	53.4	83.3	71.3
Pro	35.2	37.7	54.7	64.0	57.1	51.7	68.6	70.9	70.4	54.7	42.1	35.9	65.2	55.3	46.2	46.0	38.5	79.4	92.2
Ser	35.2	35.0	44.3	34.4	38.0	43.9	45.3	53.3	38.1	39.6	40.0	42.8	49.5	39.6	34.5	40.0	33.8	56.2	94.7
Total NEAA	366.4	361.5	439.8	405.6	435.7	443.6	444.2	501.4	397.0	408.9	419.4	448.5	554.0	437.5	385.5	413.9	378.9	435.8	480.8

Bloodmeal Ticsa (BT), Bloodmeal Neulux (BN), Soya Continental (SC), Canola Solvent (CS), Canola Cold press (CC), Extrublend (E), Soya Amminomax (SoA), Fullfat soya (FS), Soya Freestate, (SF), Soya Profile Feeds (SP), Sunflower Majesty (SuM), Sunflower Continental (SuC), Sunflower Gautenq Oil (SuG), Sunflower Aminomax (SuA), Fishmeal Namibia flame (FNF), Fishmeal Namibia steam (FNS), Fishmeal Ganslobaai (FG), Feather meal Afgri (FS) and Feather meal Medows (FC).

Table 3.10. Amino acid composition of feeds after in vitro ruminal fermentation, adjusted for microbial contamination, in g kg^{-1} CP.

	Feedstuff																		
AA	BT	BN	SC	CS	CC	E	SoA	FS	SF	SP	SuM	SuC	SuG	SuA	FNF	FNS	FG	FS	FC
Arg	77.1	74.0	58.2	50.9	37.4	72.4	53.7	60.3	37.3	60.0	30.6	49.0	31.2	40.9	91.2	74.2	63.6	109.4	129.9
His	25.7	24.9	5.5	6.1	5.6	6.4	9.0	4.8	6.4	6.0	4.3	3.0	3.1	2.1	12.5	16.3	14.1	6.1	8.1
Ile	31.4	29.8	25.7	17.1	16.4	28.3	26.7	22.9	21.1	25.9	13.0	14.3	12.7	11.1	32.5	39.2	31.9	30.4	39.3
Leu	93.1	90.2	43.1	29.5	32.8	46.6	49.4	40.0	37.0	43.3	23.1	24.3	23.9	19.4	54.7	68.3	60.6	53.4	73.0
Lys	140.5	87.5	101.8	95.4	4.3	116.0	25.3	29.5	35.3	54.7	7.3	52.1	3.4	74.5	81.6	83.8	91.5	64.2	74.6
Met	12.8	13.8	8.7	4.5	9.0	9.8	8.8	6.6	8.2	8.6	3.5	2.9	3.6	2.2	19.0	25.6	22.6	7.0	7.8
Phe	46.6	54.3	25.7	17.7	24.8	27.5	29.7	22.8	23.3	24.9	18.0	12.2	16.5	8.9	33.2	39.7	35.9	34.3	45.9
Thr	51.0	46.3	21.2	18.3	17.1	25.3	22.9	15.0	17.8	20.0	10.0	13.2	11.4	10.9	28.3	36.7	32.6	26.8	38.8
Val	53.7	52.8	28.0	20.2	21.8	30.3	29.8	27.7	25.0	29.5	15.7	17.4	16.9	14.2	37.1	43.7	37.9	40.9	56.7
Total EAA	531.8	473.6	317.8	259.7	169.2	362.5	255.3	229.5	211.4	272.9	125.6	188.5	122.7	184.3	390.1	427.4	390.7	372.4	474.0
Ala	69.6	63.5	29.4	17.6	22.4	36.4	29.3	27.9	25.7	29.0	15.4	18.7	16.7	15.5	39.8	48.4	42.5	31.1	37.6
Asp	88.9	81.5	44.2	25.4	29.5	50.4	62.4	36.3	38.8	44.6	21.2	28.3	25.6	23.7	62.8	77.7	72.6	41.5	53.8
Cys	5.2	4.6	0.7	0.0	0.0	0.0	0.9	-0.2	0.6	0.8	0.0	0.0	0.0	-0.5	2.3	2.5	2.1	4.2	12.6
Glu	107.6	98.1	57.4	34.7	34.5	63.2	93.1	50.9	49.5	55.8	26.6	35.2	32.5	31.5	74.4	102.4	94.9	61.0	81.5
Gly	33.4	32.1	23.1	16.9	23.9	27.0	27.8	22.5	22.2	24.4	18.6	17.4	19.6	14.1	39.8	45.4	40.4	47.7	61.0
Pro	30.8	36.2	19.6	13.1	18.8	20.4	27.2	19.0	18.2	21.1	13.0	10.1	12.8	8.4	30.8	36.1	31.8	60.1	85.1
Ser	34.1	33.2	16.2	11.6	14.8	18.2	25.1	12.3	15.6	15.9	9.5	9.0	9.9	7.2	25.2	33.3	25.9	36.5	70.2
Total NEAA	369.6	349.1	190.6	119.4	143.9	215.6	265.8	168.7	170.7	191.6	104.2	118.7	117.2	99.9	275.1	345.7	310.2	282.1	401.8

Bloodmeal Ticsa (BT), Bloodmeal Neulux (BN), Soya Continental (SC), Canola Solvent (CS), Canola Cold press (CC), Extrublend (E), Soya Amminomax (SoA), Fullfat soya (FS), Soya Freestate, (SF), Soya Profile Feeds (SP), Sunflower Majesty (SuM), Sunflower Continental (SuC), Sunflower Gautenq Oil (SuG), Sunflower Aminomax (SuA), Fishmeal Namibia flame (FNF), Fishmeal Namibia steam (FNS), Fishmeal Ganslobaai (FG), Feather meal Afgri (FS) and Feather meal Medows (FC).

Table 3.11. Amino acid analysis of the intestinal digested residue (uN) in g kg^{-1} CP.

	Feedstuff																		
AA	BT	BN	SC	CS	CC	E	SoA	FS	SF	SP	SuM	SuC	SuG	SuA	FNF	FNS	FG	FS	FC
Arg	36.2	36.8	20.7	46.6	21.4	31.1	24.8	19.5	24.9	24.2	34.2	16.7	20.0	16.6	22.3	28.9	24.9	39.4	70.6
His	11.4	11.1	1.2	4.7	3.2	2.8	1.7	2.1	1.8	1.9	2.7	2.0	0.0	0.0	4.6	3.1	5.0	2.8	2.7
Ile	12.8	14.0	7.7	14.9	10.4	11.1	8.8	8.9	9.0	8.8	9.2	5.7	7.7	6.6	9.9	10.3	14.5	12.1	22.4
Leu	44.7	45.7	12.2	26.0	20.2	19.0	15.5	17.8	15.3	15.3	15.8	12.0	15.5	12.8	17.0	14.7	23.4	19.7	37.8
Lys	57.9	21.6	72.7	84.5	0.5	40.9	1.1	7.7	3.0	47.9	4.6	4.2	2.1	6.4	5.7	71.9	45.2	7.6	9.6
Met	3.7	3.8	2.3	3.7	3.5	3.4	2.5	3.5	2.9	2.8	2.3	2.1	3.2	2.4	5.1	4.8	7.4	2.8	2.9
Phe	24.9	27.2	6.2	12.5	11.7	9.4	7.1	10.3	8.0	7.7	8.1	8.5	10.9	8.4	11.9	8.3	15.4	10.4	19.7
Thr	16.6	14.7	7.5	13.3	10.4	9.6	8.2	9.9	8.6	8.0	8.4	5.1	6.9	6.2	9.0	8.5	11.6	11.5	22.7
Val	26.2	26.9	8.7	18.4	13.5	12.3	9.9	11.6	10.1	10.3	11.3	8.2	10.7	9.1	12.5	11.6	16.2	17.4	35.1
Total EAA	234.5	201.9	139.3	224.4	95.0	139.5	79.6	91.4	83.5	126.8	96.4	64.5	77.0	68.5	98.1	162.0	163.5	123.7	223.6
Ala	33.6	31.1	10.2	17.2	14.1	15.0	11.7	13.5	12.3	11.5	13.3	8.2	10.4	9.3	14.4	14.6	16.9	13.2	22.2
Asp	34.6	30.1	13.4	15.7	16.7	16.6	16.4	16.8	15.2	14.8	16.8	9.2	13.4	12.1	19.7	19.2	24.8	18.3	32.8
Cys	2.8	1.8	0.4	0.4	0.3	0.1	0.2	0.1	0.3	0.4	0.2	0.1	0.1	0.0	0.3	0.6	0.7	1.9	8.6
Glu	41.4	38.0	17.8	27.9	22.4	21.8	21.5	21.4	18.8	18.4	21.4	12.6	18.5	16.1	22.7	23.5	30.1	27.6	49.0
Gly	15.8	16.1	8.5	13.0	14.7	12.4	9.8	13.4	10.3	10.0	13.9	11.8	14.5	12.1	17.3	12.6	16.8	17.2	29.0
Pro	17.2	18.8	5.4	14.1	13.1	8.4	6.4	9.4	6.5	6.7	7.1	7.4	8.3	7.4	11.7	8.0	12.6	21.9	46.6
Ser	12.1	10.8	4.5	8.1	8.5	6.0	4.9	7.1	5.3	4.8	5.1	4.2	5.3	4.7	7.4	6.8	9.2	12.4	30.2
Total NEAA	157.6	146.8	60.2	96.5	89.8	80.3	70.8	81.7	68.6	66.5	77.9	53.6	70.5	61.7	93.5	85.3	111.2	112.5	218.4

Bloodmeal Ticsa (BT), Bloodmeal Neulux (BN), Soya Continental (SC), Canola Solvent (CS), Canola Cold press (CC), Extrublend (E), Soya Amminomax (SoA), Fullfat soya (FS), Soya Freestate, (SF), Soya Profile Feeds (SP), Sunflower Majesty (SuM), Sunflower Continental (SuC), Sunflower Gautenq Oil (SuG), Sunflower Aminomax (SuA), Fishmeal Namibia flame (FNF), Fishmeal Namibia steam (FNS), Fishmeal Ganslobaai (FG), Feather meal Afgri (FS) and Feather meal Medows (FC).

3.5. Classification.

Due to the wide range of samples used we classified the samples by origin, class and protein type. We looked at the grouped samples for CP, RDP, RUP, ADIN and uCP. Furthermore, we looked at the individual AA as well as the EAA and NEAA for CP, rumen digestion (rd) and intestinal digestion (id) and uCP. Only Lys and Met are discussed below. Other AA information can be seen in Appendix 1.

3.5.1. Origin

Grouping the protein sources by origin separates the protein sources into animal and plant origin. The plant origin samples consist of canola, soya, and sunflower meal whereas the animal origin samples are blood, fish and feather meals. Looking at the parameters mentioned above, all the animal and protein sources were significantly different ($P < 0.05$) with the exception Lys for id and uCP. Interestingly, ADIP resulted higher in plant proteins as opposed to the uCP that resulted higher in animal proteins. This once again demonstrates the possible biased results given by the detergent system. Animal protein resulted in higher EAA, however most of these AA are apparently digested post-rumen (Appendix 1, Table 2).

3.5.2. Class

Grouping the protein sources by class separated the protein sources into their targeted site of digestion (rumen or intestine/bypass). Protein sources that originate from plants are generally fed as a source of rumen degradable protein. However, there are some exceptions. For instance, the Aminomax products (soya and sunflower) as well as the extrublend which is from canola are marketed and fed as bypass protein sources. On the other hand, protein sources from animal by-products are generally fed as a bypass protein source. Higher CP content (Appendix 1, Table 4) together with the process implemented to increase rumen undegradability, often justify the higher price of the animal proteins. However, in many cases our assay should be implemented to determine the "quality" of the product, apparently not necessarily related to price. We strongly believe that the assay should affect the price since some of the processed protein may not be available. Looking at the parameters the only rumen and intestine group that was not significantly different ($P > 0.05$) was uCP for EAA and Lys for CP and uCP.

3.5.3. Protein Type

Grouping the protein sources into protein type separated the protein sources into blood meal, fish meal, feather meal, soya meal, and sunflower meal. Canola and sunflower meals appeared to be the ones providing the lowest amount of CP (38.34 and 37.11%, respectively) and therefore they cannot constitute the main protein source of high producing dairy cows. However, as the CP is broken into its fraction more similar groups are found. For instance, for RDP and RUP there is not difference between fish meal and feather meal but, all the other protein types are significantly different. Looking at the uCP and ADIN groups

we would expect to find the same protein sources being significantly different or not significantly different however, this is not the case. For uCP blood meal and feather meal, soya and canola, soy and sunflower do not differ from one another ($P > 0.05$). On the other hand according to ADIP blood meal and fish meal, canola meal and feather meal do not differ from one another ($P > 0.05$). This shows that although the two fractions are supposed to provide the same information (unavailable nitrogen) different results are obtained implying that ADIN and uCP are different from one another.

Looking at the, EAA and NEAA the only protein type to show no difference for both EAA and NEAA was canola and soya meal. Furthermore, the only protein type to show no difference for both EAAid and NEAAid were canola and sunflower. However for AA, several similar values of Lys and Met were found.

3.6. CONCLUSIONS

The new laboratory assay can be used as an alternate method to determine RDP, RUP and uCP, in protein sources for dairy cows. It can therefore replace the mobile bag technique (MBT) for RDP determination as well as the ADIP procedure that has been used to determine the protein "C" fraction. Furthermore, it allows quantification of these fractions in the laboratory and reduces the need for cannulated animals. The results from this study report a drastic difference in the unavailable protein fraction when compared to ADIP and show a better prediction for RUP. Even though the aim of the new assay was to predict the uCP fraction because of the high variable results obtained using ADIP value, we believe it not only better predicts uCP but also the RDP and RUP. Furthermore, the procedure showed high sensitivity that resulted in wide range of uCP that varied more than the ADIP prediction. This will ultimately allow for better prediction of available CP in a ration which could potentially improve profitability and NUE. However, the new assay is still a novel procedure and we would recommend further tests and *in vivo* studies, to confirm the amount of N unavailable to the animal.

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Chapter 4. A comparison between the unavailable nitrogen fraction value determined by the acid detergent insoluble nitrogen approach and a new laboratory assay: implications for South African dairy farms.

ABSTRACT

The dairy industry has been known for its environmental distresses with nitrogen (N) excretion being one of the main factors. Improving nitrogen utilization efficiency (NUE) can be accomplished easily by reducing nitrogen (N) in the feed. However, even though there are numerous nutritional models available to assist with ration formulation, these models work on empirical equations and rely on continuous updates to provide the best results. Consequently, a recent update to the unavailable nitrogen (uN) fraction was implemented into the Cornell Net Carbohydrate and Protein System (CNCPS; version 6.55). Using this new update a comparison between the former acid detergent insoluble nitrogen (ADIN) procedure and the new *in vitro* assay was done assuming values obtained from a representative set of samples characterized in our laboratory. Ten rations, currently being fed in South African commercial dairy farms, were shared with us by feed companies and nutritionists. The rations were first evaluated using the uN fraction and then optimized using a CNCPS-based platform with objectives profit and N waste. The use of the updated fraction generally corresponded to a lower income over feed costs (IOFC) than expected by the feed companies, ranging from a reduction of R39.50 to an increase of R0.10 across the rations. When both ME and MP were not limiting, the use of the uCP system resulted in small amounts of extra available energy. On the other hand if the contribution of uCP was high (depending on the protein source) MP would become the limiting factor corresponding to a reduction of milk ranging from 0.31 to 7.90 l. This was mainly due to less metabolizable protein (MP) available and therefore a reduced MP allowable milk occurred. The rations were then optimized using the same diet-specific ingredients. The IOFC consistently increased across all farms resulting in values between R0.16 and R7.64 higher. Our simulations clearly show that by routinely analysing the protein sources for possible unavailable N and applying these characterizations in CNCPS-based platforms, a better formulation in terms of animal requirements can be made which will increase NUE and likely improve farm profitability.

4.1. INTRODUCTION

Human intervention has influenced the global nitrogen (N) cycle negatively with agriculture as the leading contender (Pierer, 2016). This is attributed to farms importing N in the form of fertilizer or feed supplements for their animals and not recycling this excess N via crop production. This results in a vast loss of N to the atmosphere and ground water creating a range of harmful effects to the ecosystem, human health and global climate (Erisman *et al*., 2013; Fowler *et al*., 2013; Galloway *et al*., 2004, 2008). Numerous steps have been put in place in order to reduce this N loss, such as low N fertilization, better manure management and livestock feed optimization (Dalgaard *et al*., 2014; Döhler *et al*., 2011; Newell Price *et al*., 2011; Reis *et al*., 2015) with feed optimization being highlighted as the priority area of focus in reducing N pollution (Aarnink and Verstegen 2007; Klimont and Brink 2004).

Apart from the negative environmental effects associated with using N ineffectively in feeding livestock, the surplus N also contributes to an unnecessary expense to the farmer (Pierer., 2016). Nonetheless, even though numerous studies have looked at either reducing negative environmental implications without an economical consideration (Aarnink and Verstegen 2007; Dourmad and Jondreville 2007; Nahm 2002; Ryan *et al.*, 2011) or focused on economical optimization with little environmental impact (Finneran *et al.*, 2012; Marston *et al.*, 2011; Niemi *et al.*, 2010; Vibart *et al.*, 2012) studies that focus on both economic and environmental implications are rare (Van Vuuren *et al.*, 2015).

In an attempt to reduce feed price by suppling optimal feed rations with minimal wastage and maximum production, numerous nutritional models have been developed. One of these nutritional models is the Cornell Net Carbohydrate and Protein System (CNCPS). The feed library in CNCPS lists roughly 800 ingredients which includes forages, concentrates, vitamins, minerals and commercial products. This vast range of products serves as a reference database that describes the chemical composition of a formulated ration (Higgs *et al.*, 2012). The CNCPS nutritional model depends on empirical estimations from carbohydrate (CHO) and protein degradation, as well as passage rates so that predictions of rumen fermentation, microbial growth, metabolisable energy and protein absorption through the gastrointestinal tract (GIT) can be obtained (Fox *et al.*, 2004). On account of the model depending on empirical estimations, updates are made in order to improve the accuracy of predicting an animals' requirements (Fox *et al.*, 2004, Tylutki *et al.*, 2008, Ross *et al.*, 2013). A recent update to the protein fractions, published by Ross *et al.* (2013), involves the use of a new laboratory assay to predict N that is unavailable (uN) to the whole gastro-intestinal tract. We recently implemented this new assay, with a few modifications, in the ruminant nutrition laboratory of Animal Sciences at Stellenbosch University (Venter, 2017). The work allowed us to quantify

important differences between the traditional unavailable N estimations (i.e. Acid detergent insoluble N, ADIN; Goering *et al*., 1972) and the updated values. The modified assay resulted in increased predictions for the unavailable crude protein (uCP) fraction of protein sources. Coincidentally, the risk of incurring in high uCP corresponded with some of the most expensive sources (e.g. blood meal and feathers meal) with values ranging from 34.53 g kg^{-1} CP in sunflower meal to 447.66 g kg^{-1} CP in feather meal.

The objective of this study was therefore to evaluate real commercial dairy farms diets from South Africa, with varying input level, and assess the implications on the resulting profitability and environmental impacts for a range of protein sources, when implementing the improved methodology.

4.2. MATERIALS AND METHODS

Numerous South African feed companies and independent technical advisers were asked to collaborate and share the information about TMRs' (ingredients and specifications) and animal inputs to be used in the study. Our initial objective was to have a wide range in input and management level, in milk yield, breeds and protein sources used. Unfortunately there was an apparent reluctance to share the information to avoid disclosing commercial information. We were, however, able to obtain the information relative to ten farms across the country. To respect the privacy of both the companies and the dairy farmers, we will not disclose any information other than the one needed for the study.

The 10 original rations represented the controls. Using the CNCPS-based (v. 6.55) rationing system NDS (Ru.m.&n., Ruminant Management & Nutrition, SAS, Reggio Emilia, Italy), the rations were evaluated for various parameters. The evaluation was limited by the fact that we could not have the specific ingredients of the rations to properly characterize them in the laboratory. We therefore used the average feed library values for all ingredients, except the protein sources which were substituted with specifications obtained by Venter (2017). The results from this study and conclusions should therefore be considered only simulations through use of our feed samples. Results from the feeds present in the farms may be different. The parameters initially used for the control rations were crude protein (CP), acid detergent (ADF) and neutral detergent fibre (NDF). Another 10 (treatment) rations were simulated adding the information about the uCP from Venter (2017) and basically "informing" the model to use uCP instead of ADIP as estimate of the protein fraction C (i.e. the unavailable fraction; Van Amburgh *et al*., 2015), according to the protein sources present in each diet. The highest uCP values were used to demonstrate the worst case scenario. As a consequence, the available fractions B1 and B2 (i.e. slow and medium degraded fractions) were also affected, while A1 and A2 (i.e. fast degraded fractions) were not. This resulted in a set of 10 uCP-rations

that differed in the protein fractions. The non-linear optimizer of NDS was used to formulate the best rations for maximum IOFC while considering other parameters. In fact, even if our first parameter was farm profitability, we still used constraints for metabolizable energy (ME) and protein (MP), starch. crude fibre (CF), physical effective neutral detergent fibre (peNDF), non-starch carbohydrates (NSC), non-fibre carbohydrates (NFC), total carbohydrates (CHO), acid detergent lignin (ADL), water soluble carbohydrates (WSC), ether extract (EE), ash, calcium (Ca), phosphorous (P), magnesium (Mg), potassium (K), sodium (Na), chloride (Cl) and sulfur (S). For each farm we therefore obtained 3 diets (i.e. control/original, uCP and optimized diets).

The parameters observed were crude protein (CP), milk protein, ME, MP, productive N and income over feed costs (IOFC). The productive N as well as the IOFC were calculated manually as the current version 6.55 of CNCPS is still pending an update to calculate these parameters, after linking them to the new fractionation system. The productive N was calculated from the total milk N, assuming productive N was only partitioned to milk, and the total intake N. The model does not predict milk yield but it estimates ME and MP allowable milk. We therefore set the uCP-milk yield using the lowest (i.e. limiting) between ME and MP allowable milk. Current (December 2016) feeds prices were kindly provided by the companies and technicians who shared the diets with us. Slight differences may exist between feed companies but we decided to use one single price per ingredient across farms. Milk price was set to R 5.00 per kg for all farms. At the moment, according to our knowledge, most commercial farms in South Africa do not receive a premium for milk protein and fat and therefore composition was not included in the IOFC calculation. However changes in milk composition as predicted by the NDS will be shown, even if not affecting income. The feedstuffs of the control rations, including the protein sources used to substitute into the control rations, can be seen in Tables 4.1 and 4.2.

Table 4.1. List of the feedstuffs used in the ten control rations. List of feedstuff is ordered in terms of DM contribution in each respective ration.

Control Rations									
A	B	C	D	E	F	G	H	I	J
Feedstuffs									
Maize grain	Maize grain	Maize grain	Maize grain	Maize grain	Maize grain	Maize grain	Maize grain	Oat fresh	Oat fresh
Lucerne hay	Lucerne hay	Lucerne hay	Oat silage	Lucerne hay	Maize silage	Canola meal	Oat fresh	Maize grain	Maize grain
Maize Gluten	Oat silage	Maize silage	Cotton seed whole	Wheat straw	Lucerne hay	Oat silage	Lucerne hay	Lucerne hay	Lucerne hay
Wheat straw	Canola meal	Canola meal	Wheat Bran	Maize gluten	Oat silage	Lucerne hay	Wheat Bran	Wheat Bran	Wheat Bran
Molasses	Cottonseed whole	Cotton seed whole	Feather meal	Molasses dried	Apple pomace	Maize silage	Brewers grain	Maize germ	Maize germ
Barley Malt	Wheat straw	Molasses	Soya plus	Wheat bran	Soybean meal	Cotton seed whole	Maize germ	Brewers grain	Brewers grain
Soybean meal	Blood meal	Wheat bran	Maize gluten	Feather meal	Canola meal	Wheat bran	Maize cobs	Soybean meal	Soybean meal
Potato by product meal	Wheat bran	Blood meal	Molasses	Soy plus	Wheat bran	Maize gluten	Soybean meal	Soybean hulls	Soybean hulls
Molasses	Soybean hulls	Soybean hulls	Wheat straw	Blood meal	Wheat straw	Soy plus	Soybean hulls	Maize cobs	Fish meal
Feather meal	Protected fat	Protected fat	Soybean meal	Limestone	Cotton seed whole	Feather meal	Fish meal	Fish meal	Maize cobs
Limestone	Limestone	Wheat straw	Blood meal	Salt	Lupins	Limestone	Limestone	Limestone	Limestone
Blood meal	Sodium Bicarbonate	Limestone	Limestone	Vitamin premix	Limestone	Molasses	Salt	Salt	Salt
Salt	Fish meal	Sodium Bicarbonate	Protected fat	Urea	Feather meal	Salt	Urea	Urea	Urea
Urea	Urea	Salt	Urea	Calcium phosphate	Urea	Vitamin premix	Acid Buff	Acid Buff	Acid Buff
Calcium phosphate	Acid buff	Fish meal	Salt		Fish meal	Urea	Protected fat	Protected fat	Protected fat
	Salt	Acid buff	Calcium phosphate		Calcium phosphate		Magnesium oxide	Magnesium oxide	Magnesium oxide
	Calcium phosphate	Urea			Salt		Mineral premix	Mineral premix	Mineral premix
	Protected methionine	Calcium phosphate			Vitamin premix		Calcium phosphate	Calcium phosphate	Calcium phosphate
		Protected methionine							

Table 4.2. Mean unavailable CP (uCP) for the selected protein sources using the modified Ross procedure (Venter, 2017) and acid detergent insoluble nitrogen fractions (ADIN) in g kg^{-1} of crude protein. Prices used in the calculations are also reported.

Protein Source	Processing method	Origin	Unavailable Nitrogen[2] (uCP)	ADIN[1]	Price per Tonne
Bloodmeal	Unknown	Neulux, Netherlands	386.11	14.00	R 14 500
Fish meal	Flame Dried	Ganslobaai Bay, Namibia	258.11	23.81	R 16 500
Soya meal	Solvent	Continental Oil, South Africa	85.78	33.90	R 7 500
Soya meal	Aminomax	Afgri, South Africa	92.48	24.28	R 8 500
Canola meal	Solvent	Soil-Bredasdorp, South Africa	221.43	74.00	R 4 600
Feather meal	Cooked	Medows, South Africa	447.66	20.00	R 5 000

[1] ADIN values were obtained from the latest CNCPS feed library (2016).
[2]uCP values obtained from Venter (2017).

4.3. RESULTS AND DISCUSSION

The rations varied in raw ingredients, composition, dry matter intake (DMI), targeted milk yield and were formulated for different breeds. With the exception of lucerne, the number of protein sources used in a ration ranged from 2 in ration J and I to 4 in rations F, B and D. Rations J, I and H were formulated for Ayrshire cows to produce 22, 31 and 35 l respectively. Only one of the rations (ration G) were formulated for Jersey cows and had a target milk production of 35 l. The other rations were formulated for Holstein cows with milk yield ranging from 38 to 46 l. This provided us with a broad range of information that allowed us to show implications that would be a true reflection of the industry. As a result of the broad selection of rations we grouped the rations according to targeted milk yield. We classified yields lower than 35 l (ration J and I) as low production, lower than 40 l as medium production (control H, G, E) and higher than 40 l as high production (control F, B, C, D). These groups as well as their observed changes in CNCPS can be seen in Tables 4.3, 4.4 and 4.6. Looking at the parameters, it is apparent that when the change in uCP prediction is made from the ADIN method (ADIN) to the modified Ross assay (uCP) the MP available, IOFC and productive N values all decrease. This is as a result of the drastic difference in uCP prediction from the two methods (uCP prediction is much higher Ross *et al.*, 2013; Venter 2017). The only values that increase when the uCP values change to the modified Ross assay prediction are RUP, ME % required, fraction C (supply, escape and fecal), and rarely IOFC. The changes reported in ME % required could be as a result of the protein and carbohydrate synchronization in the rumen. The uCP results in fact in extra energy available that can't be used by the microorganisms and also in less "extra" N and therefore a small urea cost. The small changes of RDP and RUP are due to the protein fractions shifting towards RUP, which also includes the fraction C.

Looking at the rations below two changes, in terms of diet protein and milk yield occur. The change in protein are caused by the higher uCP prediction using the modified Ross assay, which reduces the MP available and as a result affects the milk yield. Furthermore, due to the higher uCP there is a decrease in productive N and a decrease in IOFC would follow. In fact, the fecal N increases across all the rations when the higher uCP is used, which shows the previous underestimation of the unaivalable protein. As a result of the higher N excretion, the N efficiency will be lower than presumed. The NUE is lower in all the rations formulated using the higher uCP values with the exception of a few rations that were higher in ME than MP (J, H and G). From the low production rations (Table 4.3), these changes can be seen. For both these rations (J and I) a drop in IOFC is expected as the uCP increases however this is not the case. Both rations (I, J) actually report a slight increase in IOFC which is as a result of the initial ME being lower than the MP, and therefore limiting. Since the lower value of either ME or MP determines the milk yield prediction

the increase in uCP did not reduce the MP below ME but actually increased the ME slightly resulting in the increased IOFC. Even though the ME was lower than the MP the effect of changing the uCP to the modified assay can be seen. Both rations report a reduction in MP allowable milk with ration I having a greater reduction. Furthermore, ration I also has a bigger reduction in MP available g day^{-1}, a lower N balance, lower B1 and B2 (supply, fermented, escape, digested, fecal) N as well as a higher fraction C (supply, escape, and fecal). Therefore for rations formulated to have an initial extra amount of MP over the required, the implementation of the uCP system will not be drastic and it actually will lower the extra N (i.e. N balance) since this N is in reality part of the unavailable protein fraction. However, ration J has a bigger reduction for MP from RUP. Looking at the rations for I and J the same ingredients are fed. This implies that the composition of the ration was responsible for ration I having a bigger reduction in the named parameters, which is probably due to the higher amount of protein fed (15.75 vs. 16.39 %DM).

The mid production rations (Table 4.4) show a decrease in IOFC with ration E having the biggest loss (R17.85) followed by A (R15.90), H (R1.10) and G actually increasing by R0.30. The increase in IOFC reported in ration G is as before a result of the initial ME being lower than the MP allowable milk. Furthermore, ration E also has the biggest reduction in RDP (2.02 % CP), protein fraction B1 and B2 as well as the highest increase in N for protein fraction C. These reductions are all attributed to the higher uCP determined using the modified Ross assay and appear to be more severe in the medium production group than the low production group. The high producing rations (Table 4.5) report a consistent reduction in IOFC across all the rations. Ration D has the biggest reduction of R39.50, followed by C (R37.25), B (17.85) and farm F (R10.30). Once again in the high production rations a similar trend is seen with the ration being affected the most in terms of IOFC also reporting the biggest reductions in RDP, MP allowable milk, protein fraction B1 and B2 as well as the highest increase in protein fraction C. Looking across all the production groups the biggest change in MP milk and IOFC can be seen for the high production group. However, there are some exceptions implying that the ration composition and ingredients (protein type) have an effect on these parameters. The protein sources we believe to cause the biggest effect on these parameter are those associated with a high uCP such as blood meal and feather meal.

Table 4.3. Control rations formulated for lower production (<35 l day^{-1}) farms and the changes as in CNCPS (All values are per day).

Control Ration		J		I	
Method used to determine uN		ADIN	uN	ADIN	uN
Milk, kg		22.0		31.0	
Milk protein, %		3.4		3.4	
CP, % DM		15.8		16.4	
RDP, % CP		69.1	69.0	63.9	63.8
RUP, % CP		30.9	31.0	36.1	36.3
ME, % required		102.0	102.1	99.8	99.9
MP, % required		105.0	104.1	104.5	103.5
ME allowable milk, kg		22.8	22.8	30.9	30.9
MP allowable milk, kg		23.8	23.5	33.2	32.7
MP available, g		1775.9	1762.9	2421.1	2401.9
MP, % DMI		9.9	9.9	10.6	10.5
MP from RUP, % MP		39.8	39.3	46.7	46.2
Urea cost, Mcal day^{-1}		0.3	0.3	0.4	0.4
Protein B1, g	supply	1419.6	1413.3	2004.7	1998.1
	fermented	1036.6	1036.4	1355.3	1356.8
	escape	383.0	376.9	649.4	641.2
	digested	383.0	376.9	649.4	641.2
	fecal	0.0	0.0	0.0	0.0
Protein B2, g	supply	405.6	392.5	435.5	415.3
	fermented	246.0	241.6	226.0	219.6
	escape	159.5	150.9	209.5	195.7
	digested	127.6	120.7	167.6	156.6
	fecal	31.9	30.2	41.9	39.1
Protein C, g	supply/fecal	124.0	143.1	175.8	202.7
N efficiency	Total fecal N, g	155.9	173.3	217.7	241.8
	[1]TMP, g	719.5	720.2	976.8	977.7
	[2]TPI, g	2817.7	2817.7	3756.6	3761.5
	NUE, %	25.5	25.6	26.0	26.0
IOFC, Rand		7.8	7.9	54.2	54.4

[1]Total milk protein [2]Total protein intake.

Table 4.4. Control rations formulated for medium production (35 to 40 l day^{-1}) farms and the changes as in CNCPS (All values are per day).

Control Ration		H		A		E		G	
Method used to determine uN		ADIN	uN	ADIN	uN	ADIN	uN	ADIN	uN
Milk, kg		35.0		39.0		38.0		35.0	
Milk protein, %		3.4		3.4		3.4		3.9	
CP, % DM		16.7		16.2		15.4		17.8	
RDP, % CP		63.1	63.0	58.9	57.6	55.4	53.4	54.6	54.7
RUP, % CP		36.9	37.1	41.1	42.4	44.6	46.6	45.4	45.3
ME, % required		98.6	98.6	105.2	105.5	107.1	107.4	98.0	98.1
MP, % required		103.2	102.3	103.0	98.2	106.0	99.6	106.0	103.7
ME allowable milk, kg		34.3	34.3	41.9	42.0	41.9	42.0	34.0	34.1
MP allowable milk, kg		36.7	36.2	40.8	37.6	41.4	37.8	38.2	36.9
MP available, g		2630.1	2608.7	2802.2	2688.1	2751.9	2607.3	3031.8	2969.8
MP, % DMI		10.7	10.7	10.7	10.3	11.4	10.8	11.7	11.5
MP from RUP, % MP		48.7	47.4	52.1	50.0	48.7	45.2	56.3	55.4
Urea cost, Mcal day^{-1}		0.4	0.4	0.4	0.2	0.2	0.0	0.6	0.5
Protein B1, g	supply	2176.3	2168.5	2357.8	2192.6	2004.0	1794.7	2419.6	2503.2
	fermented	1459.0	1460.4	1419.8	1373.2	1087.2	1022.1	1386.9	1446.5
	escape	717.3	708.1	938.0	819.4	916.8	772.6	1032.7	1056.7
	digested	717.3	708.1	914.5	805.6	871.2	745.9	1032.7	1056.7
	fecal	0.0	0.0	23.5	13.8	45.6	26.7	0.0	0.0
Protein B2, g	supply	471.6	449.8	322.4	311.3	383.8	350.1	409.9	244.2
	fermented	242.4	235.8	181.2	176.3	227.9	218.0	195.1	140.0
	escape	229.2	214.0	141.2	135.0	155.9	132.1	214.8	104.3
	digested	183.3	171.2	113.0	108.0	124.7	105.7	169.3	83.4
	fecal	45.8	42.8	28.2	27.0	31.2	26.4	45.5	20.9
Protein C, g	supply/fecal	193.7	223.2	215.2	391.5	227.3	470.3	339.3	421.4
N efficiency	Total fecal N, g	239.5	266.0	418.5	418.5	258.5	496.7	384.8	442.3
	[1]TMP, g	1082.6	1083.6	1275.5	1175.9	1290.7	1179.4	1234.9	1237.1
	[2]TPI, g	4084.9	4084.9	4218.4	4218.4	3710.3	3710.3	4618.4	4618.4
	NUE, %	26.5	26.5	30.2	27.9	34.8	31.8	26.7	26.8
IOFC, Rand		66.1	65.0	19.2	3.3	111.0	93.1	81.8	82.1

[1]Total milk protein [2]Total protein intake**.**

Table 4.5. Control rations formulated for low production (>40 l day^{-1}) farms and the changes as in CNCPS (All values are per day).

Control Ration		B		C		D		F	
Method used to determine uN		ADIN	uN	ADIN	uN	ADIN	uN	ADIN	uN
Milk, kg		46.0		45.0		44.0		43.0	
Milk protein, %		3.4		3.4		3.6		3.4	
CP, % DM		17.6		18.1		18.6		16.5	
RDP, % CP		54.5	53.7	56.9	55.9	49.1	46.0	59.4	58.2
RUP, % CP		45.5	46.3	43.1	44.1	50.9	54.0	40.6	41.8
ME, % required		110.0	110.2	109.8	110.0	101.3	102.1	106.5	106.6
MP, % required		101.4	96.3	103.1	97.8	110.2	97.8	101.6	98.5
ME allowable milk, kg		52.3	52.4	51.0	51.2	44.8	45.3	46.9	46.9
MP allowable milk, kg		47.0	43.4	47.0	43.6	50.5	42.6	44.1	42.0
MP available, g		3349.0	3195.3	3229.1	3083.1	3461.6	3121.3	3160.0	3074.4
MP, % DMI		11.2	10.7	11.3	10.8	13.0	11.4	10.6	10.3
MP from RUP, % MP		55.6	53.4	54.0	51.8	62.0	57.8	50.2	48.8
Urea cost, Mcal day^{-1}		0.5	0.4	0.7	0.5	0.7	0.1	0.4	0.3
Protein B1, g	supply	2909.7	2731.3	2915.9	2742.1	2864.6	2409.6	2400.2	2335.1
	fermented	1617.5	1592.2	1713.4	1687.5	1175.7	1042.1	1413.7	1407.1
	escape	1292.2	1139.0	1202.5	1054.6	1688.9	1367.5	986.5	928.0
	digested	1184.6	1075.1	1098.8	992.9	1639.1	1338.3	986.5	928.0
	fecal	107.6	64.0	103.7	61.7	49.8	29.2	0.0	0.0
Protein B2, g	supply	322.1	245.2	284.2	208.9	326.3	258.2	321.8	270.9
	fermented	155.5	132.1	139.4	115.7	150.0	130.3	168.9	152.6
	escape	166.6	113.1	144.7	93.1	176.3	127.9	152.9	118.4
	digested	131.8	90.5	114.4	74.5	141.0	102.3	121.7	94.7
	fecal	34.8	22.6	30.4	18.6	35.3	25.6	31.1	23.7
Protein C, g	supply/fecal	364.4	619.7	327.6	576.7	257.7	780.8	336.8	452.8
N efficiency	Total fecal N, g	399.2	642.3	358.0	595.3	293.0	806.4	367.9	476.5
	[1]TMP, g	1503.0	1388.8	1505.3	1394.6	1478.1	1405.8	1380.0	1315.5
	[2]TPI, g	5279.2	5279.2	5191.0	5191.0	4950.9	4950.9	4906.1	4906.1
	NUE, %	28.5	26.3	29.0	26.9	29.9	28.4	28.1	26.8
IOFC, Rand		115.8	98.0	141.1	103.8	153.6	114.1	129.0	118.7

[1]Total milk protein [2]Total protein intake.

4.4. Optimization

The non-linear optimization of NDS was implemented using the same farm-specific feeds and therefore, each optimization is as close as possible to reality. Optimizing the same rations mentioned with IOFC being the primary objective and productive N being the secondary objective allowed us to obtain the most profitable composition with the most productive N. The non-linear optimizer of NDS was always able to find optimal solutions and IOFC increased for all the optimized rations (Table 4.6 to 4.8), with ration A reporting the highest increase of R7.64 followed by B (R7.12), D (R3.14), G (R3.12), F (R2.37), I (R1.92), J (R1.44), H (R0.62), C (R0.20) and E (R0.16). Furthermore the secondary objective for optimizing the N productivity consistently reduced the N across the protein fractions for all but two rations (A and C). Moreover, the optimized rations A,E and F were they only ones that did not show an improved NUE. An explanation for the N not being reduced could be as a result of the ration composition and the protein type. As for the ME and MP allowable milk, the values did not always increase or decrease but showed a variation of the two across all the rations. Nonetheless, even though the ME and MP values changed, the rations improved in terms of N efficiency, with less N being wasted than in the original rations, with the exception of rations A, B and F. The higher fecal N in those rations may be attributed to the ration composition. Furthermore, the IOFC also improved compensating for the slight reduction in ME and MP milk. The results prove that the optimization function in NDS together with an improved protein sources characterization can reach our main objectives through simple composition changes, i.e. having a profitable formulated diet and a low polluting farm.

Table 4.6. Optimized uN rations formulated for low production (<35 l day^{-1}) farms and the changes as in CNCPS (All values are per day).

Control Ration		J	I
Method used to determine uN		uN	uN
Milk, kg		22.00	31.00
Milk protein, %		3.40	3.40
CP, % DM		15.54	16.08
RDP, % CP		69.08	63.66
RUP, % CP		30.92	36.34
ME, % required		101.90	99.90
MP, % required		103.50	102.70
ME allowable milk, kg		22.73	30.95
MP allowable milk, kg		23.24	32.31
MP available, g		1740.91	2374.54
MP, % DMI		9.84	10.41
MP from RUP, % MP		38.74	45.69
Urea cost, Mcal day^{-1}		0.21	0.29
Protein B1, g	supply	1384.00	1952.70
	fermented	1016.30	1323.70
	escape	367.70	629.10
	digested	367.70	629.10
	fecal	0.00	0.00
Protein B2, g	supply	383.10	405.40
	fermented	236.80	214.20
	escape	146.30	191.20
	digested	117.00	153.00
	fecal	29.30	38.20
Protein C, g	supply/fecal	140.00	198.20
N efficiency	Total fecal N, g	169.30	236.40
	[1]TMP, g	718.27	978.02
	[2]TPI, g	2750.58	3666.24
	NUE, %	26.11	26.68
IOFC, Rand		9.35	56.29

[1]Total milk protein [2]Total protein intake.

Table 4.7. Optimized uN rations formulated for medium production (35 to 40 l day^{-1}) farms and the changes as in CNCPS (All values are per day).

Control Ration		H	A	E	G
Method used to determine uN		uN	uN	uN	uN
Milk, kg		35.00	39.00	38.00	35.00
Milk protein, %		3.40	3.36	3.36	3.90
CP, % DM		16.40	16.54	15.14	17.37
RDP, % CP		63.04	57.76	54.53	54.86
RUP, % CP		36.96	42.24	45.47	45.14
ME, % required		99.00	104.30	104.60	99.00
MP, % required		101.50	99.00	99.00	102.80
ME allowable milk, kg		34.52	41.36	40.50	34.53
MP allowable milk, kg		35.83	38.41	37.45	36.50
MP available, g		2582.51	2721.73	2637.60	2934.86
MP, % DMI		10.59	10.41	10.79	11.33
MP from RUP, % MP		46.74	50.42	45.17	54.30
Urea cost, Mcal day^{-1}		0.31	0.29	0.00	0.37
Protein B1, g	supply	2125.00	2242.40	1743.10	2429.10
	fermented	1432.50	1410.20	1005.70	1406.40
	escape	692.40	832.20	737.40	1022.70
	digested	692.40	817.80	720.60	1022.70
	fecal	0.00	14.40	16.80	0.00
Protein B2, g	supply	443.90	317.70	360.20	242.30
	fermented	233.80	181.00	218.50	138.60
	escape	210.10	136.70	141.70	103.70
	digested	168.10	109.40	113.40	82.90
	fecal	42.00	27.30	28.30	20.70
Protein C, g	supply/fecal	218.20	401.90	437.10	409.40
N efficiency	Total fecal N, g	260.20	429.20	465.40	430.10
	[1]TMP, g	1090.83	1202.23	1168.44	1253.44
	[2]TPI, g	4000.62	4324.52	3702.05	4498.47
	NUE, %	27.27	27.80	31.56	27.86
IOFC, Rand		65.63	10.91	93.26	85.17

[1]Total milk protein [2]Total protein intake.

Table 4.8. Optimized uN rations formulated for low production (>40 l day^{-1}) farms and the changes as in CNCPS (All values are per day).

Control Ration		B	C	D	F
Method used to determine uN		uN	uN	uN	uN
Milk, kg		46.00	45.00	44.00	43.00
Milk protein, %		3.44	3.44	3.55	3.36
CP, % DM		18.03	17.02	18.35	16.98
RDP, % CP		52.35	54.33	45.84	55.69
RUP, % CP		47.65	45.67	54.16	44.31
ME, % required		104.90	104.00	103.90	104.30
MP, % required		99.10	99.00	99.00	99.00
ME allowable milk, kg		49.14	47.45	46.33	45.54
MP allowable milk, kg		45.33	44.34	43.36	42.34
MP available, g		3341.98	3169.42	3153.07	3124.91
MP, % DMI		11.06	11.16	11.76	10.56
MP from RUP, % MP		55.55	52.00	57.45	51.34
Urea cost, Mcal day^{-1}		0.44	0.11	0.02	0.31
Protein B1, g	supply	2812.60	2681.20	2411.90	2414.30
	fermented	1592.10	1641.60	1040.50	1368.40
	escape	1220.50	1039.60	1371.40	1045.90
	digested	1170.90	1014.60	1343.10	1045.90
	fecal	49.60	25.00	28.30	0.00
Protein B2, g	supply	254.80	259.70	261.30	245.50
	fermented	133.00	134.90	130.60	133.90
	escape	121.80	124.80	130.70	111.60
	digested	97.40	99.90	104.60	89.30
	fecal	24.40	25.00	26.10	22.30
Protein C, g	supply/fecal	635.60	502.60	766.10	565.90
N efficiency	Total fecal N, g	660.00	527.60	792.20	588.20
	[1]TMP, g	1450.56	1418.88	1430.88	1325.24
	[2]TPI, g	5449.35	4833.68	4918.55	5026.08
	NUE, %	26.62	29.35	29.09	26.37
IOFC, Rand		105.10	104.03	117.25	121.02

[1]Total milk protein [2]Total protein intake.

4.5. CONCLUSIONS

Often cows do not respond how they should in terms of milk yield and composition. Before looking for "animal reasons" we should be focusing on understanding and characterizing the feeds and the diets that we are feeding to know if the diet can satisfy specific animal requirements. We and other authors have shown that there has been a large underestimation of the protein unavailable to the whole digestive system. From our simulations, it was apparent that using protein sources with higher risk of large uCP fraction can result in a drastic effect on a ration. These protein sources are coincidently associated with the most expensive products (blood meal, feather meal) resulting in a double negative factor which may affect their continued use. To avoid this we would recommend using a diverse range of protein sources which may reduce the risk on one high uCP source. However, only *in vitro* trials have been done so we would recommend *in vivo* trials to confirm the high uCP results. Furthermore, the higher uCP could be as a result of processing the products as heat is involved. Further, tests should be done to see if perhaps an improvement in the processing procedure could reduce the high uCP associated with these products.

The reduced IOFC values as well as the lower N productivity found in the rations formulated using the ADIN method in relation to the modified Ross assay is proof that the ADIN method leads to a deceitful predicted estimation in terms of supplying animal requirements. Furthermore, by continuing to use the ADIN method an unnecessary expense on feed will occur as a result of wasted N. However, with the higher fecal N values due to the higher uCP the efficiency of N is lower than presumed. Our work would strongly encourage a better feed characterization for South African protein sources utilized by dairy farms. Nowadays, dairy cows diets are formulated often considering only crude protein amount of each source. There is therefore a huge potential to increase profitability (by higher income and lower feed protein costs) just by implementing the assay described before. As previously mentioned, these are simulations and the characterization of a set of samples highly representative of feeds daily used in dairy farms was used. We therefore believe that the modified Ross assay can better satisfy animal requirements in terms of protein, which will allow for better diet fine tuning, resulting in not only in an improved nitrogen efficiency but also improved farm profitability.

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Chapter 5

GENERAL CONCLUSIONS

The environmental concern in the dairy industry is a pressing matter as it is associated with low NUE which is aggravated by the expense of protein sources. Furthermore, numerous dairy farms are feeding CP levels above the optimal level. Surpassing the optimal recommendation (16.5 % CP) has shown no benefit in terms of milk yield and leads to unnecessary N pollution as well as an extra expense to the farmer. However, formulating a ration with the optimal CP requirements depends on how accurately we categorize feed fractions. The protein fraction is characterized by RDP, RUP and uN. The unavailable nitrogen fraction of protein has been known for its fluctuating degrees of digestibility with varying amounts of AA being liberated. Consequently, a new lab assay was developed in an attempt to better predict the uN fraction in non-forage feeds.

Although the new assay was intended to only predict the uN fraction of feeds it became necessary to predict RDP and RUP to determine uN. Consequently, the new assay have reported comparable results for RDP and RUP. As for the uN fraction the new assay has provided evidence for a drastic underestimation in feed that will have an effect in the industry. The higher uN values obtained imply that we have been underestimating the amount of available N which has resulted in a high N excretion. Furthermore, the farmer has been paying for this excess uN unknowingly which has caused lower IOFCs. The implementation of this assay into CNCPS has proven this reduction in IOFC as well as the reduced N efficiency. Consequently, CNCPS allows nutritionists to better predict the protein requirements which will lead to an improved IOFC as well as an improved NUE. We therefore believe that the new laboratory assay developed to predict the uN fraction is an immense improvement that make numerous corrections that previous methods did not. However, the new assay is still a novel and further tests would be recommended both *in vitro* and *in vivo* to confirm our findings. *In vivo* studies will be needed to measure differences in N waste and corresponding milk yields when products with estimated uCP will be used and *in vitro* studies need to confirm the higher uCP values as well as establish respectable average values across all protein sources.

6. APPENDIX 1

Table 1. Classification of the protein sources based on origin.

	Origin			
	Animal protein	Plant protein	SEM	*P*-value
CP	80.54[a]	42.10[b]	1.4950	<0.0001
ADIP	1.937[b]	3.400[a]	0.3070	<0.0001
RDP	24.34[b]	65.90[a]	2.8150	<0.0001
RUP	75.58[a]	33.75[b]	2.1190	<0.0001
uCP	29.30[a]	10.09[b]	2.4670	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$).

Table 2. The classification of CP-EAA, uCP-EAA, EAA rumen digestion (rd) and EAA intestinal digestion (id) into origin.

	Origin			
	Animal	Plant	SEM	*P*-value
CP-EAA	54.65[a]	45.28[b]	0.6144	<0.0001
uCP-EAA	48.66[a]	42.56[b]	1.3567	0.0020
EAArd	17.32[b]	51.23[a]	1.4687	<0.0001
EAAid	61.80[a]	50.70[b]	1.4477	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$).

Table 3. The classification of CP-NEAA, uCP-NEAA, NEAA rumen digestion (rd) and NEAA intestinal digestion (id) into origin.

	Origin			
	Animal	Plant	SEM	*P*-value
CP-NEAA	40.33[b]	44.46[a]	0.4889	<0.0001
uCP-NEAA	36.73[a]	29.49[b]	0.4107	<0.0001
NEAA rd	17.80[b]	64.67[a]	1.3048	<0.0001
NEAA id	60.10[a]	53.96[b]	1.3589	0.0019

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$).

Table 4. Classification of the protein sources based on class.

	Class			
	Rumen	Intestines	SEM	*P*-value
CP	42.09[b]	69.02[a]	1.7945	<0.0001
ADIP	3.440[a]	2.340[b]	0.3039	0.0115
RDP	69.69[a]	33.80[b]	2.1422	<0.0001
RUP	30.31[b]	66.20[a]	2.1422	<0.0001
uCP	10.20[b]	22.85[a]	1.2796	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 5. The classification of essential aa (EAA), EAA unavailable CP (uCP), EAA rumen digestion (rd) and EAA intestinal digestion (id) into class.

	Class			
	Rumen	Intestines	SEM	*P*-value
CP	45.86[b]	51.93[a]	0.7130	<0.0001
uCP	44.41[a]	45.25[a]	1.3643	0.6648
EAArd	50.99[a]	25.13[b]	1.8429	<0.0001
EAAid	49.21[b]	60.98[a]	1.3824	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 6. The classification of nonessential aa (NEAA), NEAA unavailable CP (uCP), NEAA rumen digestion (rd) and NEAA intestinal digestion (id) into class.

	Class			
	Rumen	Intestines	SEM	*P*-value
CP	44.56[a]	41.48[b]	0.4986	<0.0001
uCP	29.42[b]	35.20[a]	0.4605	<0.0001
NEAArd	50.99[a]	25.13[b]	1.8429	<0.0001
NEAAid	51.69[b]	60.31[a]	1.2741	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 7. Classification of the protein sources based on type g kg^{-1} CP.

	Protein type							
	Blood meal	Soya meal	Canola meal	Sunflower meal	Fish meal	Feather meal	SEM	*P*-value
CP	95.31[a]	48.35[d]	38.34[e]	37.11[e]	68.54[c]	83.75[b]	1.1320	<0.0001
ADIP	0.071[d]	1.708[c]	5.23[a]	3.948[b]	0.197[d]	6.320[a]	0.4560	<0.0001
RDP	6.72[e]	55.56[c]	65.32[b]	80.17[a]	30.92[d]	30.71[d]	3.8930	<0.0001
RUP	93.28[a]	44.43[c]	34.68[d]	19.83[e]	69.08[b]	69.29[b]	3.8930	<0.0001
uCP	38.47[a]	9.20[cd]	12.97[c]	5.644[d]	20.64[b]	32.92[a]	1.9270	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$).

Table 8. The classification of essential aa (EAA), EAA unavailable CP (uCP), EAA rumen digestion (rd) and EAA intestinal digestion (id) into type.

	Protein Type							
	Blood meal	Soya meal	Canola	Sunflower meal	Fish meal	Feather meal	SEM	*P*-value
CP	59.21[a]	46.50[c]	45.81[cd]	43.37[d]	52.32[b]	53.59[b]	1.0017	<0.0001
uCP	48.07[a]	46.17[a]	48.57[a]	33.54[b]	51.27[a]	45.33[a]	2.1382	<0.0001
EAArd	8.361[d]	42.01[b]	45.78[b]	66.85[a]	21.04[c]	20.71[c]	1.6777	<0.0001
EAAid	57.56[b]	62.15[ab]	42.367[c]	42.64[c]	66.48[a]	59.01[b]	1.9333	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 9. The classification of nonessential aa (NEAA), NEAA unavailable CP (uCP), NEAA rumen digestion (rd) and NEAA intestinal digestion (id) into type.

	Protein Type							
	Blood meal	Soya meal	Canola	Sunflower meal	Fish meal	Feather meal	SEM	P-value
CP	36.39[d]	43.83[b]	42.83[b]	46.49[a]	39.28[c]	45.83[a]	0.6883	<0.0001
NEAA id	55.83[c]	63.70[b]	48.17[de]	46.15[e]	68.83[a]	51.29[cd]	1.7173	<0.0001
NEAA rd	63.21[b]	55.26[c]	63.71[b]	77.15[a]	20.91[d]	25.03[d]	1.3826	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 10. The classification of AA into class.

AA		His	Ser	Arg	Gly	Asp	Glu	Thr	Ala	Pro	Cys	Lys	Met	Val	Ile	Leu	Phe
ID	Rumen	57.21[b]	51.87[b]	41.00[b]	40.01[b]	51.87[b]	48.47[b]	43.60[b]	41.73[b]	43.60[b]	84.30[a]	52.07[b]	44.78[b]	46.15[b]	47.96[b]	46.41[b]	51.75[a]
	Intestines	67.73[a]	64.83[a]	56.98[a]	53.85[a]	61.76[a]	62.27[a]	61.24[a]	55.38[a]	54.62[a]	69.43[b]	71.89[a]	62.41[a]	55.41[a]	58.05[a]	58.28[a]	57.06[a]
	SEM	2.21	1.50	2.17	1.68	1.54	1.78	1.55	1.78	2.33	2.67	3.19	2.50	1.76	1.68	1.73	2.12
	P-value	0.0010	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0011	0.0001	<0.0001	<0.0001	0.0003	<0.0001	<0.0001	0.0785
CP	Rumen	2.120[a]	4.221[a]	10.45[b]	5.232[a]	8.501[a]	16.61[a]	1.435[b]	3.933[b]	5.721[a]	0.352[b]	10.63[a]	1.152[b]	4.211[b]	3.615[a]	7.024[b]	5.188[a]
	Intestines	2.121[a]	4.581[a]	11.62[a]	5.682[a]	7.914[b]	12.18[b]	2.846[a]	5.126[a]	5.515[a]	0.504[a]	12.29[a]	1.624[a]	4.785[a]	3.677[a]	7.633[a]	4.795[b]
	SEM	0.0702	0.1538	0.3258	0.1631	0.1402	0.3094	0.0994	0.1034	0.1771	0.0323	0.6089	0.0674	0.0696	0.0447	0.1154	0.1360
	P-value	0.9925	0.1015	0.0120	0.0510	0.0031	<0.0001	<0.0001	<0.0001	<0.0001	0.0011	0.0557	<0.0001	<0.0001	0.3574	0.0003	0.0431
RD	Rumen	74.53[a]	69.11[a]	53.87[a]	57.84[a]	61.35[a]	73.69[a]	53.04[a]	42.36[a]	71.23[a]	91.58[a]	52.77[a]	42.77[a]	45.97[a]	47.60[a]	53.36[a]	57.72[a]
	Intestines	42.81[b]	32.08[b]	31.53[b]	32.80[b]	22.24[b]	29.47[b]	22.21[b]	21.25[b]	32.62[b]	39.52[b]	27.69[b]	21.23[b]	18.95[b]	19.00[b]	22.38[b]	23.16[b]
	SEM	2.2419	2.0343	2.1861	1.9042	1.8911	2.2471	1.7373	1.7200	2.2011	3.3385	3.3902	2.9669	1.7921	1.8730	1.5924	2.3761
	P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
uCP	Rumen	0.812[b]	2.253[b]	9.811[a]	4.845[a]	5.805[b]	7.754[b]	3.344[b]	4.805[b]	3.284[b]	0.106[b]	9.562[a]	1.144[b]	4.402[b]	3.525[b]	6.414[b]	3.624[b]
	Intestines	1.315[a]	3.111[a]	10.50[a]	5.121[a]	7.063[a]	9.141[a]	3.688[a]	5.625[a]	4.681[a]	0.423[a]	8.89[a]	1.314[a]	5.352[a]	3.915[a]	7.544[a]	4.288[a]
	SEM	0.0685	0.1023	0.3024	0.1095	0.1036	0.1012	0.0554	0.0974	0.1794	0.0421	1.2123	0.0460	0.0779	0.0664	0.1214	0.0776
	P-value	<0.0001	<0.0001	0.1095	0.0609	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.6963	0.0126	<0.0001	<0.0001	<0.0001	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 11. The classification of AA into origin.

AA		His	Ser	Arg	Gly	Asp	Glu	Thr	Ala	Pro	Cys	Lys	Met	Val	Ile	Leu	Phe
ID	Animal	62.85[a]	66.58[a]	58.18[a]	57.92[a]	61.13[a]	61.62[a]	63.28[a]	56.37[a]	57.13[a]	59.96[b]	66.72[a]	69.59[a]	56.04[a]	58.92[a]	60.10[a]	60.48[a]
	Plant	62.68[a]	54.09[b]	44.30[b]	41.09[b]	54.71[b]	52.30[b]	46.82[b]	44.56[b]	44.88[b]	86.11[a]	60.04[a]	45.00[b]	48.09[b]	49.97[b]	48.31[b]	51.07[b]
	SEM	2.3538	1.5600	2.2891	1.6534	1.6463	1.9306	1.6490	1.8806	2.3785	2.4573	3.4602	2.3812	1.8371	1.7560	1.7908	2.1306
	P-value	0.9596	<0.0001	<0.0001	<0.0001	0.0072	0.0009	<0.0001	<0.0001	0.0004	<0.0001	0.1783	<0.0001	0.0029	0.0005	<0.0001	0.0024
CP	Animal	2.150[a]	4.710[a]	12.12[a]	5.980[a]	7.566[b]	10.38[b]	3.470[a]	5.752[a]	5.361[a]	0.585[a]	13.63[a]	1.824[a]	5.145[a]	3.723[a]	8.082[a]	4.516[b]
	Plant	2.112[a]	4.242[b]	10.45[b]	5.171[b]	8.556[a]	16.55[a]	1.413[b]	3.867[b]	5.764[a]	0.346b	10.26[b]	1.162[b]	4.142[b]	3.601[a]	6.915[b]	5.246[a]
	SEM	0.0720	0.1570	0.3278	0.1629	0.1371	0.2112	0.0594	0.0674	0.1808	0.0313	0.6024	0.0641	0.0542	0.0455	0.1041	0.1351
	P-value	0.6810	0.0393	0.0005	0.0006	<0.0001	<0.0001	<0.0001	<0.0001	0.1245	<0.0001	0.0001	<0.0001	<0.0001	0.0708	<0.0001	0.0002
RD	Animal	30.04[b]	19.44[b]	25.22[b]	24.79[b]	10.79[b]	15.99[b]	14.39[b]	18.81[b]	17.20[b]	17.55[b]	29.34[b]	13.78[b]	11.03[b]	10.16[b]	13.54[b]	8.381[b]
	Plant	74.04[a]	67.22[a]	51.97[a]	56.25[a]	58.26[a]	70.50[a]	49.89[a]	38.51[a]	70.57[a]	91.37[a]	45.54[a]	41.73[a]	43.83[a]	45.61[a]	50.77[a]	57.70[a]
	SEM	1.5760	1.3015	2.1073	1.6670	1.3254	1.5645	1.5454	1.8463	1.0017	1.8451	3.6678	2.8880	1.5555	1.5648	1.2237	1.4733
	P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0024	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
uCP	Animal	1.580[a]	3.450[a]	10.40[a]	5.160[a]	7.220[a]	9.290[a]	3.740[a]	5.750[a]	5.280[a]	0.578[a]	9.698[a]	1.340[a]	5.660[a]	3.900[a]	7.780[a]	4.550[a]
	Plant	0.771[b]	2.269[b]	10.04[a]	4.89[a]	6.021[b]	8.012[b]	3.40[b]	4.926[b]	3.283[b]	0.086[b]	8.924[a]	1.162[b]	4.457[b]	3.618[b]	6.556[b]	3.635[b]
	SEM	0.0605	0.0950	0.3123	0.1126	0.1102	0.1107	0.0570	0.1008	0.1664	0.0379	1.2440	0.0470	0.0696	0.0701	0.1229	0.0718
	P-value	<0.0001	<0.0001	0.4141	0.0988	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.6636	0.0075	<0.0001	0.0054	<0.0001	<0.0001

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

Table 12. The classification of AA into type.

	AA	His	Ser	Arg	Gly	Asp	Glu	Thr	Ala	Pro	Cys	Lys	Met	Val	Ile	Leu	Phe
ID	Bloodmeal	55.51^{bc}	65.88^{ab}	51.67^{ab}	51.32^{b}	62.04^{a}	61.38^{a}	67.81^{a}	51.32^{b}	45.41^{b}	53.43^{c}	67.05^{ab}	71.57^{ab}	50.05^{b}	55.93^{ab}	50.54^{c}	47.94^{b}
	Soya oilcake	71.36^{a}	66.23^{ab}	49.65^{b}	56.18^{ab}	64.98^{a}	66.56^{a}	54.82^{b}	57.73^{ab}	66.40^{a}	67.82^{b}	59.20^{b}	64.80^{b}	63.45^{a}	64.06^{a}	63.75^{ab}	68.24^{a}
	Canola	40.73^{c}	46.66^{c}	37.25^{b}	38.45^{c}	49.51^{b}	40.08^{b}	42.87^{c}	32.67^{c}	29.79^{c}	100^{a}	54.59^{b}	47.97^{c}	35.38^{d}	36.53^{d}	36.54^{d}	49.44^{b}
	Sunflower oilcake	68.31^{ab}	44.49^{c}	42.89^{b}	24.20^{d}	45.78^{b}	43.65^{b}	39.79^{c}	37.03^{c}	29.31^{c}	98.56^{a}	65.19^{ab}	18.02^{d}	38.43^{cd}	42.45^{c}	37.85^{d}	30.86^{c}
	Fishmeal	69.55^{ab}	71.56^{a}	64.78^{a}	62.19^{a}	69.77^{a}	71.45^{a}	69.71^{a}	64.54^{a}	66.78^{a}	75.48^{b}	52.58^{b}	73.70^{a}	65.66^{a}	65.77^{a}	69.54^{a}	67.00^{a}
	Feathermeal	60.16^{ab}	59.80^{b}	54.79^{ab}	58.12^{ab}	47.24^{b}	47.11^{b}	49.11^{bc}	49.16^{b}	54.39^{b}	43.21^{c}	87.60^{a}	61.44^{b}	47.60^{bc}	51.63^{bc}	55.51^{bc}	63.22^{a}
	SEM	5.087 2	3.010 6	5.662 6	2.475 3	3.224 9	3.541 5	3.529 6	3.772 5	3.900 0	4.380 4	8.432 6	3.548 6	3.152 6	3.238 6	2.964 8	3.540 5
	P-value	0.000 2	<0.00 01	0.017 3	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	0.124 0	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01
CP	Bloodmeal	2.914^{a}	3.512^{c}	8.081^{d}	3.522^{d}	8.625^{b}	9.782^{d}	4.540^{a}	6.710^{a}	3.644^{f}	0.598^{b}	18.79^{a}	1.404^{b}	5.223^{b}	3.118^{e}	9.666^{a}	5.459^{a}
	Soya oilcake	2.079^{b}	4.412^{b}	9.484^{cd}	4.609^{c}	9.413^{a}	14.96 4^{c}	1.576^{d}	3.811^{e}	6.387^{b}	0.230^{d}	11.20^{b}	1.103^{c}	4.035^{d}	3.723^{bc}	7.410^{b}	5.891^{a}
	Canola	2.275^{b}	3.877^{bc}	10.59^{bc}	4.717^{c}	7.335^{c}	16.61^{b}	1.475^{d}	4.036^{d}	5.760^{c}	0.496^{b}	11.73^{b}	1.120^{c}	4.231^{d}	3.520^{cd}	6.926^{c}	3.956^{b}
	Sunflower oilcake	2.020^{b}	4.298^{b}	11.56^{b}	6.198^{b}	8.386^{b}	18.50^{a}	1.173^{e}	3.785^{e}	4.964^{d}	0.358^{c}	7.972^{c}	1.250^{bc}	4.209^{d}	3.505^{d}	6.275^{d}	5.401^{a}
	Fishmeal	2.161^{b}	3.611^{c}	12.22^{b}	6.457^{b}	7.707^{c}	10.94^{d}	2.779^{c}	5.942^{b}	4.354^{e}	0.266^{cd}	12.33^{b}	2.718^{a}	4.608^{c}	4.034^{a}	7.520^{b}	3.948^{b}
	Feathermeal	1.346^{c}	7.547^{a}	16.03^{a}	7.729^{a}	6.296^{d}	10.14 1^{d}	3.450^{b}	4.494^{c}	8.576^{a}	1.047^{a}	10.43^{bc}	0.893^{d}	5.858^{a}	3.842^{ab}	7.333^{bc}	4.413^{b}
	SEM	0.146 0	0.222 9	0.595 1	0.227 7	0.238 1	0.388 2	0.057 0	0.066 7	0.221 5	0.047 3	1.364 3	0.070 3	0.098 5	0.088 9	0.151 4	0.268 5
	P-value	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	0.000 1	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01
RD	Bloodmeal	13.92^{e}	4.123^{e}	6.379^{d}	7.052^{d}	1.263^{e}	0.000^{f}	0.000^{e}	2.685^{d}	8.235^{e}	17.91^{c}	33.79^{ab}	5.144^{c}	0.000^{e}	3.291^{e}	5.093^{e}	7.632^{d}
	Soya oilcake	67.88^{b}	60.75^{b}	41.94^{b}	47.03^{b}	51.46^{b}	58.73^{c}	40.21^{c}	25.76^{c}	66.68^{b}	76.37^{b}	42.59^{ab}	22.35^{b}	30.47^{c}	33.67^{c}	42.29^{c}	56.65^{b}
	Canola	73.09^{b}	61.94^{b}	48.67^{b}	51.67^{b}	53.25^{b}	73.57^{b}	47.10^{b}	36.51^{b}	69.06^{b}	100^{a}	40.06^{ab}	30.18^{b}	42.79^{b}	41.90^{b}	47.92^{b}	40.34^{c}

	AA	His	Ser	Arg	Gly	Asp	Glu	Thr	Ala	Pro	Cys	Lys	Met	Val	Ile	Leu	Phe
	Sunflower oilcake	82.48[a]	79.27[a]	66.97[a]	71.21[a]	70.51[a]	82.91[a]	64.08[a]	55.93[a]	76.58[a]	103.66[a]	53.34[a]	74.63[a]	61.31[a]	63.31[a]	63.52[a]	72.04[a]
	Fishmeal	31.68[d]	22.31[d]	40.29[b]	34.12[c]	7.925[d]	17.59[e]	19.88[d]	26.75[c]	24.00[c]	13.66[c]	24.60[b]	17.39[bc]	14.23[d]	14.39[d]	18.69[d]	8.16[d]
	Feathermeal	43.71 c	30.46 c	21.45 a	28.53 c	24.61 c	29.57 d	20.55 d	23.02c	15.96 d	23.03c	31.99a b	16.99 bc	17.26 d	10.69 de	14.26 d	9.460 d
	SEM	3.111 9	1.822 8	3.924 6	2.667 7	2.074 2	2.301 5	2.570 6	2.987 9	2.011 6	3.528 1	9.309 1	4.669 1	1.975 9	2.375 7	1.935 7	2.421 5
	P-value	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	0.268 7	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01
uCP	Bloodmeal	2.478[a]	2.530[c]	8.031[c]	3.505[d]	7.132[b]	8.746[bc]	3.447[c]	7.129[a]	3.959[b]	0.507[b]	8.805[bc]	0.829[d]	5.846[b]	2.954[d]	9.953[a]	5.723[a]
	Soya oilcake	0.754[d]	2.313[d]	10.11[b]	4.531[c]	6.723[b]	8.605[c]	3.697[b]	5.199[c]	2.986[d]	0.117[cd]	12.06[ab]	1.222[b]	4.443[d]	3.806[c]	6.655[d]	3.422[d]
	Canola	1.117[c]	2.402[cd]	10.56[b]	4.371 c	5.410[c]	7.759[d]	3.555[bc]	5.038[cd]	3.722[b]	0.079[d]	13.04[ab]	1.156[bc]	4.688[c]	3.900[c]	6.968[c]	3.592[d]
	Sunflower oilcake	0.538[e]	2.115[e]	9.559[b]	5.733[b]	5.601[c]	7.461[d]	2.898[d]	4.500[e]	3.326[c]	0.052[d]	1.923[d]	1.089[c]	4.302[d]	3.172[d]	6.124[e]	3.936[c]
	Fishmeal	1.519 b	2.805 b	9.335 b	5.642 b	7.652 a	9.158 b	3.481c	5.550 b	3.859 b	0.196c	15.18a	2.049a	4.812c	4.147 b	6.548 d	4.198 b
	Feathermeal	0.784[d]	5.342[a]	14.38[a]	6.090[a]	6.677[b]	10.016[a]	4.410[a]	4.670[de]	8.737[a]	1.221[a]	2.369[cd]	0.800[d]	6.743[a]	4.479[a]	7.450[b]	3.910[c]
	SEM	0.058 9	0.067 2	0.443 6	0.107 8	0.160 4	0.165 7	0.061 2	0.122 0	0.082 8	0.039 2	1.936 5	0.028 1	0.063 5	0.080 8	0.107 1	0.075 5
	P-value	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01	<0.00 01

[ab]Means within a row not sharing the same superscript differ ($P < 0.05$)

6.1. APPENDIX 2

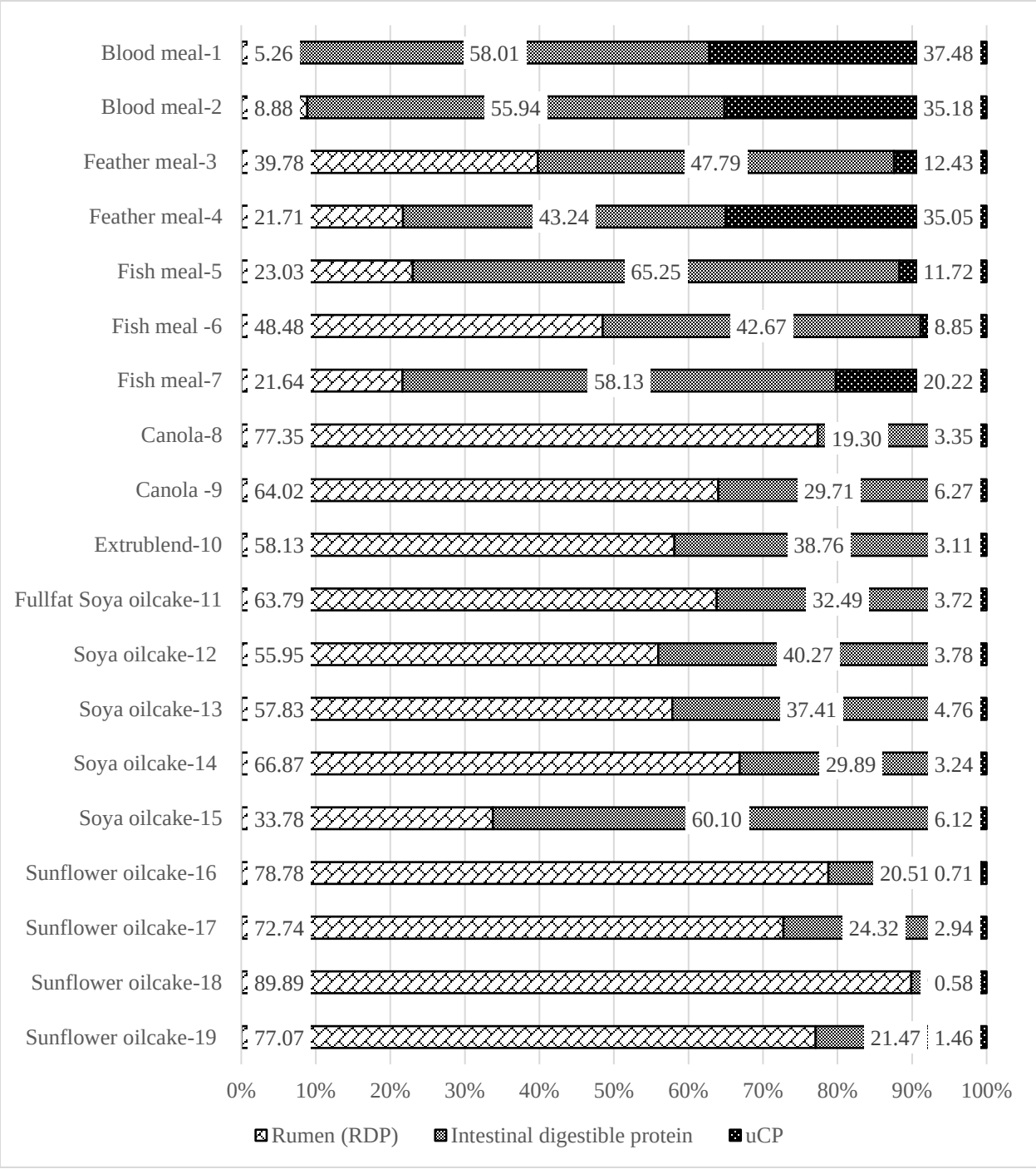

Figure 1.1. The proportion of rumen degradable protein (RDP) intestinal digestible protein (IDP) and unavailable crude protein (uCP) on CP basis.

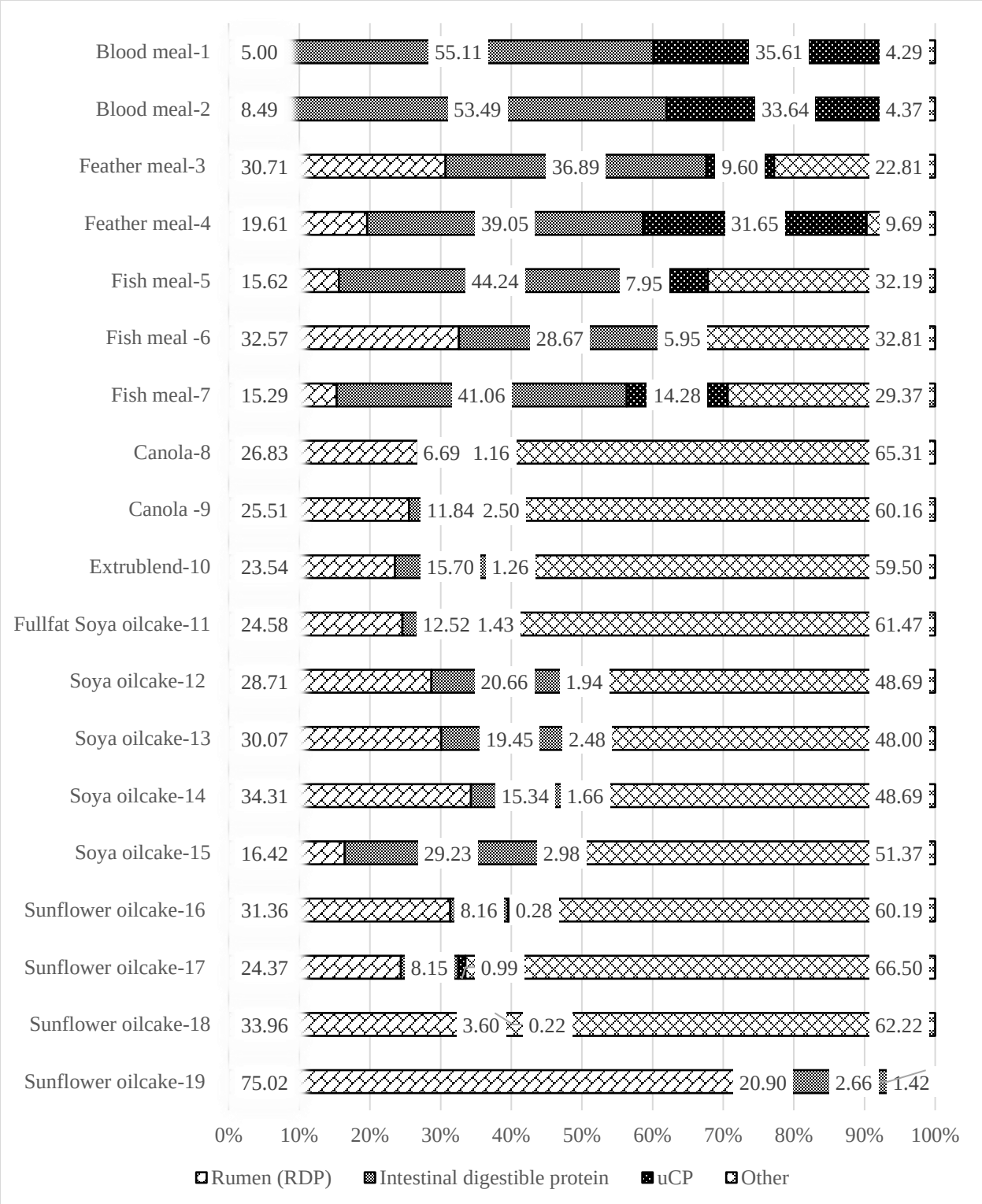

Figure 1.2. The proportion of rumen degradable protein (RDP) intestinal digestable protein (IDP) and unavailable crube protein (uCP) on DM basis.