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DIFFERENT TECHNIQUES TO EVALUATE A LIQUID RUMEN PROTECTED METHIONINE SOURCE FOR DAIRY CATTLE

By

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CONTENTS

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DECLARATION

I hereby declare that this thesis is the result of my own work and has not been submitted by me for a degree at another University.

Zeno Bester

August 2012

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SUMMARY

Different Techniques to Evaluate a Liquid Rumen Protected Methionine Source for Dairy Cattle

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Rumen protected methionine has been used in an effort to improve the amino acid composition of metabolisable protein since the early 1960's. The positive response in dairy cows in terms of milk protein composition and milk production, especially during early lactation has been well documented. Rumen protected methionine supplementation contributes to improving the protein efficiency of the dairy cow which improves the overall productivity of the dairy enterprise. Recently a locally developed liquid rumen protected methionine prototype became available. In our study this product was evaluated through a series of experiments in conjunction with two standard, well known methionine

sources, Smartamine ™ M and unprotected DL-methionine that provided a reference to the relative bioavailability of the liquid rumen protected methionine. In the first of the two studies the effect of methionine supplementation on milk yield, milk composition as well as milk protein composition was evaluated through the milk composition technique. The ability of the liquid rumen protected methionine to elevate blood plasma methionine levels was also evaluated through the blood plasma technique after oral dosing and post ruminal infusion of methionine. The liquid rumen protected methionine prototype induced no response in either milk yield or milk composition. Results suggested that the prototype is either not adequately protected against rumen degradation or it is not available for absorption in the small intestine. The inability of the liquid rumen protected methionine prototype to elevate blood plasma methionine after post ruminal infusion further proved that the product is not available for absorption at this site either. In the event that the product's mode of action or method of protection caused it not to be detected as pure methionine in the blood, an effect on milk yield would have been expected which was not the case. This product proved to have a very low or no bioavailability in comparison to the well researched and proven Smartamine ™ M.

LIST OF ABBREVIATIONS

AA	Amino acid
ADF	Acid detergent fibre
ADG	Average daily gain
AMTS	Agricultural Modelling and Training Systems
CNCPS	Cornell Net Carbohydrate and Protein System
CP	Crude protein
CPM	Cornell Penn Miner Dairy ration formulation programme
DIM	Days in milk
DLMet	DL-methionine
DMI	Dry matter intake
EAA	Essential amino acids
EE	Ether extract
FA	Fatty acids
FCR	Feed conversion ratio
FME	Fermentable metabolisable energy
GIT	Gastro intestinal tract
GLM	General Linear model of SAS
His	Histidine
HMB	2-hydroxy-4- methylthio butanoic acid
HMBi	Isopropyl ester of HMB
HPLC	High performance liquid chromatography
Ile	Isoleucine
Leu	Leucine
LRPMet	Liquid rumen protected methionine
Lys	Lysine
MAA	Metabolisable amino acids
MCP	Microbial crude protein
ME	Metabolisable energy

Met	Methionine
Met-	Methionine deficient treatment
MP	Metabolisable protein
MUN	Milk urea nitrogen
N	Nitrogen
NCP	Non casein protein
NDF	Neutral detergent fibre
NEAA	Non essential amino acids
NEL	Net energy for lactation
NFC	Non fibre carbohydrate
NPN	Non protein nitrogen
NRC	National Research Council
PDI	Protein truly digestible in the small intestine
Phe	Phenylalanine
PVC	Polyvinylchloride
RDP	Rumen degradable protein
RPAA	Rumen protected amino acid
RPLys	Rumen protected lysine
RPMet	Rumen protected methionine
RUP	Rumen undegradable protein
RR	Rulquin ratio
SEM	Standard error of the mean
SI	Small intestine
SMartM	Smartamine ™ M
TDN	Total digestible nutrients
Thr	Threonine
TMR	Total mixed ration

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

Dairy farming and animal nutrition in particular, is currently one of the most scientifically advanced disciplines which incorporates the use of complex dynamic nutritional models such as the AMTS-Cattle model (Tylutki, 2010), Amino Cow model (Evonik Degussa Industries GmbH, 2007a), CPM Dairy model (Boston *et al*., 2000), CNCPS model (Fox *et al*., 2004) or the NRC Dairy model (NRC, 2001). Some of these models also incorporate least cost formulation programmes to achieve an optimally balanced dairy ration at the lowest cost.

In vivo studies are used to estimate animal requirements and excellent management practices are aimed at optimizing animal efficiency whilst still promoting animal health. Only the best genetic material is selected for breeding purposes with dairy cows being genetically capable of a superior production output to that of their counterparts from a few decades ago. The challenge facing nutritionists today are to provide these animals with properly balanced rations in order to achieve their genetic potential.

The heightened awareness of nitrogen (N) pollution and incentives paid for increased milk protein and fat content requires an increase in protein production per unit of N consumed. Excess N excretion contributes to pollution and legislation governing the amount of N excretion by dairy operations is in place in most European countries. In South Africa (being a developing country) the focus is on mass production of animal products in order to provide enough food and the environmental impact of animal production is not of great

significance. However, in order to maximize profitable milk production the efficient utilization of crude protein (CP) provided by the dairy ration is crucial in order to maximize overall animal efficiency and production with the accompanying reduction in operational costs. This can be done through optimizing rumen function (maximizing the amount of microbial protein synthesis) and improving the overall amino acid (AA) composition of the metabolisable protein (MP) available for absorption in the lower gastro intestinal tract (GIT). Diets low in protein is an effective approach to decrease N excretion of dairy cows (Wang *et al.,* 2010) but for every 1 percent decrease in dietary protein a 0.7 to 1.2 kg/d decrease in milk yield were recorded (Broderick, 2003; Wang *et al.*, 2007). Emphasis should thus be placed on improving the overall conversion efficiency of dietary N into milk protein (Lapierre *et al.,* 2005). This can be achieved by providing a dietary AA profile closely resembling that of the AA requirement for milk synthesis (Noftsger *et al*., 2005), reducing CP content of the ration and improving N efficiency without compromising milk yield. Factors like ration composition, feed intake level and thus rumen outflow and dilution rate of microbial crude protein (MCP) dictate the contribution of microbial protein to the MP to a large degree. High producing dairy cows have high intake levels and AA requirements therefore protein should be provided postruminally with an AA profile that is consistent with the cows' requirements for metabolically absorbable protein (Robinson *et al*., 1999)

Several researchers have proven both lysine (Lys) and methionine (Met) to be first or co-limiting in dairy cows (depending on the basal ration) and the optimal ratio of Lys to Met has been shown to be 3:1 (NRC, 2001). This optimal Lys to Met ratio is difficult if not impossible to achieve with the conventional feedstuffs available. There is much variation

in the protein quality and AA composition of these available feedstuffs making it difficult to supply the dairy cow with a constant high quality diet with the desired AA composition. To achieve the recommended Lys and Met levels in MP, animal protein sources like carcass or blood meal which is high in Lys and plant proteins like maize gluten meal and maize which is high in Met but relatively low in Lys need to be included in combination at high levels. These diets are not always feasible in terms of least cost formulation, are unpalatable, and lead to a wastage of CP i.e. N pollution. Oversupplying protein to dairy cows is inefficient in that excess protein is metabolised using energy which comes at a cost to the cow (McDonald *et al.*, 1995). In most countries the use of animal by-products in ruminant diets is not permitted thereby limiting the basket of available ingredients to supplement dairy rations in order to achieve the desirable AA profile. The strategic supplementation of rumen protected AA (RPAA) sources can significantly contribute to nutritionists being able to formulate dairy diets with the desired Lys to Met ratio of 3:1.

Over the last 20 to 30 years numerous studies on the application of RPAA in ruminant nutrition has been conducted. Most of the research done with rumen protected methionine (RPMet) influenced at least milk protein composition and or milk production positively – especially when fed to early lactation, high yielding cows (Schwab & Ordway, 2001). Various technologies to protect these AA have been commercialised with some products being less successful than others in offering adequate bioavailability of Met. Rumen protected methionine needs to be protected against rumen degradation but not to the extent that it can't be disassociated from its binders or copolymers in the abomasum's unique pH environment. The ability of a RPMet source to deliver AA to the is cow is determined by a

combination of the source's AA content, ruminal stability and intestinal digestibility (Berthiaume *et al*., 2001).

Researchers have been making use of a variety of methods to evaluate the efficacy of RPMet products, with animal production trials being most common. Production trials measuring the effect on milk production, milk composition and milk protein composition in particular have been used with great success. The effect of post ruminal infusion of RPMet on blood plasma levels has also been investigated in a large number of studies. The use of in situ methods such as the mobile bag / *in sacco* techniques has also been widely applied to evaluate RPAA products (Berthiaume *et al*., 2001; Rossi *et al*., 2003).

A South African based company has developed a liquid rumen protected methionine (LRPMet) prototype and requested proper evaluation in order to prove its relative bioavailability in dairy cows. Limited technical data was made available on both the product as well as the mode of protection used; therefore it was decided to evaluate the product through a series of available techniques in order to detect the relative level of protection against rumen degradation and the availability for absorption in the small intestine (SI). The challenge was to select suitable evaluation methods to properly evaluate this LRPMet source whilst still being cost effective. Due to the large amount of technical data available on the well researched Smartamine ™ M (SMartM) (Adisseo, Inc., Antony, France) and DL-Methionine (DLMet) (Evonik Degussa, Theodore, Alabama, USA) it was decided to evaluate the LRPMet prototype in conjunction with these Met sources for comparative purposes. Smartamine™ M is adequately protected against rumen degradation whilst being available in the abomasum for absorption as opposed to the DL-methionine

which is used in monogastric nutrition and not protected at all. Smartamine ™ M is a lipid, pH-sensitive polymer protected Met containing a minimum of 75% DL-Methionine. The granules (2 mm in diameter) consist of a DLMet core with ethyl cellulose covered by a stearic acid coating containing small droplets of 2-vinyl-pyridine-co-styrene. The copolymer alters the stereochemistry of the stearic acid so enabling the surface coating to become resistant against ruminal degradation whilst allowing rapid release of Met in the abomasum (Schwab & Ordway, 2003).

The objective of this study was to evaluate the production response and relative bioavailability (ruminal degradability and intestinal availability) of a liquid rumen protected methionine product using two different methods:

1. Changes in milk protein percentage and yield in response to RPMet supplementation: The milk composition technique.
2. The effect of RPMet on blood plasma levels of Met after both oral dosing and post ruminal infusion using an adaptation of the abomasal infusion technique to determine intestinal availability of RPMet: The blood plasma technique.

The hypothesis was that the LRPMet would either elicit a response in milk composition or blood plasma Met level (rumen protected and Met available in SI for absorption) or no response (poor or no bioavailability of Met).

CHAPTER 2

LITERATURE REVIEW

2.1 Protein Digestion in the Dairy Cow

In well balanced rations 50% or more of the absorbable AA are provided by the ruminally synthesized microbial protein (Clark *et al.*, 1992; Merchen & Titgemyer, 1992; Schwab, 1995) which provides a constant source of high quality absorbable AA. Microbial protein is the cellular protein produced by a variety of fungi, protozoa and bacteria providing the majority of the duodenal AA supply from the rumen in high producing dairy cows (Schwab, 1997). The proportional contribution of rumen undegradable protein (RUP) to total protein passage and the AA composition of the RUP accounts for most of the variation in duodenal AA profiles (NRC, 2001; Rulquin & Kovalezyk, *2003)*. To maximize the efficiency of the contribution of the RUP fraction and microbial protein to improve milk yield and composition, knowledge on the ideal balance of absorbable AA and the bioavailability of potential supplementary RPAA is required.

In the process of protein degradation ruminal bacteria attaches to feed particles which are then degraded by the cell-bound microbial proteases (Brock *et al.*, 1982). Of the entire rumen microbial population approximately 80% attaches to the feed particles (Craig *et al.*, 1987) with 30% to 50% of the microbes having photolytic activity (Prins *et al.*, 1983). Different species of microbes exists forming a consortium to symbiotically degrade and ferment nutrients, including protein. Protein degradation generates peptides and amino acids. The rate of degradation depends on the proteolytic activity of the rumen micro flora

and the type and structure of the protein (Stern *et al*., 2006). Peptides are intermediates in protein degradation being broken down at different rates (Broderick *et al.*, 1988). With rapidly degradable peptides a peak concentration is reached 1-2 h after feeding, declining thereafter (Chen *et al.*, 1987a; Chen et al., 1987b). It was concluded that the hydrophobocity of peptides determines the rate of degradation whereas Wallace & McKain, (1990) found the structure of the N-terminal end of the peptide chain to have the greatest influence on peptide hydrolyses. The rate of AA degradation is usually greater than the rate of AA utilization by rumen microbes therefore AA are the major source of ammonia in the rumen (Chalupa, 1976). Rumen microorganisms can incorporate both peptides and single AA's but a preference for ammonia via diffusion across the cell membranes is generally assumed. Rumen microbes ferment a large, but variable proportion of dietary protein (60% to 90%) into ammonia which is then utilized through biosynthetic pathways to form AA.

In order to predict the amount of available MP in the SI, the requirements of the animal should be considered, in addition to knowing the amount of available AA. Both microbial protein and RUP contributes to the MP and therefore the AA composition as well as the digestibility of the microbial protein needs to be considered. Microbial protein is higher in Lys and Met than plant protein (Wallace, 1994). Earlier, microbial protein was considered to be of a constant AA composition (Purser & Buechler, 1966; Bergen *et al.*, 1967), but later Hvelplund *et al.* (2001) proved a genuine variation in microbial protein AA composition. The larger source of variation was firstly Lys and secondly Met. Ciliate protozoa contain more Lys and Met than bacteria and are selectively retained in the rumen (Veira, 1986).

Microbial protein contributes 50% to 80% of the total absorbable protein (Stern *et al.*, 2006) being affected by the availability of nutrients in the rumen and the utilization thereof by ruminal bacteria. Microbial growth rate is influenced by the availability of readily fermentable metabolisable energy (FME) as supplied by sugar and starch which are more effective than cellulose in stimulating microbial growth (Stern & Hoover, 1979). In addition to fermentable energy proteolysis of protein rendering peptides, amino acids and branched chained- volatile fatty acids dictates microbial growth rate. Improving protein capture into microbes reduces nitrogen excretion. Both the availability and the rate at which nutrients become available in the rumen dictate microbial protein production. A significant amount of nitrogen is lost in the form of ammonia when the rate of protein degradation exceeds that of carbohydrate fermentation as opposed to a higher rate of carbohydrate fermentation depressing microbial protein synthesis (Nocek & Russell, 1988).

Due to the complexity of the ruminal ecosystem the average microbial efficiency remains relatively stable due to the recycling of ammonia (through saliva and across the rumen wall) stabilising microbial growth in times of nitrogen shortage (Bach *et al.*, 2005).

The composition of the dairy ration thus dictates the rate of microbial protein synthesis based on the composition of the AA profile as well as the contribution from readily fermentable energy in various forms. About 80% of the N content of microbes is derived from ammonia nitrogen. Stern *et al*. (2006) showed that the positive response of microbial growth to AA supplementation might be due to direct incorporation of amino acids into microbial protein. In addition to the availability of protein and energy microbial growth could also be influenced by ruminal pH, availability of sulphur and dilution rate. A meta-analysis by St-Pierre, (2001) described no relationship between the efficiency of microbial

protein synthesis and a wide range of ruminal pH levels. In vitro studies by Hoover & Miller, (1991) and Calsamiglia *et al.* (2002) support this theory. However; microbial nitrogen flow has been shown to be negatively related to rumen pH in that rapid fermentation of large quantities of organic matter results in increased supply of FME, reduced pH levels and increased microbial protein synthesis (Hoover & Stokes, 1991).

Ruminal and solids dilution rates influence microbial protein synthesis and is dictated by various factors like feed intake level, dietary roughage inclusion as well as particle size or level of effective fibre. Ørskov & McDonald, (1979) showed protein degradation to be inversely related to rumen outflow rate. High dilution rates implicate a larger proportion of the microbial population to be in the exponential growth phase with a subsequent dilution of their maintenance requirements (Bach *et al.*, 2005). High dilution rates also lead to shorter rumen retention with a reduced bacterial lyses and predation by protozoa (Firkins *et al.*, 1992). Much research has been conducted in order to predict microbial growth and the AA composition of the microbial mass. In the NRC (2001) publication a new prediction equation for microbial yield was developed. This is a vast improvement on the earlier published equation (NRC, 1989) due to the much larger database, including higher producing cows with significantly higher dry matter intakes.

- Microbial Yield: Crude protein (g/day) = 130g x kg discounted total digestible nutrients (TDN) intake

Both the CNCPS and CPM Dairy models predict microbial yield from two equations that include microbial maintenance requirements and microbial growth efficiency. These are

based on fibre, starch, soluble fibre and sugars. This approach leads to a more sensitive prediction of yield in that yield for all substrates are predicted.

In a recent study Pacheco et al. (2012) compared four commercially available dairy ration programmes (Amino Cow, AMTS, CPM and NRC 2001) to predict duodenal flow of protein and EAA (essential amino acids) in dairy cows. Models tended to predict rumen flows of EAA more accurately on lucerne and maize silage based diets than in grass based diets, more accurately on maize based than on non maize based diets and more accurately in the mid range of types of diets. All the models were accurate in predicting DMI (dry matter intake) and it was concluded that Amino Cow, AMTS and NRC models were all sufficiently accurate for balancing EAA in dairy rations under field conditions.

2.2 Milk Protein Synthesis

Efficiency for milk protein synthesis is 0.67 (NRC, 2001) and a value of 0.65 is used by CPM-dairy (Boston *et al.*, 2000). The efficiency of milk protein synthesis is generally increased by increased concentrations of Met and Lys in MP (Piepenbrink *et al.*, 1999; Sloan, 2002). Using the CPM Dairy model, MP efficiency can be increased to 0.69, but this limitation on the MP as well as limiting the supply of non-essential amino acids (NEAA) in transition cows is not recommended. Milk volume is determined to a large extent by glucose uptake of the mammary gland. Alanine conversion to glucose exceeds propionate conversion to glucose post calving.

Milk protein consists of casein and whey of which α-lactoalbumin and β-lactoalbumin are the major whey proteins. Milk protein synthesis depends on the appropriate amount and

availability of amino acids and energy at the mammary gland. More than 60% of the cow's dietary AA requirements are met by digestion of microbial protein with breakdown of carbohydrates supplying energy in the form of volatile fatty acids. The pattern of fermentation (dependable on factors like ration composition, intake level etc.) will dictate the composition and level of available nutrients in the mammary gland.

2.3 Estimation of Amino Acid Requirements

The ideal AA profile has been identified in poultry (NRC, 1994) and swine (NRC, 1994) for maintenance, growth and lactation but is yet to be established for dairy cattle. The NRC (2001) focus on predicting the flow of essential amino acids (EAA) to the SI since there is no evidence that NEAA will ever become more limiting than EAA. Over the last decade several nutritional models like the CNCPS, CPM Dairy and AMTS-Cattle have been developed to allow for diet formulation on the basis of AA's (Pacheco *et al*., 2012). Rulquin & Verite, (1993), concluded that Met needs to be 2.5% of MAA (metabolisable amino acids) and Lys 7.3% of MAA to obtain optimum milk protein levels. Models like CPM Dairy, CNCPS and the more recent AMTS-Cattle model predict the duodenal flow of EAA as well as NEAA. In dairy cattle Lys, Met and Histidine (His) have been identified as the most limiting AA. The amount of microbial protein synthesis and the amount of RUP consumed as well as the AA composition thereof dictates the extent and sequence of limitation for these 3 AA's. Methionine has been shown to be first limiting for milk protein production and growth in dairy cattle fed soybean hull based diets where RUP intakes were low. In fact when most of the RUP was supplemented from soybean sources Met was the first limiting AA (Lundquist *et al*., 1988). In maize based diets Lys has been shown to be first limiting for diary animals. In most dairy rations soybean and maize are included so

that Lys and Met would nearly always be co-limiting. Histidine has been identified as first limiting for milk protein production of cows fed grass silage-cereal (small grains) based diets with feather meal as the sole source of RUP (Kim *et al.*, 1999; Vanhatalo *et al.*, 1999). Feed ingredients commonly used in the industry are inferior in their AA composition when compared to that of microbial protein and with increased intakes of RUP the imbalance in EAA could even be exacerbated (Schwab & Ordway, 2001).

With increased intakes in high producing dairy cows the RUP fraction should provide a larger proportion of the total absorbable AA's. The current approach to optimizing the amount of Met and Lys in MP involves the maximization of ruminal microbial protein synthesis, avoiding overfeeding of RUP, inclusion of feedstuffs high in Lys (e.g. fishmeal, blood meal, and soybean products) and the inclusion of RPAA. Feed protein sources vary in AA composition and the majority are not complementary to ruminally synthesized microbial protein, affecting the AA composition of duodenal protein (Schwab & Ordway, 2001). It's important to note the inherent error (Vyas *et al.,* 2009) associated with expressing AA requirements in dietary percentages since these might be greatly affected by the variability in feed intake.

Feed intake should thus be considered when comparing results obtained from RPMet supplementation as percentage of dietary requirement. The NRC (2001) protein model indicates the optimal use of MP for milk protein production when Lys in MP is 7.2% and Met 2.4 %. The optimum ratio for Lys to Met in MP is 3:1 using this model. It is not possible to achieve the optimum ratio of 7.2 % Lys and 2.4 % Met (as recommended by the model) when using conventional feedstuffs (Schwab & Ordway, 2001). Feed sources with an AA profile that compliments that of the ruminal microbes beneficial to milk

production are blood meal, fish meal, meat and bone meal of which inclusion levels or the use of is strictly controlled or prohibited entirely for use in ruminant feeds in many countries. Rumen protected amino acids can be used successfully to achieve the "Rulquin ratio" of 3:1 in the diet with an accompanying increase in milk yield, milk protein yield and content and feed intake. By increasing Met in MP from 1.89 to 2.35% and Lys in MP from 6.38 to 7.45% to achieve a Lys to Met ratio of 3:1 in the enriched diet, Chalupa *et al.* (1999) reported a 5.1 % increase in milk production, 8.0 % increase in milk protein and an 18 % increase in milk protein yield.

- **Ideal Protein Method**

The ideal protein method to estimate AA requirement is based on dose responses of milk protein to Met and Lys supplementation expressed as a percentage of MP. This dose response approach involves quantification of the EAA content of MP.

Rulquin *et al.* (1993) and Schwab, (1995) used the dose response approach in measuring lactational response to feeding and post ruminal infusion of graded levels of Lys and Met in rumen protected form combined with an estimation of AA flow into the SI. Dairy ration evaluation models like the NRC (2001), CPM Dairy and Amino Cow has incorporated these requirements into their models. Information on the post absorptive metabolism of AA including Met and its impact on the efficiency of conversion of absorbed AA into milk protein is still lacking. Balancing dairy rations adequately for both MP and AA levels allow nutritionists to reduce the overall CP content of rations which reduce N excretion to a large degree.

Historically rations has been balanced based on RUP and RDP fractions of feedstuffs, whilst rations are now being balanced not only for MP yield but also for the AA composition of this protein with Lys and Met being of significant importance. Both the level of contribution from RUP to MP as well as Lys and Met content were found to be the two most limiting factors in dairy nutrition (Schwab *et al.*, 1976). Over the last decade several nutritional models like the CNCPS Model, CPM Dairy, AMTS-Cattle and NRC (2001) (which all incorporates AA into its protein model) have been developed to allow for diet formulation on the basis of AA's. Rulquin & Verite, (1993) concluded that Met needs to be 2.5% of MAA and Lys 7.3% of MAA to obtain optimum milk protein levels. Metabolisable amino acids incorporate both EAA and NEAA. The summary by Schwab, published in the NRC (2001) came to approximately the same conclusion: 2.2% Met and 7.2% Lys as a percentage of MAA. Chalupa, (1976) and Schwab, (1995) detected that the profile of absorbed AA (the lack of sufficient Met) was not optimal in ruminants. This research indicated the importance of sulphur containing AA's (Met and cysteine) as being first limiting for wool growth and body weight gains and Met being first limiting in dairy cattle and growing animals. Sniffen et al. (2001) made used of multiple regression analyses to study the efficacy of RPMet and RPLys in transition cows whilst Rulquin *et al.* (2001) used an AA profile prediction system of intestinal matter in order to incorporate experimental data where AA were not infused post ruminally or fed in protected form.

- **Factorial Approach**

This is a mathematical approach where requirements are calculated based on transfer coefficients and rates of nutrient movement through digestive and metabolic pools. The CNCPS system is currently the best know factorial model as described by Chalupa &

Sniffen, (1996). The maintenance, conceptus growth, mammary depletion and milk protein production requirements are used to calculate the g of tissue or milk protein to be produced times the AA composition corrected for the efficiency for each AA giving the required metabolisable or absorbed AA. This approach does, however, not account for the fact that AA are taken up mainly via active transport sites and that excess AA can negatively impact on uptake of other AA. Estimation of the efficiency of AA use with this approach is difficult and variable (Chalupa & Sniffen, 2006). This method also lacks accuracy due to the level of milk production not being considered when predicting the requirements for EAA (Schwab, 1995; Sniffen, 2002)

For the latest improvements and changes in the CPM, CNCPS and AMTS models, readers are referred to Tedeschi *et al*. (2008); Tylutki *et al*. (2008) and Tylutki & Van Amburgh, (2010).

- **Indirect Dose Response Approach**

Rulquin *et al*. (1993) used this approach in estimating digestible Lys and digestible Met in duodenal contents as a percentage of total digestible protein (PDI) which were incorporated into the French PDI system. Socha *et al*. (1994b) used regression equations to estimate the duodenal Lys and Met as a % of EAA.

Three Steps are involved in this approach:

1. Establishing Lys and Met as % of EAA in digesta for treatment and control groups when either Lys or Met or both are increased and production responses determined.

2. Extrapolating reference production values in individual experiments for Lys and Met in digesta intermediate between high and low values as calculated for the experiments.
3. Calculation of production responses, positive or negative, for control and treatment groups relative to the reference production values (Schwab, 1996)

Comparison between Ideal protein and Factorial Approach

- The Factorial approach describes production responses accurately in the absence of nutrient limitations but overestimate response when nutrients are over supplied. This is due to the constant transfer coefficients for Met and Lys which dictates production responses in a linear fashion regardless of the amount of metabolisable Lys and Met.
- Responses to increasing amounts of Lys and Met might be underestimated by the ideal protein method for early and peak production cows. This method was calibrated with data obtained from cows beyond peak production.

The different approaches used in estimating AA concentrations for Lys and Met have delivered similar results and estimates of Lys and Met at 7.1% and 2.3% of total AA when feeding in general, conventional rations (Schwab, 1997).

2.4 Commercially Available RPMet Products and Modes of Protection

Rumen protected amino acids have been used successfully in dairy diets to supplement the most limiting AA (Lys and Met) and since the early 1960's several technologies have been

investigated to protect Met against rumen degradation with initial attempts combining Met with a combination of lipids, inorganic materials, carbohydrates, softening agents and fillers (Schwab & Ordway, 2001). Technologies have been developed to protect these AA, chemically and or physically against degradation in the rumen without compromising digestion in the small intestine, improving the AA composition of the MP. Increasing the amount of Met supply to the SI effectively increase protein synthesis in the mammary gland.

There are three different technological approaches to protect free AA against rumen degradation:

(i) Surface coating with a fatty acid pH sensitive polymer mixture.

(ii) Surface coating or matrices involving fatty acids or minerals.

(iii) Liquid sources of methionine hydroxy analogue (NRC, 2001)

Commercially available RPMet sources include Megalac Plus (Church and Dwight Co, Inc), Mepron® M85 (Degussa Corp.), Smartamine ™ M (Adisseo), Meta-Smart® (Adisseo) and Met- Plus™ (Nisso America). 2-hydroxy-methylthiobutanoic acid (HMB) is available as Rhodimet® AT-88 (Adisseo) and Alimet® (Novus International). Noftsger *et al.* (2005) reported that HMB is a rumen degradable source of Met, positively affecting microbial growth (in particular protozoa). Koenig & Rode (2001) reported Alimet to escape rumen degradation at 40% only whilst Rhodimet® AT-88 was cited in a study by St-Pierre *et al*. (2003) as only escaping the rumen at 5%.

- Lipid- Protection

Met-Plus™ is a lipid protected product. The matrix compound contains 65% DLMet embedded in a network of calcium salts and long-chain fatty acids (FA), lauric acid and butylated hydroxytoluene which is a preservative for the FA. This technology aims to achieve a balance between rumen protection and the amount of Met available for absorption in the SI whilst minimizing losses in the faeces and rumen (Schwab & Ordway, 2001).

- Surface Coating

Mepron® M85 pellets are coated with a carbohydrate, 1.8 mm in diameter, 3-4 mm long and with a density of 1.2 g/cm^3. The core consists of DLMet and starch coated with thin layers of ethyl cellulose and stearic acid. The Met content is 85%. Enzymatic degradation of ethyl cellulose is minimal and degradation would occur primarily due to physical action and abrasion. The product is thus slowly degraded in the rumen and has a slow intestinal release of Met too.

- pH-sensitive Polymer Coating

Smartamine™ M is an example of a lipid/pH-sensitive polymer protected RPMet. It contains minimum 75% DLMet with the core consisting of ethyl cellulose covered by stearic acid which contains small droplets of poly (2-vinylpyridine-co-styrene). The copolymer alters the stereochemistry of the stearic acid, enhancing the surface coating. The copolymer solubilises at a low pH level which allows for rapid release in the abomasum.

The most effective mode of protection has been this surface coated Met with enzyme-resistant, pH-sensitive synthetic polymers which are insoluble at higher ruminal pH levels and highly soluble in the acidic environment of the abomasum. This technology purely relies on the difference in pH levels between the rumen and SI and is not affected by enzyme function.

- Met Hydroxyl Analogues

Amino acid analogues have been created by adding a chemical blocking group to the α-amino group of the Met or removing the acyl group. These Met derivatives like isopropyl-DL-Met, t-butyl-DL-Met, N-stearoyl-DL-Met, N-oleyl-DL-Met and capryl-caprolytic-DL-Met has shown some resistance to ruminal degradation (Loerch & Oke, 1989). Studies by Robert *et al.* (2001b) and Schwab & Ordway (2001) showed a methionine hydroxy analogue (MHA- DL-α-hydroxy-γ-mercaptobutyrate), commonly called HMB to have a good replacement value for absorbed Met. The HMB is absorbed over the rumen and omasum wall via passive diffusion. Koenig *et al.*, (1999) reported 50% of HMB to be available for postruminal absorption after escaping rumen degradation in early lactation cows. Two-Hydroxy-4-methylthio butanoic acid has also been show to be converted to Met after absorption across the ruminal and omasal epithelium (Belasco, 1972; Papas *et al.*, 1974). 2-Hydroxy-4 methylthio butanoic acid is thus being used as a substitute for RPMet. However, based on studies done by Polan *et al.* (1970); Papas *et al.* (1974); Robert *et al.* (1997) and Johnson *et al*. (1999) HMB has been shown to have no or little effect on blood Met concentrations questioning the use of HMB to substitute RPMet in achieving the desired Met level in MP. More recent studies by Schwab *et al.* (2001), however,

indicated the isopropyl ester of HMB (HMBi) to be at least 53% as effective as SMartM to increase milk protein percentages.

It is extremely important to note that the different RPAA products differ significantly in the postruminal delivery of AA due to differences in ruminal stability, mode of protection and AA inclusion level. Products should therefore rather be compared on the basis of duodenal delivery of grams of available or absorbable AA's.

2.5 Methods Available for the Evaluation of RPMet Sources

Rumen protected methionine supplements are expensive and should be included and formulated into dairy diets precisely according to requirements. The requirements for the limiting AA have been well researched, but the relative value of the RPAA products in terms of ruminal stability and postruminal delivery of AA needs to be determined before inclusion. The relative bioavailability of a RPAA product is defined as its resistance to ruminal degradation and availability for absorption in the small intestine. There are various techniques to determine the value of RPAA sources. Most of the studies thus far have been conducted with either Lys or Met.

- The *in Vivo* Technique

This method requires rumen and duodenally cannulated cows and is still the standard technique but is expensive, time consuming and allows for a high margin of error. Currently the *in situ* technique is the most widely used and is also recommended by the NRC (2001) as the preferred technique. This technique, however, involves *in situ* bags

with pore size +/- 50 µm to be inserted into the rumen. The *in situ* incubation of feedstuffs in the rumen can't be used to evaluate availability of soluble RPMet products because they escape from the bag independently of their ruminal degradation (Patterson & Kung, 1988).

- The Milk Composition Technique

The value of the liquid RPMet product can be established by feeding a diet correctly balanced for Lys and the other limiting amino acids but with a shortage of Met. Milk production, protein yield and percentage can then be monitored to determine the effectiveness of the RPMet source in positively influencing milk composition and or yield (Schwab & Ordway, 2001).

- *In Vitro* Incubation of RPMet

In vitro techniques also offer the opportunity to evaluate rumen-protected amino acids. Ammonia is a major protein metabolite in the rumen: it is the principal end product of microbial protein degradation and form of nitrogen required by most strains of ruminal bacteria especially structural carbohydrate fermenting bacteria (Calsamiglia *et al*., 2002). Ruminal ammonia concentration can be used as an indicator of microbial protein degradation *in vivo* and *in vitro* and of non-protein nitrogen utilisation.

- Blood Plasma Methionine Levels Altered by RPMet

Amino acid concentrations in plasma as an indication of ruminal degradation and intestinal digestion have been used as an alternative for measuring the bioavailability of soluble amino acids (Brookes *et al.*, 1973; Reis *et al.*, 1978; Strath & Shelford, 1978). A quantitative measure can be obtained by relating the plasma Met response of RPMet supplementation to the response of a known quantity of Met delivered directly to the intestine, the major site of AA absorption. When the provision of the essential AA is below the requirement, the AA concentration in plasma may either increase marginally or not at all, but once the requirement for the AA has been met, plasma concentration increases more rapidly (Broderick *et al.*, 1974; Bergen, 1979). Measuring Met concentration in blood plasma is a direct less invasive method where cannulated animals are not needed

2.6 Responses to RPMet Supplementation

The use of RPMet and or RPLys is a more sophisticated approach to reach the optimum concentrations of AA for milk protein synthesis than using only conventional feedstuffs. When including RPAA in a ration, knowledge on the value or relative bioavailability of the amino acid sources is needed. The relative bioavailability of a RPAA product is defined as the resistance to degradation in the rumen and availability for absorption in the small intestine. To determine the bioavailability of any rumen protected amino acid, the resistance to ruminal degradation and intestinal availability need to be measured.

Responses in dairy cattle to post ruminal infusion of Lys and Met have been thoroughly reviewed by the NRC (2001). Growing animals respond in terms of feed conversion ratio

(FCR) and average daily gain (ADG) whilst dairy cattle respond in terms of milk yield, milk protein content, and feed intake.

Schwab *et al.* (2001) made the following observations:

- Milk protein is more responsive to RPLys and RPMet supplementation than milk yield especially in post peak cows.
- Milk protein increases are independent of milk yield
- Responses in milk protein content are equal to if not greater for cows during late lactation than mid lactation
- Increases in milk protein in response to increased MP due to either Lys or Met supplementation are most predictable when the other AA in MP are near or at estimated requirements
- Milk yield responses to increased amounts of Lys and Met in MP are most pronounced for cows in their first 2-3 months of lactation

Conflicting results have been reported for numerous studies conducted with RPMet supplementation. Yang *et al.* (2010) reported a positive response on milk yield and milk fat percentage for cows 4 months post calving fed RPMet at 42 g/day. A meta-analyses performed by Patton, (2010) summarised the findings of 35 studies. Rumen protected methionine supplementation increased true milk protein production by 27 g/d. Milk production was increased slightly with a slight decrease in milk fat percentage and feed intake. Milk protein response to RPMet was not related to AA as percentage of MP, predicted AA deficiency or calculated Met deficiency. Differences in results reported for a great number of lactation trials to study the effect of RPMet supplementation could be attributed to the variations in lactation stage, the experimental design (Latin square or

continuous lactation trial) the amount of supplemental RPMet provided as well as the proportions of other limiting AA in the MP (Wang *et al.*, 2010).

Philips *et al*. (2003) indicated RPMet supplementation pre and post partum had reduced body protein loss. With increased Met in MP or Met and Lys in MP increases in milk fat percentage in conjunction with increases in milk protein percentage have been reported (NRC, 2001). Some studies have shown that milk protein fractionation might even be a more sensitive method than milk protein percentage to monitor dietary AA responses. Several reports on responses in milk fat after RPAA supplementation has also been documented, but these nearly always occurred in conjunction with a response in milk protein content and have not been predictable. Increases in milk fat content to improved AA balance in MP could be due to enhanced *de novo* synthesis of short and medium chain fatty acids in the mammary gland as suggested by Pisulewski *et al. (*1996). In contradiction to the recommendation by the NRC, (2001) a meta-analyses was done by Patton, (2010) using more than 35 lactation studies where RPMet was supplemented. Results showed that a high level of Lys is not necessarily a prerequisite to obtain a true milk production response to feeding RPMet.

In a summary on supplementation of early lactation cows with RPLys and or RPMet Garthwaite *et al.* (1998) reported the following responses when compared to the control:

- 1.7 kg milk, 0.06% milk protein, 79 g milk protein, 0.1% milk fat, 85 g milk fat for cows supplemented from 2 to 21 days pre calving, measured over the first 28 to 118 days in milk (DIM).

- 0.7 kg milk, 0.16% milk protein, 79 g milk protein, 0.02% milk fat, 48 g milk fat for cows supplemented only from 0-35 days post calving, measured over days 21 to 119 DIM.

Literature reviews confirms that greater production responses to RPAA supplementation is achieved when the basal diet is imbalanced with regard to the Lys and Met content of the RUP, when RUP constitutes a large proportion of the MP and when cows are in early lactation and are high yielding rather than low producing animals (Socha *et al.*, 2005).

Various studies confirmed that milk casein is affected positively to a greater degree than the whey fraction and non-protein nitrogen (NPN) fractions (Donkin *et al*, 1989; Chow *et al.*, 1990; Armentano *et al.*, 1993). The NRC (2001) confirmed that increases in milk protein percentage are greater when supplementing RPAA as opposed to merely increasing the CP.

A response in milk yield to Lys and Met supplementation is generally limited to cows in early lactation when the requirement for absorbable AA is greatest (Polan *et al.*, 1991; Schwab *et al.*, 1992 a; Schwab *et al.*, 1992 b; Rulquin & Verite, 1993).

Chow *et al.,* (1990) reported increased yield of total N and casein N percentage in cows fed rations with additional fat as opposed to no effect in the absence of added dietary fat.

Berthiaume *et al.* (2006) evaluated the effect of RPMet on splanchnic metabolism and reported no effect on milk and milk protein yield; however, the true protein content increased linearly. Arterial Met concentration increased in response to RPMet

supplementation. This corresponds to data reported by Overton *et al.* (1996); Blum *et al.* (1999) and Berthiaume *et al.* (2001). The linear increase in total splanchnic output of isoleucine (Ile), leucine (Leu), phenylalanine (Phe) and threonine (Thr) suggested RPMet to trigger a homeostatic response resulting in decreased utilization of specific AA by the GIT and liver. Mammary extraction of Met decreased linearly in response to increased arterial inflow. Pisulewski *et al.* (1996); Varvikko *et al.* (1999) and Rulquin & Kovalezyk (2003) reported elevated blood Met after postruminal infusion of RPMet.

Graulet *et al.* (2005) evaluated HMBi which is absorbed and hydrolyzed into isopropyl alcohol and HMB which is then converted to acetone and Met respectively. The isopropyl ester of HMB was found to be of acceptable bioavailability in comparison with SMartM which has a 80% bioavailability. Ordway *et al.* (2009) demonstrated Metasmart™ and SMartM to both affect milk protein content positively for post partum cows.

Rumen protected methionine has been shown to positively affects fertility in dairy cows when supplemented in addition to choline. Methionine is a methyl donor and can also be metabolized into choline. Increased Met in MP could increase milk production and could also have a sparing effect on protein loss as demonstrated by Philips *et al*. (2003). This effect during the post calving phase could contribute to the general wellbeing and longevity of the cow. An additional benefit to RPMet supplementation is that the total amount of RUP in the ration can be reduced (NRC, 2001.)

Vyas & Erdman, (2009) performed a meta-analysis in which a regression model was fitted to performance data in response to postruminal supplementation of Lys and Met. Their conclusions based on the 23 studies included were as follows:

- Milk protein response to RPMet decreased from 16 to 4 g of milk protein/g metabolisable Met intake as Met intake varied from 25 to 70 g/cow
- Milk protein response to supplemental RPLys decreased from 5 to 3.2 g of milk protein/g of metabolisable Lys intake as Lys intake varied from 80 to 203 g/cow.
- Assuming Met and Lys at 2.75 and 7.63 g/100 g of milk protein respectively the implied marginal efficiencies of MAA used for milk protein yield decreased from 44 to 12% for Met and from 39 to 25% for Lys over the range of MAA intakes.

A low marginal efficiency of AA utilisation is to be expected when AA supply is at or near requirements (as in these experiments). This suggests current models assuming a constant AA utilisation efficiency and constant milk protein yield to be inadequate. These models will overestimate production responses to individual AA when high levels of MAA are fed. Although efficiencies are static each AA has individual efficiencies for maintenance, growth and lactation (Tylutki & Van Amburgh, 2010).

2.7 Commercial Implication of Incorporating RPMet into Dairy Rations

The "payback' or cost to benefit ratio of using RPMet products will depend on various factors and is unique for each dairy operation. Feeding of RPMet is particularly cost effective when the milk producer is being paid a premium for milk protein content. When using RPMet the entire ingredient complement, in particular those feeds contributing toward the RUP fraction should carefully be selected to enhance the profile of EAA in MP. When using RPMet in a well balanced ration the crude protein level of the ration can and

should be reduced in order to gain a cost benefit from the RPMet as well as reap the benefits in terms of improved animal performance and reduced nitrogen output.

Locally most of the RPMet sources are imported and has to compete against a variety of locally produced proteinaceous ingredients. The use of fishmeal being the only animal protein source allowed for inclusion in dairy rations which are also well balanced for both Lys and Met are limited due to the high cost in comparison to alternative plant protein sources. The locally produced LRPMet prototype from SA Bioproducts (1 Dickens Road, Umbogintwini, South Africa) would thus, if proven to be bio-available, fill a gap in the animal feed additive market in that it is locally produced, therefore allowing cost effective inclusion in dairy rations.

In the following two chapters two different methods to evaluate a LRPMet additive will be described and discussed namely the milk composition technique and the blood plasma technique.

CHAPTER 3

MILK PROTEIN COMPOSITION AS A METHOD TO EVALUATE THE RELATIVE BIOAVAILABILLITY OF A LIQUID RUMEN PROTECTED METHIONINE SOURCE.

3.1 Introduction

There are various techniques to determine the rumen stability and bioavailability of RPAA sources. The *in vivo* technique, which requires rumen and duodenally cannulated cows is still the standard technique against which other techniques should be compared, but is an invasive, expensive, time consuming process allowing for a high margin of error. Furthermore the use of markers to estimate lower GIT dry matter flow and markers to estimate microbial protein flow further complicates procedures. Currently the *in situ* technique is the most widely used and is also recommended by the NRC (2001) as the preferred technique to estimate ruminal degradability of feed or additives. This technique, however, involves *in situ* bags with pore size +/- 50 µm to be inserted into the rumen. The technique is therefore unsuitable for the evaluation of liquid RPAA sources because of the loss of the liquid from the porous bags. When various prototypes of any new RPAA product are evaluated, simple, economical and less invasive techniques are needed to first screen the prototypes for relative bio-availability before expensive long-term studies are conducted.

An alternative method to evaluate the relative bioavailability of RPAA is to formulate a diet deficient in the AA to be evaluated. This diet can then be supplemented with the RPAA product to be evaluated, which is deficient in the control diet as well as proven

RPAA products with known bioavailability. Production responses and changes in milk composition can then be compared. For the purposes of this study this technique is called the milk composition technique.

A liquid methionine prototype (methionine hydroxy analogue) protected from rumen degradation by a chemical process was developed by a local company (SA Bioproducts, 1 Dickens Road, Umbogintwini, South Africa). The objective of this study was the evaluation of this LRPMet product through the milk composition technique when compared to the tried and tested rumen protected methionine product, Smartamine^{TM} M (Adisseo, Inc., Antony, France) and feed grade DL-Methionine (Evonik Degussa, Theodore, Alabama, USA) which is not protected against rumen degradation.

3.2 Experimental Procedures

3.2.1 Animals and Diet

This study of which the protocol was approved by the Animal Use and Care Ethics Committee of the University of Pretoria was conducted at the Hatfield Experimental Farm of the University of Pretoria in accordance with the Guide for the Care and Use of Animals in Agricultural Research and Teaching (1999).

Forty high-producing mid to late lactation multiparous Holstein cows of comparable production and body weight were used in a complete randomised block design to evaluate the effect of a LRPMet source on milk production and milk composition, when added to a Met-deficient diet, in order to correct the Met:Lys RR (rulquin ratio). The CPM–dairy

formulation programme was used to formulate and evaluate the methionine deficient diet and determine the amount of post ruminal available methionine needed to achieve a RR of 3:1. Three RPMet sources providing a similar amount of potentially available methionine (23.6 g) were fed. The methionine deficient diet (Met -) with a Lys to Met ratio of 4.2:1 in MP was compared to the same diet supplemented with either a liquid rumen protected methionine source (LRPMet), DL-Methionine (DLMet) or Smartamine M (SMartM). The ingredient and chemical composition of the four experimental diets are shown in Table 3.1. The CPM Dairy prediction parameters for peptides, MP balance, bacterial and RUP contributions and the RR are shown in Table 3.2. In addition the AA profile of duodenal MP is also shown. The RR of Lys: Met is 7.2: 1.72 compared to the desired ratio of 7.2: 2.4.

Table 3.1 Ingredient and Chemical Composition of the Experimental Diets (g/kg DM)

	[1]Experimental Diets			
Ingredient Composition	**Met-**	**LRPMet**	**SMartM**	**DLMet**
Lucerne Hay	313	313	313	313
Sorghum	255	255	255	255
Whole Cottonseed	102	102	102	102
Wheaten bran	98	98	98	98
Citrus Pulp (Dried)	59	59	59	59
Molasses (Sugar Cane)	51	51	51	51
Blood Meal	43	43	43	43
Soya bean Oilcake	39	39	39	39
Megalac ™	24	24	24	24
Sodium Bicarbonate	8	8	8	8
Salt	5	5	5	5
[2]Vitamin and Mineral Premix	4	4	4	4
LRPMet	-	145 ml/cow/day	-	-
Smartamine ™ M	-	-	37.3 g/cow/day	-
DL-Methionine	-	-	-	23.8 g/cow/day
Chemical Composition	**g/kg of DM**			
Crude Protein (CP)	192.5			
Rumen undegradable protein (% CP)	50.2			
Rumen degradable protein (% CP)	49.8			
Metabolisable energy (MJ/kg)	11.12			
Net energy for lactation (MJ/kg)	7.16			
Acid detergent fibre	195			
Neutral detergent fibre	316			
[3] Non fibre carbohydrates	381.6			
Starch	164.2			
Ether extract total	65.7			
Ash	80.5			
Calcium	8.9			
Phosphorus	4.1			
Magnesium	2.6			
Potassium	16.4			
Sulphur	2.8			

[1] Experimental Diets: Methionine deficient diet (Met-); Met- diet supplemented with liquid rumen protected methionine (LRPMet); Met- diet supplemented with Smartamine ™ M; Met- diet supplemented with DL-Methionine (DLMet)

[2]Vitamin and mineral content per kg DM: 45 mg Zn, 15 mg Cu, 22 mg Mn, 0.25 mg Se, 0.1 mg Co, 0.28 mg I, 2900 IU Vitamin A, 800 IU Vitamin D, 20 mg Vitamin E

[3] NFC (% DM) = 100 - ((CP + Fat + Ash + (NDF-NDIP))

Table 3.2 CPM Prediction Parameters and AA profile for Cows Consuming the Methionine deficient diet

CPM Parameter	CPM Prediction
Peptide and NH_3 Balance (g/d)	135
Peptide Balance (g/d)	120
MP Balance (g)	280.3
NP/MP (%)	56.6
MP from Bacteria (g/d)	1386
MP from RUP (g/d)	1891
Met:% Req	102
Lys: % Req	135

Amino Acid	(AA as % MP)
Methionine	1.72
Lysine	7.2
Arginine	6.31
Threonine	4.81
Leucine	9.42
Isoleucine	4.27
Valine	6.53
Histidine	3.47
Phenylalanine	5.84
Tryptophan	1.59
Rulquin ratio	4.18:1

Cows were fed the commercial TMR which was the standard production diet fed in the herd for a period of 14 days prior to the study in order to adapt the cows to the Calan® Headgate feeding system (American Calan Inc., Northwood, NH, USA). During this period cows were assigned to one of 10 blocks of four cows each based upon firstly milk production and secondly days in milk. Within each block the forty cows were randomly allocated to one of the four treatments: Control: Met deficient diet (Met-), DL-Met supplemented diet (DLMet), Liquid Rumen protected supplemented diet (LRPMet), and Smartamine™ M supplemented diet (SMartM).

The product specifications of the LRPMet product to be evaluated are shown in Table 3.3. DL-methionine was considered to contain 99.0 % methionine thus 23.84 grams were supplemented. Smartamine™ M was considered to contain 63.3% available methionine thus 37.3 g were supplemented. The LRPMet product was considered to contain 16.23% available Met and 145 ml were supplemented. The specifications of the LRPMet product are shown in Table 3.3. Supplementation with the rumen protected sources resulted in a Lys: Met ratio of 7.2:2.4 in MP, which is considered optimal (NRC, 2001).

Table 3.3 Specifications for the Liquid Rumen Protected Methionine Source Evaluated

Description	Result	Units	Comments
Physical Form			Liquid at room temperature
Physical Form	7.8		Mildly alkaline for stability
density	1159	g/l	
Total Solids in Product	30%	% m/m	
% total methionine in product	14%	% m/m	
% sodium in product	6%	% m/m	
Bypass value	100%	% m/m	All methionine is protected from analysis of residual Met
Hydrolysability	100%	% m/m	% of hydrolysable bypass Met at pH 2/37 °C/2 hours

The experiment consisted of three experimental periods namely an adaptation period of 7 days and two experimental periods of 21 days each during which production was monitored and samples were taken. During period 1, the adaptation phase, all 40 cows were fed the standard commercial TMR for a period of 7 days. Feed was presented as a total mixed ration, (water was added to feed to allow for a dry matter content of 60 %) and fed 3 times a day to ensure fresh feed in front of the cows all day maximising intake.

Cows were milked three times daily in a 10 point herringbone system equipped with a DeLaval Alpro milking system (DeLaval (Pty) Ltd, Pinetown, South Africa). Milk production and feed intake were recorded on a daily basis. Milk samples were taken during the afternoon milking every second day and analysed for protein, milk fat, lactose and somatic cell count using the System 4000 Infrared Analyzer (Foss Electric, Hillerod, Denmark). During period 2 the Met- diet was fed for 21 days. Milk production and feed intakes were recorded daily. Milk samples were taken every second day and analysed for protein, milk fat, lactose and somatic cell count. Additional milk samples were taken on day 21 of period 2 and analysed for nitrogen fractions (casein, whey and NPN). During period 3, three of the four groups receiving the Met- diet (control diet) during period 2 were supplemented with DLMet, SMartM or LRPMet for 21 days. Supplements were mixed into a small amount of feed twice daily and fed directly after the cows returned from the milking parlour. Care was taken to ensure that cows ingested all of the supplements. Milk production and feed intake monitoring as well as the sampling procedure were similar to that of period 2 with milk samples taken every second day to analyse milk composition and samples taken on day 21 of period 3 to analyse milk nitrogen fractions.

3.2.2 Statistical Analyses

Data were analyzed as a randomized complete block design with the General Linear Model (GLM), (Statistical Analysis Systems, 2006) for an analysis of variance to determine difference between treatments and periods. Means and standard error of the means (SEM) were calculated. The significance of difference between means was determined by the Fischer's protective test (Samuels, 1989). Significance was declared at $p < 0.05$ and tendencies at $p < 0.10$.

The linear model used is described by the following equation:

$$Y_{ijk} = \mu + T_i + P_j + B_k + TP_{ij} + e_{ijk}$$

Where Y_{ijk} = variable studied during the period

μ = overall mean of the population

T_i = effect of the i^{the} treatment

P_j = effect of the j^{the} period

B_k = effect of the k^{the} block

E_{ijk} = error associated with each Y

3.3 Results and Discussion

3.3.1 Experimental Diets

The theoretical composition of the diet is shown in Table 3.1 and fulfils the nutrient requirements of a cow producing 40 kg of milk (NRC, 2001). The RR was ideal at 7.2:2.4 after supplementation. The TMR fed during the trial was supplied by a commercial feed company which analysed each batch to ensure its nutrient specifications conformed to the specifications as indicated in Table 3.1. The proximate analyses on the TMR were not made available due to the feed company's strict confidentiality policy.

3.3.2 Milk Composition Technique

Results on the effect of RPMet on the various parameters measured are presented in Tables 3.4, 3.5 and 3.6. Means, standard error of the means and significant difference were

determined for periods 1 to 3 and for the change observed for parameters measured between the last week of period 2 and period 1 and the change between the last week of period 3 and period 2 respectively. Results were thus compared within periods 1 to 3 as well as over periods 1, 2 and 3.

Table 3.4 The Effect of RPMet Supplementation on Milk Yield, Milk Composition and Feed Intake (Average Over Periods)

Parameter	[1]Treatments				SEM[E]
	Met-	LRPMet	DLMet	SMartM	
Milk Yield (kg/day)					
P1	35.72	36.05	36.48	35.36	0.99
P2	35.74	36.55	36.96	35.65	0.99
P3	35.02	35.13	36.34	35.07	0.99
Milk Composition					
Milk Protein (%)					
P1	3.10 ^{ab}	3.16 ^{ab}	3.00 ^{b}	3.23 $^{a}{}_{1}$	0.07
P2	2.98 ^{b}	3.15 ^{a}	2.94 ^{b}	3.22 $^{a}{}_{1}$	0.07
P3	3.06 ^{b}	3.25 ^{c}	2.95 ^{b}	3.46 $^{a}{}_{2}$	0.07
Fat (%)					
P1	3.54	3.65	3.62	3.80 $_{3}$	0.14
P2	3.79 ^{ab}	3.97 ^{ad}	3.82 ^{bc}	4.14 $^{d}{}_{1}$	0.14
P3	3.84 ^{ad}	3.93 ^{ab}	3.75 ^{d}	4.27 $^{c}{}_{2}$	0.14
Lactose (%)					
P1	4.83 $_{1}$	4.76 $_{1}$	4.80 $_{1}$	4.76 $_{1}$	0.06
P2	4.70 $_{12}$	4.64 $_{1}$	4.73 $_{12}$	4.63 $_{1}$	0.06
P3	4.65 $^{a}{}_{2}$	4.48$^{bc}{}_{2}$	4.62 $^{ac}{}_{2}$	4.43 $^{b}{}_{2}$	0.06
DMI (kg/cow/day)					
P1	25.03 $^{a}{}_{1}$	23.91$^{ac}{}_{1}$	22.7$^{bc}{}_{1}$	22.55 $^{ab}{}_{1}$	0.53
P2	19.82 $_{2}$	20.40 $_{2}$	20.47 $_{2}$	19.51 $_{2}$	0.53
P3	19.41$_{2}$	18.79 $_{3}$	19.49 $_{2}$	18.73 $_{2}$	0.53

[1] Treatments: Methionine deficient diet (Met-); Met- diet supplemented with liquid rumen protected methionine (LRPMet); Met- diet supplemented with Smartamine ™ M (SMartM); Met- diet supplemented with DL-Methionine (DLMet)

^{abcd} Row means with different superscript differ ($p < 0.05$)

$_{123}$ Column Means with different subscripts differ ($p < 0.05$)

^{E} Standard error of least square means

* During period 1 (P1) cows received a standard TMR, during period 2 (P2) the Met- diet and during period 3 (P3) the respective Met supplements in addition to the Met- diet

Table 3.5 The Effect of RPMet Supplementation on Milk Yield, Milk Composition and Feed Intake (Difference Between Periods).

	[1]Treatments				SEM[E]
	Met-	LRPMet	DLMet	SMartM	
Milk Yield (kg/day)					
P1-2	0.01	0.50 $_1$	0.48	0.29	0.66
P2-3	-0.72	-1.42 $_2$	-0.62	-0.58	0.66
Milk Composition					
Milk Protein (%)					
P1-2	-0.12 $_1$	-0.01	-0.06	-0.01 $_1$	0.054
P2-3	0.08 $^{a}{}_2$	0.09 ^{a}	0.01 ^{a}	0.24 $^{b}{}_2$	0.054
Fat (%)					
P1-2	0.25	0.32 $_1$	0.21 $_1$	0.34	0.09
P2-3	0.05	-0.04 $_2$	-0.07 $_2$	0.13	0.09
Lactose (%)					
P1-2	-0.13	-0.13	-0.07	-0.14	0.06
P2-3	-0.05	-0.16	-0.11	-0.19	0.06
DMI (kg/cow/day)					
P1-2	-5.22 $^{a}{}_1$	-3.50 $^{b}{}_1$	-2.23 ^{b}	-3.05 $^{b}{}_1$	0.58
P2-3	-0.41 $_2$	-1.61 $_2$	-0.98	-0.78 $_2$	0.58

[1] Treatments: Methionine deficient diet (Met-); Met- diet supplemented with liquid rumen protected methionine (LRPMet); Met- diet supplemented with Smartamine ™ M (SMartM); Met- diet supplemented with DL-Methionine (DLMet)

[ab] Row means with different superscript differ ($p < 0.05$)

[12] Column Means with different subscripts differ ($p < 0.05$)

[E] Standard error of least square means

* During period1 (P1) cows received a standard TMR, during period 2 (P2) the Met- diet and during period 3 (P3) the respective Met supplements in addition to the Met- diet.

Table 3.6 The Effect of RPMet Supplementation on Milk Protein Fractions

Parameter	[1]Treatments				SEM[E]
	Met-	LRPMet	DLMet	SMartM	
Milk Urea Content (mg/dl)					
P2	16.65	18.28 $_1$	17.00	17.09 $_1$	0.65
P3	15.72 ^{ab}	16.52 ^{b} $_2$	16.54 ^{b}	14.39 ^{a} $_2$	0.65
Difference**	-0.93 ^{ab}	-1.76 ^{ab}	-0.47 ^{b}	-2.70 ^{a}	0.71
Casein (%)					
P2	2.04 ^{bc} $_1$	2.11 ^{ac} $_1$	2.02 ^{bc} $_1$	2.24 ^{a} $_1$	0.07
P3	2.29 ^{b} $_2$	2.49 ^{a} $_2$	2.27 ^{b} $_2$	2.64 ^{a} $_2$	0.07
Difference**	0.25 ^{bd}	0.38 ^{acd}	0.25 ^{bc}	0.40 ^{a}	0.07
Non Casein Protein (%)					
P2	0.91 ^{ac}	0.91 ^{ac}	0.83 ^{bc}	0.98 ^{a}	0.04
P3	0.80 ^{b}	0.82 ^{b}	0.75 ^{b}	0.99 ^{a}	0.04
Difference**	-0.11	-0.09	-0.08	0.01	0.05
Whey (%)					
P2	0.67 $_1$	0.67	0.60	0.71	0.04
P3	0.53 $_2$	0.60	0.50	0.76	0.04
Difference**	-0.15 ^{bc}	-0.07 ^{ac}	-0.10 ^{bc}	0.05 ^{a}	0.05
Non-protein Nitrogen (%)					
P2	0.24 $_1$	0.24	0.23	0.24	0.01
P3	0.28 ^{a} $_2$	0.23 ^{bc}	0.25 ^{ac}	0.23 ^{bc}	0.01
Difference**	0.04 ^{a}	-0.02 ^{b}	0.03 ^{a}	-0.02 ^{b}	0.02

[1] Treatments: Methionine deficient diet (Met-); Met- diet supplemented with liquid rumen protected methionine (LRPMet); Met- diet supplemented with Smartamine ™ M (SMartM); Met- diet supplemented with DL-Methionine (DLMet)

^{abcd} Row means with different superscript differ ($p < 0.05$)

$_{12}$ Column Means with different subscripts differ ($p < 0.05$)

^{E} Standard error of least square means

* During period 1 (P1) cows received a standard TMR, during period 2 (P2) the Met- diet and during period 3 (P3) the respective Met supplements in addition to the Met- diet.

** Difference between P2 and P3

Milk yield was not affected by any of the Met treatments (Figure 3.1). This was to be expected since supplementation with RPMet sources impacts positively upon milk production mainly during the first 2 to 3 months of lactation (Schwab & Ordway, 2001). The cows included in this experiment were all post peak production.

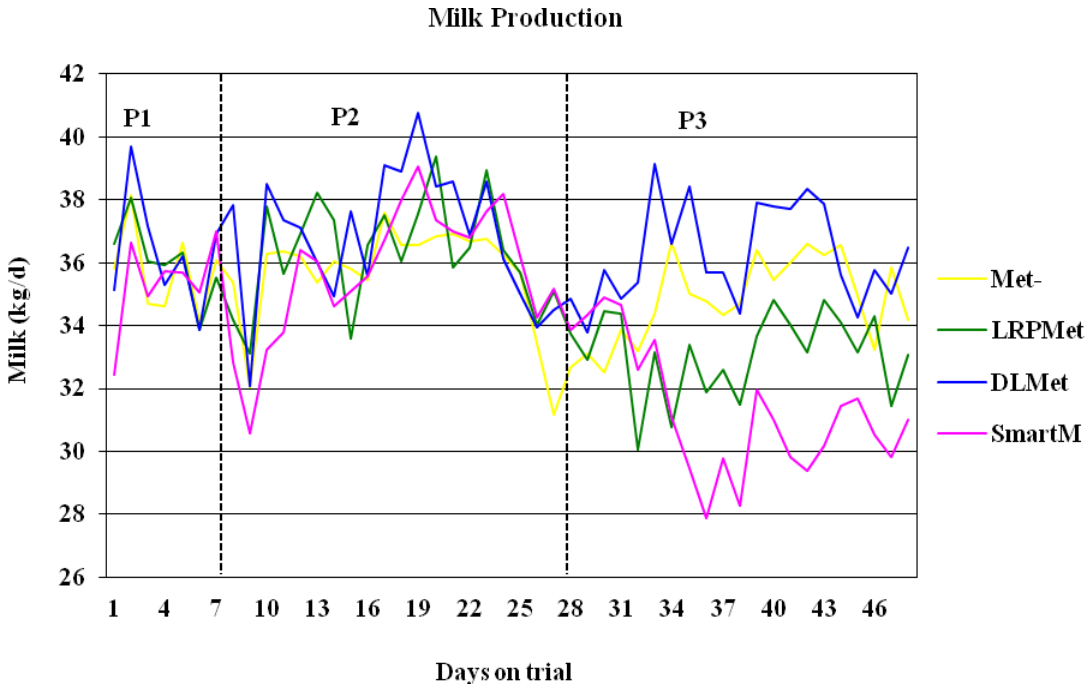

Figure 3.1 Milk Yield with Met Supplemented During Period 3

Results obtained from a recent study by Yang *et al.* (2010) contradicts this statement in that a significant increase in milk production with an accompanying significant increase in milk fat percentage were recorded for post peak cows supplemented with RPMet. The basal ration fed during the study by Yang *et al.* (2010) consisted mainly of maize, maize silage and soybean meal with a Lys: Met of 2.89:1. The basal ration fed during that study was probably higher in Lys and it can be argued that Lys did not become first limiting in response to RPMet supplementation which resulted in increased milk production.

Shingoethe *et al*. (1988) reported no response in milk yield but an increase in milk protein percentage of early lactation cows supplemented with RPMet on a barely based diet. The authors contributed the non responsiveness of milk production to a restriction on dry matter intake during early lactation but also indicated that Lys is most likely to become the first limiting AA in barley based diets when supplemented with RPMet. In the event that the Lys level is relatively low milk production would thus not be increased by RPMet. In our study however Lys was supplemented primarily through blood meal resulting in a Lys: Met of 4.2:1. Lys could thus not have become first limiting in response to Met supplementation but Ile which contributed only 128% of requirements (CPM Dairy analyses) might have become limiting. In the study conducted by Shingoethe *et al*. (1988) Ile was also ranked as one of the 5 AA's to become first limiting in response to RPMet supplementation.

In a study conducted by Pisulewski *et al*. (1996) high levels of Met supplementation was implicated in constricting blood flow to the mammary gland, reducing milk production regardless of supplementation. Cant *et al.* (1993a) proposed that individual AA's can increase the total MP production without affecting the AA supply to the mammary gland.

Numerous studies with lactating cows indicated milk protein content to be more responsive than milk yield to small changes in concentrations of Met in MP (Schwab & Ordway, 2001). According to Donkin *et al*. (1989) and Rogers *et al*. (1989) providing RPAA with low CP diets increases milk yield but supplementing high CP diets with RPAA do not affect milk yield. In this trial supplementing a diet of a CP content of 19.2 % with RPMet did not increase milk yield, suggesting that the AA flow to the duodenum probably weren't limiting to milk production.

Dry matter intake (DMI) declined significantly for all the treatments between period 1 and period 2 ($p < 0.05$) (Figure 3.2). Cows supplemented with LRPMet had a further reduction in feed intake between period 2 to 3 ($p < 0.05$) compared to the DMI for the other treatments which remained constant between period 2 to 3. DMI for the Met- treatment was much higher than for the DLMet treatment during period 1 ($p < 0.05$) but during periods 2 and 3 DMI did not differ between treatments. The Met- diet fed during period 2 and supplemented during period 3 contained 4% blood meal which were included in the diet due to its high level of Lys. The blood meal in addition to the 40% moisture content of the ration were unpalatable in comparison to the commercial TMR fed during period 1, probably depressing feed intake during period 2 as opposed to period 1 for all treatments ($p < 0.05$). Feed intake probably remained constant between periods 2 and 3 because the cows got accustomed to the taste of the blood meal. Donkin *et al*. (1989) and Rogers *et al*. (1989) reported no increase in DMI after supplementation with RPAA. The high nutrient density, especially the high level of CP in the Met- diet fed in this trial could possibly explain the lack in response of DMI. In general the supplements did not affect DMI.

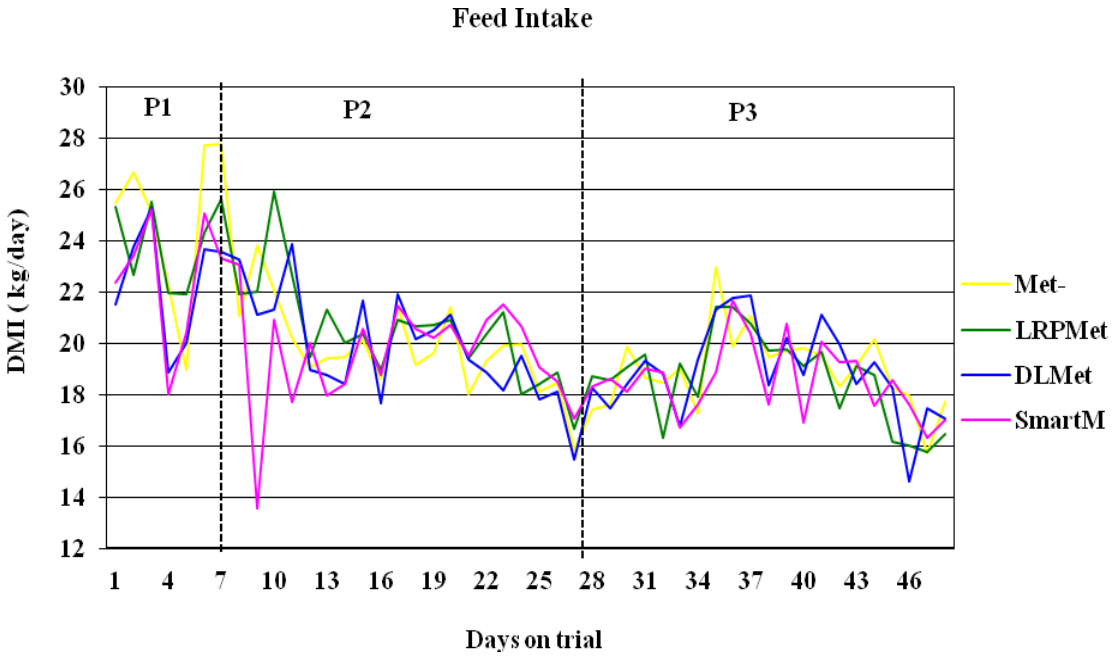

Figure 3.2 Dry Matter Intakes with Methionine Supplemented During Period 3

Smartamine™ M supplementation increased milk protein percentage (Figure 3.3) during period 3 ($p < 0.05$) with 0.24 units compared to period 2. Milk protein percentage also increased for cows receiving the Met- treatment ($p < 0.05$), however the change in milk protein percentage during period 2 to 3 for the Met- treatment was similar to that of the DLMet and LRPMet treatments. The average milk protein percentage for the SMartM group was higher than that of the other treatments during period 3 ($p < 0.05$). Chapoutot *et al*. (1992) demonstrated milk protein percentage to be more sensitive to improvements in AA composition in MP than milk yield. Benefield *et al*. (2009) reported a strong tendency for RPMet supplementation to increase milk true protein content. According to Schwab & Ordway (2001), milk protein is more responsive to small changes in Met concentrations in MP than milk yield. It should also be noted that the magnitude in change of milk protein %

between periods 2 and 3 were much larger for cows supplemented with SMartM ($p < 0.05$) when compared to the other treatments (Table 3.5).

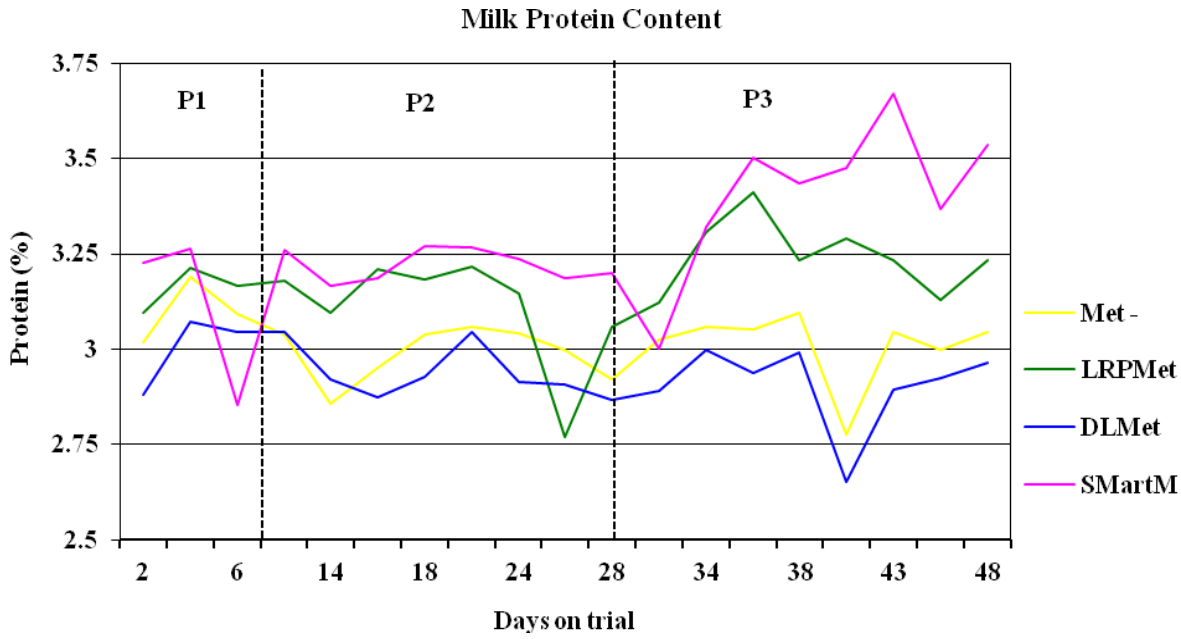

Figure 3.3 Milk Protein Percentages with Methionine Supplementation during Period 3

Smartamine™ M positively affected milk fat percentage with a 0.13 unit increase from period 2 to 3 ($p < 0.05$). Milk fat percentages remained the same between periods 2 and 3 for the other treatments ($p > 0.05$). The average milk fat percentage for the SMartM cows during period 3 was higher than the other treatments for the same period ($p < 0.05$). Milk fat content of cows receiving the SMartM treatment was higher than that of cows on the other treatments during period 3 ($p < 0.05$). There are numerous reports of increases in milk fat percentage when feeding increased amounts of Met or Met and Lys (NRC, 2001). These increases in milk fat are usually observed in conjunction with increases in milk protein. Pisulewski *et al*. (1996) suggested that improving a Met deficiency may enhance *de novo* synthesis of short – and medium chain fatty acids in the mammary gland. Limited

evidence exists of increased secretion of chylomicrons and very low density lipoprotein with improved Met and Lys levels in MP. Both arguments suggest an increase in the supply of FA to the mammary gland.

The average lactose percentage decreased between periods 2 and 3 for cows on all treatments. However, it was only a significant reduction for the LRPMet and SMartM treatments ($p < 0.05$). There were no differences between treatments for lactose content during periods 1 to 2, however, during period 3 both the LRPMet and the SMartM treatments expressed a lower lactose % compared to the Met- and DLMet treatments ($p < 0.05$). The lower milk lactose concentrations in the SMartM supplemented cows compared to the other treatments correspond with the findings of Blum *et al*. (1999) who reported a lower milk lactose content of cows on SMartM compared to control treatments. The reason for the decreased lactose content is not clear.

There was a significant drop in milk urea concentration (MUN) from period 2 to 3 in cows on both the SMartM and LRPMet treatments ($p < 0.05$). The Met- and DLMet treatments did not affect the MUN concentration of milk. The improved nitrogen efficiency of the SMartM supplemented cows is probably due to the improved AA balance in MP and the higher level of available Met. A decreased MUN value could have a beneficiary effect on cow health and fertility. Balancing the dietary AA profile according to requirements decreases AA breakdown in the liver and reduce plasma urea concentration with an accompanying energy saving effect.

There was a significant increase in milk casein % for cows on all treatments between periods 2 and 3 ($p < 0.05$) (Figure 3. 4). The increase in casein % between periods 2 and 3

was most pronounced for cows on the SMartM treatment from 2.24 to 2.64 % ($p < 0.05$) and differed from the Met- and DLMet treatments. The LRPMet increased the casein % from 2.11 to 2.49% ($p < 0.05$) which was similar to the SMartM treatment. The number of samples taken was probably not sufficient to make meaningful conclusions on the potential of milk nitrogen fractions to evaluate and screen products.

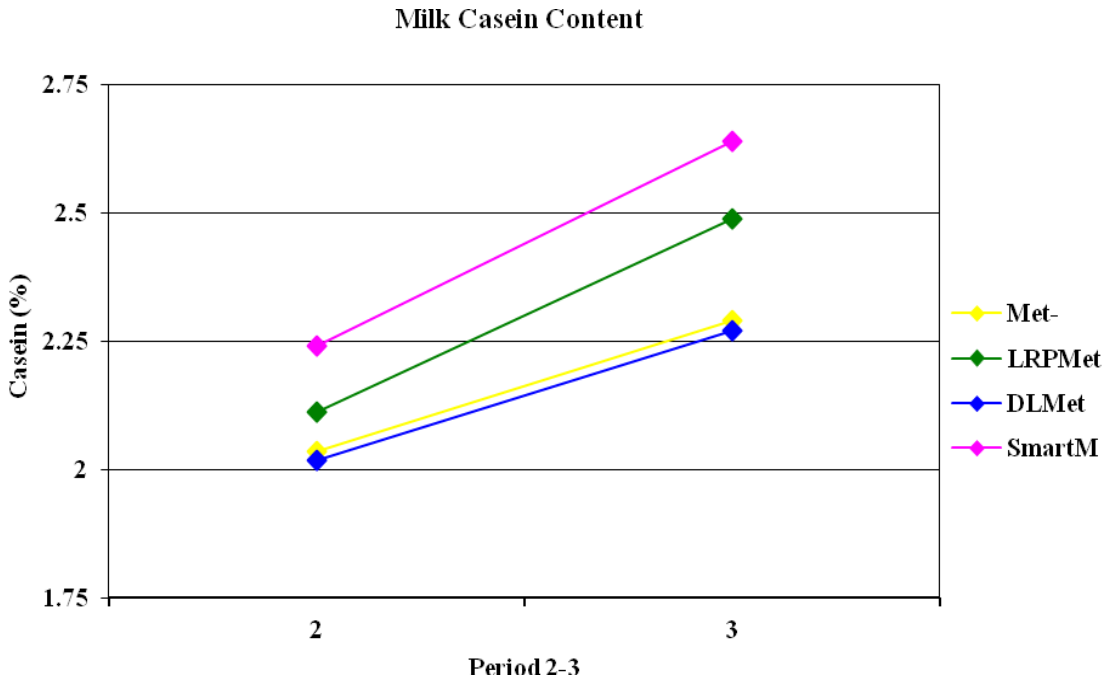

Figure 3.4 Casein Content of Milk with Methionine Supplementation during Period 3

Milk nitrogen fractions and casein % in particular is important for cheese yield. When comparing the milk whey content between periods 2 and 3 there was a significant decrease in the whey fraction of cows on the Met– treatment ($p < 0.05$) (Figure 3.5). Only the SMartM supplemented cows had an increase in whey concentration of the milk ($p < 0.05$) which differed significantly from the Met – and DLMet treatments ($p < 0.05$).

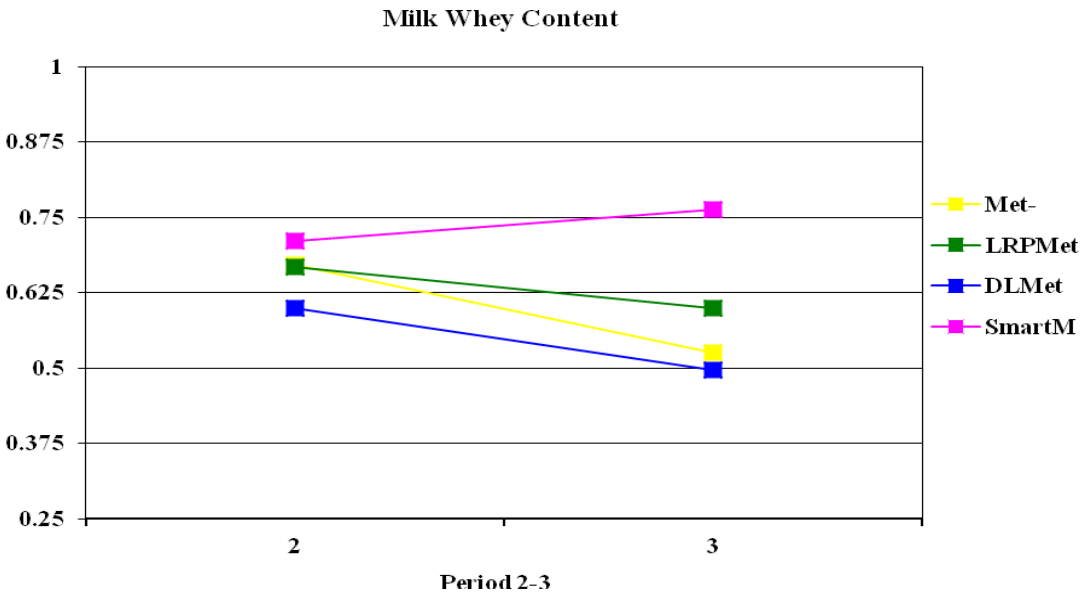

Figure 3.5 Whey Content of Milk with Methionine Supplementation during Period 3

The NPN concentration of milk is a result of changes in the other milk protein fractions. The NPN concentration of milk remained constant between periods 2 and 3 for all treatment groups ($p > 0.05$). The NPN content of the SMartM and LRPMet supplemented cows differed from the other treatments ($p < 0.05$). The slight decrease ($p > 0.05$) in the NPN values of the SMartM supplemented cows could be due to the improved AA balance in the MP decreasing the amount of MUN and plasma urea production. The decreased NPN value of the SMartM supplemented cows is reflected in their lower MUN content. The LRPMet treatment group also had a decrease in NPN similar to that of the SMartM cows from 0.24 to 0.23 % which was not significant. The LRPMet treated cows furthermore expressed a decrease in their MUN value which did not differ from the other treatments ($p > 0.05$). The SMartM treatment expressed the highest non casein protein % (NCP) fraction during period 3 compared to the other treatments ($p < 0.05$).

The results showed no effect of the Met– or DLMet treatments on any of the parameters excluding casein % measured, which is to be expected due to DLMet not being protected against rumen degradation and the Met- diet unbalanced for the required Lys to Met ratio in MP. The LRPMet treatment affected the casein % positively ($p < 0.05$) similar to the response from all the treatments, although the magnitude of change in casein percentage between periods 2 and 3 was higher for cows receiving SMartM and LRPMet. The positive response in the casein % for all treatments during period 3 ($p < 0.05$) might be explained by a lack of samples taken to accurately predict the affect of Met supplementation on casein % and no meaningful conclusion pertaining to the response in casein % can probably be drawn. Smartamine™ M increased both protein % ($p < 0.05$) and milk fat % during period 3 and differed from the other treatments ($p < 0.05$) which did not did not affect either milk protein percentage ($p >0.05$) or milk fat percentage ($p > 0.05$). The fact that SMartM positively affected casein %, milk protein % and milk fat % suggests that the treatment was successful in improving the Lys: Met ratio in MP and thus confirms that SMartM is an effective RPAA. Due to the lack of response it can be concluded with certainty that the LRPMet product had no effect on milk production and composition and appeared to have no potential as a RPAA. This could be due to the LRPMet product either not being protected against ruminal degradation, not being available for absorption in the small intestine or a combination of both factors.

3.4 Conclusion

All treatments affected casein % positively but only the rumen protected SMartM treatment increased both milk protein percentage and milk fat % ($p < 0.05$). Furthermore, the magnitude of change in milk fat and protein % between periods 2 and 3 was higher for

cows supplemented with SMartM ($p < 0.05$) when compared to other treatments. Milk yield was not affected by any of the treatments. The LRPMet product was either not protected against ruminal degradation or not available for absorption in the small intestine. Further evaluation on this RPMet source is needed to establish whether the problem is under protection in the rumen or overprotection in the abomasum i.e. low bioavailability.

This experiment proved milk composition, especially milk protein percentage and milk nitrogen fractions to be an effective technique to evaluate the relative efficiency of a RPMet product in either solid or liquid form in improving the Lys to Met ratio in MP. This technique could be used as a screening process on different RPMet prototypes before the onset of long and expensive lactation studies. The milk component technique proved to be a relatively simple and cost effective technique to evaluate the liquid RPAA in this study. The use of this technique to evaluate other RPAA prototypes, perhaps RPLys, should be investigated further.

CHAPTER 4

METHIONINE CONCENTRATION IN BLOOD PLASMA AS AN INDICATOR OF THE RELATIVE BIOAVAILABILITY OF A LIQUID RUMEN PROTECTED METHIONINE SOURCE

4.1 Introduction

Protein contributes significantly to the overall feed cost in dairy rations. Crude protein is degraded to ammonia, peptides and AA's in the rumen and is used for microbial growth in the rumen and the resulting microbial protein supplements the RUP fraction flowing to the SI. In well balanced rations 50 % or more of the absorbable amino acids are provided by the ruminally synthesized microbial protein (Clark *et al*., 1992; Merchen & Titgemyer, 1992; Schwab, 1995) which provides a constant source of high quality absorbable amino acids. With high producing dairy cows the contribution of the RUP to the absorbable AA becomes more pronounced especially due to the increased feed intake, increased rumen outflow rate and the expectation of increased milk yield and improved milk composition. The excretion of excess nitrogen should also be considered. Feeding diets lower in crude protein has been used to overcome this problem but could not supply the AA balance which has been proved to be optimal for milk production. Methionine and lysine have been proved to be the two most limiting AA in rations formulated for high yielding cows (Schwab *et al*., 1992; Rulquin *et al*., 1993). Methionine is thought to be the first limiting AA in lucerne based rations and or rations containing soybean or cottonseed meals as major protein sources (Shingoethe, 1996). A model developed by Rulquin *et al*. (1993) which was implemented in the NRC (2001) publication, indicates the optimal use of MP for milk protein production is when Lys in MP is 7.2% and Met 2.4 %. The optimum ratio

for Lys vs. Met in MP is 3: 1 when using this model. The Met contribution to the MP can be increased with the use of RPMet supplements. Dairy producers and nutritionists should select the RPMet product based on its resistance to ruminal degradation and availability for absorption in the small intestine (Robinson, 1996).

The supply of AA by these RPMet products can be evaluated using multi-cannulated animals but this is time consuming, labour intensive, expensive and prone to experimental error. The use of plasma AA methionine concentration is an alternative which involves the withdrawal of blood samples from the jugular vein without permanent catheterization. This technique has been used successfully to establish the bioavailability of RPMET products (Papas *et al*., 1984; Koenig & Rode, 2001; Sudekum *et al*., 2004; Graulet *et al*., 2005).

Plasma AA concentrations can be taken as estimates of intestinal AA supply (Dhiman & Satter, 1997) and the bioavailability of RPMet in dairy cows (Robert *et al*., 1997; Blum *et al*., 1999; Bach & Stern, 2000; Rulquin & Kowalczyk, 2000). A linear relationship was calculated between the increases of plasma Met concentrations (y) (in % over the control) in response to the amount of orally RPMet supplemented (x; $y = 3.2153\ x - 0.957$; $r = 0.87$) (Jochmann *et al*., 1996). Splanchnic metabolism does not alter peripheral methionine supply to the animal since no difference in arterial Met concentration after infusion of 15 AA, including Met into the abomasum or the jugular vein were recorded (Thivierge *et al*., 2002).

The objective of this study was to evaluate a liquid ruminally protected methionine prototype in comparison to two other methionine sources (one completely rumen degradable and the other rumen protected) using a standardised blood test.

4.2 Experimental Procedures

In this study two experiments were conducted. During experiment one three sources of Met were orally administered to cows in order to detect the blood Met levels at 0, 12 and 48 hours post supplementation as an indication of the relative bioavailability of the Met. In experiment 2 the three Met sources were infused into the abomasums of the same 3 cows and blood Met levels measured at 0, 3, 7, 12 and 48 hours post infusion. This second experiment aimed to establish the availability of the Met sources for absorption in the SI. Only after completion of both the oral dosing and the postruminal infusion of the LRPMet prototype would it be possible to make conclusions related to the relative bioavailability of the prototype. Should the LRPMet not be resistant to ruminal degradation the blood Met levels will not increase after oral dosing. A prototype not resistant to ruminal degradation should, however, increase blood Met levels after post ruminal infusion if it is available for absorption in the SI. In the event that the prototype is neither resistant to degradation in the rumen nor available for absorption post ruminally no response in blood Met is expected during either the oral dosing or post ruminal infusion of the Met.

4.2.1 Animals and Diet: Experiment 1

Three mid- lactation, high producing dairy cows of similar production and body weight were used in a 3 x 3 Latin square design. The cows received a standard lucerne based total mixed ration for *ad lib* consumption and were milked three times daily. The purpose of this trial was to evaluate the relative bioavailability of a liquid RPMet source. The free methionine in blood plasma was measured after administering the product via oral drenching. The effect of the LRPMet product on plasma Met levels was compared to that

of Smartamine™ M (a rumen protected Met source) and DL-Methionine (an unprotected Met source).

The three treatments were: Smartamine™ M (SMartM), DL-Methionine (DLMet) and the liquid RPMet product (LRPMet).

The three treatments each provided 40 g of potentially available methionine calculated as follows:

- SMartM with an assumed Met availability of 63.2 % = 63.3 g of the product to supply 40 g of available Met.
- DLMet with an assumed Met availability of 99 % = 40.4 g of the product to supply 40 g of available Met.
- LRPMet with an assumed Met availability of 16.23 % = 246.5 ml of the product to supply 40 g of available Met.

The three experimental periods were 48 hrs each. On the morning of day 1 at 08:00 the supplements were administered as an oral drench. After the cows received the supplement the daily TMR were provided. Blood samples were taken at 07:00 (zero hour samples) and 20:00 on day 1 and at 07:00 on day 3. Samples were therefore taken at 0, 12 and 48 hours. On day 3 and day 5 the same procedure were repeated with the same cow, only with a different supplement. Blood was analyzed for plasma methionine.

Due to limited data available on the mode of protection of the LRPMet product there was uncertainty as to whether the product will affect the free Met levels in the blood plasma or whether it would only be detected as methionine sulfoxide. Blood drawn from cows fed the LRPMet product during Experiment 1 were thus analyzed for free Met as well as Met sulfoxide using the HPLC. Due to there being no differences in the results it was decided

to only analyze free methionine. The data of this screening test were not statistically analyzed.

4.2.2 Sampling procedure: Experiment 1

Blood samples were taken via jugular veni-puncture into sterile tubes containing Li-heparin. The samples were placed on ice until centrifuged (1500 x g at 4˚C for 10 minutes) and the plasma were transferred into Ependorf tubes where after it was frozen at -20˚C. Samples were analysed for physiological methionine (free amino acids) at the Department of Biomedical Sciences; Tshwane University of Technology, Pretoria.

4.2.3 Animals and Diet: Experiment 2

Three high producing, ruminally cannulated Holstein cows in mid lactation were used in a 3 by 3 Latin square design. The aim of this trial was to evaluate the availability of a liquid rumen protected methionine product for absorption in the small intestine. The effect of the RPMet product on the free blood plasma methionine levels was determined after the product was delivered directly to the abomasum via the abomasal infusion technique (explained in detail in chapter 5) using a trans-ruminal abomasal terminating infusion tube (Spires *et al*, 1975). In light of a similar study done by Robinson *et al*. (1999) a discontinuous infusion of RPMet was decided upon in order to minimize the impact on animal performance and comfort and ensure accurately delivery of exact doses of the treatments. The effect of the liquid RPMet product on plasma Met levels were compared to that of Smartamine™ M and DL-Methionine. The three treatments each provided 40 g of potentially available methionine. For the three treatments 40.4 g DLMet, 63.3 g SMartM

and 246.46 ml of LRPMet were infused into the abomasum respectively. The RPMet products were infused into the abomasum as a pulse dose. Blood samples were taken at 0, 3, 7, 12 and 48 hours after abomasal infusion. The procedure was repeated on days 3 and 5 but with different products infused into each cow.

The cows received a lucerne based total mixed ration *ad libitum* and were milked three times daily.

4.2.4 Sampling Procedure: Experiment 2

Blood samples were taken via jugular veni-puncture into sterile tubes containing Li-heparin. The samples were placed on ice until centrifuged (1500 x g at 4˚C for 10 minutes) and the plasma were transferred into Ependorf tubes where after it was frozen at -20˚C. Samples were analysed for physiological methionine (free amino acids) at the Department of Biomedical Sciences; Tshwane University of Technology, Pretoria.

4.2.5 Blood Analyses

Blood samples were filtrated through a 10 000 molecular filter to remove the protein. The samples were handled as physiological samples and were thus not hydrolyzed. The instrument used to analyze for blood methionine was a Waters HPLC with two Model 510 pumps, UV detector Model 440, Auto sampler Model 710 and Waters Millennium 32 software. The PICO Tag method was used for the analysis as per: (Bindlingmeyer *et al.*, (1984).

4.3 Statistical Analyses

Data from both experiments 1 and 2 were analyzed statistically as a 3 x3 Latin square design with the GLM model (Statistical Analysis Systems, 2006) for the repeated measures analysis of variance to determine the significant difference between treatments, blocks and time periods. Means and standard error of the means (SEM) were calculated. The significance of difference (5 %) between means was determined by Fischer's protective test (Samuels, 1989).

4.4 Results and Discussion

4.4.1 Experiment 1

According to availability of Met from product specification data the three Met products each supplied 40 g of potentially available methionine. The base plasma Met levels were similar for the three treatments. The SMartM treatment increased the blood plasma Met levels significantly by > 81 µmol / l within12 hours after supplementation ($p < 0.01$) (Table 4.1). The DLMet and LRPMet products had no effect in elevating blood plasma Met after oral dosing ($p > 0.01$). SMartM differed significantly from the other treatments at 12 hours after supplementation ($p < 0.01$). The magnitude of the response in blood plasma Met levels for the SMartM in comparison to the other two treatments could possibly be explained by a difference in the kinetics of release of Met from the matrix in which it is imbedded (Sudekum *et al*., 2004). This does not relate to the DLMet treatment because it's not protected against ruminal degradation and was included in this experiment as a standard against which the LRPMet and SMartM products could be measured. At 48 hours

after supplementation the blood plasma values for all the treatments were similar to their basal Met values (Table 4.1).

Table 4.1 Blood Plasma Met Levels (µmol/l) at Different Intervals after Pulse Dosing 40 g Available Met

Time (hr)	[1]Treatments			SEM [E]	p value
	SMartM	**DLMet**	**LRPMet**		
0	**23.56** $_{1}$	**18.68**	**25.28**	3.23	0.800
12	**104.56** $^{a}{}_{2}$	**28.47** ^{b}	**25.87** ^{b}	7.61	0.078
48	**18.22** $_{1}$	**22.24**	**24.49**	1.88	0.510

[1] Treatments: Methionine deficient diet (Met-); Met- diet supplemented with liquid rumen protected methionine (LRPMet); Met- diet supplemented with Smartamine ™ M (SMartM); Met- diet supplemented with DL-Methionine (DLMet)
[ab] Row means with different superscripts differ ($p < 0.05$)
[12] Column means with different subscripts differ ($p < 0.01$)
[E] Standard error of least square means

Smartamine™ M is a well known rumen protected product whilst DL-Methionine is not protected against rumen degradation. The results on SMartM from this study are in agreement with results reported by Blum *et al*. (1999); Sudekum *et al*. (2004) and Graulet *et al*. (2005). The slight increase in blood plasma Met levels of cows supplemented with DLMet could be due to a fast outflow rate of the rumen causing some of the Met to be released into the abomasum before being degraded in the rumen. The LRPMet source was a commercial product designed to be chemically protected against rumen degradation and available in the small intestine for absorption. There was however no increase in blood plasma Met levels after LRPMet supplementation suggesting that the product was either not protected against rumen degradation or not available for absorption in the small intestine (Figure 4.1).

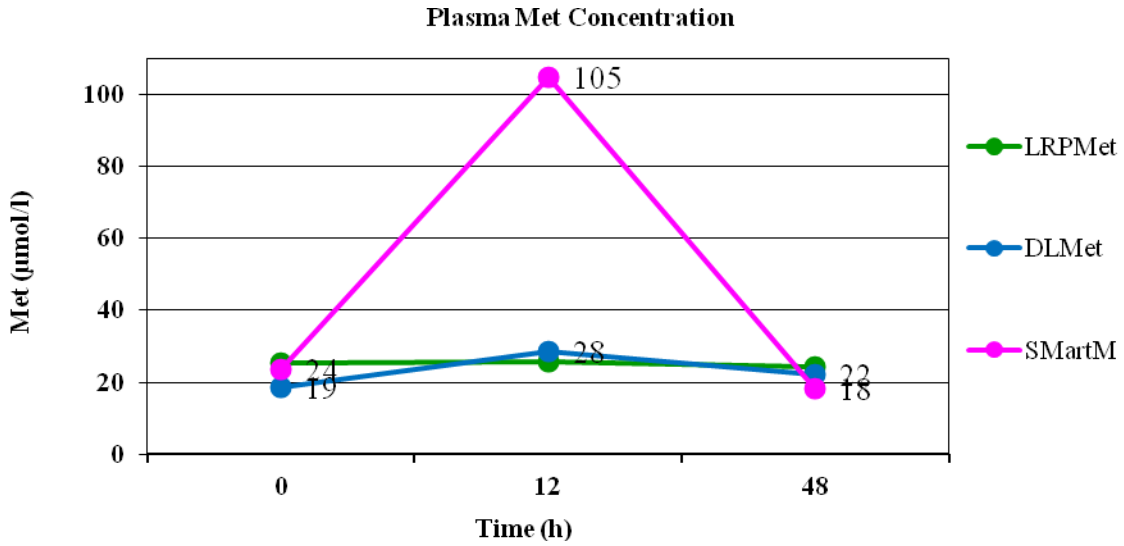

Figure 4.1 Plasma Methionine Concentration after Oral Supplementation of Met

Evaluating this LRPMet product through abomasal infusion would determine whether the product is available for absorption in the small intestine. The blood plasma Met levels of the SMartM treated cows peaked at 12 hours after oral administration. The peak in Met levels could have been earlier which require the measuring of blood plasma Met levels at more regular intervals between 0 and 12 hrs after supplementation. Bach & Stern (2000) concluded the faster the rate of ruminal degradation of a RPMet source the faster the peak plasma Met levels will be achieved. Their study showed a slow release RPMet source to induce peak plasma levels 12 hrs after dosing whereas moderately release RPMet had peak plasma levels between 6 and 12 hrs post dosing. It is thus unlikely that during this experiment the peak plasma level of any of the treatments were missed. It could be argued that the LRPMet product had a different method of protection and could therefore perhaps not be detected in the blood plasma as free Met, but instead as Met sulfoxide. This possibility was ruled out by the screening done on the six cows before the onset of the trial

feeding cows a pulse dose of LRPMET and analyzing blood for free Met as well as Met sulfoxide which delivered the same results (Figure 4.2).

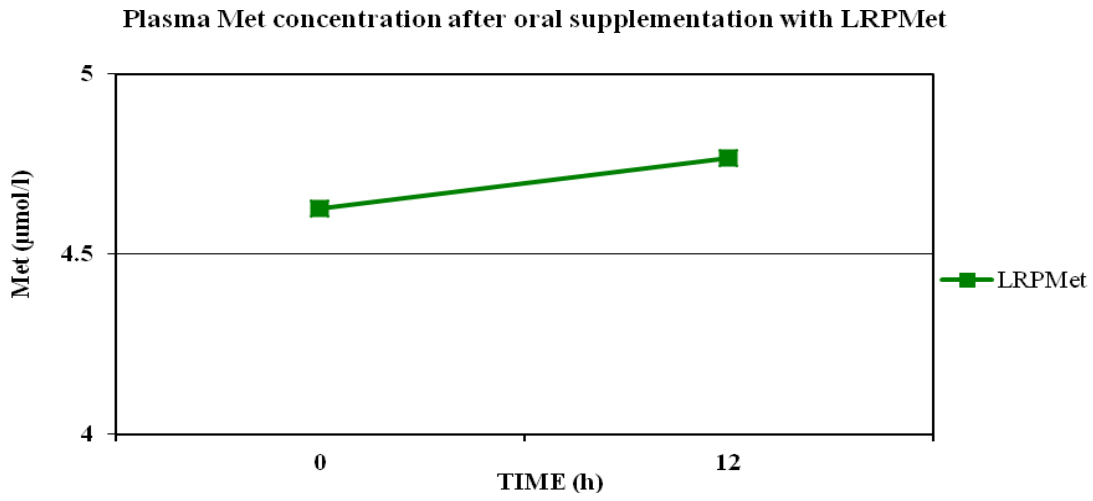

Figure 4.2 Plasma Methionine Concentration after Oral Supplementation of LRPMet as a Pulse Dose

Papas *et al.* (1984) stated 3 possible reasons for failure of RPMet to increase plasma Met concentrations: Incomplete ruminal protection, incomplete release for absorption in SI or inadequate supplementation of the RPMet. Failure of the LRPMet to increase plasma Met indicated incomplete release for absorption. Failure to increase milk production or affect any milk composition parameter indicated incomplete rumen protection. Providing an insufficient amount of RPMet to induce a response is unlikely since the SMartM treatment and the DLMet treatment provided the same amount of Met and both induced an elevated plasma Met concentration.

4.4.2 Experiment 2

The three RPMet products were infused as a pulse dose providing 40 g of potentially available Met into the rumen. The abomasal infusion technique was adapted (as described in chapter 5) and used to infuse the RPMet sources into the abomasum. The SMartM and DLMet treatments caused increases in the plasma Met levels at 3, 7 and 12 hours after infusion compared to the basal Met levels ($p < 0.01$) (Table 4.2). Both SMartM and DLMet differed significantly from the LRPMet treatment at 3 hours after infusion ($p < 0.05$). The LRPMet had a similar value of 32 µmol/l within 3 hours post infusion to the basal value of 23 µmol/l ($p > 0.01$). The SMartM and DLMet levels peaked at 3 hours after infusion but had relatively high plasma Met levels at 7 hours after infusion that differed from the basal values. There was a sharp decline in the plasma Met levels in both the SMartM and DLMet treatments at 12 hours after infusion and at 48 hours after infusion all the plasma Met levels have returned to their basal values before RPMet infusion (Figure 4.3). The Met levels of the LRPMet infused cows remained constant throughout the 48 hour period, suggesting that this liquid RPMet product was not available for absorption in the small intestine. These results suggest that the standardized blood assay on cows using samples obtained from the jugular vein at 0, 3, 7, 12 and 48 hours after post ruminal infusion with RPMet was used successful in assessing the qualitative response to post ruminal infusion of RPMet sources. Yang *et al.* (2010) also reported elevated blood Met levels when administering RPMet with a decrease in other AA recorded accompanied with an increase in milk yield, proving Met to be first limiting.

Table 4.2 Blood Plasma Met Levels (µmol/l) at Different Intervals after Abomasal Infusion of 40 g of Available Met.

	[1]Treatments				
Time (hr)	**SMartM**	**DLMet**	**LRPMet**	SEM [E]	p value
0	**26.30** $_{1}$	**31.55** $_{1}$	**22.99**	2.66	0.325
3	**542.36** $^{a}{}_{2}$	**442.43** $^{b}{}_{2}$	**32.06** ^{c}	9.75	0.004
7	**304.04** $^{a}{}_{3}$	**268.77** $^{a}{}_{3}$	**26.61** ^{b}	10.49	0.014
12	**166.76** $^{a}{}_{4}$	**129.69** $^{a}{}_{4}$	**21.92** ^{b}	11.68	0.068
48	**26.23** $_{1}$	**20.93** $_{1}$	**22.41**	1.65	0.384

[1] Treatments: Methionine deficient diet (Met-); Met- diet supplemented with liquid rumen protected methionine (LRPMet); Met- diet supplemented with Smartamine ™ M (SMartM); Met- diet supplemented with DL-Methionine (DLMet)
^{abc} Row means with different superscript differ ($p < 0.05$)
$_{1234}$ Column means with different subscript differ ($p < 0.01$)
[E] Standard error of least square means

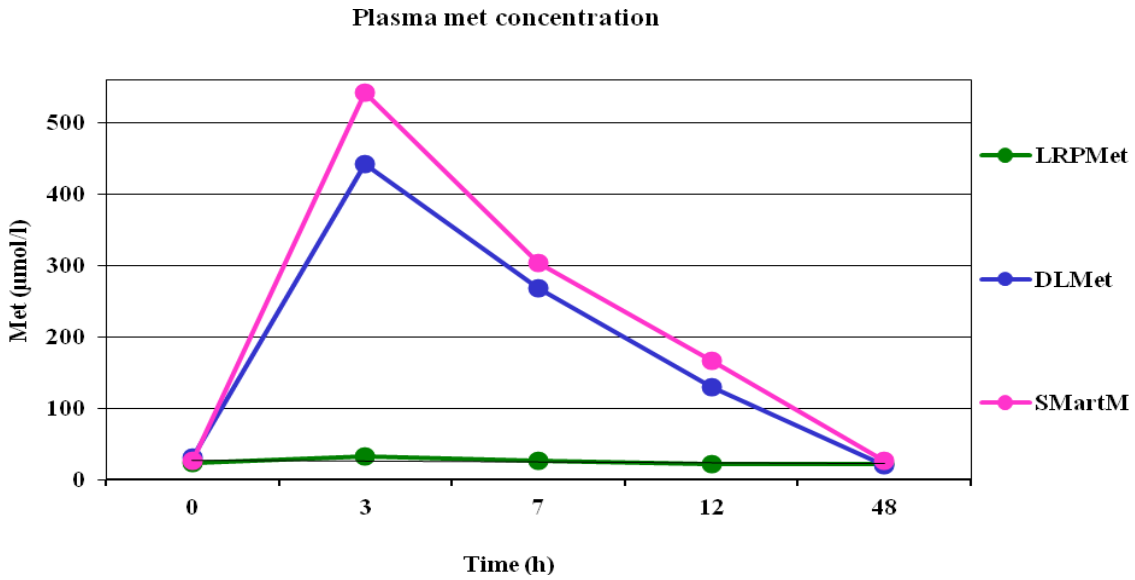

Figure 4.3 Plasma Methionine concentration after abomasal infusion of Met

Dia (2007) confirmed the level of free Met in blood serum to be positively related to the level of Met available in the SI. Therefore measuring blood Met is an accurate indication

of absorption of this AA. Both Overton *et al*. (1996) and Blum *et al*. (1999) reported increased blood Met levels when feeding RPMet. Blum *et al*. (1999) showed elevated sulphur AA levels when feeding RPMet sources coated with a pH- sensitive polymer as opposed to an increase in plasma Met levels when feeding fat coated RPMet prototypes. During the blood trials only Met were measured, but it would be advisable for future studies to analyze for the entire AA pool in the blood since the concentration of blood plasma AA reflects the supply of AA from the SI as well as endogenous synthesis as opposed to the demand for protein synthesis and degradative metabolism (Harper *et al*., 1984). When Met in the diet exceeds the animal requirement RPMet has been shown not to decrease the level of other plasma AA's. Blood plasma concentration is an accurate and sensitive measurement of the postruminal delivery of RPMet (Yang *et al*., 2010).

Quantification of the exact amount of Met delivered is difficult since the concentration of AA remains constant or increase steadily until tissue demands are met. In monogastric animals inflection points of blood response curves defining the points of change in the rate of accumulation in AA has been used to estimate AA requirements. This is not done currently for ruminants. In order to draw final conclusions as to the efficacy of any RPMet source numerous measurements, i.e. production responses, blood plasma levels etc. should be used before drawing any final conclusions as to the bioavailability of a RPMet product. Kröber *et al*. (2001) indicated that changes in other AA levels could affect the efficacy of the supplemented AA (in this case RPMet) due to antagonistic interactions, therefore the need to not only supply the limiting AA but also other AA's. Trinacity *et al*. (2009), however, reported no interaction or change in other AA levels when supplementing RPMet.

Judging by the meta-analyses of a large number of RPMet studies by Patton, (2010) one cannot unequivocally conclude from this study only that the LRPMet prototype is not available for absorption in the small intestine. Blood plasma values are an indication of the inertness or level of protection of RPMet sources (Bach & Stern, 2000). It is possible that blood values reflect the rate of release of the RPMet and not the level of protection and Patton, (2010) proposed that as DLMet release approaches utilization, blood levels might even stay constant. If this was applicable to the LRPMet source one would, however, expect a production response or at least a response in the milk protein fraction composition. This was not the case which led us to the conclusion that the LRPMet is not available for absorption in the SI. It might be overprotected to such an extent that the pH in the abomasum does not promote release of the DLMet. An interesting observation by Vanhatalo *et al.* (1999) was that infusion of Met restricted blood flow which could explain the lack of response in milk production during experiment 1. This is mainly due to the intermediate of Met metabolism, taurine that suppresses blood vessels.

An alternative method to evaluate efficacy of RPMet to deliver Met has been evaluated by Weiss & St-Pierre (2009). Using selenium as a tracer of Met, cows are given a flat rate of selenium – methionine yeast and RPMet is then supplemented. Dilution of Met in milk is used as indication of the crossover of the RPMet into milk. This method could be a more cost effective and less invasive technique to evaluate availability of RPMet sources.

Figure 4.4 Blood Collection from Jugular Vein of Cows after Post Ruminal Infusion of Met

4.5 Conclusion

Koenig & Rode, (2001) reported that measuring the increase in plasma Met relative to the increase of a known quantity of RPMet delivered into the duodenum is a sensitive indicator of the bioavailability of RPMet assuming no change in tissue utilization of Met induced by the amount of the RPMet dose administered. The LRPMet prototype used in this study was evaluated by means of both oral and post ruminal supplementation through its effect on blood plasma Met levels using a standardized blood test. The LRPMet were compared to DLMet which is not protected against rumen degradation and rumen protected SMartM. After orally supplementing the RPMet sources only SMartM caused a significant increase in blood plasma Met levels. After infusing the Met products into the abomasums, cows supplemented with DLMet and SMartM had significantly higher plasma Met levels ($p < 0.01$). The LRPMet product thus had no effect at all. The use of blood plasma Met levels as a technique to evaluate the efficiency of a RPMet product in improving the AA balance in the MP proved to be a reliable, sensitive and sophisticated technique that can be used with success to establish the relative bioavailability of RPMet products, including liquid products as in this study.

CHAPER 5

A RESEARCH NOTE ON THE ABOMASAL INFUSION TECHNIQUE

5.1 Introduction

Abomasal and duodenal fistulas have been used in cattle and sheep as infusion and digesta sampling sites. Surgical procedures to fit abomasal and duodenal cannulas are more complicated than rumen cannulation. When fitted with abomasal cannulas (especially re-entrant cannulas) animal recovery times are longer and subjected to more complications like poor appetite and general unthriftness (Wenham & Wyburn, 1980). Ruminally cannulated animals require less invasive surgery and are easier and less expensive to maintain, making it more cost effective to use a larger number of ruminally cannulated animals in experiments thereby increasing the possibility of detecting treatment differences.

With the newfound interest in the research field of amino acid nutrition of dairy cows, the abomasal infusion technique is used frequently for post ruminal infusions. Unfortunately there is no proper description of this technique which details all the major and minor aspects to perform this procedure. Gressley *et al.* (2006) described the dimensions of a post ruminal infusion line and d flange but did not describe the physical insertion of the flange into the abomasum in detail. The earliest description we could find on the abomasal infusion technique was in a study done by Spires *et al.* (1975). They infused sodium caseinate continuously into the abomasums of rumen-fistulated Holstein cows, passing a 6 mm polyethylene tube through the rumen cannula and sulcus omasi.

A 60 ml perforated polyethylene bottle was fixed to the end of the tube to prevent retraction from the abomasum. Solutions were infused via a peristaltic pump.

Huthanen *et al*. (1997) gave a detailed description of the omasal sampling technique and the equipment used to collect digesta samples from the omasum. Their sampling device was moulded from polyvinyl chloride and consisted of a round plate (6 cm i.d x 1.5 cm thick) with a perpendicular ring (15 cm o.d. x 12 cm i.d.) fused to the edge of the plate. Two polyvinylchloride (PVC) rods (1.6 cm i.d.) were placed in front of the opening to the sampling tube (1.2 m long by 1.6 cm o.d. and 0.95 cm i.d.) to prevent the omasal leaves from blocking the tube. The sampling device was inserted into the omasum compressing the ring, which was released once it was in place in the omasum. Knowlton *et al*. (1998) inserted a 6.35 mm i.d tube through the rumen cannula into the abomasum held in place by a 15 cm i.d. disk to abomasally infuse a starch solution.

Varvikko *et al.* (1999) adopted the abomasal infusion technique to achieve data on the lactation responses of supplying Met and Lys postruminally. The cows were fitted with rumen cannulas and infusion catheters (0.2 mm plastic tube) were installed via the rumen cannula and reticulo-omasal orifice into the abomasum. A peristaltic pump was attached for continuous infusions. Robinson *et al*. (1999) used discontinuous manual infusion to infuse various amino acids into the abomasum via a transruminal infusion tube terminating in the abomasum. They selected discontinuous manual infusion because it ensures precise daily delivery of amino acids and renders the use of continuous infusion lines and pumps which interfere with normal daily animal activity unnecessary. Mackle *et al*. (2003) delivered conjugated linoleic acid solutions postruminally via polyvinyl chloride tubing, using peristaltic pumps to deliver the infusate continuously. Kay *et al, (2002)* used the

same abomasal infusion technique to deliver a sterulic oil emulsion postruminally and secured the polyvinyl chloride tube with a 10 cm rubber flange in the abomasum.

In 2004 Prof. P.H. Robinson (Department Animal Science, University of California, Davis, CA) supplied the author through personal correspondence with information on an abomasal infusion technique (Robinson *et al*., 1999). Initially we used his technique to infuse liquid rumen protected amino acids into the abomasum. After experimenting with different methods of infusion and various dimensions of the apparatus used for abomasal infusion we adapted the technique for the abomasal infusion of liquid rumen protected amino acids and made some changes to the infusion apparatus.

5.2 Discussion

The cows used to administer rumen protected amino acids to (both liquid and solid sources) postruminally, were fitted with ruminal cannulas of 10.2 cm i.d. (Bar-Diamond, Parma, ID). The abomasal infusion device used to infuse liquid rumen protected amino acids discontinuously consisted of a 2.0 m long, 12 mm in diameter polyethylene infusion tube, a round flexible 120 mm in diameter plastic flange and polyvinyl chloride flange position maintainers (Figure 5.1).

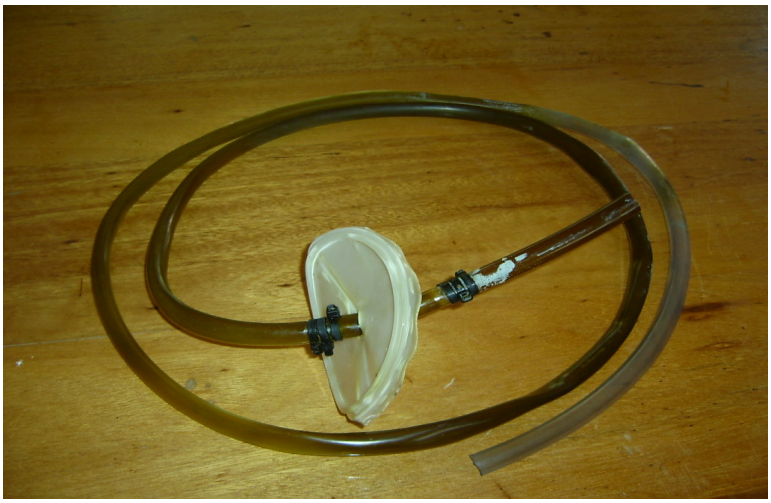

Figure 5.1 Abomasal Infusion Line

A small plastic bottle was cut open at the bottom and secured onto the end of the infusion tube to retain the apparatus in the abomasum however; the digesta accumulated in the bottle and blocked the tube. The flange is sufficient in preventing the infusion line from retracting from the abomasum. A perforated Dacron bag filled plastic bottle can be attached to the end of the infusion line when infusing liquids continuously.

An 8 mm in diameter rubber infusion tube was used at first, but the tube was too flexible and bended and blocked around corners when infusing solid sources especially when the animal‘s rumen was filled with feed. By using a larger 12 mm in diameter plastic tube (which is less flexible) it is easier to infuse large cows (650 kg +) without having to empty out the rumen which is time consuming and could interfere with normal digestion and ultimately abomasum infusion results. An acid resistant rubber flange can be used but the material is difficult to get hold of. After experimenting with various materials (most of which was degraded in the abomasum) the rubber flange was replaced with one made of Polyvinyl Chloride Plastic of 120 mm in diameter. A smaller flange of 100 mm in diameter came undone from the abomasums of the larger cows especially if the device was left in

the cows for more than a week. The flange was attached 190 mm from the end of the tube with PVC cable ties. The flange was secured with at least 3 large cable/plastic ties in front of it and 3 at the back to prevent it from sliding down the infusion tube during regular digestive tract contractions.

The great obstacle in applying this infusion technique is pushing the infusion apparatus together with one's hand through the reticulo-omasal orifice. This sphincter is a strong muscle and a lot of strength is needed from the researcher to open the omasal orifice with his fingers. When enough force is applied the orifice only relaxes after about 10 minutes but not sufficiently to go through without considerable strain. Once inside the omasum it is necessary to push through the omasal laminae, which provides a lot of resistance, towards the omaso-abomasal-orifice which fortunately opens easily and into the abomasum. Once you have succeeded in entering the omasum there is a lot of pressure on the hand and arm making it very difficult to continue pushing through towards the abomasum.

An attempt to solve the problem was made by injecting the animal intra-muscularly with an anti-spasmodic drug 15 minutes before placing the infusion line. There was a slight weakening in the contractions of the reticulo-omasal orifice but not sufficient for effortless entry. A further disadvantage of using the anti-spasmodic drug was that it weakened contractions throughout the digestive system over a period of 24 hours possibly affecting digestion and rumen outflow rate adversely. The problem was solved by injecting the animal with a local anaesthetic inside the rumen at the ventral border of the oesophagus as well as around the reticulo omasal orifice itself. The anaesthetic (Lignocaine 2 %; Bayer) was injected in individual doses of 0.5 ml each around the ventral border of the oesophagus to anaesthetise the branches of the vagus nerve located in that area, which is responsible for contraction of the digestive system. Relaxation of the reticulo-omasal sphincter was

increased by injecting small amounts of the anaesthetic directly into the muscle wall of the reticulo-omasal sphincter. The contractions of the omasal orifice weakened considerably after 5 minutes and the relaxation lasted for about 20 minutes after administering the drug thus imposing no adverse long term effect on digestion. A small syringe with a thin needle should be used to inject the drug. Any length of arm can be used for the injection because it is not necessary to reach deep down into the digestive system of the animal. It helps to first locate the oesophagus (it feels glandular and slimy) at the cranial dorsal wall of the rumen and then to pull the ruminal folds at the base of the oesophagus towards the caudal part of the rumen. It takes some exercise to locate the oesophagus and inject the drug with one hand. About 2 ml of anaesthetic (Lignocaine 2%) can then be injected around the border of the reticulo-omasal orifice. In total about 6 ml of anaesthetic was administered. Depending on the type of drug that has been injected the infusion device can then be inserted effortlessly about 5 minutes after administering the drug.

Some experience is needed to find the reticulo-omasal orifice the first time (it is a strong frequently contracting muscle which differs in diameter between cows) and it is advisable to locate the omasal orifice to familiarize oneself with its exact location before returning with the flange .Using a well gloved left arm reaching through the rumen cannula (which have been removed first) towards the cranial part of the rumen. Thereafter reach through the reticulo-rumen and locate the reticulo-omasal orifice. Once the orifice has been located one can return with the flange folded in the left hand. The orifice will easily open when pressure is applied with the two forefingers and the flange (still folded) can then gently be pushed through. Do not try to push the flange in a parallel line to the caudal part of the animal. Once inside the omasum which is easily recognised by the leaf - like laminae the flange should slowly be moved downwards to the ventral border of the omasum and then

slightly to the caudal part until it slides effortlessly into the abomasum which is lined with glandular tissue. The flange can then be opened and pushed around which feels like a bend. It is not possible to pull the opened flange out of the abomasum when it is positioned behind the curve. If it is a struggle to keep the flange folded and then to unfold the flange with one hand in the abomasum an elastic band can be used to roll the flange onto itself. Once inside the abomasum the band can be released and moved back up the infusion line to the outside. Caution should be taken not to leave the elastic band in the abomasum. When the flange is secured return to the rumen and make sure enough of the infusion line is present in the rumen (to prevent the apparatus retracting from the abomasum during digestive contractions) before running the infusion line through a punched hole in the cap of the ruminal cannula (not cut, otherwise the infusion line is folded). The end of the infusion line can then be attached to an infusion end of choice.

The infusion line should be filled with water immediately after placement (and after every infusion) to prevent it from blocking (Figure 5.2). It is recommended to check the position of the infusion apparatus at 48 hour intervals since some rations contains longer fibrous material which can wrap around the infusion line and force it from the abomasum. This device has been left in cows for 6 weeks without any adverts affects and upon removal the device was still perfectly intact.

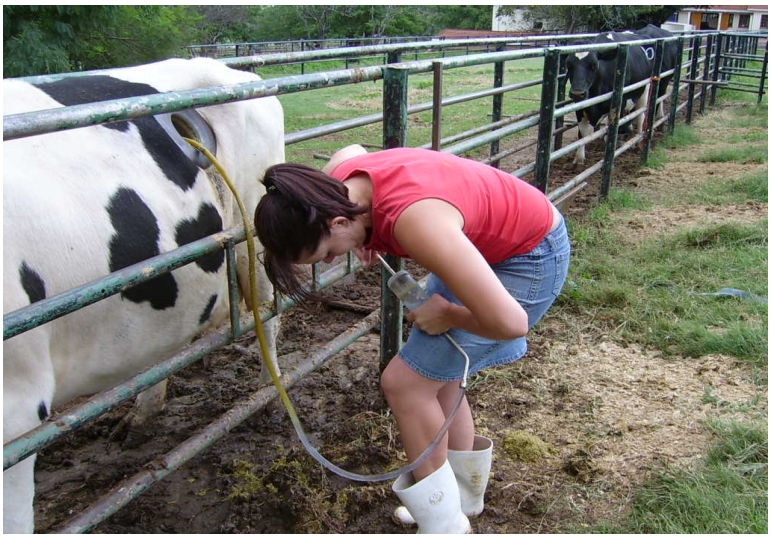

Figure 5.2 Filling the Infusion Line with Water after Placement

5.3 Conclusion

The abomasal infusion technique has been used for postruminal infusions since 1975, but only since 1997 has it been used frequently in especially amino acid infusion studies. This technique is much more cost effective than the use of intestinally cannulated animals (especially re-entrant cannulated animals) which is predisposed to longer recovery times and general unthriftness. With the newfound interest in amino acid and conjugated linoleic acid research the use of the abomasal infusion technique is essential for post ruminal infusions. However a detailed description of the abomasal infusion technique is not available in the literature. It is imperative for this technique to be accessible to all researchers. The purpose of this short paper is to describe the abomasal infusion technique in detail providing researchers with a step by step description of the placement of the abomasal infusion equipment without having to struggle through all the obstacles themselves.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

A liquid rumen protected methionine prototype was evaluated for ruminal stability and relative postruminal availability using two different techniques. These methods were selected based on the methods being suitable to evaluate a liquid product whilst remaining cost effective. Judging by results obtained from both the reference Met sources, protected and unprotected, and the LRPMet source these methods can successfully be applied in evaluation studies of RPMet sources or prototypes.

The milk composition technique was used to establish the effect of three Met treatments on milk yield and composition. Cows post peak production is expected to be responsive to RPMet supplementation in terms of milk protein composition (Schwab *et al*., 2001). None of the treatments positively affected milk yield ($p > 0.05$) which might be due to the fact that the cows were past peak production and in the declining phase of their lactation curve therefore experiencing a decline in the average milk production. Another reason for the non responsiveness of milk yield to Met supplementation during this study could be the fact that increased levels of Met restricts blood flow (Vanhatalo *et al*. (1999). This is mainly due to the intermediate of Met metabolism, taurine that constricts blood vessels. All treatments positively affected casein % ($p < 0.05$) during period 3, when compared to period 2, with SMartM differing from the Met- and DLMet treatments. Whey content decreased during period 3 but this change was only significant for the Met- treatment with the magnitude of change for SMartM being larger compared to that of the DLMet and Met- treatments ($p < 0.05$). A reduction in whey content was expected for the SMartM treatment too, however, there was an increase in whey content. The samples taken were probably too

few to draw a meaningful conclusion regarding milk protein fractions. Only the SMartM treatment positively affected milk protein and milk fat percentage ($p < 0.05$). The DLMet had no effect ($p > 0.05$) on milk composition as could be expected due to it not being protected against rumen degradation. The LRPMet failed to initiate any response in milk composition too. The LRPMet was thus either not sufficiently protected in the rumen and or not available for release in the abomasum.

The blood plasma technique was used in order to establish the reason for the LRPMet not initiating any response in terms of milk composition. Bioavailability of RPMet can be determined through measuring the response in blood Met levels to feeding RPMet (Rulquin & Kovalzcyk, 2000). The three Met treatments were thus subjected to an experiment measuring blood plasma Met in response to both oral dosing and postruminal infusion of the Met products. Cows supplemented with SMartM had a significant response ($p < 0.01$) in terms of elevated blood plasma Met after both oral dosing and postruminal infusion whilst cows supplemented with DLMet only expressed significantly higher blood Met levels after postruminal infusion ($p < 0.01$) compared to the cows receiving the LRPMet and SMartM treatments. These responses were to be expected due to the level of protection of the two sources. The LRPMet treatment, however, had no effect on blood plasma Met after either oral or postruminal infusion ($p > 0.01$). This method proved successful and very sensitive (also noted by Koenig & Rode, 2001) to determine or screen bioavailability of a RPMet relative to that of other products.

After evaluating the LRPMet prototype through both the milk composition and blood plasma techniques the primary reason for the LRPMet being ineffective in elevating blood plasma Met remains unclear. Judging by the meta-analyses of a large number of studies

done by Patton (2010) one cannot unequivocally conclude from only this study that the LRPMet prototype is not available for absorption in the SI.

Blood plasma values are an indication of the inertness or level of protection of RPMet sources (Bach & Stern, 2000). It is possible that blood values reflect the rate of release of the RPMet and not the level of protection. Patton (2010) proposed that as DLMet release approaches utilisation, blood Met levels might even stay constant. If this theory was applicable to the LRPMet source one would, however, expect a production response or at least a response in the milk protein concentration. This was not the case which strongly suggests that the LRPMet is not available for absorption in the SI. It might be overprotected to such an extent that the pH in the abomasum does not promote release of the DLMet.

An alternative method to evaluate efficacy of RPMet to deliver Met has been evaluated by Weis *et al*. (2009). Using selenium as a tracer of Met, cows are given a flat rate of selenium-methionine yeast and RPMet is then supplemented. Dilution of Met in milk is used as an indication of the crossover of the RPMet into milk. This method could be an alternative and less invasive technique to evaluate availability of RPMet sources.

In conclusion the LRPMet product evaluated proved to be either not adequately protected or overprotected from ruminal degradation leading to unavailability of Met in the SI. Both techniques used namely the milk composition technique and the blood plasma techniques proved to be simple and effective techniques to evaluate or screen different rumen protected amino acid sources or prototypes.

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