Stability of rumen protected nutrient supplements in lactating Jersey cows

By

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Chapter 3 - Materials & Methods

Declaration

I hereby affirm that this dissertation, for the degree MSc (Agric) Animal Science: Nutrition Science, at the University of Pretoria, South Africa is my own work and that it has not been submitted by myself for a degree at any other University.

M. Sakkers

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Summary/ Abstract

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Determination of rumen escape of rumen protected nutrients is needed to accurately assess the amount of nutrients that can be absorbed and utilised from the intestinal tract of dairy cows. This assessment allows more precise feeding of specific nutrients, thereby increasing metabolic efficiency and reducing production of animal wastes. The currently used method of choice to determine the rumen escape of rumen protected nutrients is ruminal *in situ* evaluation, which cannot measure actual rumen escape, as the experimentalist can only estimate the rate at which a rumen protected product (RPP) will exit the rumen. The two-part objective of the study was to use an *in vivo* dual liquid phase marker system to determine ruminal stability of RPP and to determine the stability of the three RPP, as well as determine the best *in sacco* incubation time to match the determined stability.

Four multiparous, ruminally cannulated, lactating Jersey cows [body weight 384 kg ± 28.0 kg, milk yield 24 ± 4.0 L, days in milk 69 ± 42 d, parity 4.5 ± 1.29 (mean ± standard deviation)] were used in a 4 x 4 Latin square design. Cows were assigned to one of four groups with one group being the control group and the other three each receiving a different RPP. The study was composed of four 14 d periods with a 10 d adaptation period before the start of the study to allow the cows to adjust to the individual stalls, diets and conditions. Days 1-6 of each period were for recovery/ rest, days 7-8 for an *in sacco* measurement to determine the stability of the RPP, days 8-11 for pH logging, and

days 11-14 to determine the *in vivo* stability of the RPP. Cows were fed *ad libitum* a common total mixed ration (TMR) composed of chopped lucerne hay, maize stover, maize meal, soybean oilcake, hominy chop, molasses, urea, rumen inert fat and a vitamin/mineral premix containing 180 g/kg crude protein, 317 g/kg neutral detergent fibre and 213 g/kg starch on a dry matter basis. Cows were fed twice daily at 2 kg above their daily voluntary feed intake level and kept in individual stalls of 6 x 6 m with wood chips on the floor as bedding. The three RPP were: RP Ascorbic Acid (A), RP Lysine (L) and RP Niacin (N). The RP A and RP N were both composed of 623 g/kg nutrient (Ascorbic acid/ Niacin respectively), 89 g/kg Co-EDTA and 288 g/kg fat matrix, with a measured specific gravity of 1.21. The RP Lysine was composed of 518.7 g/kg Lysine, 86.5 g/kg of Co-EDTA and 394.8 g/kg fat matrix, with a measured specific gravity of 1.21. The fat matrix used in all the RPP's was the same.

The method used in this study aimed to create an accurate quantitative value of true ruminal stability, which traditional methods lack. Stability of the RPP was measured as the proportion of the area under the curve from the ruminal clearance of Co (included in the RPP as Co-EDTA) relative to the clearance of the Cr (as free Cr-EDTA).

In sacco measures consisted of insertion of six Dacron bags into each treatment cow (i.e. A, L, N), with each Dacron bag containing 5 g of the relevant product and each cow receiving a different product. Two bags were collected after 12, 24 and 30 h of incubation and then weighed back to determine the stability of the RPP as well as disappearance of the RPP over the 30 h period. Ruminal pH logging occurred directly after and the pH loggers were left in the rumens for 48 h to measure pH every 10 min in each cow.

During *in vivo* measurements each cow was dosed simultaneously with 150 g of the relevant RPP (calculated to contain 15g Co-EDTA) as well as 16.679 g of Cr-EDTA (Control group was dosed with 16.679 g Cr-EDTA and 15 g Co-EDTA) calculated to deliver 2.4 g of Co and 2.4 g of Cr respectively, into the rumen of each cow. Pre-dosing rumen fluid samples were collected and samples were then collected, starting one hour post dosing, every 2 h through 25 h post-dosing, then every 4 h until 49 h post dosing, and thereafter every 6 h until 73 h post dosing. These samples were analyzed for Co, Cr and pH. Samples were also collected every 6 h throughout the 74 h test period for nitrogen ammonia and volatile fatty acid analysis.

Rumen pH was within normal ranges and showed normal diurnal variations during sampling. Ruminal pH was unaffected by *in vivo* treatment and averaged 5.88, with a diurnal variation between 5.65 and 6.40. Animal performance was unaffected by treatment with average milk production of 24.6 L/ day, milk fat of 41.8 g/ kg and milk crude protein of 35.6 g/ kg. The rumen stability of the RPP differed, despite having the same fat matrix, presumably due to differences in the chemical interactions of the nutrients with the fat cover; for example lysine is known to be more reactive. The rumen stability of RP Niacin was the highest ($p = 0.06$) at 66.7% relative to RP Lysine at 55.0%, with RP Ascorbic acid at 58.7%. *In sacco* incubations of the RPP showed variation in results. This *in vivo* method can be utilised to quantitate rumen stability of RPP, although it can not indicate the most appropriate rumen *in sacco* incubation time to reflect that measurement.

Keywords: ruminal stability, fluid marker, clearance curve

List of Abbreviations

AA	Amino Acid
AAS	Atomic Absorption Spectrophotometry
ADF	Acid Detergent Fibre
AUC	Area Under Curve
BCS	Body Condition Score
ΔBCS	Change in Body Condition Score
BW	Body Weight
ΔBW	Change in Body Weight
Co-EDTA	Cobalt-EDTA
Cr-EDTA	Chromium-EDTA
CP	Crude Protein
DDDH$_2$0	Double Distilled De-ionised Water
dH$_2$O	Distilled water
DIM	Days In Milk
DM	Dry Matter
DMI	Dry Matter Intake
dNDF$_{30h}$	Digestible NDF after 30 h in vitro incubation
EDTA	Ethylene Diamine Tetra Acetic Acid
EE	Ether Extract
EP	Experimental Period
FA	Fatty Acid
FPM	Fluid Phase Marker
GE	Gross Energy
GIT	Gastrointestinal Tract
GLM	General Linear Model of SAS
*iv*OMD	*in vitro* Organic Matter Digestibility
LCFA	Long Chain Fatty Acid
ME	Metabolisable Energy
MF	Milk Fat
MFP	Milk Fat Proportion
NDF	Neutral Detergent Fibre
NH$_3$-N	Ammonia Nitrogen
OBP	Onderstepoort Biological Products
OM	Organic Matter
RH	Relative Humidity
RP A	Rumen Protected Ascorbic Acid
RP L	Rumen Protected Lysine
RP N	Rumen Protected Niacin
RPP	Rumen Protected Product

SCC	Somatic Cell Count
SG	Specific Gravity
SEM	Standard Error of the Mean
TMR	Total Mixed Ration
VFA	Volatile Fatty Acids
WOL	Weeks of Lactation

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Chapter 1: Introduction

1.1 Background Information

The FAO (2009a) reported that the world population is anticipated to reach 9.1 billion by the year 2050. This 34% increase in global human population will require a 70% increase in global food production due to the increase in living standards in many parts of the world (FAO, 2009a). The resultant increase in global livestock production and development of systems to attain the required production, has become known as the "Livestock Revolution" (Delgado, 1999). As urbanization continues, the increased food requirement may result in direct competition for cereal production between humans and livestock, as well as for land and water resources. Livestock product consumption is steadily increasing, with global milk consumption doubling since 1960. Developing countries such as China and Brazil have undergone increases in milk consumption of 1000% and 40% respectively since 1980 (FAO, 2009b). In order to reach future target production levels, livestock production will have to increase in terms of numbers of animals, production per animal and overall efficiency of production. Current dairy production has shifted away from low input grazing systems with low outputs, to high concentrate intensive feeding systems using total mixed rations and state-of-the-art farming practices in order to maximise production, increase efficiency of nutrient utilisation and ensure more precise feeding to support high animal production levels (Capper *et al*, 2009).

According to Steinfeld *et al* (2006), 26% of the earth's ice-free land is currently exploited by grazing systems. In a recent study it was found that 15 out of 24 investigated ecosystems were experiencing degradation from soil erosion, deforestation, desertification and/ or salinisation (Pitesky *et al*, 2009). The United Nations Environmental Program (UNEP) has estimated that 35% of global land area is at risk of desertification. Although it is difficult to determine to what extent livestock farming is responsible for soil damage and lack of renewal of soil reserves due to overgrazing, it is known that grazed regions have much more soil damage than non-grazed regions (Pitesky *et al*, 2009). In order to supply growing population demands for food, it will be necessary to protect natural resources and ensure that systems are put into place to allow for growth in agricultural production to meet growing demands and reduce damage to natural resources (Tamminga, 1991; De Haan & Steinfeld, 2008). This will require intensification of livestock production systems, but with an increased awareness on sustainable production practices. In order to achieve

intensification and reduce production of animal wastes, precision feeding will be required to ensure the most efficient use of feed resources, by meeting exact nutrient requirements of livestock.

Diets formulated to meet animal production requirements are designed to deliver each required nutrient to exact requirements, for maximum production and lowest waste production. Many feed ingredients contain limited quantities of key nutrients required for production (NRC, 2001). Use of precision feeding and development of specific nutrient supplements has allowed more accurate diet formulation. There are many aspects of this field of interest, including different raw ingredient processing methods to improve digestibility and nutrient availability (such as micronisation and extrusion), feeding specific dietary components, which may be in short supply within the diet (such as specific amino acids or vitamins essential for production), as well as modulators of rumen fermentation (such as pH stabilisation products, buffers, yeast products, essential oils, ionophores, probiotics and enzymes) to control the digestive environment to ensure its' optimal functionality. Using supplemental feed ingredients has become increasingly popular, as these products have often shown proof of efficacy and economic returns for farmers, making them feasible and practical for use.

Development of rumen protected products (RPP) using fat encapsulation or coatings to deliver limiting nutrients post-ruminally, where absorption occurs and rumen microbes will not degrade the nutrients, has become an option for decreasing methane/ waste excretion due to feeding excess nutrients in the diet. Rumen protected nutrients, such as vitamins and amino acids, can correct nutrient gaps in diets and feed ingredients by allowing addition of specific nutrients instead of raw ingredients, such as grains, which supply a range of nutrients, but with some in excess and others in deficit.

The diet consumed by an animal and the level of each nutrient within that diet, is not the same as the nutrients reaching the intestinal absorptive site, or the amount of nutrients digested and absorbed as it cannot be assumed that the diet fed and the nutrient profile reaching the small intestines will precisely meet the nutrient requirements of the animal (France *et al*, 2000). It is therefore important to determine the levels of nutrients entering the small intestine in order to determine the true level of nutrients available for metabolism. With the use of rumen protected nutrients in a precise feeding system it is important to

determine the actual amount of the nutrient in the product escaping ruminal degradation and entering the small intestines.

Supplementation of high producing dairy cows with ruminally protected amino acids has been researched extensively, with some beneficial effects. However vitamin requirements have largely not been investigated for over 50 years. Research is scarce and outdated, with modern dairy cows receiving different diets and producing far higher levels of milk than those during previous studies, which may increase requirements above levels synthesised (Santschi *et al*, 2005). Most supplemental vitamins and amino acids have been shown to be extensively degraded within the rumen, making supplementation difficult, although supplementing as rumen protected products can solve this problem (Zimbelman *et al*, 2010).

Ascorbic acid (Vitamin C) is not considered an essential vitamin in ruminants, as it is synthesised within the animal, although the mechanism and site of this synthesis is unknown. It is an antioxidant involved in immune function as a component of leukocytes (Weiss & Ferreira, 2006; Thurnham, 1997) and metabolism. Studies using ascorbic acid have focused on milk and blood concentrations of ascorbic acid after dosing either orally, via fistula or intravenously. The only studies shown to increase blood and milk ascorbic acid concentrations are those where intravenous ascorbic acid was dosed. It has been shown in a number of studies that ascorbic acid is degraded within the rumen (Vavich *et al,* 1945; Knight e*t al*, 1941). With new insights being shown on potential health benefits of ascorbic acid in humans, there is a need for up-to-date research on this vitamin, utilising a form that will not be degraded within the rumen.

Niacin (Vitamin B3) is another vitamin not considered essential (and no daily requirement has been determined), although currently there is research into potential benefits to dairy cows, particularly during heat stress (Yuan *et al*, 2011; Zimbelman *et al*, 2010; Di Constanzo *et al*, 1997), ketosis and fatty liver syndrome (Weiss & Ferreira, 2006). Niacin is involved in energy metabolism, particularly as a co-enzyme in NAD/NADP pathways. Niacin has been shown to increase skin vasodilation and heat dissipation (Di Constanzo *et al*, 1997). Niacin is virtually completely degraded (98.5%) within the rumen as shown by Santschi *et al* (2004), making rumen protected forms needed to establish effects of supplementation (Weiss & Ferreira, 2006).

Lysine is an essential amino acid considered to be deficient in many diets and feed ingredients for high producing dairy cows (Polan *et al*, 1991; NRC, 2001). Lysine is involved in milk protein synthesis and thus considered essential to high milk production (King *et al*, 1991).

With rumen microbes degrading proteins and amino acids within the rumen, it is necessary to supplement amino acids in a rumen protected form in order to supply high quality amino acids to the intestines and reduce wastage of amino acids (Robinson *et al*, 1995). It is thus necessary to more accurately determine the delivery of nutrients post ruminally.

There is a need for reliable and inexpensive RPP to fill the nutrient gaps in animal diets to ensure optimal and precise ration formulation. This will ensure animal waste products are minimised and that the best possible diet is obtained for sustainable livestock production.

1.2 Problem Statement

In order to enhance delivery of nutrients within the digestive tract, it is necessary to determine the proportion of RPP delivered to the intestinal tract from the rumen (i.e. the "payload"). This can only be estimated using an estimated fractional passage rate (K_p) divided by the *in sacco* measurement of the fractional digestion rate (K_d), to predict ruminal escape of a RPP. Thus evaluating ruminal escape with this method does not provide an accurate measure of the payload of a RPP, primarily because there are so few objective criteria upon which to estimate K_p. A method to determine the RPP "payload" is needed in order to exactly meet dietary requirements for animal production and maintenance in a precision feeding system.

1.3 Thesis Statement

The aim of this work was to:

1. Determine the ruminal stability of the three RPP (ascorbic acid, lysine and niacin) using a novel *in vivo* dual fluid phase marker technique
2. Examine the *in vivo* method as a possible method for determining the "payload" of RPP
3. Establish the most relevant *in sacco* incubation time to predict stability as determined by the *in vivo* fluid marker technique.

1.4 Delineations & Limitations

This study did not deal with effects of RPP under practical feeding conditions and did not assess the RPP under these conditions, as the RPP were not dosed on a daily basis, but used to evaluate their ruminal stability using a single pulse dose. This study was conducted in ruminally cannulated Jersey cows in the Cape region of South Africa and therefore results from this study may not be relevant to other areas or breeds of cows.

1.5 Definition of Terms

The term 'rumen stability' used in this document refers to the degree to which a RPP is able to withstand degradation in the rumen over a specific period of time (its stability) within *in sacco* bags (the k_d). Estimated rumen escape of the RPP considers rumen stability, specific gravity (SG), particle size and physical stability, which are all used to estimate k_p. Combining k_d and k_p as: $k_p/(k_d+k_p)$ estimates ruminal escape of RPP with the *in sacco* procedure.

The term "payload" refers to the actual amount of a nutrient entering from the rumen into the small intestines. This term was used in place of "rumen escape" in order to account for the inclusion of rumen kinetics and distinguish between *in sacco* escape (normally used as rumen escape) and actual escape (the payload).

The term Experimental Period (EP) refers to a 14 day test period, where each of the four EPs are composed of six days recovery (no sampling, days 1 - 6), two days of *in sacco* sampling (days 7 & 8), three days of pH logging (days 8 (starting afternoon), 9, 10 and 11 (morning only) and four days of *in vivo* sampling (days 11 - 14).

1.6 Assumptions

Assumptions upon which this thesis is based are that:

1) The fluid marker Co-EDTA, included within the RPP, is assumed to be released within the rumen fluid (completely water soluble) and behave in a similar manner and rate as the nutrient under investigation.

2) The characteristics of the RPP are unchanged by the addition of Co-EDTA.

3) The results and conditions under which the study was conducted are situation specific and should not be assumed to be the same for all conditions, environments or animals.

1.7 Significance/ Rationale

This is the first study to provide actual RPP "payload" rather than estimates with *in sacco* approaches for three RPP. Determination of "payload" will allow for RPP with known payloads to be developed, to more accurately feed dairy cows for reduced waste and optimum performance.

1.8 Chapter Overviews

This document reviews literature regarding the knowledge and methods used in determining ruminal stability of products in Chapter 2. Chapter 3 provides specific explanations detailing the methods and the materials used in study procedures; starting with the farm, cows and management. Treatments, procedures and sampling methods used are described, followed by analyses and results. In Chapter 4 results are shown, starting with the descriptional data of the rumen and performance data of the cows, followed by *in sacco* results, then Cobalt and Chromium clearance curves from *in vivo* rumen samples and lastly calculations of stability. In Chapter 5 results are discussed and compared with other research. In the final chapter, Chapter 6, conclusions are drawn and explained, with recommendations for future research.

Chapter 2: Literature Review

2.1 Methods Used to Quantitatively Assess Feed Constituents

López (2005) states "For the quantitative description of digestive and metabolic processes, appropriate biological data are required and can be obtained using *in vivo*, *in situ* and *in vitro* methods." This forms the central triangle whereby animal data can be obtained. A brief overview of these methods and their advantages and disadvantages are displayed in Table 2.1 and *in vivo* and *in situ* methods will be discussed in detail within this chapter, with particular reference to *in vivo* methods and fluid phase markers.

Table 2.1 A Summary of the three branches of methods used to determine digestive and metabolic processes in ruminants (Modified from López (2005))

Method	Description	Accuracy	Advantages	Disadvantages
In Vivo	Determination of responses within the animal using cannulated animals	High	Most reliable, good to use as reference, high correlation with *in sacco* results, requires little equipment	Expensive, time-consuming, labour intensive, requires high level of feeds, not good with high numbers of different feed types, results are restricted to specific experimental conditions of trial, requires cannulated animals, high level of animal variation, possible unreliable markers, requires some replications for accuracy
In Situ	A biological model of responses within specific region of animal	Medium	Good agreement with *in vivo* responses, requires little equipment, and is fairly rapid	Variation between laboratories regarding methods used, bag pore sizes, different incubation sites within the rumen, aerobic contamination during collections, fill effect of bags within the rumen, loss of bags in rumen and limited number of samples as well as knotting of strings and bags making collections difficult, lack of standardisation
In Vitro	Simulation of *in vivo* environmental conditions in a laboratory	Low	Doesn't require fistulated animals, useful to rank different feed digestibilities, rapid and reproducible	High variation between inoculums used and laboratory procedures, enzymatic activity variations, does not replicate true ruminal environments and microbial populations or the biological process of digestion in the rumen, cannot assess rumen kinetics

2.2 Animal Based Techniques

2.2.1 In Vivo Methods to Determine Rumen Fluid Passage

In vivo methods are generally considered the best methods to describe nutrient digestibility, as they are performed under the most similar conditions to practical farming conditions and occur within animals. These methods are, however, prone to high variation, due to animal variation (Nocek, 1988) and unknown factors, making it necessary to perform replications in order to increase accuracy (Broderick & Cochran, 2000). The need for replications also makes this method the most expensive as it requires cannulated animals, large amounts of feed and time. There is also the need for ethical considerations to be adhered to, as animal welfare is a factor with this approach and can often restrict studies of this nature (Broderick & Cochran, 2000). Although this method is subject to large variation, it is still considered the standard against which other methods are compared (McDonald *et al*, 2002).

2.2.2 Digesta Flow Markers

Markers are used in animal nutrition in order to evaluate two objectives (MacRae, 1974):
1 – to measure the time taken for digesta to move from one point to another
2 – to measure digestibility, volume or 24 h flow rate of digesta

Some of the flow rate aspects which have been investigated are particulate flow (movement of undigested particles), solute flow (movement of dietary or microbial solutes) and microbial flow (movement of microbes). Flow markers fall into a number of functional categories based upon their ability to be used for different types of flow rate measurements. Categories of markers include water soluble flow markers, water insoluble flow markers, metabolic isotope markers, particulate bound markers, radioisotopes, metals and rare earth metals (Ellis & Lascano, 1980). Markers are also categorised into two types based upon whether or not they are added to the feed to be evaluated (external markers) or are intrinsically contained within the feeds (internal markers) (Van Soest, 1994). External markers include stains and dyes, plastic and rubber, metal oxides, rare earth and other metals/ metal chelates, isotopes and soluble markers. Internal markers include

lignin, acid insoluble ash and silica, indigestible NDF or ADF, chromogens, waxes, faecal nitrogen purines and diaminopimelic acid (DAPA) (Van Soest, 1994).

2.2.3 Properties of Digesta and Flow Markers

Markers should meet a number of criteria before being considered for use in experimental procedures and should encompass the following properties for effective use as markers (Kotb & Luckey, 1972):

1. Must be inert, with no toxic physiological or psychological effects
2. Be neither absorbed nor metabolised within the gastrointestinal tract (GIT)
3. Have no appreciable bulk
4. Mix intimately with and remain uniformly distributed in the digesta
5. Have no influence on GIT secretions, digestion, absorption, or normal motility
6. Have no effect on the microflora of the GIT
7. Have physico-chemical properties, readily discernible throughout the GIT, which allow ready, precise, quantitative measurement

Although no marker is able to completely fulfill all these requirements, the ideal is established in order to keep the goals of markers clear and achieve the best marker. Currently used markers tend to fall short of the ideal in some key aspects; generally adherence to unlabelled particles (marker migration), uneven labelling and some digestibility or disappearance within the rumen and/ or intestines (Owens & Hanson, 1992; Marais, 2000). Whilst not making the marker ideal, these shortcomings can generally be adjusted for, provided they are known. Liquid markers generally have fewer problems, and tend to have a much lower marker migration than particulate markers (Udén *et al,* 1980). Markers should thus be chosen with care, taking the specifics of the components to be assessed into account, as well as the inherent shortcomings of the marker, to ensure the best marker possible is chosen for the specific situation (Kotb & Luckey, 1972; MacRae, 1974; Marais, 2000).

2.2.4 Fluid Phase Markers

In the 1920's using dyes such as Anthroquinone violet as digesta fluid phase markers (FPM) was adopted (Kotb & Luckey, 1972). Analysis of these substances was difficult and unreliable as sieving faecal and rumen fluid samples often resulted in loss of markers depending on sieve size and so quantitative assessment of dyes was never achieved. Alternative, more accurate markers that were easily quantifiable and recoverable were the next step towards determining rate of passage in the digestive tract. This led to the development of other digesta fluid phase markers (FPM). Little further research was conducted until the 1980's to identify further FPM, compare FPM or determine the effectiveness of current FPM in estimating fluid passage in the digestive tract. It was determined by Teeter and Owens (1983) that all water soluble markers behave in a similar biological manner, although there is variability in results shown from some studies (e.g. Goodal & Kay, 1973). There have been no published studies to determine the effectiveness of a FPM combined into a nutrient pellet. Only one comparison of two FPM used simultaneously within the same environment to determine similarity of fluid passage out of the rumen was found, a study by Downes & McDonald (1964) where PEG and ^{51}Cr-EDTA were compared in sheep and found to be similar.

a. Polyethylene Glycol

Polyethylene Glycol (PEG) was first introduced as a digesta fluid phase marker (FPM) in ruminants by Sperber, Hydén & Ekman (1953). Polyethylene glycol was introduced as a marker at a molecular weight of 4000, with approximately 95% recovery of the dosage in faeces (Downes & McDonald, 1964). The PEG was found to be 92% available to fluid in the rumen (Goodal & Kay, 1973). It has been shown (Kay, 1969) that PEG concentrations are influenced by high tannin levels, by binding to them, as well as showing adsorption to particulate matter (Sutherland, 1962) and decreased recovery in rumen fluid for feeds with higher digestibility (Teeter *et al*, 1979). Analysis of PEG has been debated, as initially it was difficult to accurately assess the level of PEG in a sample with turbidimetry, which was shown to be highly variable (Hydén, 1955) and tended to lose measurability over time of storage of samples and standards. Determination of PEG in samples is labour intensive and lacks repeatability. It was thought that labelling PEG with H^+ (H-PEG) would overcome the difficulties of analysis, however it was found to adhere to particulate matter and give

highly variable and poor recovery (Neudoerffer *et al*, 1973). It was later shown by Teeter *et al* (1979) that an increase in feedstuff digestibility results in decreased recovery of PEG.

b. Chromium-EDTA

Chromium-EDTA was proposed as an alternative fluid phase marker to PEG by Downes & McDonald (1964). It was initially suggested that radioactive Cr be used to measure lower levels of Cr (Downes & McDonald, 1964), but it was later suggested by Binnerts *et al* (1968) that use of atomic absorption spectroscopy instead of radioactively labelling the Cr was a safer alternative, as well as being a rapid and accurate method of analysis. Cr-EDTA has the advantage over PEG of being 99% available to the fluid in the rumen (Goodal & Kay, 1973). Although Cr-EDTA shows some binding to particulate matter and partial digestion, at a dosage level of 10 g per cow this would be negligible within the volume of the rumen fluid, and would only be important at very low dosage levels (Teeter *et al*, 1983).

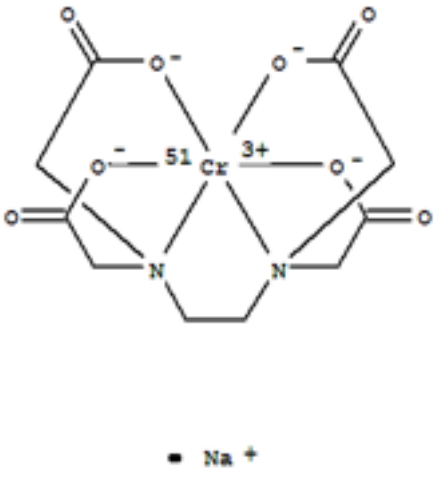

Figure 2.1 Chemical Structure of ^{51}Chromium-EDTA (Sodium Salt)

c. Cobalt-EDTA

An alternative marker to Cr-EDTA was suggested by Udén *et al* (1980) for use in combination with Cr-mordanted fibre, as Cr-EDTA and Cr-mordanted fibre could not be used together and, at the time, Cr mordanted fibre was the best particulate and Cr-EDTA the best fluid phase marker. Thus Co-EDTA allowed for the combination of a fluid (Co-EDTA) and particulate (Cr mordant) marker to determine rates of passage of liquids and particulates simultaneously.

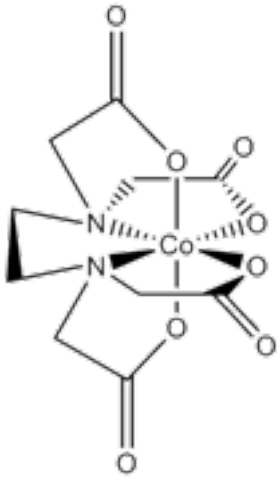

Figure 2.2 Chemical Structure of Co-EDTA

d. Co-EDTA vs. Cr-EDTA

The first comparison of Cr-EDTA and Co-EDTA was completed by Udén *et al* (1980), who determined that the two markers behaved in a biologically similar manner in the digestive tract of cows, horses, rabbits, goats and sheep. Both markers showed a 2 - 3% loss (recovered in urine) during comparison by Udén *et al* (1980). Some variability has been shown between Co-EDTA and Cr-EDTA results (Stern *et al*, 1983; Teeter & Owens, 1983).

e. Other Markers

Metal chelates of EDTA such as Fe and Yb have also been used and analysed, although in very few experiments. In a study by Teeter & Owens (1983) Fe-EDTA and Yb-EDTA were compared with other FPM and the findings were in agreement with each other. The use of either of these FPM has not been popular due to the high cost of these metal chelates in comparison with Co or Cr.

f. Advantages of *In Vivo* Techniques (Passage Rate Determination)

The major advantage of this method is that samples can be taken as frequently as desired, as rumen fluid samples are easily collected from the rumen of a cannulated animal to provide a detailed picture of rumen clearance rate and rumen movement of particles/ nutrients out of the rumen, by analysing the samples for marker concentration. It is also possible to apply this method within the digestive system and is thus considered to be the most accurate indicator of true ruminal conditions and events. In this manner, one is able to create a detailed explanation of passage within the rumen as well as determine flow rates and digesta kinetics.

g. Disadvantages of *In Vivo* Techniques (Passage Rate Determination)

In vivo methods have disadvantages which include the high expense, as it requires several cannulated animals, and requires large amounts of feed and time. Thus it is labour intensive to perform and researchers have to apply for ethical approval of the procedures to be used.

2.3 *In Situ* Methods to Measure Particle Disappearance Rate

In situ methods involve incubation of samples in the rumen of a living animal as opposed to simulated rumen conditions in a laboratory, as used for *in vitro* techniques (López, 2005). Feed samples are placed within porous bags and loss is measured from the bags after different periods of incubation (López, 2005). It is assumed that disappearance from the bags is equivalent to digestion within the rumen. This is a method currently in use to determine ruminal stability of nutrients using an estimation of rate of passage (Ørskov &

McDonald, 1979). In many studies using this method, the extent of digestion of nutrients in the bags is estimated by incubating them for the estimated mean retention time. However this cannot account for rumination, mastication or passage of particles out of the rumen as they are digested (Nocek, 1988) and will thus tend to overestimate digestion. *In situ* methods are unable to provide an accurate assessment of the events occurring within the rumen and gives no indication of flow rate or movement of particles out of the rumen. Owens & Hanson (1992) recorded a 12 – 32% overestimation of digestibility for passage rates of 2 – 10 % / h. Passage of particles from the rumen is not a uniform process and is selective, making its kinetic simulation more complex (Owens & Hanson, 1992).

a. Advantages of in situ techniques (particle disappearance)

These techniques provide relative results of rumen degradability of forages and proteins within the rumen and results generally correlate with *in vivo* results (i.e. it is a good qualitative assessment among feeds). It is also a rapid and reproducible method, with very little equipment required. *In situ* methods can be used to assess animal and dietary effects on ruminal conditions as well as microbial populations within the rumen. It is a good method to assess associative effects between forages and fermentable carbohydrates (López, 2005).

b. Disadvantages of in situ techniques (particle disappearance)

The biggest disadvantages to *in situ* techniques are the lack of methodology standardisation, the restriction on the number of samples that can be incubated and the unknown accuracy of the results. There is a limit to the number of bags that can be placed within the rumen and there is also the problem of entanglement, with bags and strings/ stockings becoming entwined within the rumen as the rumen compresses and contracts. The other disadvantage is the fill effect that bags may have upon feed intake during sampling. The assumption that loss from the bags is the same as digestion is ambiguous as pore size plays a large role in loss of matter and smaller particles may exit the pores, but are not digested (i.e. it is not a good quantitative assessment of feeds). There is also microbial contamination, as microbes attach within and onto the bags, thus contributing to the weight of matter in the residues. Another issue is that most feeds must be dried and/ or ground prior to incubation, thereby changing particle size and degradability. Thus there is no way to assess *in situ* accuracy of individual feeds in a diet.

2.4 *In Vitro* Methods to Measure Rumen Digestion

In vitro methods are laboratory based techniques that were introduced to provide a cheaper and quicker method for the evaluation of feeds (Tilley & Terry, 1963; Nocek, 1988). These methods involve the incubation of samples in simulated rumen conditions (i.e. within rumen liquor extracts or within simulated rumen solutions containing enzymes) for a set period of time (Tilley & Terry, 1963). Residues are then analysed and digestion determined from values obtained (Tilley & Terry, 1963).

a. Advantages of in vitro techniques (rumen digestion)

The use of simple, standardised solutions and methods make the technique fairly accurate for the ranking of feeds (Khan *et al*, 2003). It is a fairly quick method with well defined and controlled conditions (Slyter & Putnam, 1967).

b. Disadvantages of in vitro techniques (rumen digestion)

I*n vitro* methods are unable to simulate true rumen environmental conditions and cannot account for rumen kinetics and changes which occur within the rumen (i.e. pH changes, mastication, rumination, rate of flow, rumen fill). This method is also not very reproducible, as variations in rumen liquor extract (activity and composition) are high, due to variations in diets fed and animal variation (Khan *et al*, 2003). Another major problem with this technique is the microbial populations present, as protozoal populations in particular have been shown to differ with extracted liquor samples vs. *in vivo* samples. The use of enzymes for digestion often result in varying levels of enzymatic activity, which create variations in results (Nocek, 1988).

Chapter 3: Methods and Materials

3.1 Introduction

This chapter details the Research Design used for the experiment with respect to location, duration, animals used, housing, management, treatments and feeding methods. The Methodology section describes details pertaining to collection of samples and analytical methods implemented, as well as calculations and statistical methods employed. Samples were collected, prepared, stored and analysed according to the recommendations of Rutherford and Moughan (2000). Analyses were performed at the University of Pretoria, Nutrilab, which is accredited by AgriLASA. Milk samples were analysed by Lactolab, a SANAS accredited laboratory. This study was approved by the Animal Use and Care committee of the University of Pretoria and cows were at all times handled and managed according to recommended ethical practices (FASS, 2010). Rumen fistulas were checked before the start of the study for hardening and cracking, as well as for leakage. All fistulas not deemed acceptable were replaced with new ones (#1C, Bar Diamond, Inc., Parma, ID, USA).

3.2 Research Design

3.2.1 Farm Site & Location

The study was conducted at the Outeniqua Research Farm in George within the Western Cape region of South Africa. The farm is at an altitude of 201 m above sea level, and the GPS co-ordinates are 33^{o} 58’38” S, 22^{o} 25’16” E. The average minimum and maximum daily temperatures during the study were 12 and 20° C, respectively, with a daily average of 3 mm rainfall and relative humidity (RH) of about 78% during the experiment.

3.2.2 Duration of the Study

The study was conducted from 12 October to 11 December 2010. Experimental Period 1 was from 15 to 28 October, Experimental Period 2 from 29 October to 11 November, Experimental Period 3 from 12 November to 24 November and Experimental Period 4 was from 25 November until 10 December.

3.2.3 Cows

Four multiparous, lactating, rumen cannulated Jersey cows [BW 384 ± 28.0 kg, milk yield 24.0 ± 4.01 L, parity 4.5 ± 1.29, Days in Milk (DIM) 69 ± 42 d (mean ± SD)] were selected from the Outeniqua Research Farm herd. Cows were selected based on milk production, lactation number and DIM, and were all milked together twice daily and kept in individual camps. Cows wore light chains around their necks with coloured and labelled tags to indicate their camp and treatment. Control treatment was a white tag labelled C; Ascorbic acid treatment was a yellow tag labelled A; Lysine treatment was a pink tag labelled L; and Niacin was a blue tag labelled N. Tags were swopped after each experimental period. Details of each cow are in Appendix table A1.

3.2.4 Housing & Camp Layout

A calving camp situated close to the milking parlour (approximately 30 x 15 m) was converted into temporary individual pens. Half of the camp area was sectioned off and four steel pens were constructed to create four individual pens of 6 x 6 m for each cow. Cows were organized alphabetically (i.e. Bella137 – Pen 1, Firefly52 – Pen 2, Greta34 – Pen 3, Marta178 – Pen 4).

Cows were kept in their individual pens for the duration of the study and did not switch pens, to allow the fewest possible changes, with the size of the pens being sufficient for movement, eating and sleeping (recommended minimum sizes 2400 length x 1200 mm width (Lindeque, 2003)). Wooden chips were used as bedding material and refreshed as needed. Pens were cleaned daily and cows had *ad libitum* access to TMR and water at all times. Each pen was labelled with the cows name and ID number as well as the treatment.

Cows were placed in the pens at the start of the adaptation period following the afternoon milking as recommended by Lindeque (2003).

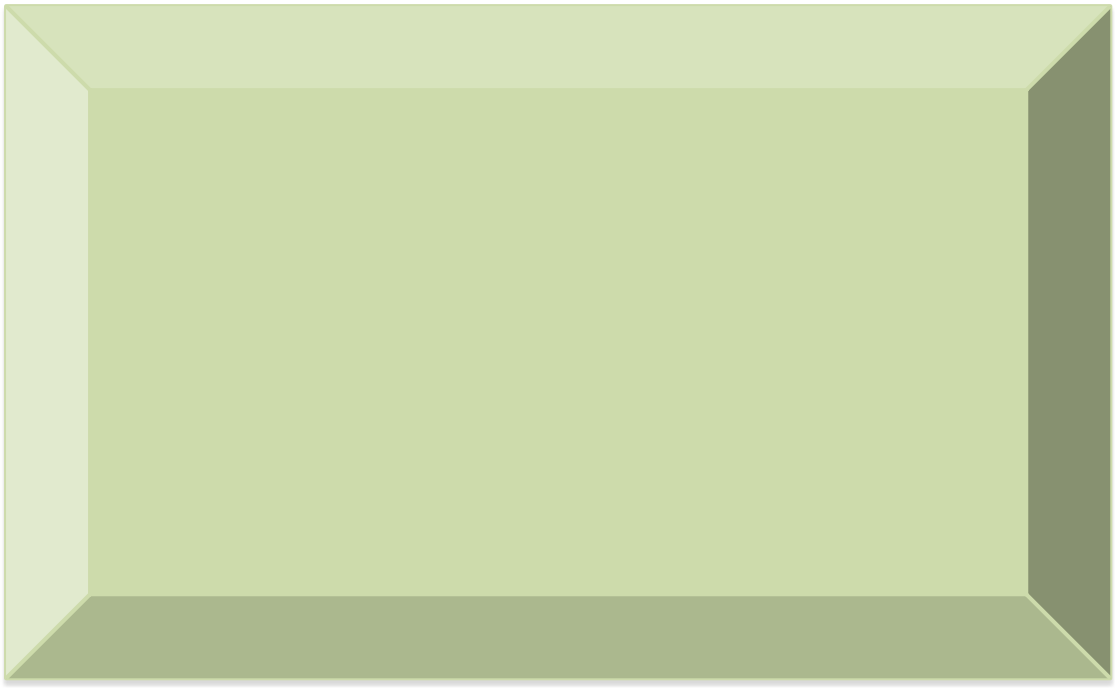

Figure 3.2.4.1 Layout of pens and cow placement

Figure 3.2.4.2 Construction of cow pens

Figure 3.2.4.3 Cow pens completed, showing the green feed bucket on the left and a green half drum automatic water trough on the right

3.2.5 Animal Management

3.2.5.1 Vaccinations

Cows were vaccinated against Lumpy Skin Disease on the 15th November 2010 after the afternoon milking (Onderstepoort Biological Products (OBP), Onderstepoort, SA), and against Rift Valley Fever (RVF) on the 1st December 2010 after the afternoon milking, with RVF Clone 13 vaccine, Batch number 9 (OBP, Onderstepoort, SA) as part of normal herd practice.

3.2.5.2 Artificial Insemination (AI) & Pregnancy Diagnosis (PD)

Greta34 was inseminated prior to the study on 22 August 2010 and was confirmed pregnant on 3 October 2010 (during Experimental Period 1).
Firefly52 was inseminated on 24 October 2010 during the study (Experimental Period 1).
Marta178 was inseminated on 13 November 2010 during the study (Experimental Period 3).
Bella137 was inseminated on 24 November 2010 during the study (Experimental Period 3).

Artificial Inseminations (AI) were conducted by one of the farm managers 6 h after oestrus was observed as cows were taken from their pens to the milking parlour. On all occasions oestrus was observed before the morning milking and AI was performed just before, or directly after, the afternoon milking.

3.2.5.3 Milking procedures

Cows were milked in a 20-point Dairy Master swing-over dairy unit with weigh-all electronic milk meters. Daily milk production was recorded on milk meters (Total Pipeline Industries, Heidelberg, SA) and then automatically uploaded onto the herd computer database. Mean milk production was calculated from the accumulated daily milk production during the 4 d of *in vivo* sampling during each experimental period (EP). Milk production data was monitored as an indication of any possible period, cow or treatment interactions. Cows were milked twice daily at 7:30 and 16:30 h.

3.2.5.4 Body Weight & Body Condition Score

Before commencement of the study and after each EP all cows were body condition scored (BCS) and body weight (BW) determined gravimetrically. Values were used as an aid in evaluating the general wellness of the cows.

Weighing was performed over two successive days, always after milking, to reduce daily variations in BW that occur due to water intake, milk removal, and excrement output. The two BW measurements were then averaged. Cows were weighed and body condition scored on 14 and 15 October 2010 (start of test run 1), 28 and 29 October (end of test run 1, start of test run 2), 11 and 12 November (end of test run 2, start of test run 3), on the 25 and 26 November (end of test run 3, start of test run 4), and on the 9 and 10 of December 2010 (end of test run 4).

Body condition score was determined on the second day of weighing, after milking. The BCS was evaluated by observation of cows and palpation of various bony regions (ribs and hind quarter). The BCS was then allocated based upon the amount of fat covering these bony regions between 1.00 and 5.00, where 1.00 is emaciated and 5.00 obese, with increments of 0.25 being used to describe the condition (Edmonson *et al*, 1989; Elanco Animal Health, 1996).

3.2.6 Experimental Treatments

The experimental design utilised a 4 x 4 Latin square with four 14 d periods (EP), in which the first 6 d were for adjustment/ recovery between periods with 2 d (day 7 & 8) for *in situ* incubation, 4 d for pH logging (days 8,9, 10 & 11) and the final 4 d for *in vivo* measurements (rumen fluid sample collections, days 11-14).

For *in situ* incubations, six nylon bags were placed in the rumen using a stocking method (Cruywagen, 2006), with each RPP in a different cow during each EP (i.e. cows receiving RP Lysine treatment during the EP would have 6 bags containing RP Lysine inserted). Three sample collection periods were utilised after incubation for 12 , 24 and 30 h. Five grams of RPP were placed in each Dacron bag and two bags were used for each time period to provide duplicate incubations.

For pH logging, pH loggers (TruTrack Data Loggers, Model pH-HR mark 4, Intech Instruments LTD, Christchurch, NZ) were placed in the rumen of each cow immediately after the *in sacco* incubation and left in to record rumen pH values every 10 min. This data was then condensed to provide 48 h of rumen pH data.

For *in vivo* measurements, cows were dosed via the rumen immediately prior to the morning feeding (directly after morning milking) on day 11 of each EP with one of the RPP containing Co-EDTA, as well as with Cr-EDTA in a free form. The experimental treatments were:

1) Control: 16.697 g Cr-EDTA; 15 g Co-EDTA
2) Product 1: 16.697 g Cr-EDTA; 150 g Bovi-C (RP Ascorbic acid)
3) Product 2: 16.697 g Cr-EDTA; 150 g Bovi-Lysine (RP Lysine)
4) Product 3: 16.697 g Cr-EDTA; 150 g Bovi-Niacin (RP Niacin)

The 150 g of the RPP were designed to deliver 15 g of Co-EDTA. Levels within treatment were designed to deliver equal molar quantities of Co and Cr to facilitate estimation of the disappearance of Co and Cr, as well as the ruminal stability of the RPP (See calculations in Appendix A2).

Cows were allocated to treatment groups alphabetically (by cow name and treatment name), and then rotated one treatment forward each successive test period. (i.e. Bella 137 = Cow 1, Control = Treatment 1; Firefly52 = Cow 2, Ascorbic Acid= Treatment 2). Treatments for each cow during each EP are in Table 3.2.6.

Each of the three RPP were manufactured with Co-EDTA marker and coated with a fat matrix to form pellets of approximately 2 mm diameter, with varying lengths of up to 10 mm.

Treatments were conducted in the same manner for all four EP and for each cow, with two pouches being physically made within the rumen by shifting the ruminal contents to allow each component to be accurately placed within the rumen. This allowed for the Cr-EDTA to be placed on the left side within the rumen, and either the RPP or the free Co-EDTA to be placed on the right side. Cannulas were then closed and treatments allowed to freely mix within the rumen. The Co-EDTA and the Cr-EDTA were dosed as is (i.e. in crystal

powder form), and not pre-mixed with water before dosage. The dosages of each RP product as well as the Cr-EDTA and Co-EDTA were weighed before each *in vivo* session into labelled and sealed plastic containers until dosage.

Table 3.2.6. A 4x4 Latin Square arrangement of treatments for each cow, for each experimental period. Where treatments were: Control, RP Ascorbic acid, RP Lysine and RP Niacin

EP⇒ Cows ⇓	Experimental Period 1	Experimental Period 2	Experimental Period 3	Experimental Period 4
Bella137	Control	Niacin	Lysine	Ascorbic Acid
Firefly52	Ascorbic Acid	Control	Niacin	Lysine
Greta34	Lysine	Ascorbic Acid	Control	Niacin
Marta178	Niacin	Lysine	Ascorbic Acid	Control

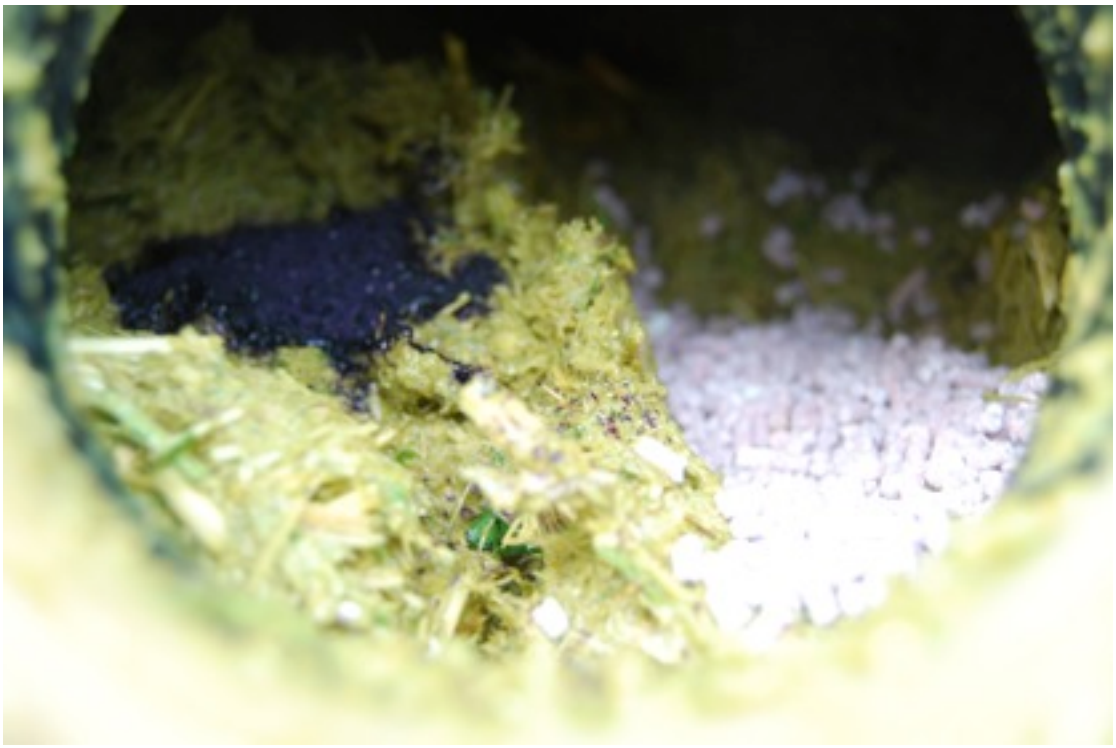

Figure 3.2.6 Dosage of Cr-EDTA on the left and RPP on the right in the fistula

3.2.6.1 Fluid Markers

The Co-EDTA and Cr-EDTA markers were selected as being water soluble with similar properties and molecular weights, giving the most similar ruminal clearance curves. Udén *et al* (1980) stated that Co-EDTA and Cr-EDTA are suitable liquid markers with essentially similar characteristics. Their composition is shown in Table 3.2.6.1.

Table 3.2.6.1 Fluid Marker Composition

Marker Name	Manufacturer	Marker Description	Composition
Co-EDTA	AVA Chemicals (P) Ltd.	Cobalt Ethylene Diamine Tetra Acetic Acid crystals (magenta-purple colour)	134 g Co/ kg Co-EDTA
Cr-EDTA	University of Pretoria	Chromium Ethylene Diamine Tetra Acetic Acid crystals (deep purple/ indigo colour)	121 g Cr/ kg Cr-EDTA

3.2.6.2 Rumen Protected Products (RPP)

The chemical composition of the three RPP is in table 3.2.6.2 with the fatty acid matrix composition in table 3.2.6.3. All fatty acids are rumen stable saturates.

Table 3.2.6.2 Rumen Protected Products and their Chemical Composition

Product Name	Manufacturer	Product description	Composition	Specific Gravity (SG)
Bovi-C	QualiTech Inc.	Ascorbic acid	Ascorbic Acid, 623.2 g/kg Co-EDTA 89 g/kg Fat matrix 287.8 g/kg	>1.207
Bovi-Lysine	QualiTech Inc.	Lysine	Lysine HCl, 518.7 g/kg Co-EDTA 86.5 g/kg Fat matrix 394.8 g/kg	>1.207
Bovi-Niacin	QualiTech Inc.	Niacin	Niacin, 623 g/kg Co-EDTA 89 g/kg Fat matrix 288 g/kg	>1.207

Table 3.2.6.3 Fatty Acid Profile of the RPP Fat Cover

Fatty acids	**g/kg**
<C14	1.8
C14:0	3.5
C14:1 trans	411.4
C14:1 cis	0.6
C16:0	278.3
C17:0	1.2
C18:0	246.7
C18:1	1.5
C18:2	1.8
C20:0	4.0
C20:5	0.4
C22:5	37.3
Unidentified	11.7

3.2.7 Experimental Diets

3.2.7.1 Total Mixed Ration

The total mixed ration (TMR) fed to the cows during the study was formulated by Nova Feeds (George, Western Cape, South Africa). The dietary ingredients and chemical composition of the diet are listed in Table 3.2.7.1. The chemical composition was determined by analyses at Nutrilab (Department of Animal & Wildlife Science, University of Pretoria, Pretoria, South Africa). The TMR was fed to each cow twice daily at 7:45 and 16:45 h and orts were removed each morning before milking. Fresh TMR was fed after milking and was adjusted daily to maintain *ad libitum* feeding at 2 kg (approximately 10%) above daily voluntary intake.

Table 3.2.7.1 Ingredients and chemical composition of the experimental diet fed to the rumen cannulated Jersey cows as determined from analysis and supplied information

	Total Mixed Ration
Inclusion levels of feed ingredients	g/kg DM
Lucerne hay	448
Maize residues	85
Maize meal	226
Hominy chop	66
Molasses, liquid	61
Soya oilcake meal	85
Urea	3
Rumen inert fat	11
Mineral & vitamin premix	14
Chemical composition by analysis	g/kg (DM basis)
Dry matter	902
Organic matter	918
In vitro organic matter digestibility	756
Starch	205
Neutral detergent fibre	369
$dNDF_{30hr}$	142
Ether extract	34
Crude protein	174
Ca	11
P	2
Co	0.2
Cr	0.00
ME (MJ/kg DM) *	10.8

* ME = 0.82 x (*iv*OMD x GE)

3.2.7.2 Feed Intake

Feed intake was measured daily by removing all orts within the feed buckets and transferring residues into plastic bags. Plastic bags containing feed residues were weighed. Weights of orts were then subtracted from weight fed to the cow to calculate daily feed intake:

Daily Feed Intake (kg) = Total TMR Fed (kg) – Orts (kg)

3.3 Methodology

3.3.1 Sample Collection

3.3.1.1 *In Vivo* Rumen fluid

Rumen fluid samples were collected by hand, by sampling pieces of rumen particles and then squeezing the fluid from the particles into a numbered sample container with residual particles placed back in the rumen. Samples were collected from at least ten locations within the rumen in a systematic order starting from left to right. A sample was considered sufficient when the 425 ml bottle was completely filled with fluid. Samples were then measured for pH using a hand-held device placed into the sample (WTW pH 340i pH meter with a WTW SenTix 41 pH electrode (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) and recording the value from the device. Samples were then pressed through 6 layers of mutton cloth and placed into labelled bottles. Samples for Co/Cr analysis were performed in duplicate and thus 150 ml was placed into each of two bottles to make up A and B pool samples. Both A and B pool samples were frozen immediately until further analysis at Nutrilab. Samples for NH_3-N analysis were measured with a syringe to allot 30 ml into a container and preserved with 5 ml of 50% sulphuric acid (De Bruyn, 1995), whilst VFA samples were measured into 20 ml samples and put into bottles with 4 ml of 25% orthophosphoric acid as preservative (Beauchemin *et al*, 2003). All rumen fluid samples were frozen immediately and stored until analysis in the laboratory.

Figure 3.3.1.1 Squeezing rumen fluid from rumen contents into numbered container

Figure 3.3.1.2 Pressing rumen fluid through six layers of mutton cloth

3.3.1.2 Milk

Milk samples were obtained from two successive milkings (an afternoon and then the following morning) on days 10-14 of each experimental period, to supply five daily milk samples per cow for each experimental period (therefore one milk sample per cow was pre-pulse dosing as a comparison and the remaining four samples were during the *in vivo* sample collection period). During milking the machine allotted small portions of milk into the test bottle to give a composite sample of the entire milking. Once milking was complete, each test bottle was gently mixed by swirling the bottle in order to mix the milk components and then pouring a representative sample into a syringe. During the afternoon milking, 8 ml of milk was poured from the test bottle into a marked syringe and then poured into the Lactolab sample container with a preservative pellet (Broad Spectrum Microtabs II (BSM II) containing Bronopol and Natmycin to prevent bacteria, yeast and mould growth) and the following morning a 16 ml sample was collected, as for the afternoon milking and added to the 8 ml sample to give a complete 24 ml sample (where 24 ml represents 1 ml/ h to give a 24 h milk sample). After collection, milk samples were refrigerated and once an experimental period (EP) was complete, samples were couriered to Lactolab for analysis (Lactolab, Irene, South Africa).

Figure 3.3.1.2 Measuring milk sample into sample bottle containing preservative pellet

3.3.1.3 Total Mixed Ration (TMR)

The TMR was sampled by taking random grab samples from feed bags on days 11 and 13 of each experimental period and placing a representative sealed sample into a bag. Samples were a minimum of 1 kg and were milled through a 1 mm screen and mixed. Representative samples were then placed into sealed, labelled containers for chemical analysis.

3.3.1.4 *In Sacco* Residues

Six Dacron bags were incubated in each cow (i.e. two bags for each of the three time periods). Figure 3.3.1.4 shows the filled stockings. Only RPP were placed in the Dacron bags (5 g each), with the control cow receiving no *in sacco* bags (ie one cow receiving no bags and the other three cows each receiving a specific RPP). Dacron bags were labelled by test period, time and treatment type (e.g. Experimental Period 3, 24 h (second collection), treatment ascorbic acid (1) = 3-2-1) *In sacco* samples were inserted at 9:00 h on day 7, and 12 h samples were removed at 21:00 h on day 7, at 9:00 h on day 8 (24 h samples) and again at 15:00 h on day 8 (30 h samples). Incubated samples were removed from the rumen via the rumen cannula and washed three times using cold water in a bucket by placing bags in the bucket of water and swirling (3 circles each direction, then the same in the opposite direction, repeated thrice) and then pouring the water out and refilling the bucket, at which point the water ran clear. Bags containing residues were frozen until analysis.

Figure 3.3.1.4 *In sacco* samples in receptacles, using "Stocking Method"

3.3.1.5 Ruminal pH logging

During each EP rumen pH was measured for 48 h using automated pH probes mounted onto a rumen cannula plug, recording rumen pH every 10 min. Ruminal pH loggers were calibrated before each insertion using the Omnilog Data Management Program (Version 1.64) with buffered pH standards of pH 4 and 7. Once the computer and logger had been calibrated, the loggers were activated and inserted into each cow. The same logger was used in the same cow for every EP (TruTrack Data Logger, Model pH-HR Mark 4 with pH probe PT100 temperature sensor (Website www.intech.co.mz)). An example of the calibration setup is shown in Figure 3.3.1.5.1. Ruminal pH loggers were inserted directly after *in sacco* incubations were completed (Figure 3.3.1.5.2). When the 30 h *in sacco* bags were removed, the pH loggers were inserted. The pH loggers allow for continual contact with ruminal contents due to the positioning of the loggers onto the cannula plugs and the water-tight casing around the probe, allowing only the electrode to come in contact with ruminal fluid.

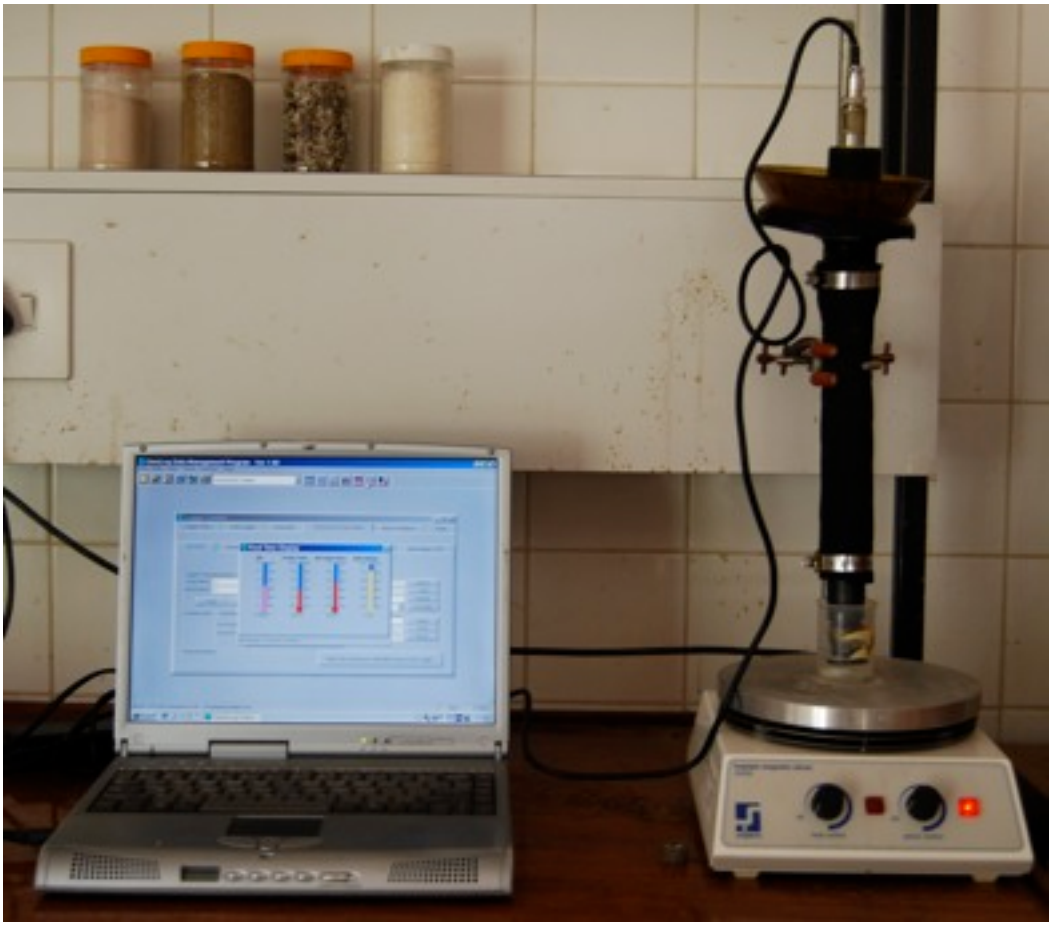

Figure 3.3.1.5.1 pH logger calibration setup using Omnilog Data Management program and buffer pH solution

Figure 3.3.1.5.2 pH logger insertion into rumen fistula

3.3.2 Analytical Methods – Chemical Analysis

3.3.2.1 Nutritive Value Determination

All collected feed samples were ground through a 1 mm screen using Beaver Lab 2682 mill (Asbestos Grading Equipment Co, Johannesburg, Gauteng, South Africa) followed by a second milling through a 1 mm screen using Retsch XM 200 mill (Retsch, Haan, Germany) at the Animal and Wildlife Sciences laboratory at the University of Pretoria (Nutrilab, SA). Milled feed samples were thoroughly mixed and subsampled for chemical analysis. Feed samples from each test period were composited to give one feed sample per experimental period. Feed samples were analysed for: dry matter (DM), ash, nitrogen (N) for crude protein (CP) determination, *in vitro* organic matter digestibility (*iv*OMD), starch, neutral detergent fibre (NDF), acid detergent fibre (ADF), gross energy (GE), ether extract (EE), 30 h digestible NDF ($dNDF_{30\ h}$), and calcium (Ca), phosphorus (P), cobalt (Co) and chromium (Cr).

The DM was determined as gravimetric loss of free water from two 1 g samples placed into a forced air oven (Scientific Series 2000 oven) at 105^{o} C for 24 h (AOAC 2000, procedure 934.01), Ash was determined as the gravimetric loss of OM from feed samples placed into a muffle furnace (Brainchild KTC-103) at 550^{o} C for 4 h (AOAC 2000, procedure 942.05). Nitrogen (N) was determined using a Leco N analyzer (Model FP-428, Leco Corporation, St Joseph, MI, USA) and CP was calculated as: CP = N x 6.25 (AOAC 2000, procedure 990.03). The *iv*OMD was determined by incubation of feed samples (Tilley & Terry, 1963; using rumen fluid extracted from a ruminally cannulated sheep fed Lucerne hay),

Ether Extract was determined by extraction of fat from feed samples using boiling petroleum ether in a standard Soxhlet procedure with the dried residues gravimetrically determined (AOAC 1984, procedure 7.06). Starch was ascertained by gelatinisation of starch within samples, using an autoclave followed by enzymatic hydrolysis of starch to glucose, where glucose was quantified by the glucose oxidase method (Faichney & White, 1983; MacRae & Armstrong, 1968; AOAC, 1984, procedure 996.11).

Neutral detergent fibre was quantified as gravimetric residue of ashing of fibrous residues of neutral detergent solution extraction in a Fibertec 500 (Foss Electric, Hilderød, Denmark) (Robertson & Van Soest, 1981), Acid detergent fibre was quantified as the gravimetric residue of ashing (Labcon Furnace L-1200) of fibrous residues from the acid detergent solution and acetone extraction of feed samples (Goering & Van Soest, 1970).

Samples were prepared for mineral analysis by acid digestion using nitric and perchloric acids.

Mineral levels were quantified by atomic absorption spectroscopy (AAS) where Ca (Perkin-Elmer S100 PC Atomic Absorption Spectrophotometer) (AOAC 2000, procedure 965.09), P (AOAC 2000, procedure 965.17), Co (Varian SpectrAA 50 Atomic Absorption Spectrophotometer) (Analytical methods, Varian Australia, 1989) and Cr (Varian SpectrAA 50 Atomic Absorption Spectrophotometer) (Analytical methods, Varian Australia, 1989) were analysed.

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Appendix A1

Table A1 Individual cow information

Number	Name & number	Cow ID	DIM	Parity	Calved	Average milk production during trial (L/ day)	Average body weight during trial (kg)
1	Bella 137	155	36	5	07/09/2010	28.1	401.7
2	Firefly 52	105	59	3	15/08/2010	22.2	373
3	Greta 34	96	127	6	08/06/2010	19.2	414.4
4	Marta 178	116	41	4	02/09/2010	26.3	348.1

Table A2 Individual cow weights (kg) per period

Experimental Period	Bella	Firefly	Greta	Marta
Start of Study	377	347.5	406.5	339
EP1	407	367	412	347
EP2	411.5	382	408	351.5
EP3	402	377.5	414	356
EP4	411	391	431.5	347

Appendix A2

Calculations for Cr-EDTA and Co-EDTA inclusion levels

Cr-EDTA = $CrC_{10}H_{12}Na^{+}N_2O_8$

Atomic weight EDTA = 120 + 12 + 23 + 28 + 128

= 311 g (1 mol EDTA)

Therefore: Cr-EDTA = 52 + 311 = 363 g (1 mol Cr-EDTA)

Co-EDTA = 59 + 311 = 370 g (1 mol Co-EDTA)

Initial inclusion level of Co-EDTA within RP products was set at 10%, based on this assumption it was calculated that:

150 g (RP product) x 10% = 15 g Co-EDTA within the RP products

Thus 15 g Co-EDTA dosed in Control

To determine Cr-EDTA dosage:

15 x 59/370 = 2.391891892 mol

2.39189182 x 363/52 = 16.69724532g ≈ 16.697 g Cr-EDTA dosed

Thus: 15 g Co-EDTA and 16,697 g Cr-EDTA both contain 2.392 mol Co/Cr respectively.

In actuality 8.9% Co-EDTA was included in RP Ascorbic Acid and RP Niacin and 8,65% Co-EDTA in RP Lysine, therefore:

150 g RPP x 0.089 = 13.35 g Co-EDTA within RP A and RP N

150 g RP L x 0.0865 = 12.975 g Co-EDTA within RP L

Thus: 13.35g x 59/370 = 2.12878378378 mol Co-EDTA in RL A and RP N

12.975 x 59/370 = 2.0689864864864 mol Co-EDTA in RP L

Therefore number of mol of Co and Cr were not equal in the actual experiment due to the change in Co-EDTA included within the RP products from initial calculations.

Appendix A3

Preparation of Cr-EDTA (Robinson, 1992):

Day 1: Mix 244 g Cr (III) acetate hydroxide, 350 g ethylene diamine tetra-acetic acid (EDTA), 48 g sodium hydroxide and 2 L distilled water into a 4 L glass beaker and stir continuously over moderate heat for 4-5 h. Turn off heat and replenish with distilled water to starting volume, then stir continuously overnight, covering beaker with aluminum foil.

Day 2: Slowly add 240 ml 30% hydrogen peroxide and continue stirring for 5-6 h. Divide into two 4 L beakers and add 1.8 L of 99% ethanol to each. Leave at room temperature for 1-2 h and refrigerate covered in aluminum foil for 72 h.

Day 5: Filter on a large Buchner funnel under vacuum, then oven dry at 60º C for 48 h.

Day 6: Air equilibrate for 24 h

Day 7: Place in a storage container, take 5 g sub-sample for Cr testing, label and store until use.

Appendix A4

GNU Octave input code used for the calculation of area under curve (AUC) of Cobalt and Chromium clearance curves from *in vivo* sample analyses.

Calculate Area file:

```
clear all;
% to remove all previously used data from the GNU Octave in use

a = dlmread('TR4L.csv',';');
% where: a specifies the specific values to utilise at each interval from the .csv file specified, where comma separated values are ordered by time interval within the file

delta_time = 0.001;
% the increment spacing between each time step (altered to suit specific requirements for accuracy)

time = a(:,1);
% the specific time at which the measurement was taken
absorbancy1 = a(:,2);
% the absorbancy reading from the Cobalt at a specific time
absorbancy2 = a(:,3);
% the absorbancy reading from the Chromium at a specific time

time_new = min(time):delta_time:max(time);
absorbancy_new1 = interp1(time,absorbancy1,time_new);
absorbancy_new2 = interp1(time,absorbancy2,time_new);

% Integration
area1 = 0;
```

```
% to calculate the area under the curve of the Cobalt
area2 = 0;
% to calculate the area under the curve of the Chromium


for i = 1:length(time_new)
    area1 = area1 + absorbancy_new1(i)*delta_time;
    area2 = area2 + absorbancy_new2(i)*delta_time;


end
% indicates where end of code is


disp(['Area under first curve is  = ',num2str(area1)])
disp(['Area under second curve is = ',num2str(area2)])
% to display the area under the curve of the Cobalt and then the Chromium curves respectively
```

Appendix B1

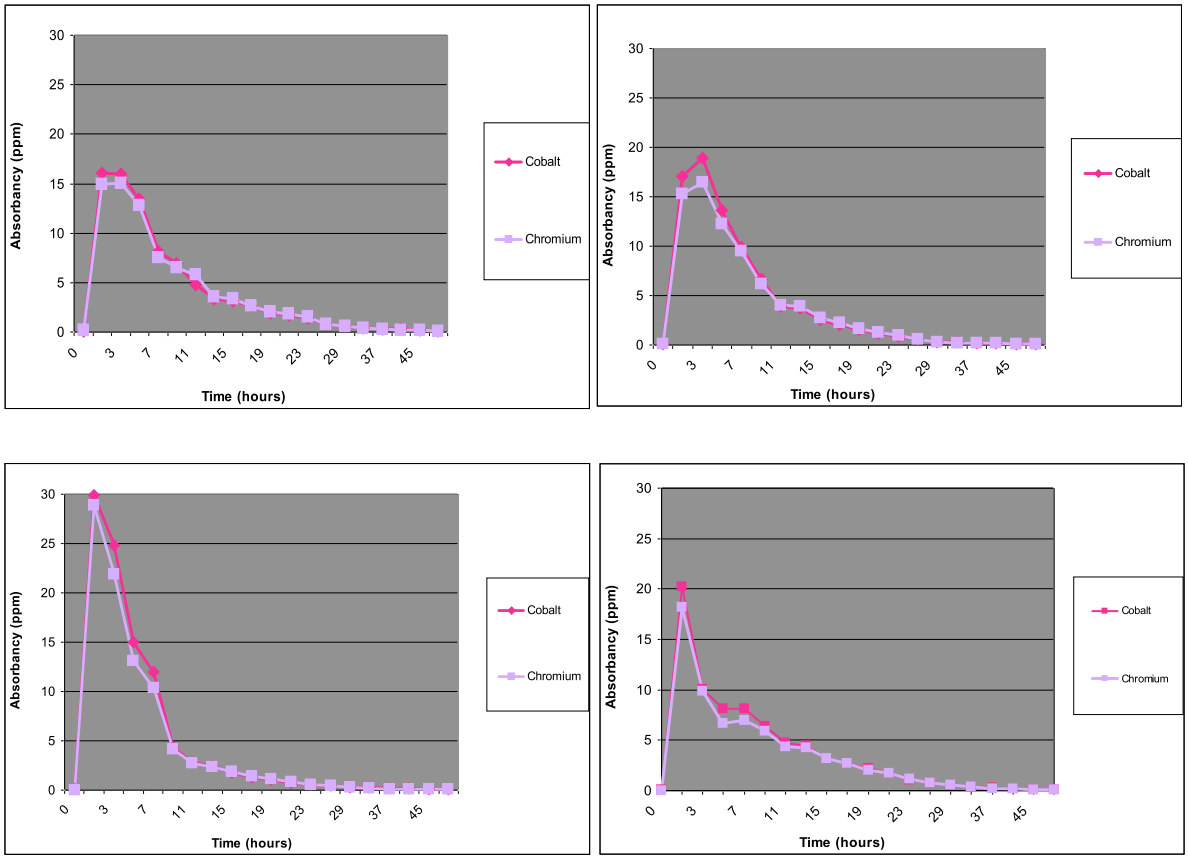

Figure B1 Clearance curves of Cobalt and Chromium from Control groups during each period

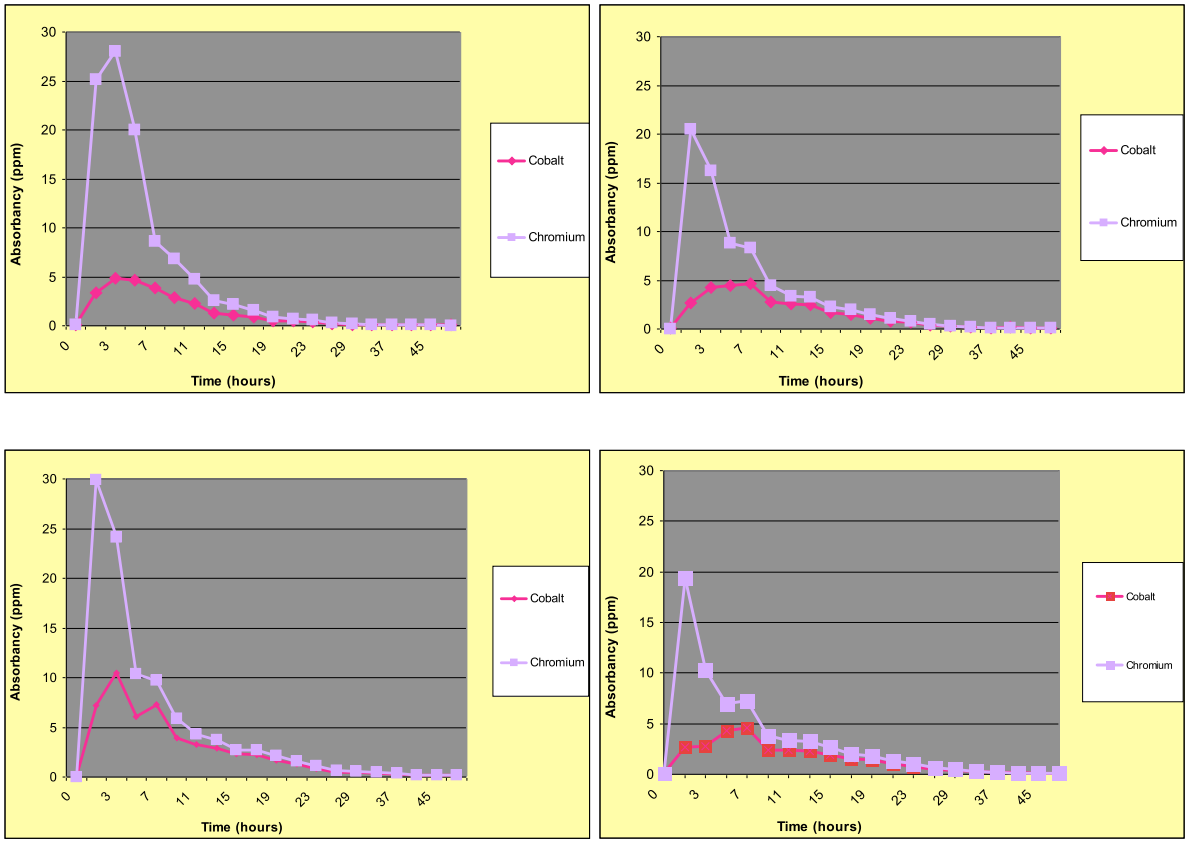

Figure B2 Clearance curves of Cobalt and Chromium from RP Ascorbic acid groups during each period

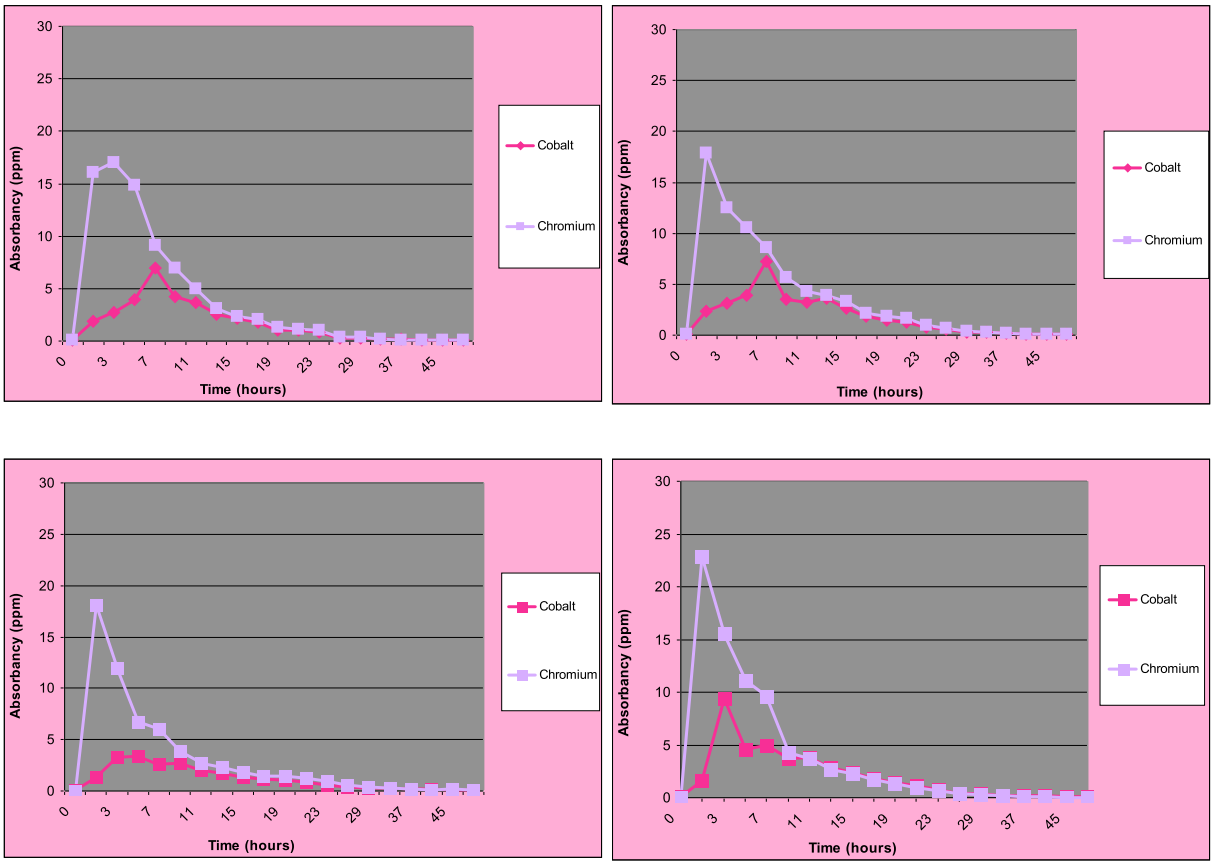

Figure B3 Clearance curves of Cobalt and Chromium from RP Lysine groups during each period

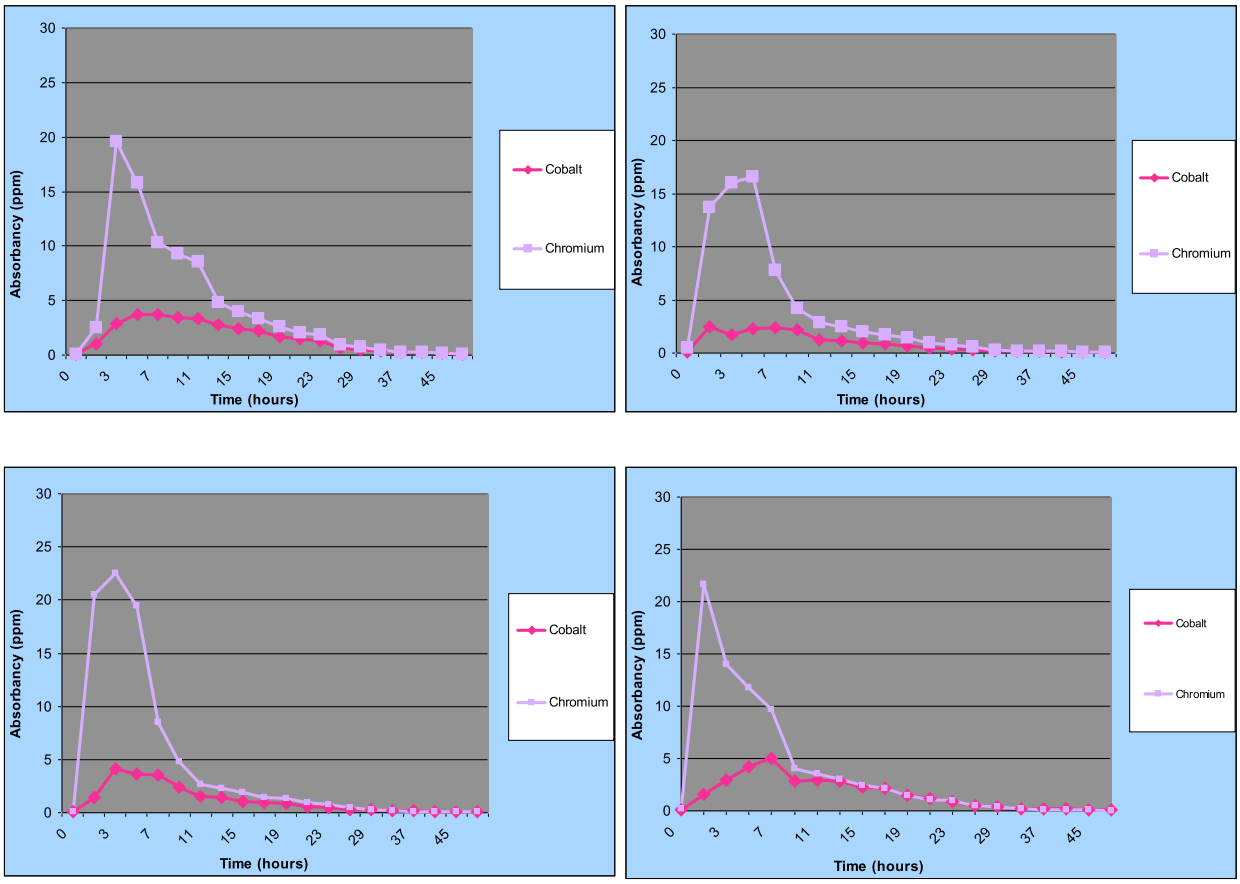

Figure B4 Clearance curves of Cobalt and Chromium from RP Niacin groups during each period

Table B5 Average SAS results for RPP. Where [a] indicates $p < 0.05$, and [b] indicates $p < 0.1$

Experimental Period	Stability
Experimental Period 1	66.1937500
Experimental Period 2	60.1342500
Experimental Period 3	60.4032500
Experimental Period 4	45.2567500[a]

Table B6 Average cow effect on product stability. Where [a] indicates $p < 0.05$ and [b] indicates $p < 0.1$

Cow	**Stability**
Bella137 – cow 1	66.06625
Firefly52- cow 2	62.06475
Greta34 – cow 3	55.89375
Marta178 – cow 4	47.96325[a,b]

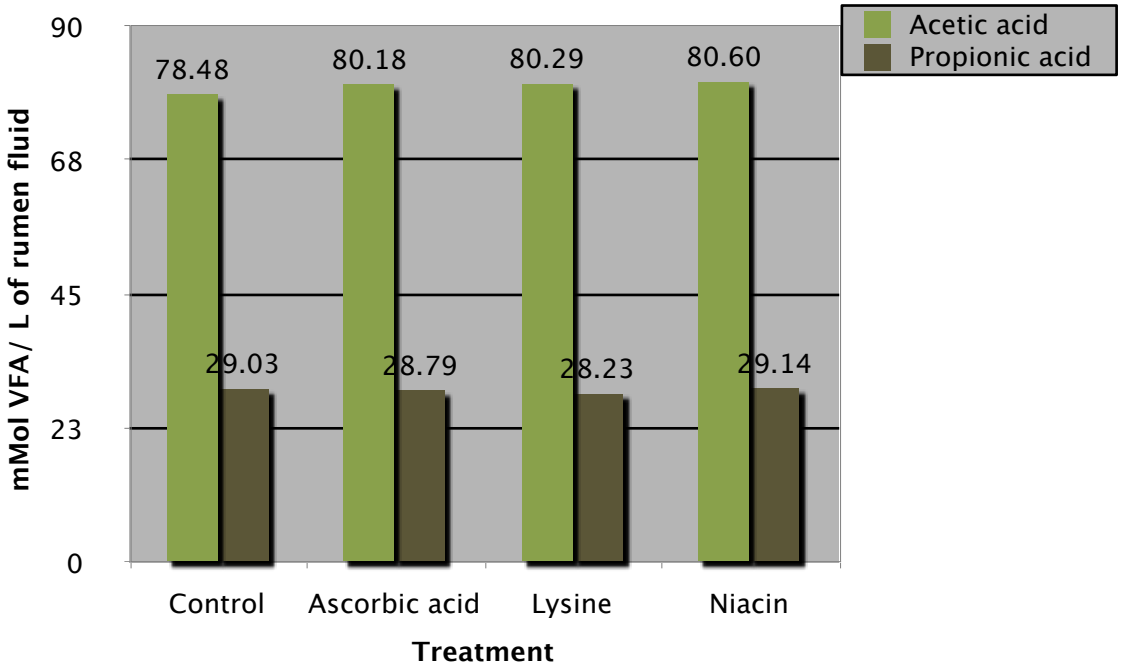

Figure B7 Average acetic acid: propionic acid ratio for each treatment

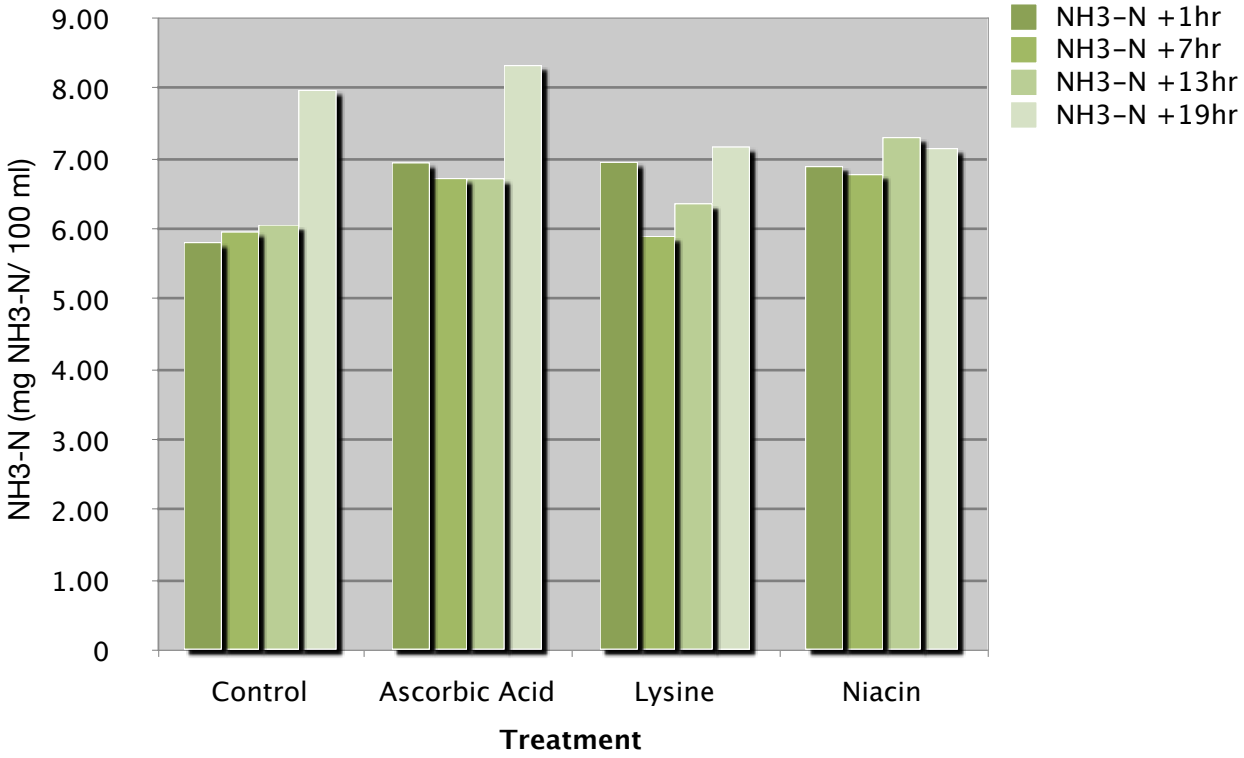

Figure B8 Average nitrogen ammonia levels for different treatments at different times relative to feeding

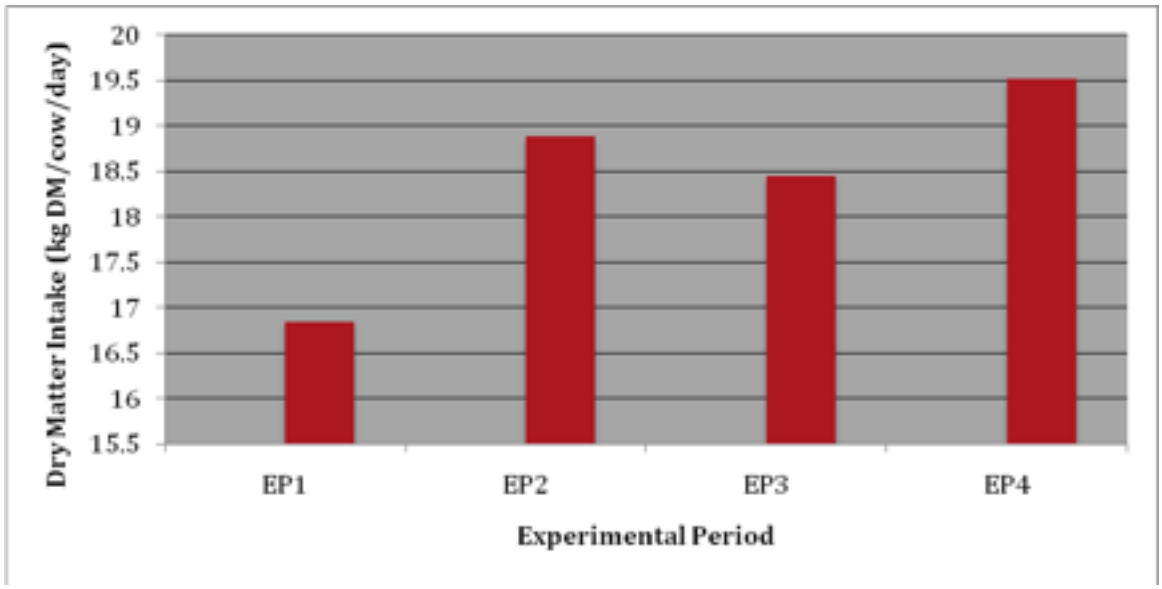

Figure B9 Average dry matter intake per experimental period

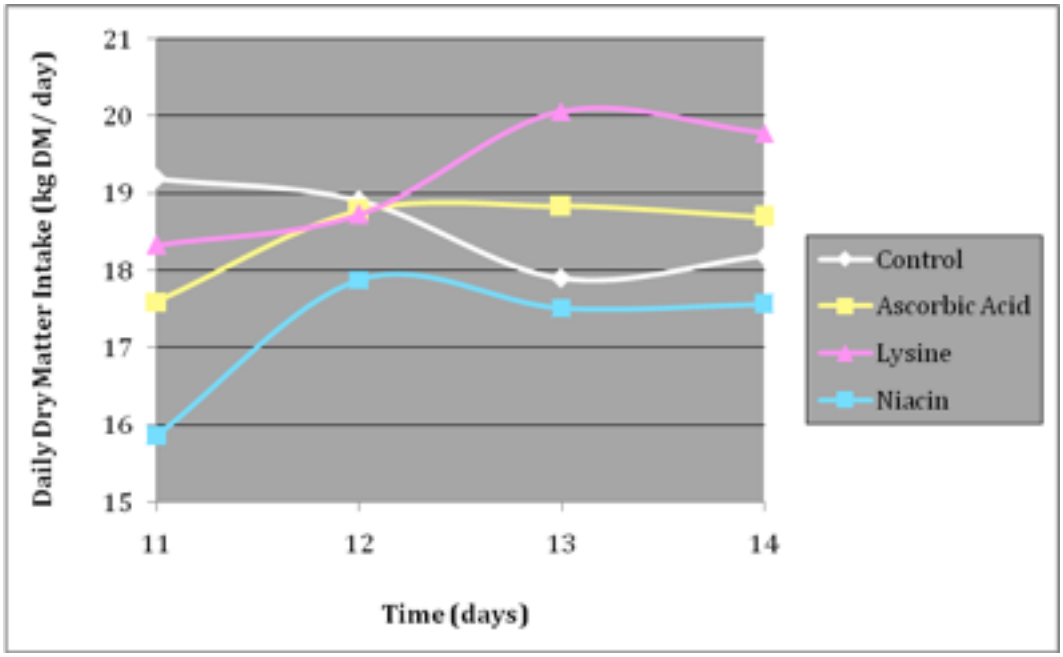

Figure B10 Average daily dry matter intake during *in vivo* testing

Table C1 *In sacco* disappearance of DM and Cobalt from RP Ascorbic acid product

Time	Co	DM
EP 1		
12 h	43.40	28.15
24 h	78.45	42.89
30 h	90.90	50.69
EP 2		
12 h	42.50	26.04
24 h	64.95	39.41
30 h	83.55	44.95
EP 3		
12 h	53.10	29.43
24 h	77.65	43.09
30 h	90.95	49.33
EP 4		
12 h	53.10	34.95
24 h	52.80	39.77
30 h	73.90	48.16

Table C2 *In sacco* disappearance of DM and Cobalt from RP Lysine product

Time	Co	DM
EP 1		
12 h	90.95	43.69
24 h	90.0	42.73
30 h	98.55	45.42
EP 2		
12 h	80.25	40.41
24 h	95.56	44.85
30 h	97.63	46.34
EP 3		
12 h	80.70	41.85
24 h	60.45	45.68
30 h	87.70	49.04
EP 4		
12 h	83.80	41.83
24 h	98.09	44.47
30 h	98.52	46.58

Table C3 *In sacco* disappearance of DM and Cobalt from RP Niacin product

Time	Co	DM
EP 1	Co	DM
12 h	39.40	19.03
24 h	61.25	28.51
30 h	71.25	41.15
EP 2		
12 h	30.70	14.14
24 h	72.45	31.05
30 h	69.95	32.71
EP 3		
12 h	33.30	12.28
24 h	62.30	26.00
30 h	82.20	39.03
EP 4		
12 h	53.50	17.86
24 h	61.90	27.23
30 h	78.30	38.30

3.3.2.2 Energy Determination (ME)

The GE value of the TMR was determined by bomb calorimeter (MC-1000 Modular Calorimeter, Energy Instrumentation, Sandton, SA), as the amount of heat produced from a 0.2 g sample (Mettler AC100 scale) through combustion (MC-1000 Modular Calorimeter, Operators manual). The Metabolisable Energy (ME) was then calculated as:

MJ ME/ kg = 0.82 x (ivOMD x GE)

Using the equation by ADAS cited by Robinson *et al* (2004).

3.3.3 In Vivo Rumen Fluid Analysis

3.3.3.1 Cobalt & Chromium Analysis

Rumen fluid samples from the *in vivo* collections were analysed using AAS (Varian SpectrAA 50 Atomic Absorption Spectrophotometer) (Analytical methods, Varian Australia, 1989). Experimental Period 4 B-pool samples were used as the initial test samples to determine the time where Co and Cr levels were extinguished and to determine the end point for analysis of samples. It was deduced from EP 4 samples that 49 h was sufficient for Co and Cr to no longer be present in the rumen fluid samples at measurable levels. Sample preparation involved thawing of samples, followed by centrifugation at 943 xg for 15 min at 90º (Hettich Zentrifugen Rotofix 32 A) to precipitate solids, dead bacteria and particulate matter. Samples were tested using AAS and any samples exceeding range detectable by AAS were diluted (Gilson Dilutor 401) using 5 or 10 times dilution depending on the level above normal range, and then retested using AAS.

Figure 3.3.3.1 Atomic absorption spectrophotometry on Co and Cr rumen fluid samples

3.3.3.2 Volatile Fatty Acid Analysis

Volatile Fatty Acids were determined from samples pooled relative to feeding times to create four samples/ cow/ EP, at time intervals relative to feeding of +1 and +7 h relative to morning feeding and +1 and +7 h relative to afternoon feeding (or +13 and +19 h relative to morning feeding). Using the +1 h post feeding sample indicates the early active part of fermentation and the +7 h shows the middle of fermentation. This was done to evaluate feeding effects on VFA production. The VFA assayed were: acetic, butyric, isobutyric, propionic, valeric and total VFA determined by gas chromatography (Webb, 1994, with modifications)

3.3.3.3 Nitrogen Ammonia Analysis

Nitrogen ammonia rumen fluid samples were pooled as for VFA samples and assayed by spectrophotometry with Analytik Jena Spekol 1300 (Analytik Jena UK, Wembley, UK) using the method of Broderick & Kang (1980).

3.3.4 In Sacco Analyses

In sacco residues were analysed for Cobalt (Co) (RPP containing Co-EDTA marker), N (RP Lysine residues only), and DM. Frozen samples were dried in an oven (Scientific Series 2000 oven) at 60º C for 24 h. DM (AOAC 2000, procedure 934.01) of the *in sacco* bag residues were then gravimetrically determined (Mettler Toledo, Classic PB303-L) after air equilibration (Duran desiccator). Dried residues were then removed from the Dacron bags and pooled by treatment and incubation period, and then ground with a mortar and pestle (Morgan Technical Ceramics Haldenwanger, Waldkraiburg, Germany), and transferred into airtight, labelled containers. Ground lysine samples were analysed for N using a Leco FP-428 (Leco Instruction Manual). Levels of niacin and ascorbic acid could not be measured by nitrogen determination as they do not contain nitrogen atoms, and were not tested for vitamin levels as the fat coating interfered with tests.

3.3.5 Specific Gravity Testing

The specific gravity (SG) of each of the RPP was determined by placing a specific amount of each into saline solutions of different concentrations. This analysis was conducted at the laboratories at the University of Pretoria, South Africa and the SG (g/ cm^3) was determined by calculating the weight of the solution divided by the volume of the solution. Different weights of pure sea salt (heavy metal free) were added to 100 ml of double distilled de-ionised water ($DDDH_2O$), with an increase in salt resulting in an increase of the SG of the fluid. The salt was weighed out and then added to the 100 ml of measured $DDDH_2O$, stirred vigorously until completely mixed and the volume of the final solution was measured and weighed. The highest possible saline concentration that can be achieved is 1.207, where 35 g of salt was added to 100 ml of water. Twenty particles of each product were placed in different SG solutions and if the particles all sank, their SG was greater than the SG of the fluid. If particles remained floating in the solution, then the particles had a SG less than that of the solution. Testing started by using a solution with a SG of 1.00 ($DDDH_2O$ with no salt added), with all products sinking, thus indicating that all products had a SG > 1.00. It was found that all products had a SG > 1.207, thus the exact SG of each product could not be accurately determined using the salt solutions, although Lysine had the SG closest to 1.207, with some smaller particles floating at SG 1.207 similar to Swanepoel (2009).

3.3.6 Milk Sample Analysis

Milk samples were analysed by Lactolab SA (SANAS accreditation laboratory number T0154, Irene, SA) for fat, protein, lactose and milk urea N (MUN) using infrared technology with a Milkoscan FT6000 (Foss Electric, Hillerød, Denmark), as well as somatic cell counts (SCC) by flow cytometry using a Fossomatic 5000 (Foss Electric, Hillerød, Denmark).

3.3.7 Manufacture of Products and Chemicals

3.3.7.1 Manufacture of Co-EDTA

Co-EDTA was manufactured by AVA Chemicals (P) Ltd (ISO 9001 Certified, Mumbai, India) as a Colbalt EDTA chelate.

3.3.7.2 Chemical Manufacture of Cr-EDTA

Cr-EDTA was manufactured at the Nutrilab laboratories at the University of Pretoria (Pretoria, SA), using the method of Robinson (1992, provided in Appendix A3), as modified from Udén *et al*, 1980. A detailed description of the method is shown in Appendix A3.

3.3.7.3 Manufacture of Rumen Protected Products

The RPP were manufactured by Quali Tech Inc., by creating a homogenous mixture of Co-EDTA and nutrient (i.e. ascorbic acid, lysine or niacin), pelleting the mixture and then spraying a fat coating (Table 3.2.6.3) over the pellets (all rumen stable, saturated fatty acids).

3.3.8 Calculations - Area Under Curve

The ruminal stability of the three Co-EDTA/ RPP matrices was estimated as the difference between the areas under the Co and Cr clearance curves.

The area under the curve (AUC) was determined from data obtained during the *in vivo* sampling periods using Co and Cr residues from the rumen fluid samples, using AAS. Data was converted into AUC for the first 49 h only, by two separate methods; firstly by approximation (where time intervals are separated into rectangular areas) using Microsoft Excel (Microsoft Office, 2008) using the formula for area as:

Area = Base x Height (i.e. Area = time interval x absorbency value)

or secondly by integration using GNU Octave (GNU Octave Version 3.2.3, configuration i386-apple-darwin8.11.1, 2009), an engineering program designed to integrate large volumes of data (Eaton, 2002), to determine area under curves. The code used to perform the calculations in GNU Octave is in Appendix A4.

An example of the method used to calculate the stability from the area under the curve (AUC) is given below in Figure 3.3.3.2. The area under each curve is determined as shown in the second graph of Figure 3.3.3.2 by integration. The area of the green curve (area 1) is calculated first and then the area under the blue curve (area 2) and combined in the following method to provide a stability value:.

(Area 2/ Area 1) x 100% = z%

100 - z% = stability [i.e. if green curve represents 100% instability, and blue curve is level of product instability (how much degraded in the rumen), then this is the products ruminal stability (difference between curves)].

The integration method allows for a higher level of accuracy as time intervals can be made as small as desired (a time interval of 0.1 was used) thereby allowing area to be more precisely determined.

Chapter 4: Results

4.1 Descriptional Data

4.1.1 Rumen conditions

a. pH

Diurnal pH variation among the cows fed the different RPP shows a range in ruminal pH between 5.65 and 6.40, with an average of 5.88 (Figure 4.1.1). Observation of results shows that cows receiving the RP ascorbic acid treatment had a slightly elevated pH level over the 24 h period, with cows receiving RP niacin inducing the most variation in pH levels and control cows having the least pH variation, as well as the lowest overall pH levels.

Statistical analysis of each of the treatments and their effect on rumen pH is shown in Table 4.1. The pH levels indicate that the niacin product influenced rumen pH (p = 0.05) but none of the treatments had any effect on the Co or Cr-clearance curves (p = 0.05).

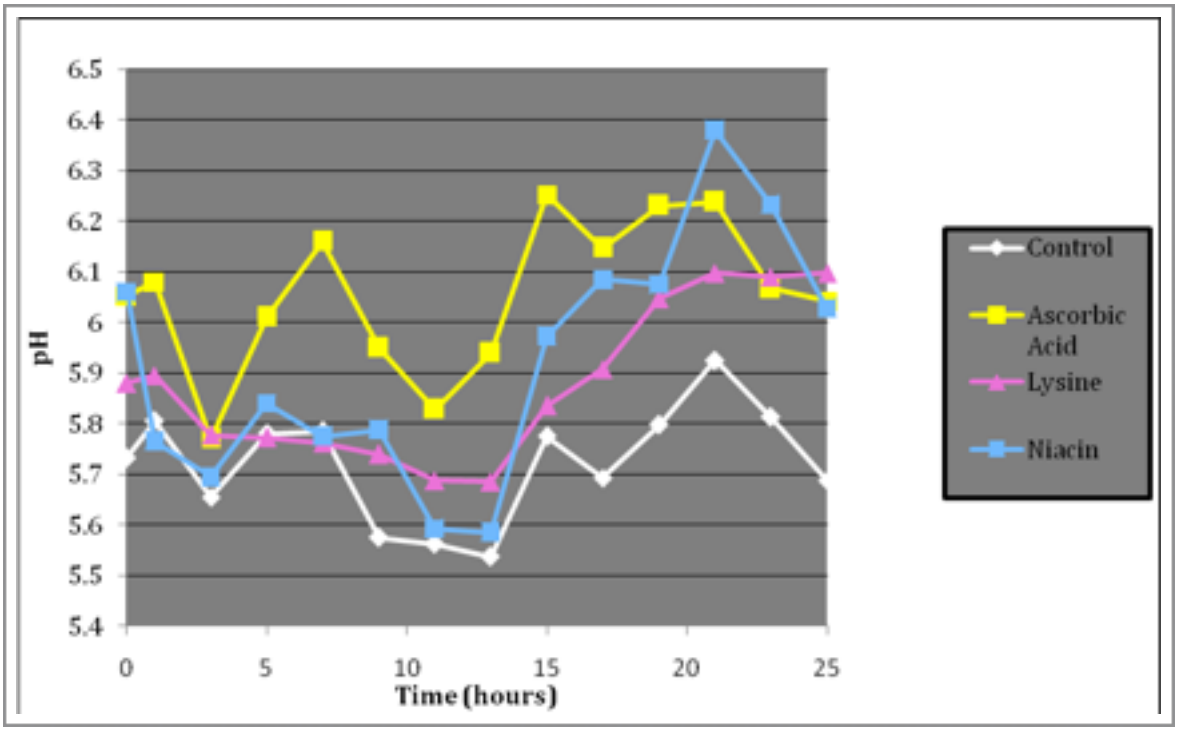

Figure 4.1.1 Average diurnal pH variation during *in vivo* sampling over 25 h for each treatment, recorded with a hand-held pH recording device

Table 4.1 Effect of experimental treatments against control on average ruminal pH of cows, as measured by hand-held pH meter. Where [a] indicates $p < 0.05$ and [b] indicates $p < 0.1$

Treatment	Control	Ascorbic acid	Lysine	Niacin	SEM
pH	5.84	5.88	5.85	5.91 [a]	0.076

pH logging

The continuous pH evaluation is illustrated below in Figure 4.1.2. The data describes the rumen environment between testing phases (i.e. between *in sacco* and *in vivo* testing). The values indicate that the lowest ruminal pH occurred between 19:00 and 20:00 h daily and the highest pH values between 04:00 ad 05:00 h. These values indicate average values from all cows over 2 d combined to produce a 24 h curve of pH variation and are assumed to be indicative of normal rumen environment during the experimental period. The average ruminal pH during the pH-logging was 5.92, with the control group cows having the lowest average pH value of 6.00 and cows receiving RP lysine having the lowest pH at 5.81. The cows receiving RP niacin was the closest to the control group pH at 5.95 and the cows receiving RP ascorbic acid had the second lowest pH average of 5.91.

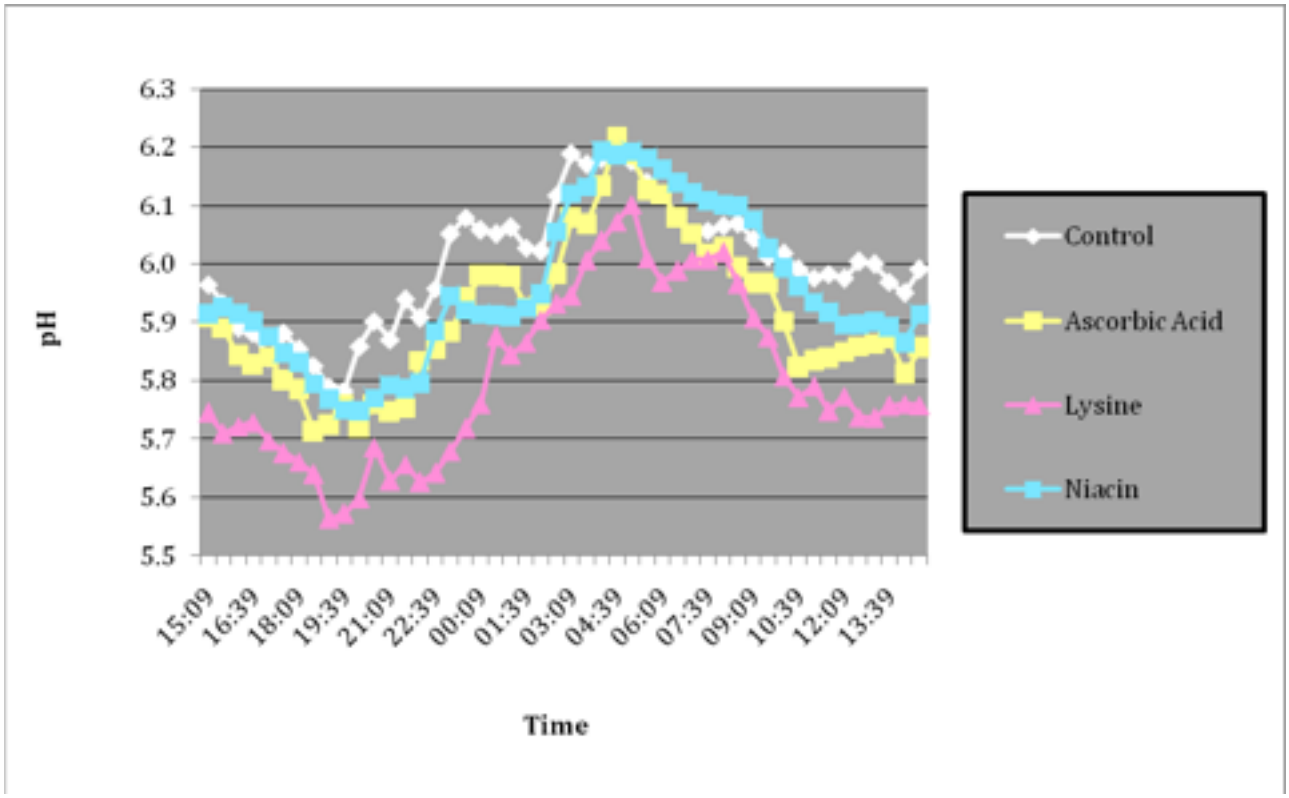

Figure 4.1.2 Average daily pH variations for each RP product and control after *in sacco* incubations (taken over 48 h and averaged for every 24 h using pH data loggers)

b. Volatile Fatty Acids

The predominant VFA was acetic acid, averaging 79.89 mMol/L, followed by propionic acid at 28.80 mMol/L and butyric acid at 15.58 mMol/L. Isobutyric and valeric acid had very low levels relative to total VFA at 0.84 and 1.91 mMol/L respectively (Table 4.2).

It is also shown in Table 4.2 that the level of butyric acid relative to total VFA was decreased by dosing the RP lysine, whilst increased by both the RP ascorbic acid and the RP niacin relative to control. The cows receiving the RP niacin treatment had lower butyric acid levels ($p = 0.011$ and $p = 0.039$) at +1 h and +7 h relative to morning feeding in comparison with the control group. The RP lysine treatment had a tendency ($p = 0.065$) to a slightly elevated total VFA acid level compared to the control group at + 7 h relative to morning feeding. These effects are not biologically significant.

Table 4.2 Volatile fatty acid levels (mMol/L) according to treatment and time relative to morning feeding. Where $p < 0.05$, and [b] indicates $p < 0.1$ (compared with the control)

VFA	Treatment																SEM
	Control				Ascorbic Acid				Lysine				Niacin				
Time ~>	+1 h	+7 h	+13 h	+19 h	+1 h	+7 h	+13 h	+19 h	+1 h	+7 h	+13 h	+19 h	+1 h	+7 h	+13 h	+19 h	
Acetic acid	74.12	78.54	84.57	76.68	74.83	79.05	85.72	81.14	76.56	84.56	85.60	74.45	74.68	81.87	85.51	80.34	3.19
Butyric acid	15.64	16.64	17.57	16.16	15.27	15.70	17.22	16.58	15.11	16.13	15.99	14.03	13.11[a]	14.63[a]	14.98	14.50	0.79
Isobutyric acid	0.63	0.66	0.67	2.58	0.76	0.72	0.75	0.83	0.75	0.77	0.73	0.66	0.71	0.75	0.73	0.81	0.50
Propionic acid	27.50	28.15	32.67	27.80	25.45	28.15	33.57	28.02	25.28	29.32	32.83	25.50	25.89	29.61	32.93	28.12	2.04
Valeric acid	1.89	1.94	2.02	1.86	1.91	1.87	2.08	1.95	1.85	1.87	2.01	1.70	1.87	1.89	1.96	1.89	0.04
Total VFA	119.78	125.94	137.50	125.08	118.22	125.49	139.34	128.51	119.55	132.64[b]	137.16	116.34	116.26	128.75	136.11	125.67	4.05

b. Ammonia Nitrogen (NH_3-N)

Treatment effects occurred only at +1 h and +7 h relative to morning feeding with RP ascorbic acid ($p = 0.0468$) and RP lysine ($p = 0.0458$) showing higher levels of NH_3-N and RP niacin showing slightly higher ($p = 0.0548$) NH_3-N levels than the control at +1 h. At +7 h relative to morning feeding, RP ascorbic acid ($p = 0.0879$) and RP niacin ($p = 0.0724$) had a tendency to higher levels of ammonia N than the control. Both cow variation and period variation had significant effects on the NH_3-N levels (particularly at +1 h and +7 h time periods), where cow effects ($p = 0.0118$ for RP A, $p = 0.0275$ for RP L and $p = 0.0223$ for RP N) and

period effects ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$) were assessed. These effects are also not biologically significant.

Table 4.3 Nitrogen ammonia (mMol/L) levels for each treatment and time relative to morning feeding

Where [a] indicates $p < 0.05$, and [b] indicates $p < 0.1$ (compared with the control)

Time after feeding	Control	Ascorbic Acid	Lysine	Niacin	SEM
+1 h	5.81	6.94[a]	6.95[a]	6.89[b]	0.279
+7 h	5.96	6.72[b]	5.89	6.78[b]	0.237
+13 h	6.06	6.72	6.36	7.30	0.267
+19 h	7.98	8.33	7.17	7.15	0.296

4.1.2 Performance - Feed Intake & Milk Production

Daily Dry Matter Intake (DMI) was about 18.5 kg of DM/ cow/ day and there were no treatment effects ($p > 0.1$) (Table 4.4).

Dry matter consumption of cows during the first experimental period (EP 1) was lower ($p = 0.0399$) compared with the last period (EP 4), and tended to be higher in EP 2 ($p = 0.0943$). This suggests that there was an improvement in feed intake as the experiment progressed.

Milk production and milk components from the daily monitoring of milk is shown in Table 4.4. There were no changes in milk production due to treatments ($p > 0.1$). Milk lactose tended to be higher with RP ascorbic acid ($p = 0.045$) and milk protein proportion tended to decrease ($p = 0.1$) for RP ascorbic acid as well. The increase in SCC during the final EP was due to one cow having mild mastitis and was not biologically significant. Daily milk production improved for all cows, most likely due to increased nutrient dense feed.

All cows gained weight during the trial, increasing with each EP (Table A2). This is most likely due to an increase in nutrient dense feed from the TMR and less activity/ movement due to their removal from grazing.

Table 4.4 Production performance parameters measured during the experiment. Where [a] indicates $p < 0.05$, and [b] indicates $p < 0.1$ (compared with the control)

	Treatment				
	Control	**Ascorbic Acid**	**Lysine**	**Niacin**	**SEM**
DMI (kg DM/d)	**18.61**	**18.53**	**19.29**	**17.27**	**0.72**
Milk (kg/d)	**24.04**	**23.21**	**24.75**	**24.04**	**0.54**
Fat (%)	**4.09**	**4.21**	**4.25**	**4.18**	**0.11**
True Protein (%)	**3.59**	**3.49** [b]	**3.52**	**3.63**	**0.04**
Lactose (%)	**4.90**	**5.00** [a]	**4.94**	**4.95**	**0.03**
SCC (x 1000 cells/ml)	**582.00**	**100.00**	**119.00**	**101.00**	**207.80**
MUN (mgN/dl)	**14.28**	**14.44**	**15.05**	**14.49**	**0.61**
BCS (units)	**2.38**	**2.38**	**2.31**	**2.31**	**0.06**
ΔBCS (units/ treatment)	**0.00**	**0.06**	**0.00**	**0.00**	**0.07**
BW (kg)	**387.5**	**385.5**	**389.1**	**391.9**	**3.86**
ΔBW (kg/treatment)	**15.0**	**7.3**	**3.5**	**8.6**	**3.96**

4.2 *In Sacco* Data

In sacco residues were assayed for Co and DM disappearance after 12, 24 and 30 h of ruminal incubation (Table 4.5).

RP ascorbic acid treatment had a lower DM disappearance than RP lysine ($p = 0.0004$) and RP niacin had a lower DM disappearance than both RP ascorbic acid ($p = 0.0002$) and RP lysine ($p < 0.0001$) after 12 h incubation. After 24 h of incubation RP ascorbic acid had a tendency for a lower DM disappearance than RP lysine ($p = 0.0827$) and RP niacin had a lower DM disappearance than both RP ascorbic acid ($p = 0.0001$) and the RP lysine ($p < 0.0001$). At 30 h, RP niacin had a lower DM disappearance than RP ascorbic acid ($p = 0.0003$) and RP lysine ($p = 0.0007$). There were some cow effects noted at 12 h incubation, where cow 1 (Bella) had a higher DM disappearance than cow 2 (Firefly) ($p = 0.0068$) and cow 3 (Greta) ($p = 0.0494$) and cow 2 had a lower DM disappearance than cow 3 ($p = 0.0397$) and cow 4 (Marta) ($p = 0.0083$). Period effects or tendencies were also noted for DM disappearance again at 12 h incubation for all periods, except EP1 v. EP 4.

The Co disappearance at 12 h incubation showed RP ascorbic acid treatment to have a lower Co disappearance than RP lysine (p = 0.0002) and RP niacin had a lower disappearance than RP lysine (p < 0.0001). At 24 and 30 h, RP niacin had a tendency for a lower disappearance than RP lysine (p = 0.0726) and a lower disappearance (p = 0.0096) respectively. At 30 h RP ascorbic acid had a tendency for a lower disappearance than RP lysine (p = 0.0912). Cow effects for cow 2 vs. cow 3 (lower disappearance) were shown (p = 0.0293) at 12 h incubation and for cow 1 vs cow 2 (lower disappearance) at 30 h incubation (p = 0.0417). Period effects were noted for 12 h incubation for EP 1 vs EP 4 (p = 0.0138) with EP 4 having lower disappearance than EP 1.

Table 4.5 *In sacco* results of DM and Co disappearance (g/ kg) from RPP incubations. Where [a] indicates p < 0.05 and [b] indicates p < 0.1

Time	Ascorbic acid	Lysine	Niacin	SEM
DM disappearance (g/ kg)				
12 h	296 [a]	419	158 [a]	6.5
24 h	400 [b]	444	282 [a]	4.2
30 h	483	468	378 [a]	2.8
Co disappearance (g/ kg)				
12 h	480 [a]	839	392 [a, b]	11.8
24 h	685	862	645 [b]	5.8
30 h	848 [b]	956	754 [a]	5.1

4.3 Stability (Co release data relative to Cr release data *in vivo*)

Analysis of rumen fluid samples are shown in Figure 4.3.1 (Control) and Figure 4.3.2 (the RPP) as the average curve of the Co and Cr release over time, after ruminal dosing. The control graph indicates good agreement between the liquid markers in terms of clearance from the rumen, which was in agreement with Udén's (1980) statement that Co-EDTA and Cr-EDTA act in the same manner within the rumen. All four EP's had highly similar curves (Figure 4.3.2), with no outliers (individual graphs by cow and period are in Appendices B1 - B4). The clearance curves of Co and Cr for the RPP's showed consistent results suggesting no interference between the product and the Cr-EDTA. Co-EDTA release from the RPP was also similar for all products and periods (Figure 4.3.2). There were no treatment effects ($p > 0.3$) on Cr-EDTA levels between treatments and control. There were no cow effects on Co-EDTA, although there was a tendency for cow effects for Cr-EDTA clearance in Firefly52 ($p = 0.086$) and Marta178 ($p = 0.0518$) (the 2 smaller cows).

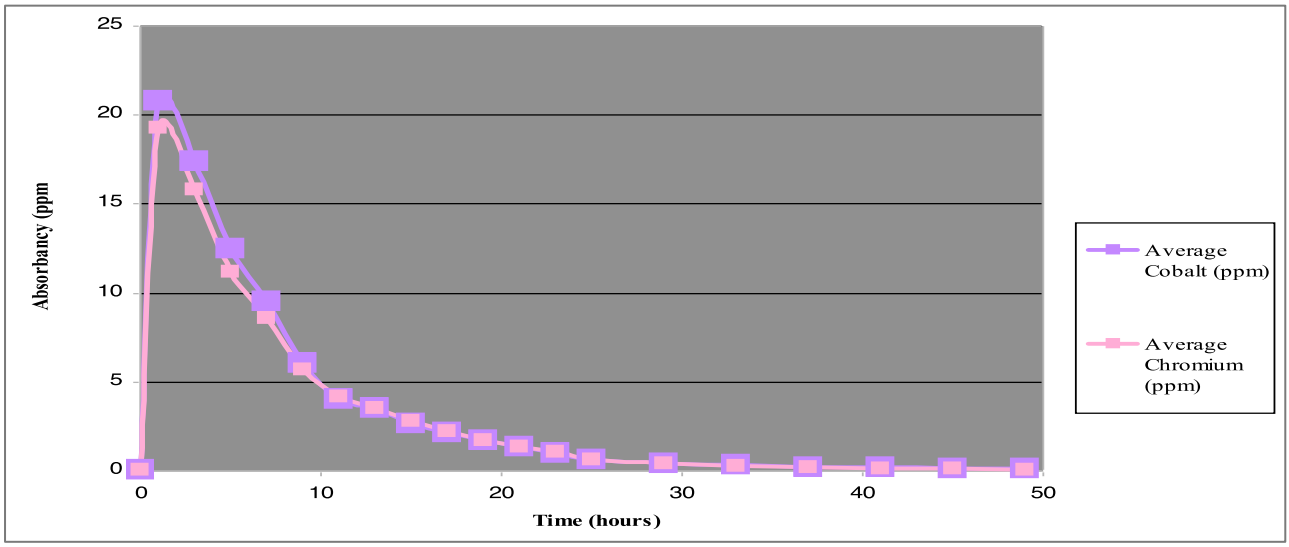

Figure 4.3.1 Average clearance curves of Cobalt and Chromium for Control

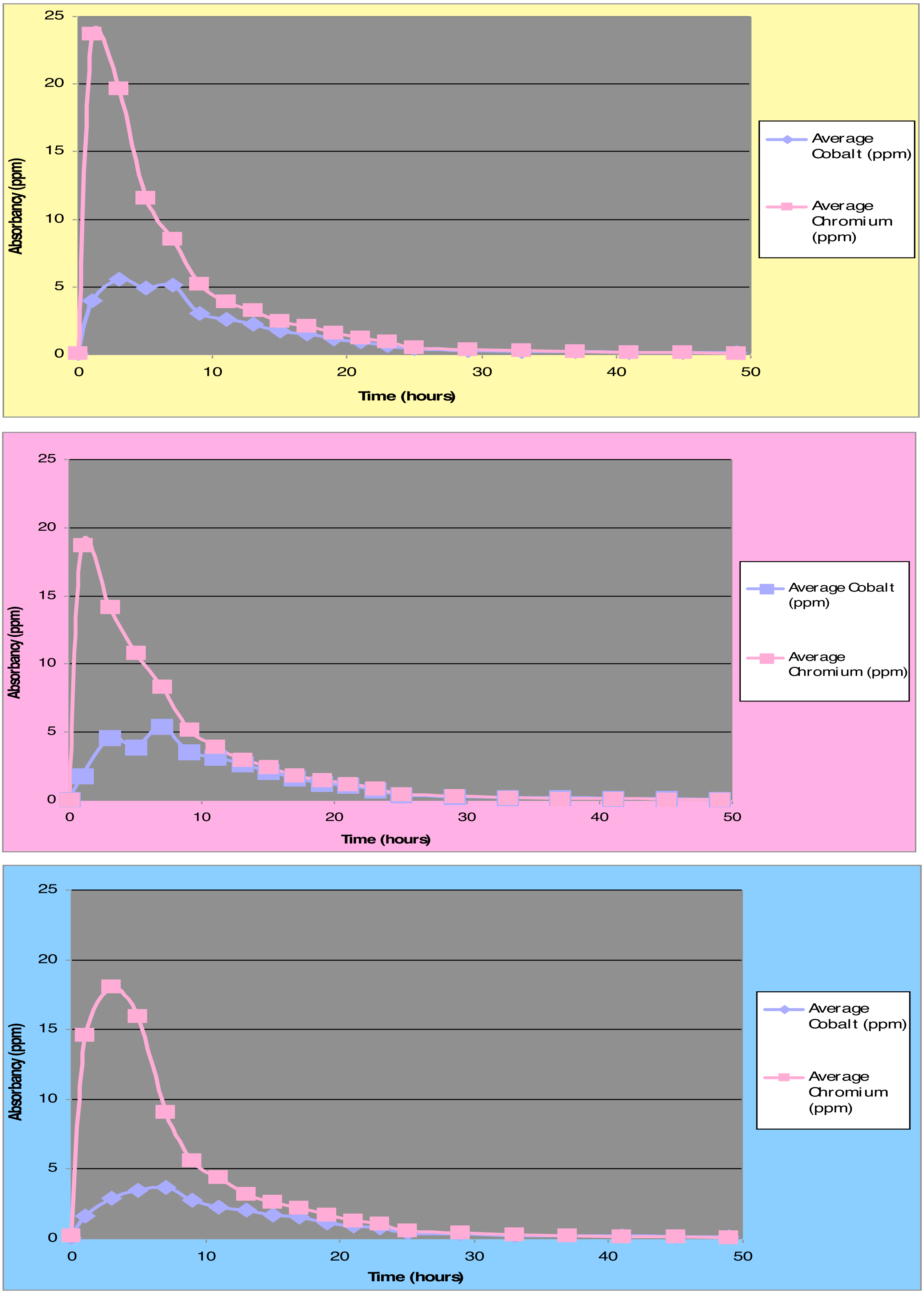

Figure 4.3.2 Average clearance curves for Cobalt and Chromium for RPP

4.3.4. Clinical Notes

During each *in vivo* EP the cows were monitored for health changes and possible treatment complications, with all occurrences being recorded. During the first EP Greta34, who was then dosed with the RP Lysine, had frothy bloat, which made sampling rumen fluid quite difficult as the foam, which occurred was very bubbly and tended to cause a lot of rumen matter to exit the rumen when the plug was opened and made squeezing fluid from this matter difficult. After 49 h with no change in bloat, it was decided that she be dosed with propylene glycol (150 ml *per os*) after the morning milking and sampling. After dosing with propylene glycol Greta34 was much improved although the frothiness was still present during the night samplings. The frothy bloat was generally improved after milking and after dosing with propylene glycol there was some improvement. Greta34 had intermittent bloat during all the *in vivo* samplings, with the first being the worst and the only serious bloat requiring treatment. Successive bloats were mild and involved only some frothiness/ bubbliness of the rumen contents. During EP2, two of the cows (Bella137 and Greta34) had some mild diarrhoea after 24 h, though the diarrhoea was not excessive or completely runny. During EP 4 all treatment cows got mild diarrhoea (Bella137, Firefly52 and Greta34), with both Greta34 and Firefly52 having frothy/bubbly bloat (neither required treatment). It appeared that the RP Lysine treatment tended to induce a frothy bloat the most severely and that the RP niacin did so to a smaller extent. It was also observed during EP3 and EP4 that the cows started licking and eating soil (geophagia), which may have been due to loss of sodium from the intensive samplings (Stobbs & Minson, 1983)

4.5 Calculations - Area Under Curve (AUC)

The areas under the Co and Cr curves were calculated for each EP with the area under the Co curve being deducted from the area under the Cr curve to obtain the average of each treatment. The table below (Table 4.6) shows the results as calculated by GNU Octave as this was the most accurate measure, where integration was performed to the level 0.1 (a much smaller increment than calculated using Excel). This then gave the percentage stability of the product when multiplied by 100 (Table 4.6), The table also shows the percentage similarity between the two fluid markers as 95.4 %. The RP niacin had the highest stability of 65.6 %, followed by the RP ascorbic acid at 55.8 % and lastly the RP lysine at 52.7 %. There was a significant period effect (Table B5) on the stability of the products, with EP 4 showing a lower stability for all three products in

comparison to EP 1, EP 2 and EP 3 (p = 0.0205 for EP 4 vs. EP 1, p = 0.0478 for EP 4 vs. EP 2 and p = 0.0468 for EP 4 vs. EP 3). There was also a cow effect (Table B6) from cow 4 (Marta178) showing lower stability (p = 0.0302) than cow 1 (Bella137), this may have been due to the fact that cow 1 was the largest of all the test cows and cow 4 was the smallest, which may have affected rumen passage rate and thereby rumen stability. It was also determined that the RP lysine had a lower (p = 0.0408) rumen stability than the RP niacin and a tendency (p = 0.084) for a higher stability than the RP ascorbic acid.

Table 4.6 Summary of results from stability evaluation using GNU Octave, where [a] indicates $p < 0.05$, and [b] indicates $p < 0.1$

	Treatment				
	Control	Ascorbic Acid	Lysine	Niacin	SEM
Average Area Under Curve (Cobalt)	161.0	70.4	68.6	54.6	24.37
Average Area Under Curve (Chromium)	154.0	162.5	145.2	158.6	3.72
Average Area Under Curves (as % of Cr)	95.66% (% similarity between curves)	43.34%	47.28%	34.42%	
Average Ruminal Stability (%)	--	56.66	52.72	65.58 [a,b]	0.039

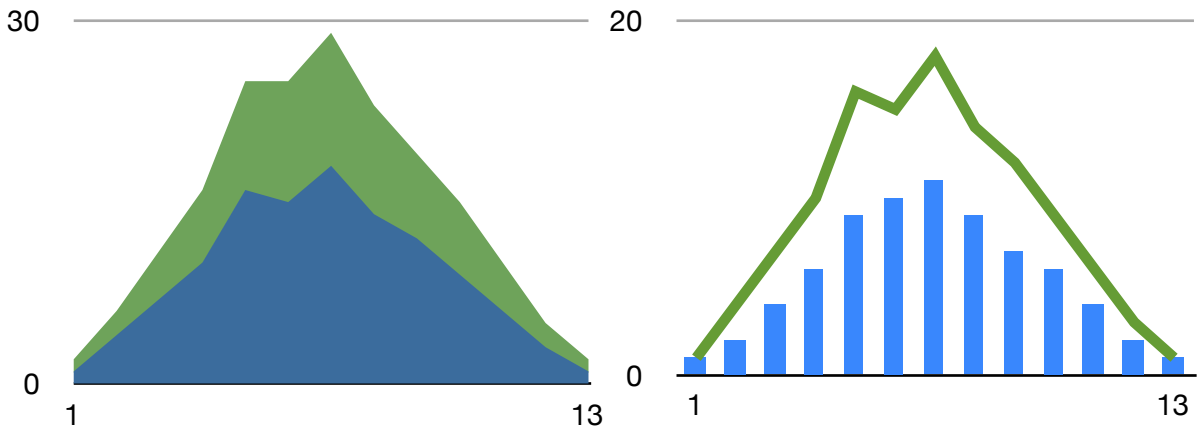

Figure 3.3.3.2 Graphical depiction of two area curves where the total area under the green line subtracted from the total area under the blue line

3.3.9 Statistical Analysis

All response parameters were analysed using the PROC GLM option of SAS for a Latin square design. Orthogonal contrasts were used to compare the treatment to the control (SAS, 2008). Significance due to treatment, cow and experimental period effects were determined. Significance was declared at $p < 0.05$ and tendencies if $p < 0.10$.

Chapter 5: Discussion

5.1 Rumen Protected Products

Coating

The three rumen protected products (RPP) utilised a fat coating consisting of saturated, rumen stable, long-chain fatty acids (LCFA). Using saturated fatty acids allows for a higher melting temperature and a longer shelf life of products as saturation decreases rancidity and prevents auto-oxidation wherein unsaturated FA's react with oxygen. LCFA's are insoluble in water and commonly used in soap production, as well as for dietary supplements where controlled release in a specific location is required (i.e. intestinal tract). The three major fatty acids used for all three RPP were C14:1 trans, C16:0 and C18:0, which are all inexpensive, readily available LCFA's with no adverse effects and many are used as dietary supplements. The major fatty acids in the RPP were C14:1 trans fatty acids (411.4 g/kg), C16:0 fatty acids (278.3 g/kg) and C18:0 fatty acids (246.7 g/kg) which were different from those in other RP lysine products (Robinson *et al*, 2011) where the major fatty acids were C18:0 (846.0 g/kg) and C16:0 (112 g/kg). Research by Wu *et al* (1991) showed that more than 90% of LCFA's less than C14 (14 carbons in the chain) disappeared in the rumen and about 30% of all dietary LCFA's disappear in the rumen. It was also found that selective disappearance occurred with the lowest rumen disappearance of LCFA's occurring when only C16 or only C18 LCFA's were fed. Thus it is possible that the highest rumen protection would occur when only one type of FA is used for coating. The use of C14 FA's in our RPP may have contributed to the lower stability than that estimated by Robinson *et al* (2011) where C16 and C18 predominated, which were shown by Wu *et al* (1991) to be the more stable FA within the rumen.

Rumen protected products incorporate a number of mechanisms to prevent ruminal degradation, including encapsulation, coatings, matrices, pH sensitive polymers, metal chelates and accumulation yeasts (Rode & Kung, 1996). These forms allow for ruminal stability, but release within the intestinal tract, by enzymatic degradation and/ or pH sensitivity. Thus a pH sensitive RPP will remain stable at a rumen pH, but degrade at a duodenal pH. Utilising fat to prevent ruminal degradation as the rumen contains no enzymes to break down

fats, but the intestinal tract does, can occur from various strategies offering differing levels of protection. However there is always a tradeoff between rumen stability level and digestibility within the intestines as high rumen protection often results in low intestinal availability.

Comparison of our RPP with Commercially Marketed RPP

Currently commercially available products similar to those tested are described below and compared with our results.

RP ascorbic acid containing products are relatively few, with only Rumen-C® (Bioscreen Technologies) and Vitamine C50% RP™ (Jefo Species-specific additives & Vetagro S.p.A.) commercially available and with VitaShure®-C (Balchem Animal Nutrition & Health) soon to be available. All three of these products have no published research on their effectiveness or stability and there is currently no published research on RP ascorbic acid products. A study was performed by MacLeod *et al* (1996) on Ascorbyl-2-polyphosphate as a stable form of supplemental vitamin C, which showed 75% disappearance after 12 h and 80% disappearance after 24 h of rumen incubation, although it does not indicate how it was dosed. This is a much higher disappearance than the *in sacco* stability level determined in our study of 44.2%, although without knowing how this was measured, an accurate comparison cannot be made. Rumen-C claims a 75% rumen escape after 8 h rumen *in sacco* incubation, although without knowing the specific gravity it is difficult to estimate its rumen mean retention time (MRT). The Rumen-C is formed into granules with fatty acid coatings, but intestinal availability and rumen stability is not known and thus it cannot be directly compared with our RP ascorbic acid product. No technical or research information on Vitamine C50% RP is provided.

There are a number of RP lysine products, as both lysine and methionine have been available and researched for a few years as they are considered first limiting amino acids (NRC, 2001; Schwab, 2003). Currently commercially available products are: AminoShure™-L (Balchem Animal Nutrition & Health), AjiPro™-L (Ajinomoto Heartland LLC), Fatrix® Lysine (Bewital GmbH & Co KG), SmartAmine®-ML (Rhone-Poulenc Animal Nutrition), CuPLEX® (Zinpro Performance Minerals), Biolys® (Evonik Industries AG), LysiPEARL™ (Kemin Industries, Inc.), Megamine-L™ (Arm & Hammer Animal Nutrition, a subsidiary of

Church & Dwight Co., Inc), Relys® (Vetagro S.p.A. and Jefo Species-specific additives) and MetaboLys® (H.J. Baker & Bro., Inc)

A prototype AjiPro-L was examined by Swanepoel *et al* (2010), which showed a specific gravity of 1.09g/ cm^3 utilising a matrix protection technology. Swanepoel *et al* (2010) incorporated an HP-Arg marker method similar to our Co/Cr-EDTA method, where graphs are determined from rumen samples at various time intervals after dosing to calculate the difference in the areas under the curves for the two measured substrates. Using this method, the prototype AjiPro-L RP lysine was determined to provide 0.45 ruminal escape, lower than the 0.53 determined in our study. Thus despite having a much higher estimated rumen stability than the RPP in our study, our RPP had a slightly higher rumen escape value than that of Swanepoel *et al* (2010). AjiPro-L showed an 85-90% predicted ruminal stability after 24 h *in sacco* ruminal incubation, with an intestinal availability of about 80% of fed amount, using the method of Swanepoel *et al* (2010), with 50% intestinal digestibility.

Robinson *et al* (2011) used production quality AjiPro-L, which also incorporated a matrix RP technology, with a rumen escape of 0.72, which was higher than the product tested by Swanepoel *et al* (2010), likely due to the reduced particle size of this RPP vs. the prototype, as well as the RPP we evaluated. AjiPro-L has a higher predicted rumen stability using *in sacco* methods and a lower specific gravity than our RP lysine.

CuPLEX is a metal specific amino acid complex (one amino acid ion bound to one metal ion), which has been used in dairy cows by Chase *et al* (2000) and Nocek *et al* (2006), although only the Cu effects were determined on claw health and performance and rumen stability was not evaluated. There is no published research on the rumen stability, specific gravity or coating evaluation of CuPLEX.

Fatrix Lysine is a matrix and encapsulated product, which claims effectiveness, based on a thesis by Pieter Obin, but neither the thesis nor any published work on this product and its stability could be obtained.

Biolys claims 100% digestibility, but gives no indication of rumen stability or specific gravity and has no scientific data supporting the values. Biolys has been used in pigs, but only for growth and performance effects, and did not evaluate product properties. A study by Bernard *et al* (2004), using L-Lysine-HCL (Biolys) showed no ruminal escape of lysine from duodenal samples, which suggests it is not a suitable form for supplementation as a rumen escape lysine for ruminants.

LysiPEARL is an encapsulated form of RP lysine utilising MicroPEARLS®, a freeze spraying process using liquid nitrogen, to provide rumen stability and intestinal release. There is no specified rumen stability or

intestinal availability provided, although it claims results are backed by *in vitro* and dairy cow research. No published research could be obtained on this product, thus it cannot be compared with our product.

Megamine-L is a calcium fatty acid salt matrix, pelleted product containing rumen protected lysine and bypass fat. The main FA's present are C16:0 (50.8%) and C18:1 C (35.7%), thus there is a larger proportion of C16:0 in this product than in ours (27.8%) and our product contained a minimal amount of C18:1 (0.15% as C and T form). These LCFA's have been shown to be more stable in the rumen although without a rumen stability value and intestinal release this cannot be assessed or compared with our product. Research conducted on Megamine-L was not published and only assesses production effects, with no evaluation of the products' rumen stability or intestinal availability being described. Arm & Hammer state that the Lysine is 46% metabolisable, although they do not state how this was determined, only that it was based upon performance improvements. Arm & Hammer also state that *in vitro* assessment of bypass rate and digestibility was determined, but only state an *in sacco* bypass rate after 12 h incubation of 58%, although without the specific gravity of the product MRT can not be determined and thus the product can not be compared with our product.

No technical information or published research could be found for Relys.

MetaboLys is a lipid coated Lysine Sulphate, but no further information is provided and no published research could be found.

A liquid RP lysine was evaluated by Erasmus *et al* (2004), although rumen stability and intestinal release was not evaluated, thus this product cannot be compared with our RP lysine product.

RP niacin products marketed include Niashure™ (Balchem Animal Nutrition & Health), which is a micro coated granule (fat/lipid coating) with a predicted 85% stability in the rumen after 24 h rumen incubation with 40% intestinal bioavailability. Most published research on this product evaluated production and metabolic responses (Morey *et al*, 2011) and heat stress effects (Zimbelman *et al*, 2007). The stability value is higher than the stability determined for our RP niacin product of 65.6%, although the actual intestinal bioavailability of our product was not evaluated.

This research suggests that there is some degradation of fatty acid coatings of the RPP. It is possible that the rumen microbes are able to dissociate the product from the fatty acids (rather than degrade the fatty acids, although with a high level of C14 FA's there may be some degradation). Although all our RPP had the same

fat coating, the rumen stabilities differed, indicating that the protected nutrients had different levels of association with the fatty acid matrix coating. There is no published research comparing different RPP with the same fatty acid matrix technology and their corresponding stabilities. Although RPP have generally been evaluated at set pH levels *in vitro* to determine release of nutrient at estimated intestinal (2.9) vs ruminal pH (5.4) (Papas *et al*, 1984). This is not a true indication of the protection level of the nutrient within the rumen as microbial interference is not considered.

5.2 Rumen Conditions & Performance

pH

Woodley & Metcalf (2005) recommend maintaining rumen pH between 5.8 and 6.2 to support optimal rumen function. Average pH of 5.88 and variation between 5.65 and 6.40 during our study falls within or near this range. Average pH levels for each treatment were within the recommended range and pH levels among treatments averaged 5.92, slightly higher than those found during the sampling period, but still within the ideal range.

VFA

Total volatile fatty acid levels were marginally higher than in other studies conducted at our research centre (Malleson, 2008 and Lingnau, 2011), probably due to differences in the diets fed, as cows are normally kept pastured on kikuyu/ ryegrass and fed a small amount of supplementary concentrate. Comparison of results shows a higher level of propionic and butyric acids than the other studies, which is expected with TMR based feeding (Balch and Rowland, 1957).

NH_3-N

Ammonia nitrogen levels were higher than those observed by Malleson (2008) at our research centre, most likely due to the TMR diet fed.

Dry Matter Intake

Hutjens (2000) completed an analysis of top producing Jersey cows in the USA and found that they consumed about 20 kg DM/ day. Feed intake of our cows was thus acceptable as research cows do not always consume feed as readily under research conditions as they would under normal conditions. Study cows achieved an average intake of 18.42 kg DM/ d. The average BW of our cows (384 kg) is lower than the average US Jersey cow (431 kg) (AJCA, 2008) which may account for the slightly lower DMI as well.

Feed intake increased with each successive EP, thus it is surmised that either a longer period was needed for adjustment of the cows to the experimental conditions than was provided, or that the initial change from the adjustment phase to the sampling phase during treatments had a negative impact on feed intake, which then improved with each successive EP as the cows adapted to the conditions, or that cows adapted to experimental conditions and thus intake improved in line with adaptation.

Milk and Milk Composition

Comparison of milk production (24.2 kg/ day) with other studies at our research centre using Jersey cows shows an average daily milk production of 21.5 kg/cow/day on pasture supplemented with concentrate and fishmeal (Malleson, 2008) and 20.2 kg/cow/d on pasture supplemented with concentrate and medium starch levels (Lingnau, 2011). Thus milk production of our TMR fed cows is higher. Milk production of Jersey cows fed concentrates in South Africa who participated in the Dairy Cattle Improvement Scheme for 2007 was 21.3 L/ d for a 300 d lactation (Theron & Mostert, 2009). Thus study cows performed above expected levels for cannulated cows, most likely due to feeding of the TMR. Any nutritional impact from the RPP seems unlikely. Milk yield did improve during the trial, with a starting milk yield of 21.2 L/ d. This increase is most likely due to the change from pasture to a TMR diet and adaptation to the diet over time.

Milk component yields were similar to cows from other studies (Malleson, 2008 and Lingnau, 2011) at our research centre. Malleson (2008) found a fat proportion of 3.93% and a milk protein proportion of 3.35% with fish meal supplementation, whilst Lingnau (2011) found a fat of 4.49% and a milk protein of 3.63% with medium starch supplementation levels. A fat proportion of 4.77% and protein proportion of 3.74% were the averages for concentrate fed Jersey cows tested in the Dairy Cattle Improvement Scheme (Theron & Mostert, 2009), whilst a fat of 4.18% and milk protein of 3.89% were the values for our study, showing a lower fat, but a higher protein level than average as well as in comparison with other studies at our research centre (except for Malleson, where a lower milk fat occurred as well). As the other studies used a higher

number of cows, it is possible that the cows used in our study had genetically higher milk protein levels than average, or that the TMR fed improved milk protein production in comparison with the other concentrates or pastures.

The milk production parameters were consistent with expected outcomes, as the products were dosed once every two weeks and would not be expected to influence milk parameters or production within such a short time span.

In Sacco

Dry matter *in sacco* disappearance has been shown by Kung *et al* (2003) to have limitations in accuracy when assessing the quality of RPP, particularly in comparison with stability evaluations (*in vitro* rumen fermentation) of the same product, as results tend to differ from one another, giving misleading results. Our results are in agreement with Kung *et al* (2003), as there was considerable variation between EP's with both the DM and the Co disappearance for all three treatments. The RP ascorbic acid residues indicated a high level of variation between Co loss in residues for all EP's, with no EP showing a similar pattern to any other EP (Table C1). The DM loss from RP ascorbic acid residues was fairly consistent, indicating about a 50% loss of DM after 30 h of ruminal incubation. RP lysine residues showed variation in values for all time periods, and showed inconsistent results for Co in particular, with no EP showing similar results or trends. Losses of N from RP lysine showed a high N loss from the product, except in EP 1 which showed a much smaller loss of N over 24 h, than the other EP's, thus these results were discarded due to very high levels of variation and lack of agreement with other test results (Table C2). This could indicate one of two occurrences; either the lysine was released at a higher rate than the cobalt marker from the fat matrix, or there are inaccuracies with the N analysis wherein the fat may have interfered with the assay. Cobalt analyses were completed on acid digested samples to remove fatty acid interference, whilst the N samples were not. RP niacin showed minor variations between Co loss from *in sacco* residues, with the 12 h loss being much higher for EP 4, which may indicate some loss in stability with age of products, or possible oxidation or degradation of products with time. DM loss from RP niacin residues shows fairly consistent results between EP's and for each incubation period, with the lowest of all treatments losses from both DM and Co residues (Table C3). This concurs with the *in vivo* results insofar as it shows higher stability for the RP niacin, then RP ascorbic acid and lastly RP lysine, as well as indicating a lower loss of substrate from products after 24 h.

Period and cow effects were shown, mostly for 12 h incubation times in both DM and Co disappearance, which were again not consistent, with no similar trends at 24 or 30 h incubations. The cow effects may have been due to differences in rumen activity and microbial populations.

Initial Cobalt levels within the RPP were analytically determined, but are not shown as they were lower than the final levels within the *in sacco* residues, which indicates an interference between the fatty acid matrix of the products and the Co assays in the RPP before incubation. The RP ascorbic acid showed a relatively even loss of Co over each successive incubation period with a relatively high level of Co still present after 12 h of incubation. The RP lysine residues indicated much lower levels of Co than the other two products, with a small loss in Co between the 12 and 24 h intervals. The RP lysine had a slightly lower level of Co included than the other two RPP, but only a small percentage difference. The difference in percentage of Co included in the RPP would not account for such low Co levels in the residues, and it is presumed that a large portion of the Co in the products is lost within 12 h of rumen incubation based on in vivo results. The RP niacin showed the highest Co levels after 12 h of rumen incubation and thus the highest stability and resistance to degradation in the rumen, suggesting a high level of stability during incubations of less than 24 h. During the *in vivo* measurements, there were no visible pellets from the treatments seen in the rumen after 12 h, and it is speculated that the RP products exit the rumen in less than 12 h based upon rumen observation during sampling of *in vivo* experiments and with levels measured by AAS showing a large drop by 12 h (levels measured after 12 h may be due to Co remaining in the rumen fluid rather than RPP in the rumen) and the specific gravity and size of the pellets. A specific gravity of 1.17 g/cm^3 is considered the ideal for maximum escape from the rumen for inert particles (Siciliano-Jones & Murphy, 1986).

5.3 Markers

Both markers were water soluble and showed similar trends over the 49 h of post-dosing. Clearance curves for both markers showed sharp peaks within an hour of dosing, followed by gradual decline until 30 h where most of the marker had exited the rumen. The clearance curves for Control groups were very consistent, with very similar clearance curves for all EP's (95.4% similarity), indicating little absorption of either marker and no interference among cows. These results suggest that the combined usage of the two markers would be an effective combination for use as a control model, when using fluid markers for evaluation of feeds. Use of

one of the markers (Co-EDTA) in combination with an RPP, and measurement of release of the marker from the RPP, could therefore allow for a comparison to be made when Cr-EDTA is pulse dosed at the same time as the RPP.

The clearance curves in EP 1 and EP 2 were very similar. The Cr-EDTA curves in cows dosed with the RPP also show high similarity with the control Cr curves, thus indicating that the RPP combined with the Co-EDTA and the freely dosed Cr-EDTA did not interact or cause abnormal results.

The two liquid markers Co-EDTA and Cr-EDTA were shown to behave in a similar manner. These results are in agreement with studies performed by Teeter & Owens (1983), which concluded that water soluble markers have biologically similar flow properties. A study by Udén *et al* (1980) also showed that Co-EDTA and Cr-EDTA behave in a biologically similar manner with 2-3% loss in urine for both. Our study showed a 95.4% similarity between the levels of marker determined from rumen fluid samples. The slightly higher levels of Cr-EDTA in rumen fluid samples than Co-EDTA may have been due to differences between animals, rather than differences between markers as suggested by Teeter & Owens (1983) as there were differences in rumen volume among cows, or due to slight differences between dosages of each marker when dosed, as markers were dosed in solid form and then allowed to mix, thus some of the marker remained in the dosing containers after dosing. As this is the first evaluation of a fluid phase marker being incorporated into a RPP, there is no literature to be compared to. However results suggest good agreement between markers with consistently similar disappearance curves for the markers when dosed separately as well as for the Co-EDTA combined with the various RPP showing similar trends within RPP for all EP's.

Co/Cr Area Under Curve Comparison

Although no published studies could be found using this method, there are studies which have used other markers, such as HP-Arg, to determine stabilities of RPP from area under curve (AUC) graphs. Stability for RP ascorbic acid was 55.8%, for RP lysine 52.7% and for RP niacin 65.6%. Swanepoel *et al* (2010) determined the stability of RP lysine (prototype AjiPro-L) as the difference between the area for RP lysine and arginine (highly protected (HP)-arginine, which was assumed to be 100% undigested in the rumen) and determined the rumen stability to be 45%. It is difficult to compare these results with ours as Swanepoel *et*

al. (2010) fed the RP lysine mixed with the feed and there was some breakdown of particles during mixing and chewing, thus potentially lowering values relative to those obtained in our study where the RPP were directly inserted into the rumen thereby ensuring correct dosages. There are currently no other studies with a similar approach. It may also be possible to create a database with this method and link up new products by correlating them with the closest tested product and completing the appropriate corresponding *in vivo* incubations to determine the RPP stability and compare with other products of similar specific gravity and size and determine correlations between characteristics and stability values.

Chapter 6: Conclusions and Recommendations

6.1 Conclusions

Rumen study

The ruminal data indicates that the three RP products had very minor influences on ruminal parameters and rumen environment. However these results may not be replicated under normal feeding conditions, where a much lower dosage level will be offered on a continuous basis.

In Vivo Dual Fluid Phase Marker Technique

The *in vivo* method resulted in very similar rumen marker clearance curves for control groups dosed with both Co-EDTA and Cr-EDTA. The Co-EDTA showed repeatable results and release rates *in vivo* when combined with different RPP with the same fat coating. There was some variation among test periods, which could be due to cow variation, as well as variation among products, with some oxidation of RPP over time. None of the three products impacted milk production parameters, most probably because they were given only once every two weeks, which was not sufficient to cause milk responses. The different RPP had slightly different ruminal stabilities despite having the exact same coating, most likely due to variations caused by the interaction of the coated nutrients with the fat coating.

6.2 Summary of Findings

The *in vivo* analyses indicate that the RPP and markers were effective in allowing determination of ruminal clearance curves and thereby the stability of the RPP within the rumen of dairy cows. The control groups showed high levels of similarity between marker rumen clearance curves, indicating good agreement of the markers. The RP niacin had the highest ruminal stability, followed by the RP ascorbic acid and then the RP lysine. The *in sacco* analyses showed mixed results for DM and Co disappearance of the RP products, with the RP niacin again showing the lowest DM and Co disappearances reaffirming its highest ruminal stability

of the three products. It was determined that the best rumen *in sacco* incubation time to reflect the *in vivo* stability was indeterminate for this study. The continuous pH measurement showed normal pH variation over 24 hour periods, with slight differences among RPP. Production parameters remained constant during testing, suggesting no influence of treatment on production.

6.3 Suggestions for Future Research

Further research to investigate the dual fluid marker system is recommended. Studies with other RPP would further this method. Future studies could examine the same RPP under different production systems (i.e. different cow types, diets and production levels). Assessing different types of RPP under different conditions may then be used to create a database of experimental conditions and stabilities based on *in vivo* clearance curves. This would allow for future RPP to be assessed based upon similarity with RPP already tested with this method. It is also suggested that the intestinal availability of the nutrient in each RPP be determined.

The *in vivo* sampling was conducted over 72 h as it was unknown how long the rumen protected products with the Co-EDTA and Cr-EDTA would remain within the rumen. However, with the results now obtained, it is evident that approximately 49 h sampling would suffice for future research conducted in a similar method and under similar conditions to those used in our study. The inclusion of a sodium chloride lick during experiments similar to this one to overcome possible sodium deficiencies from extensive sampling should also be considered.

For *in sacco* incubations it would be advised to insert additional incubation samples around the 12 h period to improve precision and that this method, based on results presented should only be considered for ranking of products and not stability determination.

6.4 Implications for Existing Theory

Results of this study are consistent with research performed by Udén *et al* (1980), who determined that Co-EDTA and Cr-EDTA are suitable rumen fluid markers. However this dual liquid phase marker technique is likely not practical for all RPP under all situations due to the need to manufacture the Co-EDTA containing products. An additional application of the method to determine the optimal *in sacco* incubation time for similar conditions and RPP to create a simple ruminal *in sacco* method for determining ruminal stability of RPP was found to be inadvisable due to the high level of variation from *in sacco* results for both DM and Co.

6.5 Recommendations for Implementation of Techniques

For improved efficiency it is suggested that a 49 h *in vivo* rumen fluid collection period and potentially 25 h *in sacco* incubation is sufficient for the evaluation of *in vivo* ruminal stability of products. It is also recommended that duplicate samples should be taken for all procedures, as was the case in this study, to ensure sufficient sample for testing and if supplementary samples for analysis should be required. About 100 - 150 ml per rumen fluid sample would be suggested to ensure enough for all ruminal fluid analyses. An *in sacco* incubation time corresponding with the *in vivo* rumen stability is not advised to provide a simplified methodology to determine ruminal stability of RPP under similar conditions due to lack of agreement of results. The *in vivo* method alone is recommended for further research.