Determining *in vivo* ruminal stability of three ruminally protected nutrients in lactating Jersey cows

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Submitted to Animal Feed Science and Technology in August of 2012.

Revised and resubmitted in April of 2013.

Revised and resubmitted in June of 2013.

Abstract

Current methods to determine rumen escape (as stability) of rumen protected nutrients (RPNU), such as ruminal *in situ* evaluation, only estimate the rate at which nutrients leave the *in situ* bag (rather than the rumen) and thus can provide only a rough estimate of actual rumen stability, which is also impacted by actual ruminal RPNU release rate, rate of ruminal passage of the RPNU, as well as impacts of ruminative chewing on the RPNU product; all of which can only be estimated. The aim of our study was to use a novel *in vivo* dual liquid phase marker technique to measure actual ruminal stability of three fat coated RPNU products, and to determine if a common *in situ* incubation time could match the *in vivo* values determined among products. The three RPNU products were RP ascorbic acid, RP lysine and RP niacin, which were manufactured to contain Co-EDTA, and pulse dosed into the rumen at a single time for each experimental period, concomitant with an equal weight of Cr which was pulse dosed as free Cr-EDTA. The study was a 4 x 4 Latin square with 14 d periods using 4 multiparous ruminally cannulated lactating Jersey cows. Rumen instability of the RPNU products was measured as the proportion of the area under the curve from ruminal *in vivo* clearance of Co (manufactured into each RPNU product as Co-EDTA) relative to the clearance of Cr (simultaneously rumen dosed as free crystalline Cr-EDTA). The measured rumen payload of the three RPNU products differed despite having the same fat matrix coating and general characteristics, likely due to differences in the chemical interactions of the nutrients with the fat covering, with the *in vivo* measured payload of RP niacin highest at 656 g/kg, relative to RP lysine at 527 (P<0.05) and to RP ascorbic acid at 558 g/kg (P<0.10). *In situ* incubations of the RPNU products in the same cows, at the same time, suggested that *in situ* 30 h dry matter stability was the best predictor of their *in vivo* measured rumen stability. This novel *in vivo* dual liquid phase marker technique can be used to determine the actual rumen stability of any ruminally protected product under any target feeding scenario.

Key words: fluid phase marker, clearance curve, fat coating

Abbreviations: AA, amino acid; AAS, atomic absorption spectrophotometry; AUC, area under the curve; BCS, body condition score; DM, dry matter; LCFA, long chain fatty acids; RPNU, ruminally protected nutrient; SG, specific gravity; TMR, total mixed ration.

1. Introduction

Supplementation of high producing dairy cows with ruminally protected amino acids (AA) has received attention in research studies for over 40 yrs, with a few showing beneficial effects (Robinson, 2010). However water soluble vitamin requirements of ruminants have not been investigated to the same extent. Research in this area is limited and outdated, with modern dairy cows fed different diets, and producing far higher levels of milk, than those in earlier studies. Animal requirements for some nutrients may be much higher than previous estimates based upon concentrations in their diets and microbial biomass (Santschi et al., 2005). As most supplemental AA and water soluble vitamins have been shown to be extensively degraded in the rumen (Niehoff et al., 2009; Zimbelman et al., 2010), dietary supplementation without rumen protection is a very inefficient nutrient delivery mechanism to the intestinal absorptive site.

In order to know the delivery of nutrients to the post-ruminal digestive tract, it is necessary to determine the proportion of ruminally protected nutrients (RPNU) delivered post ruminally (*i.e.,* the ‘payload’). This value can be estimated using an estimated fractional rumen passage rate (k_p) divided by an *in situ* measurement of the fractional bag rumen release rate (k_d) of the products payload in order to predict ruminal escape of the RPNU. However evaluating ruminal escape using this method does not provide an accurate measure of the RPNU ‘payload’, primarily because there are few objective criteria upon which to convert an *in situ* k_d into an *in vivo* k_d, or to estimate k_p of the RPNU products payload.

Plasma responses to feeding a ruminally protected nutrient has been used to estimate post-ruminal delivery of some RPAA (Südekum et al., 2004) by feeding a known quantity of

an AA in an RP form and calculating its relative rumen stability as a proportion of the plasma response. However this procedure requires serial plasma samples, is analytically very costly and has, to date, been limited to AA.

The main purpose of our study was to use an *in vivo* technique which inherently takes into account rate of RPNU release, its rate of ruminal passage and all other factors which impact ruminal stability, such as ruminative chewing, to determine the actual ruminal stability of RPNU, and its payload. The technique is based on the premise that the liquid marker (*i.e.,* Co-EDTA) manufactured into the RPNU product is released from the RPNU products at the same rate as the protected nutrients. However, as this technique cannot be expected to be used on a regular basis due to high costs, a secondary objective was to determine if a common time of *in situ* ruminal incubation existed among the three RPNU products in order to allow a rapid (and relatively simple) *in situ* determination of the actual rumen stability of RPNU products.

2. Materials and Methods

2.1. Cows and Diets

Four lactating multiparous ruminally cannulated Jersey cows [body weight 384 ± 28.0 kg, milk yield 24.0 ± 4.0 L/d, parity 4.5 ± 1.29, days in milk 69 ± 42 d] were used (± denotes standard deviation of means). All cows were milked together and kept in individual pens of 6 x 6 m using wood chips as bedding. Pens were cleaned daily with bedding replenished as needed and cows had *ad libitum* access to a total mixed ration (TMR; Nova Feeds, George, Republic of South Africa; Table 1) and water at all times. Cows were milked in a 20 point Dairy Master swing-over dairy unit with weigh-all electronic milk meters. Daily milk production was recorded using automated milk meters (Total Pipeline Industries, Heidelberg, RSA). Cows were milked twice daily at 7:30 and 16:30 h.

2.2. Experimental Design

The experimental design was a 4 x 4 Latin square with 14 d experimental periods, with the first 6 d for adjustment between periods, d 7 and 8 for *in situ* incubation, and days 11 to 14 for *in vivo* measurements, including rumen fluid sample collection (results of which are not presented here).

This study was approved by the Animal Use and Care committee of the University of Pretoria and cows were handled and managed according to recommended ethical practices (FASS, 2010) at all times.

2.3. Measurements

For ruminal *in situ* incubations, 6 nylon bags were placed in the rumen via cannula using a stocking retainer method (Cruywagen, 2006). Each RPNU product was placed in the rumen of the cow which was to be pulse dosed with that RPNU product during the *in vivo* part of that experimental period, with *in situ* sample removals at 12, 24 and 30 h of incubation. Five grams of each RPNU product was placed in a Dacron bag with 2 bags used for each time period to provide duplicate incubations. Control cows in each experimental period received empty *in situ* bags. Incubated samples were removed from the rumen via the rumen cannula and washed thrice using cold water. Bags were washed by swirling and then pouring the water out. This process was repeated twice, at which time the water was clear. Bags containing RPNU residues were frozen until analysis.

For *in vivo* measurements, cows were dosed via the rumen cannula, immediately prior to the morning feeding *(i.e.,* directly after morning milking) on day 11 of each experimental period, with 150 g of one of the RPNU products (Table 2) containing Co-EDTA, as well as 17.0 g of Cr-EDTA in a free crystal form (control cows were dosed with Co-EDTA and Cr-EDTA, both in a free form) to deliver 2.4 g/cow of Co and 2.4 g/cow of Cr respectively. All RPNU were manufactured to contain 88 g/kg Co-EDTA (87 g/kg for RP lysine), 617 g/kg nutrient (516 g/kg for RP lysine) and 295 g/kg fat matrix covering (397 g/kg for RP lysine)

by QualiTech Inc. (Chaska, MN, USA). The appropriate nutrients and Co-EDTA were combined with fat, pelleted and the pellets were sprayed with a coating of the same fat matrix. The fatty acid profile of the fat matrix is the same as that described in Wrinkle et al. (2012). The RP lysine had a higher proportion of fat coating than the RP ascorbic acid and RP niacin products due to experience by the manufacturer with lysine having higher reactivity with the fat coating. The Co-EDTA and Cr-EDTA markers were selected as being water soluble with similar properties and molecular weights, as Udén et al. (1980) found that Co-EDTA and Cr-EDTA were liquid phase markers with essentially the same ruminal flow characteristics. Rumen fluid samples of 475 ml were collected prior to dosing and thereafter every 2 h until 25 h post-dosing, and then every 4 h until 49 h post-dosing.

2.4. Analytical Methods

Rumen fluid samples were collected by hand by sampling rumen digesta, followed by squeezing the fluid from the digesta into a sample container with residual particles returned to the rumen. Samples then were squeezed through 6 layers of mutton (cheese) cloth and poured into labeled airtight containers. Samples for Co/Cr analysis were in duplicate and both samples were frozen immediately until analysis using a Varian SpectrAA 50 Atomic Absorption Spectrophotometer (Varian, 1989).

In situ residues were analyzed for Co (*i.e.,* RPNU products containing Co-EDTA) and dry matter (DM). Frozen samples were dried in an oven at 60ºC for 24 h and the DM (105ºC for 24 h) of the *in situ* bag residues was gravimetrically determined after air equilibration in a desiccator. Dried residues were removed from the Dacron bags and pooled by treatment and incubation period, ground with a mortar and pestle, and transferred into airtight containers.

All TMR assays were completed according to Malleson et al., (2008), with representative samples collected at the start and end of each experimental period. Milk samples were assayed (Malleson et al., 2008), with samples used in the ratio 9:15 ml (*i.e,* the approximate afternoon/morning milking ratios during the study) over the 4 consecutive days of *in vivo*

analysis in order to represent an average 24 h day. Body condition score (BCS) was assessed at the start and end of each experimental period (Malleson et al., 2008).

2.5. Density

The specific gravity (SG) of each RPNU was determined by placing a measured amount of each RPNU into saline solutions of different concentrations according to Swanepoel et al. (2010). Twenty particles of each RPNU product were placed in different SG solutions and, if the particles all sank, their SG was greater than the SG of the solution. If particles remained floating in the solution, then the particles had a SG less than that of the solution.

2.6. Calculations

The ruminal payload of the three Co-EDTA/RPNU products was estimated by dividing the area under the ruminal clearance curve of the Co-EDTA (manufactured into the products) by the area under the ruminal clearance curve of the Cr-EDTA (simultaneously dosed as crystalline Cr-EDTA) based upon the assumption that the protected nutrient and protected Co-EDTA were released at the same rate. The area under the curve (AUC) was determined during each ruminal *in vivo* sampling period by individual cow using Co and Cr analysis of the rumen fluid samples, and converted to AUC by integration using GNU Octave (GNU Octave Version 3.2.3, configuration i386-apple-darwin8.11.1, 2009), which is an engineering program designed to integrate large volumes of data to determine AUC (Eaton, 2002).

2.7. Statistical Analysis

Response parameters were analyzed using the GLM option of SAS (2004) for a Latin square design with treatment, cow and period as factors. Contrasts were used to compare treatments to control. Significance was accepted if $P \leq 0.05$ and tendencies accepted if $P \leq 0.10$.

3. Results

3.1. Cow Performance

Dry matter intake was 18.4 kg/cow/d (SEM = 0.72), milk production was 24.0 kg/cow/d (SEM = 0.54), milk fat proportion was 41.8 g/kg (SEM = 1.11), milk true protein proportion was 38.5 g/kg (SEM = 0.35), BCS was 2.34 units (SEM= 0.062), body weight was 388.5 kg (SEM = 3.86) and there were no treatment effects.

3.2. Product Specific Gravities

The RPNU all had a SG higher than 1.207 g/cm^3 (Table 3), likely due to the long chain fatty acids (LCFA; *i.e.,* C14, C16, C18) used for its fat coating (Wrinkle et al., 2012).

3.3. In vivo Determination of RPNU Payloads

The Co and Cr concentrations of rumen fluid (Figure 1) are presented as average curves of the Co and Cr over time after ruminal dosing by treatment. The concentration curves of Co and Cr for the RPNU products were consistent among cows and experimental periods (data not shown) suggesting no interactions between the products and the Cr-EDTA due to these factors. There were no treatment effects on ruminal Cr concentrations between any treatment and the control, and the ruminal concentration curves of Co and Cr virtually superimposed in the control treatment when both Co-EDTA and Cr-EDTA were dosed as free crystal.

The RP niacin had the highest nutrient payload at 656 g/kg (Table 3, followed by RP ascorbic acid at 560 and RP lysine at 525 (RP lysine had a lower (P<0.05) rumen stability than RP niacin, but RP ascorbic acid only tended to have (P<0.10) a lower stability). Payloads were not corrected for the 95% similarity between Co and Cr concentrations when both were fed as free crystal in the control treatment.

3.4. In Situ Prediction of RPNU Stability

Rumen protected ascorbic acid had a lower (P<0.05) *in situ* DM disappearance than RP lysine, and RP niacin had less (P<0.05) DM disappearance than both RP ascorbic acid and RP lysine at 12 h of incubation (Table 4). After 24 h of incubation, RP ascorbic acid tended

(P<0.10) to have a lower DM disappearance than RP lysine, and RP niacin had less (P<0.05) DM disappearance than RP ascorbic acid and RP lysine. At 30 h, RP niacin had less (P<0.05) DM disappearance than RP ascorbic acid and RP lysine.

The Co disappearance at 12 h of incubation with RP ascorbic acid was less (P<0.05) than with than RP lysine, and RP niacin had a lower (P<0.05) disappearance than RP lysine. At 24 and 30 h, RP niacin tended (P<0.10) to have a lower disappearance than RP lysine while, at 30 h, RP ascorbic acid also had a tendency (P<0.10) to a lower disappearance than RP lysine.

4. Discussion

4.1. Product Evaluation

The three RPNU products utilized a fat matrix consisting of saturated, rumen stable, long chain fatty acids (LCFA; Wrinkle et al., 2012). Using saturated fatty acids allows for a higher melting temperature and longer shelf life of products, because saturation decreases rancidity and prevents auto-oxidation. The major fatty acids in our RPNU products were C14:1 trans, C16:0 and C18:0 (Wrinkle et al., 2012), which differed from those in another RP lysine product (Robinson et al., 2011) where the major fatty acids were C18:0 and C16:0. Wu et al. (1991) showed that more than 0.9 of LCFA less than 14C disappear in the rumen, but only ~0.3 of all dietary LCFA disappear in the rumen. In addition, selective disappearance occurred with the lowest rumen disappearance of LCFA when only C16, or only C18, LCFA were fed. Use of C14 FA in our RPNU products may have contributed to the lower stability than that estimated by Robinson et al. (2011), where C16 and C18 predominated. It is also possible that the rumen microbes were able to dissociate the nutrient from the fatty acids (rather than degrade the fatty acids).

4.2. Evaluation of Co- and Cr-EDTA

The liquid markers Co- and Cr-EDTA behaved in a similar manner when they were both dosed as the free crystal to control cows (Figure 1), which is consistent with Teeter and

Owens (1983) who concluded that these water soluble markers have biologically similar flow properties, and Udén et al. (1980) who had earlier shown that Co- and Cr-EDTA behave in a biologically similar manner in the gastrointestinal tract. This supports the use of Co-EDTA incorporated into the RPNU product, and for measurement of its release from the RPNU product, as an index of the stability of the nutrient in that RPNU product, when compared to Cr-EDTA simultaneously dosed as free crystal.

4.3. In vivo Determination of RPNU Payloads

Although no published studies have used our *in vivo* method, there is one study which used another marker, highly protected arginine (HP-Arg), to determine stabilities of RPNU products from AUC calculations. Calculated *in vivo* stability for our RPNU products were 558, 527 and 656 g/kg for RP ascorbic acid, RP lysine and RP niacin respectively. Swanepoel et al. (2010) estimated the stability of their RP lysine as the difference between the area of RP lysine release *versus* HP-Arg, which was assumed to be totally undigested in the rumen, and determined its rumen stability to be ~450 g/kg. It is difficult to compare these results with ours, as Swanepoel et al. (2010) used a theoretical approach whereas in our study the RPNU products were directly inserted into the rumen and subject to actual rates of digestion and passage, as well as ruminative chewing.

Although all our RPNU products had the same fat matrix, their measured payloads differed, indicating that the protected nutrients had different extents of chemical and/or physical association with the fatty acid matrix. The RP lysine had a lower stability despite having a higher proportion of fat matrix, which suggests a higher level of reactivity than the other two products and possibly a weaker bond with the fat matrix. Lysine has proven difficult to incorporate into ruminally protected forms and maintain intestinal release and stability, due to its poor manufacture and handling qualities (Barton, 2010) as it is known to be sensitive to Maillard reactions (heating) and has a reactive NH_2 group (McSweeney and Fox, 2009).

4.4. In Situ Prediction of RPNU Stability

In situ disappearance of DM was shown (Kung et al., 2003) to have limited accuracy when assessing the rumen stability of RPNU products, particularly when compared with *in vitro* rumen fermentation stability evaluations of the same products. Our results are consistent with Kung et al. (2003), as there were substantial differences between DM and Co disappearance within incubation time and RPNU product. In addition, there was no time of ruminal *in situ* incubation, which created similar estimates of nutrient payloads based upon Co disappearance. The closest agreement occurred at 12 h of ruminal incubation where the payloads measured *in vivo* and predicted *in situ* were 55.8/52.0, 52.7/16.1 and 65.6/60.8 g/kg respectively for RP ascorbic acid, RP lysine and RP niacin respectively. Clearly the *in vivo* RP lysine payload was not predicted using the same *in situ* procedure, which relatively closely predicted the two other RP nutrient payloads. In contrast to Co, the estimates of nutrient payloads based upon DM disappearance occurred at 30 h of ruminal incubation where the payloads measured *in vivo* and predicted *in situ* were 55.8/51.7, 52.7/53.2 and 65.6/62.2 g/kg respectively for RP ascorbic acid, RP lysine and RP niacin respectively. The large difference in the optimal ruminal incubation time between *in situ* Co and DM disappearance as predictors of measured *in vivo* payloads has no evident explanation. However it seems that, as a general statement, 30 h *in situ* stability was the best predictor of the actual *in vivo* determined nutrient payloads for these three RPNU products, at least under the conditions of our study, and this conclusion contrasts with Kung et al. (2003), who reported that *in situ* disappearance of DM had limited accuracy when assessing the rumen stability of RPNU products.

5. Conclusions

The very similar rumen Co and Cr concentration curves for control cows dosed with both Co- and Cr-EDTA as free crystal supports the use of Co-EDTA included in the RPNU

products to determine ruminal stability of the RP nutrients by comparing the concentration curve of Co to the concentration curve of Cr simultaneously dosed as free crystal. In spite of the general similarity of the RPNU products which we used, particularly in terms of fat matrix protectant, their measured nutrient payloads differed, thereby suggesting that interactions of the payload and coating occurred to impact RPNU product stability. *In situ* incubations of the RPNU products in the same cows pulse dosed with that product suggested that *in situ* 30 h DM stability was the best predictor of the *in vivo* measured rumen stability of these RPNU products under the conditions of this study.

Acknowledgements

Funding of the project was provided by QualiTech Inc. (Chaska, MN, USA), who also provided the tested products, but played no role in collection of samples, statistical analysis or interpretation of the data, or in the layout of the manuscript for publication.

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Table 1

Ingredient and chemical composition (g/kg DM) of the experimental diet fed to the rumen cannulated Jersey cows.

Ingredient composition	
Lucerne, hay	448
Maize, stover	85
Maize, meal	226
Hominy, chop	66
Molasses, liquid	61
Soya oilcake, meal	85
Urea	3
Fat, rumen inert	11
Mineral/vitamin premix[1]	14
Chemical composition	
Dry matter	902
Organic matter	918
Starch	205
Neutral detergent fibre (aNDFom)	369
$dNDF_{30h}$[2] (as aNDFom)	142
Ether extract	34
Crude protein	174
Ca	11
P	2
Co	0.2
Cr	<0.1

[1] Contained/kg of premix: 7000 kIU of Vitamin A; 1500 kIU of Vitamin D_3; 1300 mg of niacin; 1000 mg of Co; 3000 mg of I; 375 mg of Se; 100,000 mg of Mn; 20,000 mg of Cu; 100,000 mg of Zn; 350,000 mg of S; 60,000 mg of Fe.

[2] Digestible neutral detergent fibre digested *in vitro* for 30 h.

Table 2

The rumen protected products and their chemical composition.

Product [a]	Nutrient	Composition of tested product [b]	Specific gravity[c]
Bovi-C	Ascorbic acid	Ascorbic acid, 623 g/kg Co-EDTA, 89 g/kg Fat matrix, 288 g/kg	>1.207
Bovi-Lysine	Lysine	Lysine HCl, 519 g/kg Co-EDTA, 87 g/kg Fat matrix 395 g/kg	>1.207
Bovi-Niacin	Niacin	Niacin, 623 g/kg Co-EDTA 89 g/kg Fat matrix 288 g/kg	>1.207

[a] These are the product names as manufactured by Qualitech Inc. (Chasta, MN, USA), although the actual products used in our study replaced some of the nutrient with Co-EDTA as described in the 'Composition' column of the table.

[b] As specified by the manufacturer.

[c] As determined (see text for method details).

Table 3

Stability evaluation of rumen protected nutrients using GNU Octave.

	Treatment				
	Control	Ascorbic acid	Lysine	Niacin	SEM
Area Under Curve (Co)	161	70	69	55	24.4
Area Under Curve (Cr)	154.0	162.5	145.2	158.6	3.72
Average Ruminal Stability (g/kg)[c]	-	558.4 [y]	527.2 [b]	655.8 [a, x]	0.39

Means on the same line with different [a, b] superscripts differ (P<0.05) whereas means on the same line with different [x, y] superscripts tend to differ (0.05>P<0.10).

[c] - Similarity between free Co-EDTA and Cr-EDTA curves was 95.7% in the control treatment.

Table 4

In situ dry matter and Co disappearance (g/kg) from the rumen protected products at three times of ruminal incubation.

	RP ascorbic acid	RP lysine	RP niacin	SEM
DM disappearance at:				
12 h	29.6 ^{b}	41.9 ^{a}	15.8 ^{c}	6.53
24 h	40.0 ^{x,b}	44.4 ^{y,b}	28.2 ^{a}	4.20
30 h	48.3 ^{a}	46.8 ^{a}	37.8 ^{b}	2.84
Co disappearance at:				
12 h	48.0 ^{b}	83.9 ^{a}	39.2 ^{b}	11.84
24 h	68.5	86.2 ^{x}	64.5 ^{y}	5.80
30 h	84.8 ^{y}	95.6 ^{x}	75.4 ^{y}	5.05

Means on the same line with different $^{a, b, c}$ superscripts differ (P<0.05) whereas means on the same line with different $^{x, y}$ superscripts tend to differ (0.05<P<0.10).

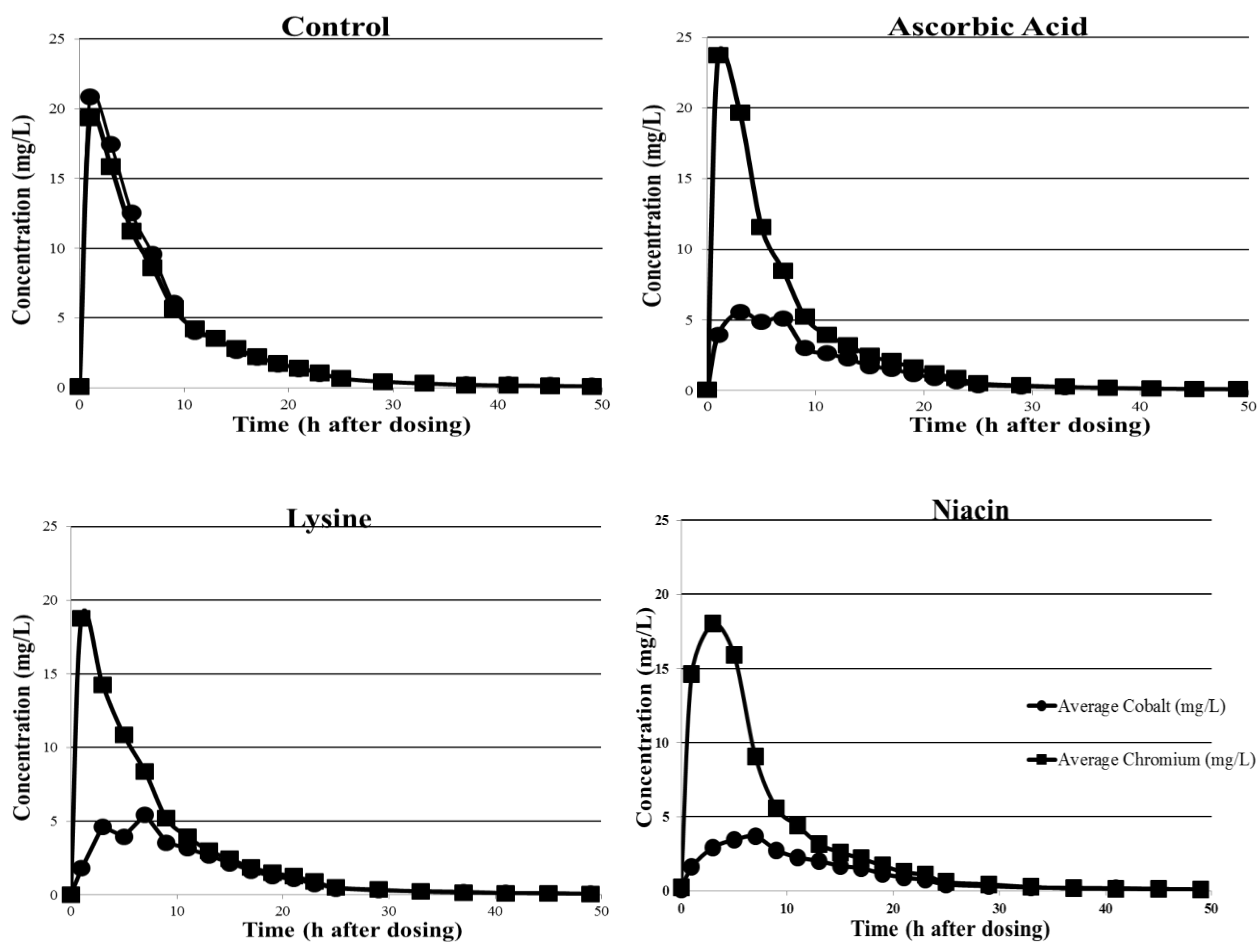

Figure 1. Average ruminal concentrations of Co (as free Co-EDTA from the RPNU product) and Cr (simultaneously dosed as free Cr-EDTA) from cows not fed a RPNU (Control – top left) and for rumen protected nutrient product dosed cows, where Co-EDTA was manufactured into the RPNU and Cr-EDTA was simultaneously dosed as free Cr-EDTA (Top right: ascorbic acid, bottom left: lysine, bottom right: niacin).