Cautions and Considerations When Bioavailability of Rumen Protected Amino Acids is Determined

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Summary

When rumen protected **(RP)** amino acid **(AA)** are included in a ration, knowing its bioavailability is critical to provide additional supply of certain AA with RP-AA that may be limiting milk protein synthesis and improve the profile of metabolizable AA.Various methods are available to determine bioavailability of RP-AA and all the methods have their own pros and cons. In this article, we discusse d particular techniques which is rumen in situ incubation of RP-AA followed by abomasal infusion of the residuals or plasma AA appearance. We conducted multiple experiments where cows were infused 15N-Lys continuously into the abomasum to understand postruminal Lys metabolism or RP-Lys prototypes that contain 15N-Lys were incubated in the rumen followed by abomasal infusion of the residuals. Results indicated that cautions are needed when rumen bypass is determined using an in situ incubation because of potential overestimation of rumen bypass rates of RP-AA. Cautions are also needed when intestinal digestibility or bioavailability of RP-AA are determined using plasma AA appearance. Results indicated that intestinal digestibility or bioavailability of RP-AA can be over- or underestimated. Especially, RP-AA that was highly protected in the rumen was digested in the small intestine slowly and the absorption of Lys released from RP-Lys in the small intestine was slow as well. In this case, plasma Lys concentration was not able to capture accurately the Lys slowly absorbed from RP-AA, resulting in underestimation of intestinal digestibility of RP-AA when plasma Lys appearance was used. We also found that taking ruminal passage rate into account is very critical when rumen bypass and bioavailability of RP-AA are determined. Furthermore, we provided evidence that free AA in the small intestine may not be 100% digestible. Although more studies are needed, if free AA are not 100% digestible in the small intestine this should be quantified and addressed when intestinal digestibility or bioavailability of RP-AA is determined.

Introduction

As protein nutrition in dairy cows is advanced, importance of formulating a ration for amino acids **(AA)** has been emphasized (Schwab and Broderick, 2017; NASEM, 2021). Formulating a ration for AA (vs. crude protein or metabolizable protein) should result in more efficient utilization of dietary N, optimizing production performance, and reducing N excretion (NASEM, 2021). Rumen-protected **(RP)** AA are commercially available (mostly Lys and Met) and have been fed to cows in practice. Feeding RP-AA may become more popular in the future because most requirement models have started switching from a protein-based requirement system to AA-based (e.g., NASEM, 2021; Van Amburgh et al., 2015).

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The purpose of feeding RP-AA is to improve the profile of metabolizable AA and provide AA that are limiting milk protein synthesis. Therefore, we may expect more production (e.g., milk protein), greater dietary N utilization, and less N excretion when a diet is balanced for AA with supplemental RP-AA. When a ration is formulated with RP-AA, nutritionists should know the bioavailability of RP-AA. Inclusion of RP-AA with incorrect information of bioavailability can create an imbalance of metabolizable AA profile, resulting in potential inefficient use of dietary N, no responses of production, and importantly increases in feed costs without returns. Therefore, efforts have been made to develop methods that accurately determine rumen bypass, intestinal digestibility, and bioavailability of RP-AA.

Various methods (in vitro, in situ, in vivo, or combination of some of these) to estimate bioavailability of RP-AA have been available (e.g., Tilley and Terry, 1963; Calsamiglia and Stern, 1995; Overton et al., 1996; Fleming et al., 2019; Räisänen et al., 2020). All the techniques available have pros and cons. One popular approach is the combination method between in situ and in vivo techniques. For example, the rumen bypass rate of RP-AA is determined using an in situ technique (i.e., ruminal incubation of nylon bags containing RP-AA; Fleming et al., 2019; Räisänen et al., 2020), followed by abomasal infusion of the residuals of RP-AA (Fleming et al., 2019) which determine intestinal digestibility using plasma AA appearance (changes in plasma Lys concentration), or plasma AA appearance is solely used to estimate bioavailability after feeding RP-AA (Whitehouse et al., 2017). In this article, we discussed cautions on results of rumen bypass, intestinal digestibility, and bioavailability obtained from these approaches. Also, we discussed potential factors that may significantly affect results of rumen bypass, intestinal digestibility, and bioavailability of RP-AA.

Cautions on Rumen Bypass of RP-AA Determined by an In Situ Method

An in situ technique has been used widely to evaluate feed quality on fractions rumen-degraded and undegraded (Ørskov and Mcdonald, 1979; Liebe et al., 2018). The quality of protein in feedstuffs has been evaluated with this method as well and the results have been accepted by various requirement models, including NRC (2001) and NASEM (2021). The in situ technique uses nylon bags containing feeds and the bags are incubated in the rumen for certain hours to determine how much feed or N disappeared from the bags over time. Therefore, this method has been adopted to determine rumen protection (bypass) of RP-AA. As mentioned previously, the in situ technique has been often used in a combination with in vivo methods to obtain intestinal digestibility in addition to rumen bypass (Robinson et al., 2011; Fleming et al., 2019; Räisänen et al., 2020). However, rumen bypass rates of RP-AA obtained from an in situ method may mislead according to our studies.

We conducted an experiment to determine bioavailability of RP-Lys prototypes where the combination technique of in situ incubation followed by abomasal infusion of the residuals was used, but the prototypes of RP-Lys labeled with ^{15}N-Lys were used. The experiment was conducted with 6 cannulated cows where 2 cows were used only for rumen in situ incubation and then 4 cows were used in a Latin square design for infusion of the residuals from rumen incubation. For treatments, L-Lys containing ^{15}N-Lys was infused into the abomasum for control **(CON)** and 3 different prototypes of RP-Lys (^{15}N-Lys labeled) were infused into the abomasum after rumen incubation. The in situ

rumen incubation of RP-Lys prototypes was conducted for 8 hours and the residuals were infused into the abomasum within 1 hour after the rumen incubation. One prototype was not labeled with ^{15}N-Lys; therefore, results of the other 2 prototypes labeled with ^{15}N-Lys are only discussed in this article. We used 2 cannulated cows only for rumen incubation of RP-Lys (i.e., separate from 4 cows for abomasal infusion) because the prototypes included ^{15}N-Lys and had potential to contaminate samples with ^{15}N (milk, feces, and urine) during rumen incubation, which could have confounded ^{15}N enrichment in samples from abomasal infusion of the residuals. The result of rumen incubation of RP-Lys prototypes is shown in Table 1. Rumen bypass of RP-Lys prototypes was determined with DM, N, and ^{15}N content before and after in situ incubation. When the prototypes were ranked, P2 had the greatest rumen bypass followed by P1 and P3 regardless of the method (i.e., DM, N, and 15N). However, the magnitude of rumen bypass was different among methods, causing an interaction of prototype by method. Overall, rumen bypass determined based on DM was overestimated compared with that based on N, and the degree of overestimation was different among prototypes. This could be a result from contamination (feed particle andmicrobes) or the coating properties. When the rumen bypass was determined with ^{15}N, still P2 was superior in rumen bypass to P3. When compared between methods, the rumen bypass rate was 14% lower for P2 when ^{15}N was used compared with N. This indicates that microbial contamination existed, and use of N content overestimated rumen bypass for P2. Fleming et al. (2019) also observed potential microbial contamination of RP-Lys after rumen incubation where the rumen bypass was greater than 100%. However, the rumen bypass for P3 was similar between N and ^{15}N for P3, indicating no microbial contamination and causing an interaction of prototype by method. The interaction indicated that microbial contamination was different among prototypes during rumen incubation and it likely depended on the amounts of residuals left in nylon bags after the incubation, i.e., more residuals with greater rumen protection had more microbial contamination. From this experiment, we found potential overestimation of rumen bypass when RP-AA were determined from DM and N with an in situ technique, and results need to be interpreted with caution. However, an in situ technique is still useful to rank prototypes in terms of rumen bypass.

Cautions on Bioavailability of RP-AA Determined by Plasma AA Appearance

Although plasma AA concentration reflects AA absorbed in the gut and AA utilization or release from body tissues, it often responds to metabolizable AA supply. When certain AA are provided with supplemental RP-AA, increases in plasma concentration of the AA have been often observed in studies (Lee et al., 2012a; Giallongo et al., 2015; Whitehouse et al., 2017), but not in others (Lee et al., 2012b; Paz and Kononoff, 2014). Because of the reflective responses of plasma AA concentration to AA supply, plasma AA concentration has been used to evaluate bioavailability of RP-AA (i.e., plasma AA appearance). The recommended procedure for the plasma AA technique has been published by Whitehouse et al. (2017). However, although the technique of plasma AA appearance provides bioavailability of RP-AA, it does not provide information of rumen bypass and intestinal digestibility. Therefore, the plasma AA appearance technique has been also used in a combination with an in situ technique mentioned above., i.e., residuals of RP-AA after rumen incubation are infused into the abomasum and plasma AA appearance is used to determine intestinal digestibility of RP-AA. As advantages of this technique, an experiment is relatively simple in design and easy to conduct and not

costly. However, we found that caution is needed when bioavailability is determined with plasma AA appearance.

In the experiment that we mentioned above (in situ rumen incubation of RP-Lys containing ^{15}N-Lys), the residuals of RP-Lys after rumen incubation were infused into the abomasum (n = 4). After infusion, blood samples were collected frequently through jugular catheters over 18 hours (Figure 1). In addition, total feces were collected for 5 days. Intestinal digestibility of RP-Lys was first determined using plasma Lys appearance, i.e., area under the curve **(AUC)** of plasma Lys concentration above the baseline (concentration at -2 h before infusion) relative to AUC of CON (L-Lys infusion assuming 100% of intestinal digestibility). The result is shown in Table 2. Secondly, ^{15}N excretion was determined from total feces collected over 5 days after abomasal infusion and the intestinal digestibility was determined by the following equation: (^{15}N from RP-Lys residuals infused – ^{15}N excreted in feces) ÷ ^{15}N from RP-Lys residuals infused × 100. Because ^{15}N excreted in feces originated 100% from ^{15}N infused (RP-Lys residuals), this equation provides true intestinal digestibility of RP-Lys residuals infused into the abomasum (Table 3). The intestinal digestibility of RP-Lys residuals was compared between plasma Lys appearance and ^{15}N excretion in feces. Plasma Lys concentration increased for a few hours after infusion and decreased until 6 to 8 hours after infusion. Based on the AUC for prototypes relative to CON, the intestinal digestibility of P2 and P3 was 10 and 138%, respectively, where the intestinal digestibility of P3 was clearly overestimated. When the bioavailability was calculated for P2 and P3 (i.e., rumen bypass in Table 1 and intestinal digestibility in Table 2), they were 10 and 8%, respectively, concluding that the bioavailability was similar between P2 and P3. However, when the intestinal digestibility was calculated using ^{15}N excretion in feces, P2 and P3 had 40 and 86% of intestinal digestibility, respectively, and those resulted in 34 and 5% of the bioavailability, respectively. Assuming estimation with ^{15}N excretion in feces is more accurate, plasma Lys appearance underestimated intestinal digestibility for P2 and overestimated that for P3. For P3, the bioavailability estimated between plasma Lys appearance and ^{15}N excretion in feces was quite comparable (8 vs. 5%), but the similar bioavailability was just coincidental because the rates of rumen bypass and intestinal digestion were largely different. Then, the following question arises: why was the intestinal digestibility of P2 underestimated by plasma Lys appearance when compared with ^{15}N excretion in feces, and why was that of P3 overestimated? For P3 being overestimated, it is difficult to explain, but the overestimation for P3 was obvious because the intestinal digestibility was greater than 100%. On the other hand, we found a reason that underestimation of intestinal digestibility for P2 occurred. When ^{15}N enrichment of plasma Lys was determined, the pattern of ^{15}N enrichment of plasma Lys over time was different between P2 and P3 (Figure 2). While the pattern of ^{15}N enrichment for P3 over 18 hours was quite similar with the pattern of plasma Lys concentration, i.e., ^{15}N enrichment of plasma Lys peaked at 2 hr after infusion and then decreased, and that for P2 was different. ^{15}N enrichment of plasma Lys for P2 increased gradually for 10 hr after infusion and then decreased (Figure 2). Compared with P3, P2 had greater rumen bypass, and it is well known that RP-AA that has highly-rumen protected has less digestibility in the intestine. The release and absorption of Lys from P2 was relatively slow in the small intestine; therefore, the slow absorption of Lys from P2 over 18 hr was not large enough to be fully reflected in plasma Lys concentration, causing underestimation of intestinal digestibility. This indicates that plasma Lys appearance might be useful to rank

prototypes for intestinal digestibility (in situ incubation of RP-AA followed by abomasal infusion) or bioavailability (feeding RP-AA), but cautions are needed when relative or absolute intestinal digestibility or bioavailability of RP-AA are determined.

Considerations When Bioavailability of RP-AA is Determined

There are several in vivo techniques available in determining bioavailability of RP-AA, including techniques mentioned previously. An in vivo technique should provide better estimation of bioavailability of RP-AA because RP-AA go through the actual digestion process. However, an experiment with in vivo techniques is usually laborious and costly. Therefore, it might be ideal to evaluate RP-AA for bioavailability with a non-in vivo technique first and then absolute bioavailability is obtained using an in vivo technique. For example, RP-AA prototypes can be screened with an in vitro or in situ technique to select the best performing prototype in terms of rumen bypass and intestinal digestibility, and then the absolute bioavailability of the selected protype is determined in vivo. As a long-term and ultimate goal, a standard in vivo method should be established, and the standard in vivo method needs to be used to improve the existing non-in vivo methods or develop a new non-in vivo method so that an in vivo experiment can be avoided to obtain bioavailability of RP-AA. Therefore, we suggest here several factors that need to be considered when a method (in vivo or non-in vivo methods) is developed or improved.

Passage rate

From our previous studies, we found that considering the ruminal passage rate seems critical when bioavailability of RP-AA is determined. In the study that we mentioned previously (in situ rumen incubation of RP-Lys followed by abomasal infusion of the residuals), we obtained 34 and 5% of bioavailability (approach of ^{15}N excretion in feces) for P2 and P3, respectively. According to the result obtained from the method, we concluded that P2 provides about 6 times more metabolizable Lys than P3, and P2 is the better performing prototype. Another experiment with the same prototypes (i.e., P2 and P3) was conducted where 2 ruminally-cannulated cows were used in a crossover design. In this experiment, cows were pulse-dosed P2 or P3 in the rumen and milk samples were collected for 6 days after the dose. Milk samples were analyzed for ^{15}N secretion and ^{15}N enrichment of milk AA. The secretions of ^{15}N in milk for 6 days after the ruminal pulse-dose of P2 or P3 are shown in Figure 3, and ^{15}N enrichment of milk AA on day 1 after dosing is present in Figure 4. According to ^{15}N secretion in milk, P3 had 4 times greater bioavailability compared with P2, which was surprisingly quite opposite to the results obtained above where P3 was 6 times less bioavailable compared with P2 (in situ rumen incubation followed by abomasal infusion). However, if P3 was largely digested in the rumen as observed during the in situ incubation of P3, ^{15}N-Lys in P3 might have been largely metabolized by rumen microbes when dosed in the rumen. If this was the case, part of ^{15}N-Lys might have ended up in milk as ^{15}N-microbial AA. If milk ^{15}N shown in Figure 3 originated mostly from ^{15}N-microbial AA, ^{15}N enrichment of milk AA should have spread out quite uniformly among milk AA because ^{15}N-NH_3 infusion in the rumen labels rumen microbes (microbial AA) with ^{15}N and labels uniformly milk casein AA with ^{15}N (Guillin et al., 2022). However, that was not the case in the current study. As shown in Figure 4, the major AA that was labeled with ^{15}N was Lys in milk for both P2 and P3. Although the enrichment was very small, other AA in milk were also labeled with ^{15}N. The ^{15}N enrichment of other AA can be explained

partly by the contribution of ^{15}N-microbial AA (originating from ^{15}N-Lys from P2 and P3) as described above. In addition, Lys is usually taken up in excess by the mammary glands and part of Lys taken up in excess is oxidized and the amino group is utilized to synthesize other AA through transamination in milk (Lapierre et al., 2009), which also contributed to ^{15}N enrichment of AA other than Lys in milk in this study. We expect that both sources contributed to ^{15}N enrichment of AA other than Lys to an extent. However, the fact that ^{15}N enrichment mainly occurred in milk Lys indicates that ^{15}N found in milk was mostly taken up by the mammary glands as Lys, suggesting that P3 reached the small intestine and provided metabolizable Lys greater than P2. Therefore, we concluded that the method of the in situ incubation of RP-Lys considerably underestimated the rumen bypass of P3 and this is explained by no consideration of ruminal passage when the in situ method was used. Future methods should consider ruminal passage when rumen bypass of RP-AA is determined. This also suggests that the rumen bypass of RP-AA can be altered to some degree by diets that affect ruminal passage rates (i.e., forage to concentrate).

Intestinal digestibility of free AA

Studies with AA infusion into the abomasum have been conducted to understand postruminal roles of AA, which advanced AA nutrition in dairy cows. In those studies, free AA infused into the abomasum have been assumed to be 100% digestible. When bioavailability of RP-AA is determined, this assumption has been also used. For example, plasma AA appearance for RP-AA is calculated relative to plasma AA appearance for free AA infused into the abomasum (i.e., control), where free AA infused are assumed to be 100% digestible. From our multiple studies where isotope labeled-AA (^{15}N-Lys or ^{13}C-Met) were infused by either a pulse dose or continuous dose into the abomasum, we consistently observed that 8 to 12% of ^{15}N infused was excreted in feces (data not shown). As an example, see Figure 5 for ^{15}N enrichment in feces during continuous infusion of ^{15}N-Lys into the abomasum. The excretion of ^{15}N in feces can be a result from: 1) recycling back to the gut after absorption followed by excretion, 2) gut tissue utilization and then excretion after tissue protein catabolism, 3) gut microbial utilization and excretion, or 4) just excretion without being metabolized. If ^{15}N-Lys was absorbed in the gut or utilized by the gut tissues, these should be considered 'digested' although excreted in feces. If ^{15}N-Lys was utilized by gut microbes and excreted in feces or excreted without being metabolized in the gut, then fecal ^{15}N from those should be considered 'undigested'. We do not know what sources contributed the most to ^{15}N excreted in feces in our studies. For further understanding, fecal samples collected on days 4 and 5 during the continuous infusion (Figure 5) were analyzed for ^{15}N enrichment in fecal free AA. Free AA were water-extracted from feces and analyzed for ^{15}N enrichment of AA. Free AA from feces were detectable and Lys was the major AA enriched with ^{15}N (Figure 6). This could be evidence that part of ^{15}N-Lys infused was excreted in feces without being absorbed or metabolized. If this was true, the results indicate that free AA in the small intestine are not 100% digestible (at least Lys), and true intestinal digestibility of free AA should be quantified and considered in the future methods that measure intestinal digestibility of RP-AA.

Further information beyond the bioavailability of RP-AA

When bioavailability of RP-AA is determined, it would be great if the method can produce additional information, such as utilization and excretion of absorbed AA that

originated from RP-AA. The information would be useful to provide RP-AA to cows more precisely in the future when sufficient data are accumulated. This is because the purpose of feeding RP-AA is to provide ideal AA profile without shortage of certain AA so that metabolizable AA are utilized more efficiently for milk protein synthesis. Then, it would be critical to know whether RP-AA provided was actually utilized more efficiently for milk production. We consistently observed that part of ^{15}N-Lys infused in the abomasum excreted in urine (data not shown), suggesting oxidation of metabolizable Lys and excretion without being used for protein synthesis. Accumulation of data about AA utilization (milk secretion) and oxidation (urinary excretion) in relation to feeding RP-AA would also help improve current AA requirement models. Current available methods that measure bioavailability of RP-AA do not provide such information. Production trials with feeding RP-AA have been conducted and measured milk yield and milk protein yield in response to RP-AA supply, which indirectly provides information of use of RP-AA. However, responses to feeding RP-AA are highly variable and inconsistent, indicating that deeper knowledge on AA utilization or oxidation after absorption in various conditions (diets, lactation stage, etc.) is necessary for precise formulation of a diet for AA. Therefore, efforts are needed to develop a method to determine fates of RP-AA after absorption in addition to bioavailability.

Conclusion

We found some evidence that cautions are needed for rumen bypass, intestinal digestibility, and bioavailability of RP-AA when determined using in situ rumen incubation of RP-AA and plasma AA appearance. Improving or developing an in vivo method as a gold standard is necessary to measure bioavailability of RP-AA, and ultimately efforts are needed to improve non-in vivo techniques to determine bioavailability of RP-AA accurately by comparing with the gold standard in vivo method. When in vivo and non-in vivo methods are improved or developed, considering rumen passage rate and true intestinal digestibility of free AA will be critical for accurate determination of bioavailability of RP-AA.

References

Calsamiglia, S., and M.D. Stern. 1995. A three-step in vitro procedure for estimating intestinal digestion of protein in ruminants. J. Anim. Sci. 73:1459–1465.

Fleming, A.J., K.A. Estes, H. Choi, B.A. Barton, C.A. Zimmerman, and M.D. Hanigan. 2019. Assessing bioavailability of ruminally protected methionine and lysine prototypes. J. Dairy Sci. 102:4014–4024.

Giallongo, F., A.N. Hristov, J. Oh, T. Frederick, H. Weeks, J. Werner, H. Lapierre, R.A. Patton, A. Gehman, and C. Parys. 2015. Effects of slow-release urea and rumen-protected methionine and histidine on performance of dairy cows. J. Dairy Sci. 98:3292-3308.

Guillin, F.M., C. Gaudichon, L. Guerin-Deremaux, C. Lefranc-Millot, G. Airinei, N. Khodorova, R. Benamouzig, P.H. Pomport, J. Martin, and J. Calvez. 2022. Real ileal amino acid digestibility of pea protein compared to casein in healthy humans: A randomized trial. Am. J. Clin. Nutr.115:353-363.

Lapierre, H., L. Doepel, E. Milne, and G.E. Lobley. 2009. Responses in mammary and splanchnic metabolism to altered lysine supply in dairy cows. Animal 3:360-371.

Lee, C., A. N. Hristov, K. S. Heyler, T. W. Cassidy, H. Lapierre, G. A. Varga, and C. Parys. 2012b. Effects of metabolizable protein supply and amino acid supplementation on nitrogen utilization, milk production, and ammonia emissions from manure in dairy cows. J. Dairy Sci. 95:5253-5268.

Lee, C., A.N. Hristov, T.W. Cassidy, K.S. Heyler, H. Lapierre, G.A. Varga, M.J. de Veth, R.A. Patton, and C. Parys. 2012a. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J. Dairy Sci. 95:6042-6056.

Liebe, D.., J.L. Firkins, H. Tran, P.J. Kononoff, and R.R. White. 2018. Technical note: Methodological and feed factors affecting measurement of protein A, B, and C fractions, degradation rate, and intestinal digestibility of rumen-undegraded protein. J. Dairy Sci. 101:8046–8053.

NASEM. 2021. National Academies of Sciences, Engineering, and Medicine. Nutrient requirements of dairy cattle: 8th rev. ed. Natl. Acad. Sci. Washington, DC.

NRC. 2001. National Research Council. Nutrient requirements of dairy cattle. 7th rev. ed. Natl. Acad. Sci. Washington, DC.

Ørskov, E.R., and I. Mcdonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. 92:499–503.

Overton, T.R., D.W. Lacount, T.M. Cicela, and J.H. Clark. 1996. Evaluation of a ruminally protected methionine product for lactating dairy cows. J. Dairy Sci. 79:631–638.

Paz, H.A. and P.J. Kononoff. 2014. Lactation responses and amino acid utilization of dairy cows fed low-fat distillers dried grains with solubles with or without rumen-protected lysine supplementation. J. Dairy Sci. 97:6519-6530.

Räisänen, S.E., C.M.M.R. Martins, K. Nedelkov, J. Oh, M.T. Harper, A. Melgar, X. Chen, C. Parys, R.A. Patton, M. Miura, and A.N. Hristov. 2020. Bioavailability of rumen-protected methionine, lysine and histidine assessed by fecal amino acid excretion. Anim. Feed Sci. Technol. 268.

Robinson, P.H., N. Swanepoel, I. Shinzato, and S.O. Juchem. 2011. Productive responses of lactating dairy cattle to supplementing high levels of ruminally protected lysine using a rumen protection technology. Anim. Feed Sci. Technol. 168:30–41.

Schwab, C.G. and G.A. Broderick. 2017. A 100-Year Review: Protein and amino acid nutrition in dairy cows. J Dairy Sci 100:10094-10112.

Tilley, J.M.A., and R.A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. Grass Forage Sci. 18:104–111.

Van Amburgh, M.E., E.A. Collao-Saenz, R.J. Higgs, D.A. Ross, E.B. Recktenwald, E. Raffrenato, L.E. Chase, T.R. Overton, J.K. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci. 98:6361-6380.

Whitehouse, N.L., C.G. Schwab, and A.F. Brito. 2017. The plasma free amino acid dose-response technique: A proposed methodology for determining lysine relative bioavailability of rumen-protected lysine supplements. J. Dairy Sci. 100:9585-9601.

Table 1. Rumen bypass rates of RP-Lys prototypes measured using DM, N, and ^{15}N from an in situ rumen incubation for 8 hours (Rebelo et al. unpublished).

Items	RP-Lys prototypes[1]			SEM	*P*-values[2]		
	P1	P2	P3		RP	M	Int
Rumen bypass, %					< 0.01	< 0.01	< 0.01
DM	69.3^{by}	101.6^{ay}	16.3^{cx}	1.28			
N	60.2^{bx}	98.9^{ay}	5.8^{cy}	1.35			
^{15}N	-	85.1^{ax}	5.4^{cy}	1.10			

^{abc}Means with different letters are significantly different within a row.
^{xyz}Means with different letters are significantly different within a column.
[1]P1 did not include ^{15}N-Lys and P2 and P3 contained ^{15}N-Lys.
[2]RP, effects of RP-Lys prototype; M, effects of method; and Int, interaction of RP × M.

Table 2. Intestinal digestibility and bioavailability of RP-Lys determined by plasma Lys concentration after abomasal infusion of RP-Lys residuals from the in situ incubation (Rebelo et al. unpublished).

Items	CON[2]	RP-Lys prototypes[1]		
		P1	P2	P3
Plasma Lys appearance				
Plasma Lys area 18 h[3]	833	421	92	71
Plasma Lys area 8 h[3]	780	279	73	54
Intestinal digestibility, %				
18 h		89	10	138
8 h		63	9	112
Bioavailability,4 %				
18 h		53	10	8
8 h		38	9	6

[1]P1 did not include ^{15}N-Lys and P2 and P3 contained ^{15}N-Lys.
[2]Abomasal infusion of L-Lys.
[3]Calculated with the trapezoidal rule.
[4]Rumen bypass rates determined with N content in Table 1 was used.

Table 3. Rumen bypass, intestinal digestibility, and bioavailability of RP-Lys determined using abomasal infusion of RP-Lys residuals containing ^{15}N-Lys (Rebelo et al. unpublished).

Items	CON[2]	RP-Lys prototypes[1]		SEM	*P*-values
		P2	P3		
Rumen bypass,[3] %	100[a]	85.1b	5.4[c]	1.91	< 0.01
^{15}N intestinal digestibility, %	92.8[a]	40.1[b]	86.0[a]	2.74	< 0.01
Bioavailability, %	92.8[a]	34.3[b]	4.6[c]	2.12	< 0.01

[abc]Means with different letters are significantly different within a row.
[1]P2 and P3 contained ^{15}N-Lys.
[2]Abomasal infusion of L-Lys.
[3]Determined by ^{15}N remaining in RP-Lys residuals after rumen incubation.

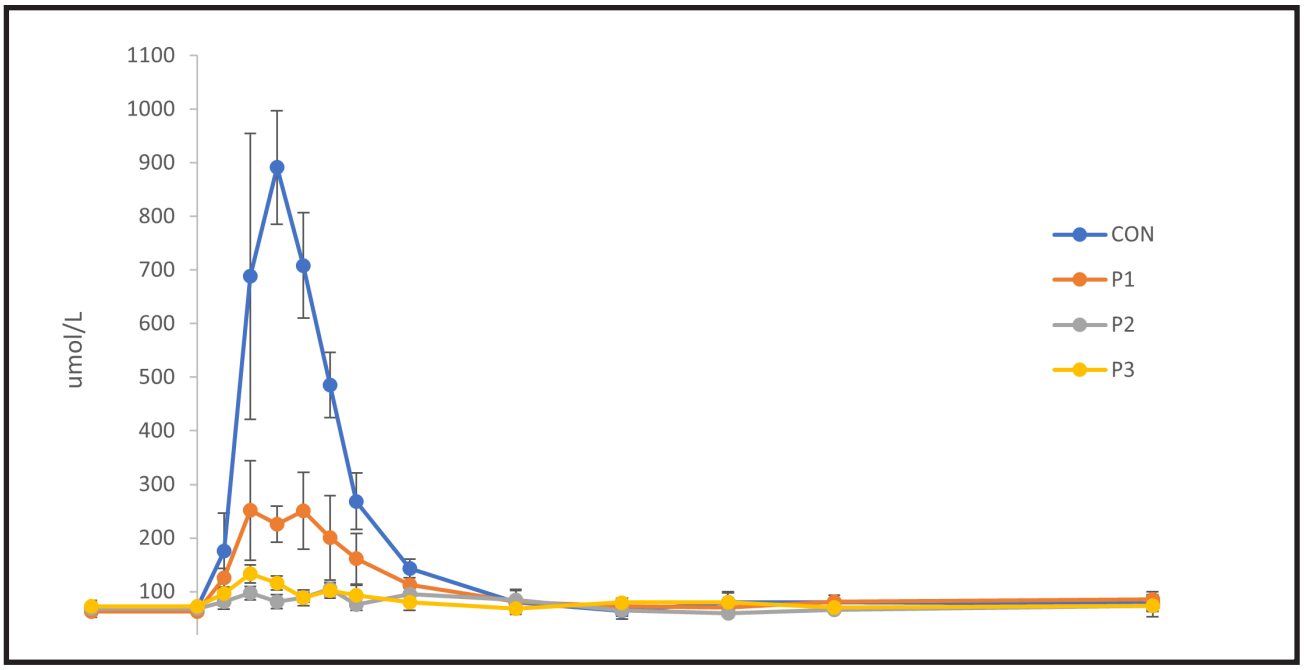

Figure 1. Plasma Lys concentration over 18 hours after abomasal infusion of RP-Lys residuals after rumen incubation. CON, abomasal infusion of L-Lys; P1, RP-Lys prototype 1 without ^{15}N-Lys; P2, RP-Lys prototype 2 with ^{15}N-Lys; P3, RP-Lys prototype 3 with ^{15}N-Lys (Rebelo et al. unpublished).

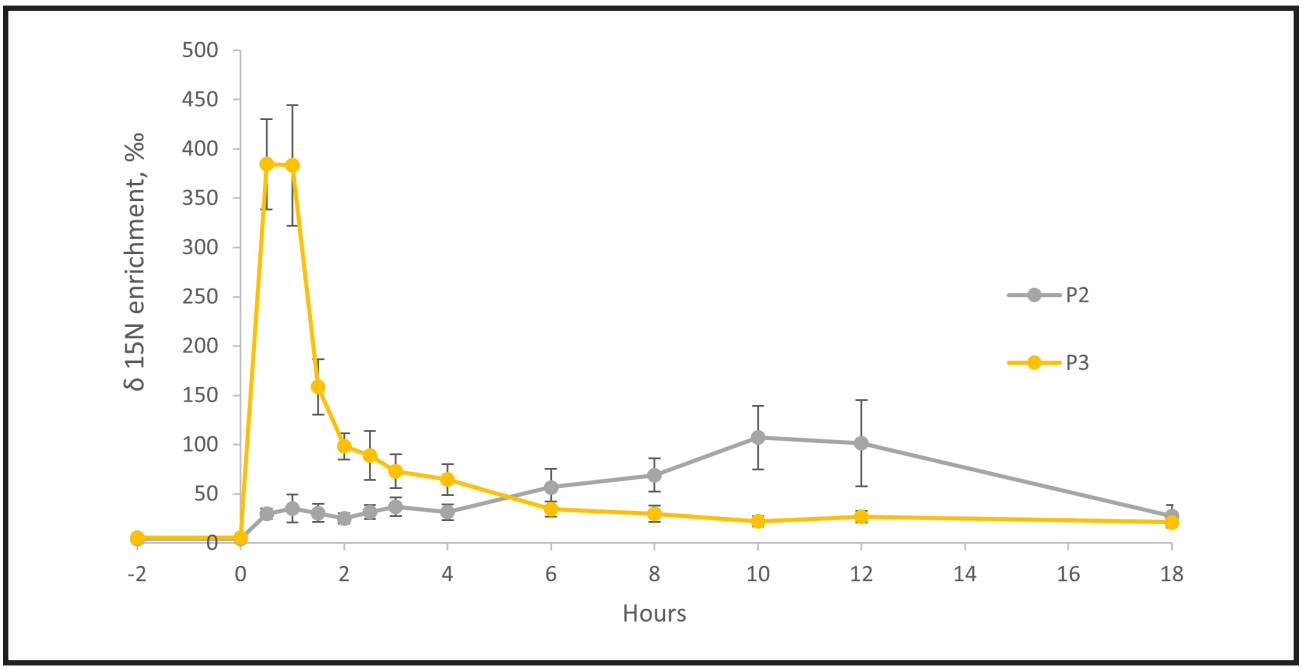

Figure 2. ^{15}N enrichment of plasma Lys over 18 hours after abomasal infusion of RP-Lys residuals (P2 and P3) after rumen incubation. P2, RP-Lys prototype 2 with ^{15}N-Lys; P3, RP-Lys prototype 3 with ^{15}N-Lys (Rebelo et al. unpublished).

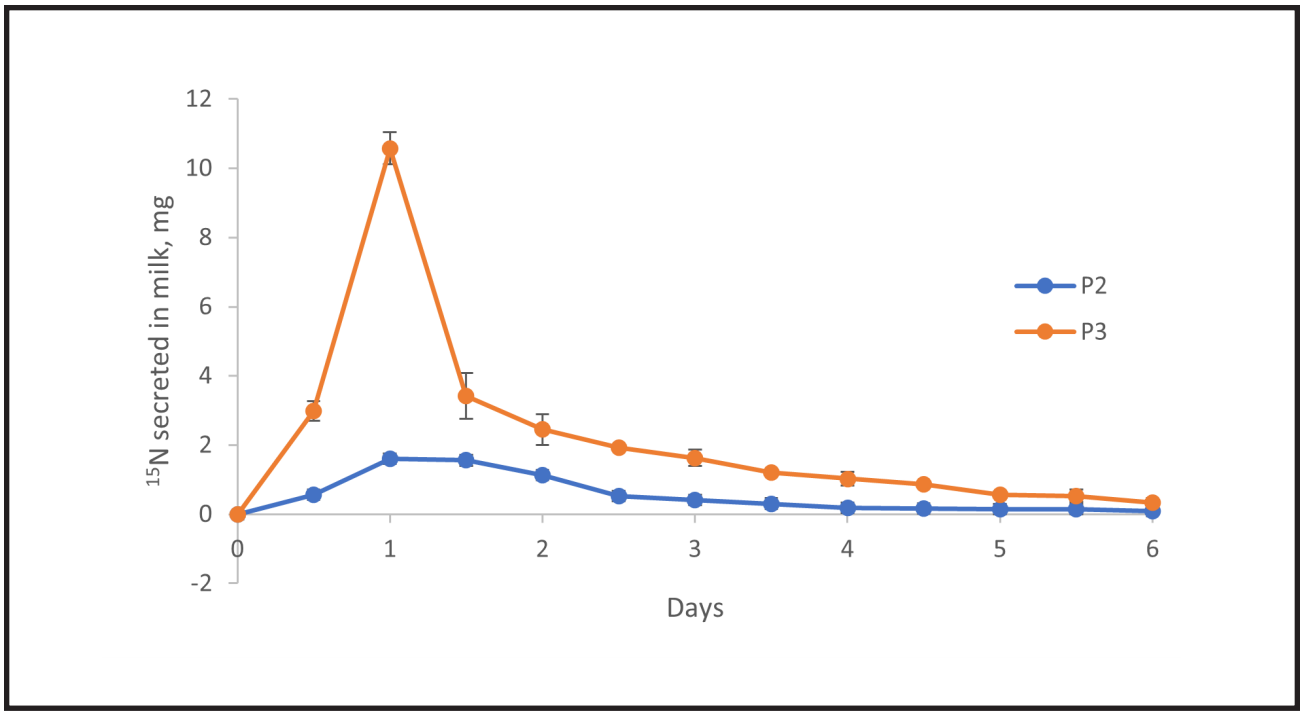

Figure 3. ^{15}N secretion in milk after a pulse-dose of RP-Lys prototypes (P2 and P3). P2, RP-Lys prototype 2 with ^{15}N-Lys; P3, RP-Lys prototype 3 with ^{15}N-Lys (Rebelo et al. unpublished).

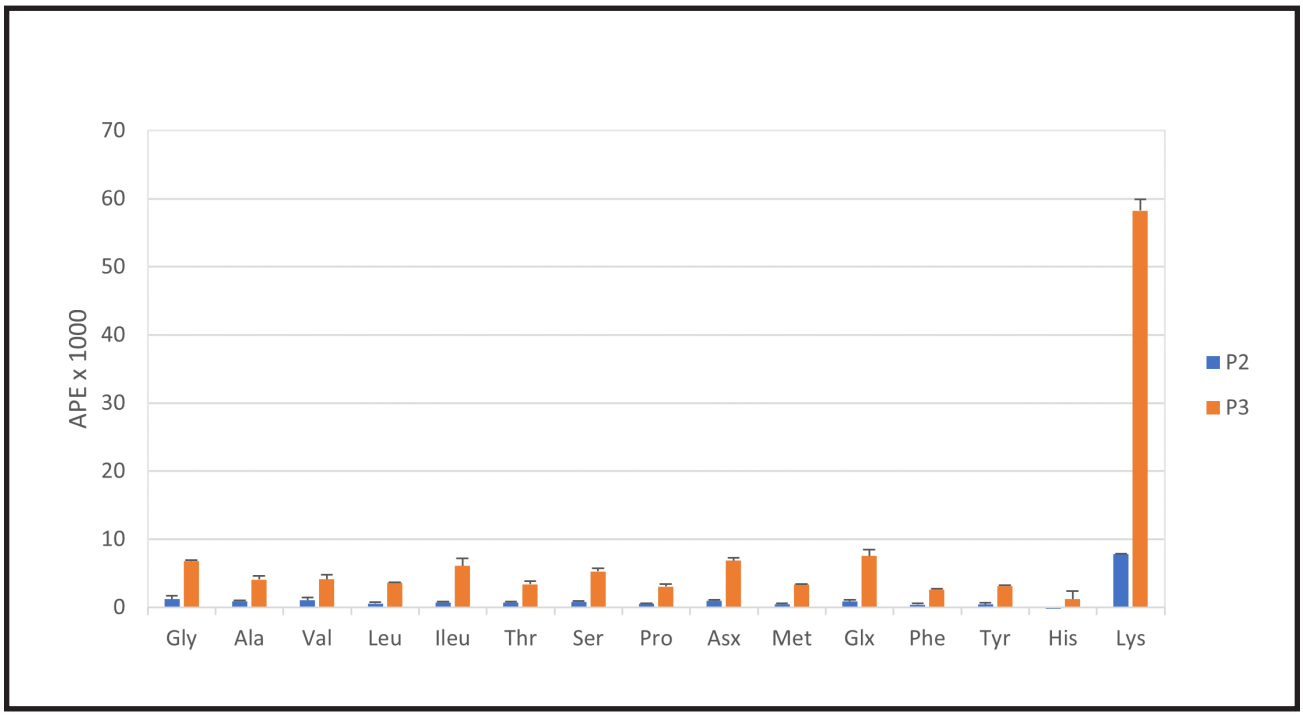

Figure 4. ^{15}N enrichment in milk AA (atom percent excess) on day 1 after a pulse-dose of RP-Lys prototypes (P2 and P3). P2, RP-Lys prototype 2 with ^{15}N-Lys; P3, RP-Lys prototype 3 with ^{15}N-Lys (Rebelo et al. unpublished).

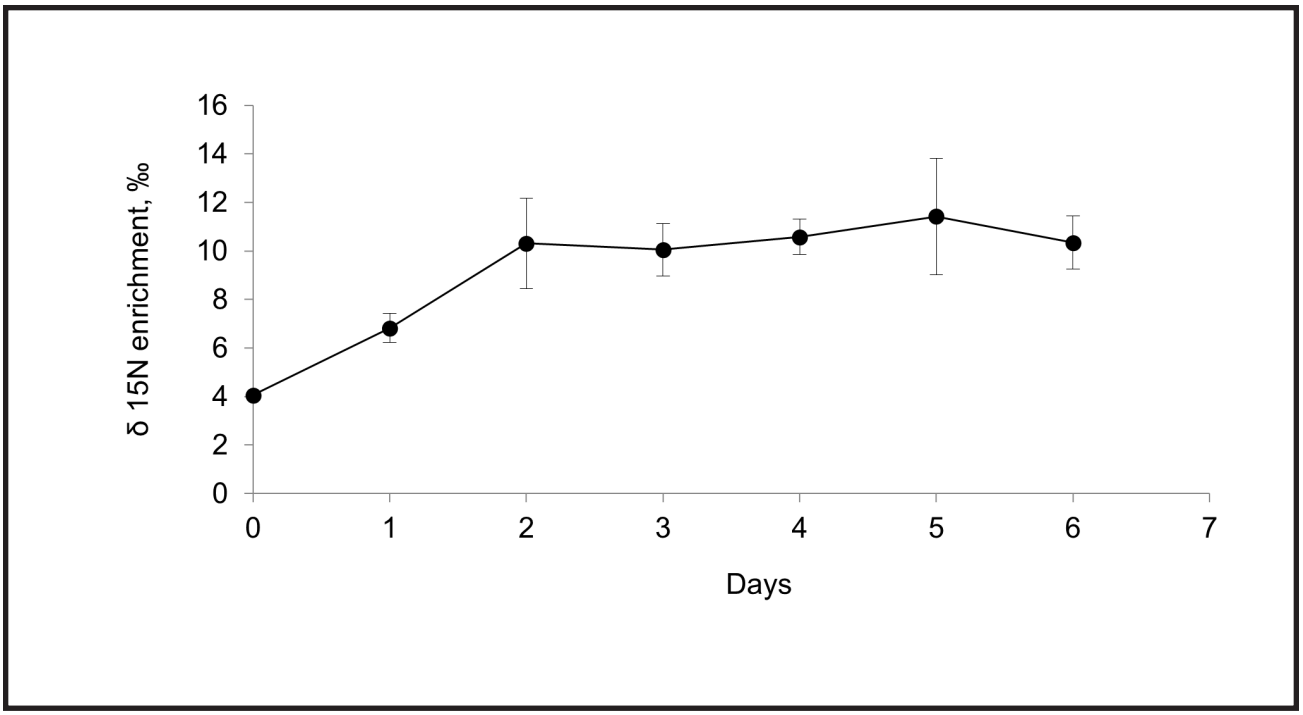

Figure 5. Fecal ^{15}N enrichment during continuous infusion of ^{15}N-Lys into the abomasum for 6 days (Rebelo et al. unpublished).

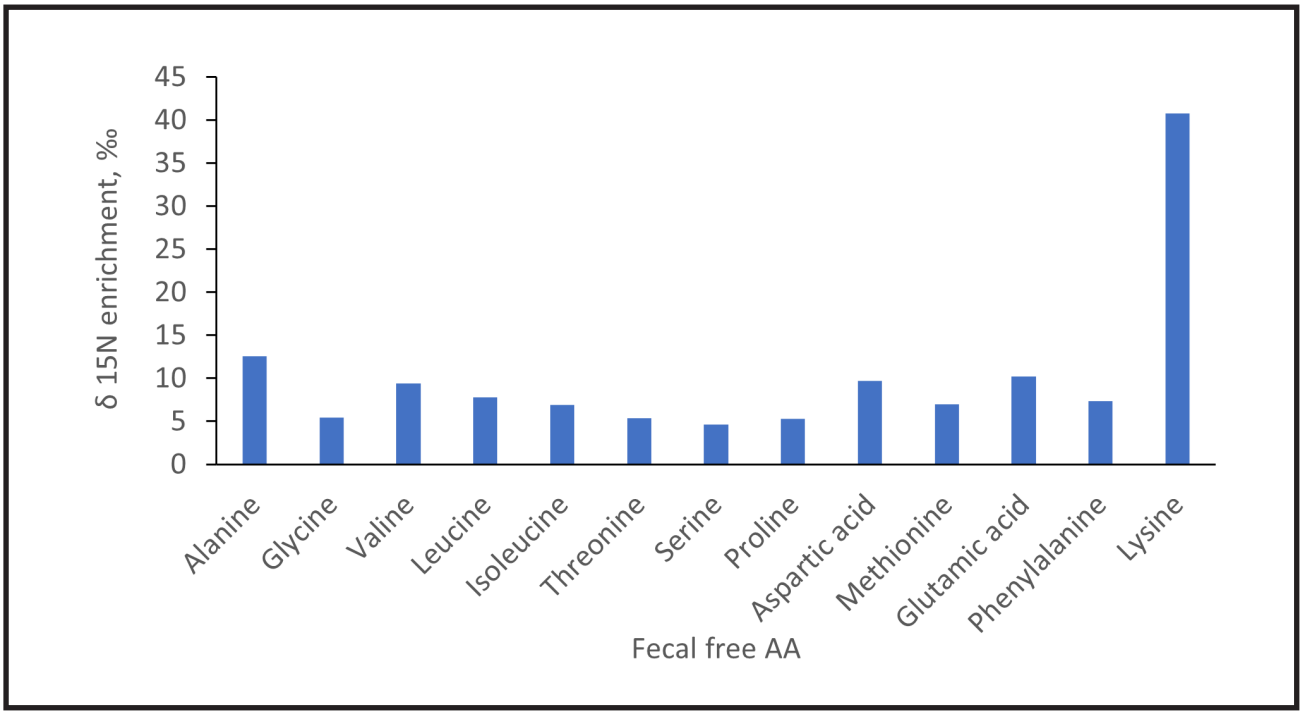

Figure 6. 15N-enrichment of free AA water-extracted from feces (Rebelo et al. unpublished).