The plasma free amino acid dose-response technique: A proposed methodology for determining lysine relative bioavailability of rumen-protected lysine supplements

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ABSTRACT

Estimates of Lys bioavailability of rumen-protected Lys (RP-Lys) supplements are often obtained using in vitro or 2-step in situ techniques, with little to no data determining efficacy and bioavailability in vivo. The objective of this study was to further evaluate and refine the use of the plasma free AA dose-response technique as a method for determining Lys relative bioavailability of RP-Lys supplements. Thirteen dose-response Latin square studies using 87 lactating, ruminally cannulated multiparous Holstein cows (days in milk from 55 to 315 and milk yield from 12 to 62 kg/d at the start of the studies) were conducted to measure the relative bioavailability of RP-Lys supplements. Intestinal (1 study) and abomasal (12 studies) infusions of Lys ranged from 0 to 84 g/d, and experimental periods ranged from 4 to 21 d. Basal diets were formulated to be adequate in metabolizable Met, but varied in predicted metabolizable Lys (5.04 to 6.81% of metabolizable protein). One to 4 daily blood samples were taken from the coccygeal vessels for 1 to 3 consecutive days in each period. Plasma Lys concentration in cows assigned to the control treatment (0 g/d Lys) ranged from 1.83 to 5.21% of total plasma AA, whereas that from cows duodenally or abomasally infused with Lys ranged from 2.53 to 7.51% of total plasma AA. Results from studies involving more than 2 amounts of infused Lys confirmed linearity of response. The following variables were regressed against the plasma Lys dose-response slopes generated from the Lys infusion treatments to examine their effects on the magnitude of the slopes: plasma Lys concentration of the control diet, plasma Lys concentration at the greatest amount of infused Lys, net energy of lactation and metabolizable protein balances, metabolizable protein supply, days in milk, milk yield, milk concentrations of fat, true protein, and lactose, milk true protein

yield, and dry matter intake. The variable having the greatest effect on the magnitude of the dose-response slope was the plasma Lys concentration at the greatest amount infused. The relative bioavailability of evaluated RP-Lys supplements using the plasma free AA dose-response technique ranged from 5 to 87%. It was concluded that plasma free Lys increases in a linear fashion to increasing amounts of absorbed Lys and that the dose-response technique is an appropriate technique for evaluating RP-Lys supplements.

Key words: dairy cow, dose-response, lysine, relative bioavailability

INTRODUCTION

Lysine and Met are the 2 most limiting AA in typical North American dairy diets (NRC, 2001). Increasing Lys in MP to more optimal concentrations by postruminal infusion (King et al., 1991; Schwab et al., 1992) or by feeding a rumen-protected Lys (**RP-Lys**) supplement (Robinson et al., 2011; Lee et al., 2012) increased milk protein yield. Several RP-Lys supplements are available for the purpose of formulating rations for increased predicted concentrations of Lys in MP. Availability, variability in nutritional composition, digestibility, and cost of blood and fish meals have made RP-Lys supplements an attractive alternative, or a partial substitute, to these 2 RUP, Lys-rich supplemental protein sources. The RP-Lys supplements currently available on the market differ not only in terms of encapsulation technology, but also in size, density, and Lys concentration. Limited research indicates that the apparent availability of Lys to ruminants from RP-Lys supplements also varies widely (Robinson et al., 2011; Wu et al., 2012; Larson et al., 2015), making reliable estimates of Lys bioavailability essential when considering the use of these products. Although most suppliers provide estimates of Lys bioavailability for their RP-Lys supplements, these bioavailability values were not obtained using standardized techniques or methodologies. Without a standardized procedure

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for differentiating RP-Lys supplements under feeding conditions closer to commercial practices, producers and industry personnel have no reliable information to make decisions about which RP-Lys supplement to use based on price and amount of absorbable Lys.

The in situ technique that was developed to measure digestion of feed DM and its constituent organic fractions in the rumen and small intestine has been used to evaluate rumen-protected AA (**RP-AA**) supplements (Overton et al., 1996; Berthiaume et al., 2000; Koenig and Rode, 2001). The in situ technique has the advantage of obtaining independent estimates of ruminal escape and intestinal absorption of an AA for an RP-AA supplement from which an overall estimate of bioavailability can be calculated. Even though feedstuffs are subjected to ruminal fermentation and intestinal digestion with the in situ technique, several limitations exist, including (1) the absence of chewing and rumination effects; (2) potential discrepancy between nutrient disappearance from a bag and nutrient digestibility or absorption (Reynal et al., 2007); and (3) low reliability when testing fine or soluble supplements because they may be washed out of the bag (NRC, 2001; Robert, 2004; Kononoff et al., 2007). Ross et al. (2013) developed an in vitro assay for isolating the RUP fraction of feedstuffs and measuring its intestinal digestibility. This in vitro technique has been adapted for obtaining rates of ruminal degradation and estimates of intestinal digestibility of RP-AA supplements. Whereas the in vitro technique from Ross et al. (2013) has some decided advantages over the in situ method, the effect of exposing the RP-AA supplement to the ration before consumption, which has been shown to affect the integrity of some RP-Lys supplements (Ji et al., 2016) as well as chewing and rumination effects on bioavailability of AA from RP-AA, are eliminated. The Ross et al. (2013) in vitro technique has also not been published in a peer-reviewed journal, whereas the other techniques have.

In vivo techniques that have been used to determine AA bioavailability include the production response approach (Schwab et al., 2001), the area-under-the-curve method (Graulet et al., 2005), and the plasma free AA dose-response technique (Rulquin and Kowalczyk, 2003; Borucki Castro et al., 2008; Hanigan et al., 2009). The major limitation for the production response approach is being able to ensure a deficiency of the AA, in all animals, over the entire range of the treatment dosages that are used so that linearity in responses (e.g., concentration or yield of milk protein) are observed. For the area-under-the-curve technique, animals receive a single pulse dose of the RP-AA in amounts not normally encountered by the ruminal microbiota, which may limit supplement degradation. For studies where

the plasma dose-response technique has been used (Rulquin and Kowalczyk, 2003; Borucki Castro et al., 2008; Hanigan et al., 2009), the limitation was that not all treatments (i.e., infused and fed doses of AA) were tested simultaneously within the same Latin square, thereby ignoring potential animal variation.

The plasma free Lys dose-response technique that we propose is a refinement of the plasma AA dose-response developed by Rulquin and Kowalczyk (2003). The technique relies on a positive linear relationship between incremental amounts of infused or fed Lys and plasma Lys concentration. In fact, a linear relationship between incremental doses of infused Lys into the omasum, abomasum, or duodenum, and plasma Lys concentration has been reported (Rulquin and Kowalczyk, 2003; Borucki Castro et al., 2008; Hanigan et al., 2009). We hypothesized that increased plasma Lys concentration would reflect increased net absorption of Lys, and that changes in plasma Lys concentration could be used to calculate reliable estimates of Lys relative bioavailability (**RBV**) in RP-Lys supplements. The objective of our study was to further evaluate and refine the use of the plasma free AA dose-response technique as a method for determining Lys RBV of RP-Lys supplements, which was achieved by (1) confirming linearity of the response between incremental amounts of infused Lys and plasma Lys concentration; (2) examining cow-to-cow variation in plasma Lys concentration in response to increasing amounts of absorbed Lys and the consequent effect on RBV of Lys in RP-Lys supplements; (3) examining animal factors, covariate analysis, and carryover effects among other variables; and (4) comparing the RBV of RP-Lys supplements using the plasma free Lys dose-response technique.

MATERIALS AND METHODS

All procedures related to animal care were conducted with approval of the University of New Hampshire Institutional Animal Care and Use Committee (protocols no. 070902, 091201, 110502, 111103, 121201, and 130904). All studies were conducted at the University of New Hampshire Fairchild Dairy Teaching and Research Center (Durham, NH). Studies were conducted in 2001 (study 1), 2008 (study 2), 2010 (studies 3 and 4), 2011 (studies 5 and 6), 2012 (study 7), 2013 (studies 8 to 11), and 2014 (studies 12 and 13).

Experimental Design and Treatments

The data set used herein came from 13 Latin square studies that were conducted at the University of New Hampshire to calculate the RBV of Lys from 27 RP-Lys supplements. It is important to note that some of the

RP-Lys supplements were prototypes and some commercially available, but due to confidentiality agreements we cannot identify or disclose all products tested. Studies were conducted as single ($n = 7$) or replicated ($n = 6$) Latin squares. Specifically, 1 study was designed as a 3×3 , 5 studies as 4×4 , 5 studies as 5×5 , and 2 studies as 6×6 Latin square design. Milk yield, DMI, and plasma concentration of AA were measured in all 13 studies. Milk composition (fat, protein, lactose, and MUN) was measured in all studies except 3 and 4. The RBV of Lys from all 27 RP-Lys supplements were calculated across the 13 studies. Details about replications and experimental conditions are presented in Table 1. Information about DMI, milk composition, and plasma concentration of AA for the infusion treatments are presented in supplemental tables (Supplemental Tables S1 to S20; <https://doi.org/10.3168/jds.2017-12695>).

In study 1, Lys was infused into the duodenum using duodenally cannulated cows following the procedure of Schwab et al. (1992), and all animals were injected with sometribove zinc suspension (Posilac; Protiva, St. Louis, MO) in the first day of each 14-d experimental period. In studies 2 to 13, Posilac was not administered and Lys was infused into the abomasum using ruminally cannulated cows following the procedure

of Gressley et al. (2006). The ingredient composition of the basal diets and the nutritional composition of individual feedstuffs are presented in Tables 2 and 3, respectively. Smartamine M (Adisseo USA Inc., Alpharetta, GA) was fed in all studies, with the exception of study 10, in which MetiPEARL (Kemin Industries Inc., Des Moines, IA) was used. The RP-Met supplements were fed to ensure that diets were Met-adequate according to the NRC (2001) so Met deficiency would not have adverse effects on the plasma AA profile (NRC, 2001). The infusion treatments consisted of Lys-HCl (80% Lys) assuming a Lys bioavailability of 100% (Izquierdo et al., 1988; Chung and Baker, 1992) or Lys sulfate (50.7% Lys), also assuming a Lys bioavailability of 100% (Smiricky-Tjardes et al., 2004). The infusion solutions were prepared daily at 1350 (study 1), 1530 (study 2), and at 1400 h (studies 3 to 13) by mixing Lys-HCl or Lys sulfate with 4 L of warm tap water. Cows assigned to the control treatment (0 g/d of infused Lys or fed RP-Lys) were infused with tap water only. Solutions were infused using peristaltic pumps (Manostat, Barnant Co., Barrington, IL; Masterflex, Cole-Parmer, Vernon Hills, IL). Pumps were turned off and the infusion lines disconnected during milking times for approximately 45 min per milking. Pumping

Table 1. Experimental design, DIM ($\pm$ SD), amounts of Lys infused, period length, and regression parameters across 13 studies with tested rumen-protected Lys supplements ($n = 224$ observations)

Study ¹	Latin square	Initial DIM	Lys, ² g/d	Period length, d	Linear <i>P</i> -value	Intercept Lys ³ (% TAA – Lys)	Slope Lys (% TAA – Lys)	Slope SE	R ²	RMSE ⁴
Lys-deficient diet										
1	5×5	93 ± 30	0, 16.5, 33, 49.5, and 66	14	<0.01	2.49 ^c	0.024 ^{ab}	0.002	0.97	0.142
2	5×5	65 ± 5	0, 8, 16, 24, and 32	21	<0.001	2.45 ^c	0.037 ^a	0.009	0.84	0.238
Lys-adequate diet										
3	4×4	210 ± 35	0, 28, 56, and 84	4	0.02	4.33 ^a	0.024 ^{ab}	0.003	0.96	0.230
4	4×4	254 ± 35	0, 14, 42, and 70	4	<0.01	4.40 ^a	0.027 ^{ab}	0.005	0.93	0.238
5	6×6	110 ± 20	0, 30, and 60	7	<0.001	3.79 ^b	0.018 ^b	0.003	0.97	0.151
6	$2-4 \times 4$	178 ± 20	0 and 60	7	<0.001	3.40 ^b	0.027 ^{ab}	0.002	—	0.173
7	$2-4 \times 4$	106 ± 20	0 and 60	7	<0.001	3.79 ^b	0.026 ^{ab}	0.004	—	0.283
8	$2-3 \times 3$	179 ± 123	0 and 60	7	<0.001	3.65 ^b	0.037 ^{a,x}	0.002	—	0.136
9	6×6	189 ± 125	0 and 60	7	<0.001	3.60 ^b	0.032 ^{ab,y}	0.003	—	0.256
10	$2-5 \times 5$	172 ± 52	0 and 60	7	<0.001	3.61 ^b	0.035 ^{a,xy}	0.001	—	0.104
11	4×4	211 ± 44	0 and 60	7	<0.001	3.83 ^b	0.027 ^{ab}	0.001	—	0.090
12	$2-5 \times 5$	117 ± 62	0 and 60	7	<0.001	3.58 ^b	0.023 ^{ab}	0.001	—	0.041
13	$2-5 \times 5$	178 ± 56	0 and 60	7	<0.001	4.22 ^a	0.022 ^b	0.002	—	0.158

^{a-c}Means within the same column differed at $P < 0.05$.

^{x,y}Slopes for these studies when analyzed separate from the other slopes, means within the same column differed at $P < 0.04$.

¹Studies were conducted in 2001 (study 1), 2008 (study 2), 2010 (studies 3 and 4), 2011 (studies 5 and 6), 2012 (study 7), 2013 (studies 8 to 11), and 2014 (studies 12 and 13).

²Lys dose infused (g/d) per 24.6 kg of DMI (study 1), 24.9 kg of DMI (study 2), and 22.7 kg of DMI (studies 3 to 13). The amounts of Lys (g/d) from rumen-protected Lys supplements fed were the same as infused Lys (g/d), except for study 5, where the amounts fed were 0, 30, 45, and 60 g/d of Lys.

³TAA = total AA.

⁴RMSE = root mean squared error.

rates (175 mL/h) were adjusted and monitored to ensure uniform and complete daily administration of Lys. The RP-Lys supplements were mixed by hand for approximately 1 min with 1.0 or 1.5 kg of as-fed TMR in studies 1 to 6 and 7 to 13, respectively, placed in rubber tubs, and fed 30 min before each of the 3 daily feedings. The TMR/RP-Lys mix not consumed within 15 min was administered to animals via the ruminal cannula. Body weights were measured on 2 consecutive days at the beginning and end of the studies using a hydraulic floor scale (Emery Winslow Scale Co., Seymour, CT), and BCS was assigned in 0.25-unit increments on a 1 to 5 scale (Ferguson et al., 1994) at the beginning and end of each experiment.

Management of Cows and Feed Sampling and Analyses

Cows were housed in a naturally ventilated tiestall barn, fed individually ad libitum with minimal orts (2 to 4%), and had access to water throughout the duration of the studies. All rations were fed as TMR. Cows were milked 3 times daily at 0550, 1350, and 2150 h in study 1, and twice daily at 0430 and 1530 h in studies 2 to 13. Milk weights were recorded during each milking. Cows were fed 3 times daily at 0600, 1400, and 2200 h in study 1, and at 0500, 1400, and 2100 h in studies 5 to 13, and twice daily at 0500 and 1500 h in studies 2 to 4. Basal diets were fed for a minimum of 2 wk before the start of the studies and were the same as those fed during the experimental periods. Dry matter intake, milk yield, concentrations and yields of milk components, and concentration of plasma free AA (μ M) in studies 2, 5, and 7 to 13 were collected the week preceding the start of the experiments and were used as covariates in the statistical model.

Samples of TMR and orts were collected daily and composited by equal weight in each period for determination of DM. The NRC (2001) evaluation of all basal diets is presented in Table 2, and nutritional composition of individual feed ingredients in Table 3. Samples of corn and haycrop silages were collected daily in all 13 studies. Alfalfa hay (studies 2 to 6) was sampled by using a Hilti model TE 7-A drill fitted with a 40-cm long metal core sampler (Hilti North America, Tulsa, OK). Blood meal, Bergafat (fractionated palm fatty acids with a high proportion of palmitic acid; Berg + Schmidt America LLC, Libertyville, IL), and mineral mixes were sampled weekly. The individual feedstuffs used to make the concentrate mixes were sampled at the feed mill (Poulin Grain, Newport, VT) and sent to the University of New Hampshire Fairchild Dairy Teaching and Research Center. Ingredients used in

studies 2 to 13 were freeze-dried (Labconco Model 5, Kansas City, MO) for 48 h, whereas ingredients used in study 1 were dried to 88% DM for a minimum of 12 h in a forced-air oven at 60°C (VWR Scientific, Radnor, PA). Samples were stored in air-tight glass jars until completion of each study and ground to pass through a 1-mm screen using a Wiley mill (Thomas Scientific; Arthur H. Thomas, Philadelphia, PA) before nutritional analysis.

Composites of each ingredient were shipped to a commercial laboratory (Dairy One Cooperative Inc., Ithaca, NY) for nutritional analyses according to the methods for DM (method 930.15; AOAC International, 2006), CP (method 990.03; AOAC International, 2006), NDF [Ankom Technology method 6 (amylase- and sodium sulfite-treated NDF in feeds-filter bag technique for A200; Ankom Technology, Fairport, NY; solutions as in Van Soest et al., 1991)], ADF [Ankom Technology method 5 (ADF in feeds-filter bag technique for A200; Ankom Technology); solutions as in method 973.18; AOAC International, 1998], ether extract (method 2003.05; AOAC International, 2006), and ash (method 942.05; AOAC International, 2006). Feeds were also analyzed for AA concentration using cation-exchange chromatography (cIEC-HPLC) coupled with postcolumn ninhydrin derivatization and quantitation and norleucine as the internal standard (method 982.30; AOAC International, 2016; University of Missouri Experimental Station Chemical Laboratories, Columbia, MO). Tryptophan was determined after alkaline hydrolysis and sulfur AA were analyzed after performic acid oxidation (method 988.15; AOAC International, 2016).

Milk Sampling and Analyses

Milk samples were collected at each milking during the last 3 d of each experimental period. No milk samples were taken for studies 3 and 4. Samples were preserved in tubes containing 2-bromo-2-nitropropan-1,3 diol (Broad Spectrum Microtabs II; Advanced Instruments Inc., Norwood, MA), pooled by cow for each day according to daily milk weights, and refrigerated at 4°C until shipped for determination of fat, true protein, lactose, and MUN by mid-infrared reflectance spectroscopy (Dairy One Cooperative Inc.).

Blood Sampling and Analyses

Blood samples were collected 3 h after the morning feeding on d 10 in study 1; 3 h after the morning feeding at d 19 and 21 in study 2; 1.5, 3, 4.5, and 6 h after the morning feeding on d 4 in studies 3 and 4; and 2, 4, 6, and 8 h after the morning feeding on

Table 2. Ingredient composition (% of diet DM), actual DMI and milk yield, and NRC (2001) evaluation of the control diets (0 g/d of infused or fed Lys) fed to lactating dairy cows across 13 studies with tested rumen-protected Lys supplements

Item	Study												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Ingredient composition													
Corn silage	39.4	32.9	36.7	36.7	36.6	36.1	36.0	34.1	34.0	33.9	34.9	32.4	32.4
Mixed mostly grass silage	9.92	10.9	12.1	12.1	13.7	13.9	13.9	13.9	14.4	14.5	14.1	16.3	16.4
Alfalfa hay	—	7.20	5.44	5.44	2.70	2.81	—	—	—	—	—	—	—
Wheat straw	—	1.03	0.93	0.93	—	—	—	—	—	—	—	—	—
Steam-flaked corn	—	—	3.76	3.76	4.25	4.33	4.46	4.67	4.58	4.57	4.58	4.21	4.22
Ground corn, dried	26.9	21.8	9.73	9.73	11.0	11.2	11.6	12.1	11.9	11.9	11.9	11.5	11.5
Corn DDGS ¹	—	4.98	0.97	0.97	0.89	0.88	1.04	1.04	1.08	1.08	1.11	1.12	1.11
Corn gluten meal	7.55	2.51	—	—	—	—	—	—	—	—	—	—	—
Corn gluten feed	11.5	14.1	—	—	—	—	—	—	—	—	—	—	—
Citrus pulp	—	—	7.12	7.12	8.07	8.24	8.48	8.85	8.71	8.70	8.71	8.43	—
Beet pulp	—	—	—	—	—	—	—	—	—	—	—	—	8.44
Soybean meal, solvent extracted	—	—	9.38	9.38	8.43	8.57	9.89	9.89	10.1	10.1	10.5	10.8	10.8
Soyhulls	—	—	3.01	3.01	3.39	3.46	3.57	3.74	3.65	3.65	3.67	3.55	3.55
Canola meal, solvent	—	—	2.96	2.96	2.67	2.72	3.13	3.13	3.21	3.21	3.33	3.39	3.39
Molasses, sugarcane	—	—	1.06	1.06	1.19	1.20	1.24	1.32	1.29	1.29	1.29	1.24	1.23
ProvAAL Elite ²	—	—	2.26	2.26	2.20	—	—	—	—	—	—	—	—
ProvAAL Basic ³	—	—	—	—	—	2.21	2.26	2.26	2.21	2.21	—	—	—
ProvAAL Lysine ⁴	—	—	—	—	—	—	—	—	—	—	1.67	1.98	1.77
Smartamine M	0.08	0.08	—	—	—	—	—	—	0.07	—	0.07	0.07	0.07
MetiPEARL ⁵	—	—	—	—	—	—	—	—	—	0.20	—	—	—
Urea	0.50	0.49	0.22	0.22	0.32	0.29	0.25	0.25	0.24	0.24	0.29	0.33	0.24
BergaFat F-100 ⁶	0.75	0.74	0.93	0.93	1.15	0.83	0.83	1.41	1.37	1.37	1.00	1.79	2.04
Mineral/vitamin mix	3.44	3.30	3.46	3.46	3.43	3.11	3.23	3.27	3.18	3.05	2.77	2.89	2.82
DMI, kg/d	24.1	24.3	22.9	21.8	22.1	21.7	24.0	24.8	24.3	22.6	22.9	24.5	21.6
Milk yield, kg/d	44.4	40.4	33.3	22.6	40.6	34.6	46.1	39.5	44.5	40.8	38.3	46.0	29.2
Milk protein yield, kg/d	1.22	1.16	1.10	0.78	1.20	1.14	1.30	1.28	1.39	1.22	1.23	1.34	1.14
NRC (2001) diet evaluation ⁷													
NDF, % diet DM	28.6	34.0	29.5	29.5	33.3	36.2	31.5	27.8	35.2	34.1	32.7	32.5	34.1
Ash, % diet DM	5.2	6.4	7.2	7.2	7.2	6.6	6.7	6.7	7.0	6.7	6.6	7.2	7.1
Ether extract, % diet DM	4.1	4.5	3.8	3.8	4.3	3.9	4.2	4.6	4.1	5.4	4.2	5.7	4.9
NE _L balance, Meal/d	-2.4	0.4	0.2	5.4	-3.6	-3.4	-4.2	0.4	-4.8	-2.3	-1.3	-2.3	2.3
MP balance, g/d	162	34.0	-26.0	361	-203	-89.0	-180	123	-151	5.00	-127	70.0	230
RDP, % diet DM	9.1	11.2	10.5	10.6	9.8	9.8	10.0	10.4	10.1	10.5	10.2	10.8	10.4
RUP, % diet DM	7.3	6.1	6.9	6.8	6.1	6.4	6.7	7.0	6.8	6.8	6.2	6.8	6.5
RDP balance, g/d	-192	357	141	136	-40	-1	11	95	82	58	24	156	55
RUP balance, g/d	187	42	-32	440	-247	-107	-218	149	-181	4	-154	-12	279
MP-Lys, % MP	5.05	5.52	6.78	6.81	6.80	6.60	6.60	6.43	6.53	6.58	6.67	6.51	6.68
MP-Met, % MP	2.35	2.43	2.06	2.06	2.08	2.37	2.26	2.08	2.08	2.17	2.13	2.01	2.09
MP-Lys, g/d	143	144	178	170	162	158	181	187	182	173	169	185	163
MP-Met, g/d	66	64	54	52	50	57	62	60	58	57	54	57	51

¹Corn DDGS = corn dried distillers grains with solubles.²ProvAAL Elite (Perdue AgriBusiness; Salisbury, MD) contains blood meal, feather meal, 2-hydroxy-4-(methylthio)butanoic acid, and 2.7% Smartamine M (rumen-protected Met; Adisseo USA Inc., Alpharetta, GA).³ProvAAL Basic (Perdue AgriBusiness) contains blood meal, feather meal, and 2-hydroxy-4-(methylthio)butanoic acid.⁴ProvAAL Lysine (Perdue AgriBusiness) contains blood meal and feather meal.⁵Rumen-protected Met (Kemin Industries Inc., Des Moines, IA).⁶Fractionated palm fatty acids with a high proportion of palmitic acid (Berg + Schmidt America LLC, Libertyville, IL).⁷Actual feed nutritional composition and animal description (e.g., DMI, milk yield and composition, BW, DIM, BCS) were entered in the NRC (2001) Ration Evaluation Software to generate these predictions.

Table 3. Nutritional composition (mean $\pm$ SD) of forages and concentrates used across 13 studies with tested rumen-protected Lys supplements (% of DM, unless otherwise noted)

Ingredient	DM, % as fed	CP	NDF	ADF	Lignin	Ether extract	Ash	Lys, % CP	Met, % CP
Corn silage	31.2 $\pm$ 1.9	7.7 $\pm$ 0.5	42.9 $\pm$ 4.3	26.1 $\pm$ 2.7	3.3 $\pm$ 0.8	3.7 $\pm$ 0.6	3.9 $\pm$ 0.5	2.82 $\pm$ 0.3	1.53 $\pm$ 0.2
Grass silage ¹	35.2 $\pm$ 3.3	15.6 $\pm$ 1.6	57.3 $\pm$ 3.9	39.2 $\pm$ 2.0	6.6 $\pm$ 1.2	4.7 $\pm$ 0.9	9.5 $\pm$ 0.9	4.33 $\pm$ 0.3	1.39 $\pm$ 0.1
Alfalfa hay	89.0 $\pm$ 3.7	19.2 $\pm$ 1.7	42.2 $\pm$ 3.3	33.1 $\pm$ 2.8	6.8 $\pm$ 1.0	1.9 $\pm$ 0.3	10.6 $\pm$ 0.6	5.32 $\pm$ 0.8	1.48 $\pm$ 0.2
Wheat straw	91.5 $\pm$ 0.2	4.7 $\pm$ 0.1	81.1 $\pm$ 2.8	56.6 $\pm$ 1.5	8.7 $\pm$ 0.9	1.1 $\pm$ 0.4	8.9 $\pm$ 2.0	3.08 $\pm$ 0.1	1.10 $\pm$ 0.1
Steam flaked corn	88.6 $\pm$ 2.8	7.9 $\pm$ 0.7	10.0 $\pm$ 2.2	3.7 $\pm$ 0.9	1.4 $\pm$ 0.5	3.5 $\pm$ 0.5	1.4 $\pm$ 0.2	3.35 $\pm$ 0.3	1.93 $\pm$ 0.1
Ground corn	89.8 $\pm$ 1.8	8.2 $\pm$ 0.8	10.0 $\pm$ 0.9	3.8 $\pm$ 0.6	1.2 $\pm$ 0.4	3.8 $\pm$ 0.3	1.4 $\pm$ 0.2	3.60 $\pm$ 0.4	1.96 $\pm$ 0.2
Corn DDGS ²	90.9 $\pm$ 1.6	29.9 $\pm$ 1.4	34.4 $\pm$ 3.4	15.2 $\pm$ 1.84	4.2 $\pm$ 1.3	12.2 $\pm$ 2.3	6.7 $\pm$ 0.7	3.57 $\pm$ 0.2	1.97 $\pm$ 0.1
Corn gluten meal	91.4 $\pm$ 1.2	67.3 $\pm$ 0.9	5.2 $\pm$ 1.1	3.6 $\pm$ 0.6	2.2 $\pm$ 0.3	2.8 $\pm$ 0.3	4.2 $\pm$ 1.5	1.81 $\pm$ 0.1	2.46 $\pm$ 0.1
Corn gluten feed	90.6 $\pm$ 4.7	28.0 $\pm$ 7.8	37.8 $\pm$ 5.4	13.5 $\pm$ 1.6	1.4 $\pm$ 0.7	4.3 $\pm$ 0.9	5.6 $\pm$ 0.6	3.20 $\pm$ 0.1	1.51 $\pm$ 0.1
Citrus pulp	91.8 $\pm$ 1.1	6.6 $\pm$ 0.5	24.0 $\pm$ 1.3	21.4 $\pm$ 1.1	2.0 $\pm$ 1.0	2.7 $\pm$ 0.3	8.2 $\pm$ 0.6	3.20 $\pm$ 0.5	1.05 $\pm$ 0.1
Soybean meal ³	91.0 $\pm$ 2.1	52.3 $\pm$ 0.9	12.0 $\pm$ 2.1	8.0 $\pm$ 1.1	1.2 $\pm$ 0.4	1.2 $\pm$ 0.2	7.2 $\pm$ 0.9	6.37 $\pm$ 0.2	1.34 $\pm$ 0.1
Beet pulp	91.0 $\pm$ 0	7.1 $\pm$ 0	33.0 $\pm$ 0	29.2 $\pm$ 0	4.5 $\pm$ 0	1.0 $\pm$ 0	12.5 $\pm$ 0	6.34 $\pm$ 0	1.33 $\pm$ 0.1
Soy hulls	91.7 $\pm$ 2.0	11.5 $\pm$ 1.7	68.4 $\pm$ 4.2	48.1 $\pm$ 1.9	3.0 $\pm$ 0.9	2.2 $\pm$ 0.7	5.1 $\pm$ 0.5	6.93 $\pm$ 0.6	1.08 $\pm$ 0.1
Canola meal ⁴	91.7 $\pm$ 1.4	41.6 $\pm$ 1.8	31.9 $\pm$ 2.7	22.8 $\pm$ 1.9	10.1 $\pm$ 0.9	3.9 $\pm$ 0.5	7.5 $\pm$ 0.3	5.66 $\pm$ 0.2	1.96 $\pm$ 0.1
Molasses ⁵	75.9 $\pm$ 1.0	7.3 $\pm$ 1.6	—	—	—	1.9 $\pm$ 0.7	15.5 $\pm$ 2.4	0.43 $\pm$ 0.1	0.11 $\pm$ 0.1
ProvAAL Elite ⁶	97.1 $\pm$ 0	87.7 $\pm$ 0	—	—	—	3.9 $\pm$ 0	5.41 $\pm$ 0	7.36 $\pm$ 0	4.02 $\pm$ 0
ProvAAL Basic ⁷	93.6 $\pm$ 1.8	90.8 $\pm$ 2.3	—	—	—	3.8 $\pm$ 1.2	5.0 $\pm$ 0.8	7.24 $\pm$ 0.3	1.14 $\pm$ 0.1
ProvAAL Lysine ⁸	94.5 $\pm$ 0.7	88.3 $\pm$ 2.6	—	—	—	3.3 $\pm$ 0.1	10.3 $\pm$ 1.9	6.47 $\pm$ 0.1	0.86 $\pm$ 0.1

¹Mixed mostly grass silage.²Corn DDGS = corn dried distillers grains with solubles.³Soybean meal, 48% CP.⁴Canola meal, solvent.⁵Molasses, sugarcane.⁶ProvAAL Elite (Perdue AgriBusiness; Salisbury, MD) contains blood meal, feather meal, 2-hydroxy-4-(methylthio)butanoic acid, and 2.7% Smartamine M (rumen-protected Met; Adisseo USA Inc., Alpharetta, GA).⁷ProvAAL Basic (Perdue AgriBusiness) contains blood meal, feather meal, and 2-hydroxy-4-(methylthio)butanoic acid.⁸ProvAAL Lysine (Perdue AgriBusiness) contains blood meal and feather meal.

d 5, 6, and 7 in studies 5 to 13. Blood was collected from the coccygeal vessels into 10-mL vacutainer tubes (Monoject, Mansfield, MA) containing 15% EDTA. Tubes were immediately placed on ice and centrifuged within 10 to 15 min at $1,200 \times g$ for 20 min at 4°C. A 4-mL aliquot of plasma from each sample was placed in glass test tubes containing 1.0 mL of 15% (wt/vol) 5-sulfosalicylic acid. The tubes were allowed to sit for 10 min in the centrifuge before the second centrifugation ($1,200 \times g$ for 20 min at 5°C). An aliquot of 0.45 to 1.8 mL (depending on the study) of deproteinized plasma was removed, placed into 1.8-mL cryovials, and stored at -80°C for AA analysis using norleucine as the internal standard and cation-exchange chromatography (cIEC-HPLC) analysis by the University of Missouri Experimental Station Chemical Laboratories, following the procedures of Deyl et al. (1986) and Fekkes (1996). The deproteinized plasma collected at 2 different days in studies 1 and 2 were composited resulting in 1 sample per cow/period. The deproteinized plasma collected at 1.5 and 3 h after feeding in studies 3 and 4 were composited using the same pooling procedure done in studies 1 and 2; this procedure was repeated for deproteinized plasma collected at 4.5 and 6 h after feeding resulting in 2 samples per cow/period. In studies 5 to 13, the 4 deproteinized plasma samples collected daily were composited yielding 3 samples per cow/period.

Statistical Analyses and Calculations

The UNIVARIATE procedure of SAS (SAS version 9.4; SAS Institute, Inc., Cary, NC) was used to determine outliers in each experiment data set. A concentration of plasma Lys (μM) >2.5 standard deviations (SD) from the mean was considered an outlier and all associated AA data were removed from the statistical analyses.

Dry matter intake, milk yield, concentrations and yields of milk components, and plasma concentrations of free AA (μM) were analyzed using the MIXED procedure of SAS (SAS version 9.4) according to the model

$$Y_{ijklm} = \mu + L_i + P_j + S_k + lC_{il} + D_m + LD_{im} + E_{ijklm},$$

where Y_{ijklm} = the dependent variable, μ = overall mean, L_i = the fixed effect of the i th Lys infused and RP-Lys fed level, P_j = the fixed effect of the j th period, S_k = the fixed effect of the k th square (studies 6, 7, 8, 10, and 12), l = the regression coefficient of the covariate C , C_{il} = the value of the covariate variable for the l th cow of the i th level (studies 2, 5, and 7 to 13), D_m = the fixed effect of the m th day (studies 5 to 13), LD_{im} = the fixed effect of the interaction between the i th level

and the m th day, and E_{ijklm} = the random residual error $\sim N(0, \sigma)$. The random effect of cow was used as the error term for the effect of infused Lys and fed RP-Lys levels on all dependent variables. Period and square (when replicated) were removed from all final models ($P \geq 0.25$). Degrees of freedom were calculated using the Kenward-Roger option of the MIXED procedure of SAS. Least squares means (LSM) were determined for Lys infused or RP-Lys fed levels. Significant effects for Lys level was noted at $P \leq 0.05$. In studies 1 to 5, linear, quadratic, and cubic effects were determined using the CONTRAST statement of SAS. The LSM for plasma Lys concentration from studies 1 to 5 were also subjected to the NLIN procedure of SAS for the threshold-plateau analysis. The data set from all 13 studies were run using a meta-analytical approach with study as a random effect. As the effect of study was not significant, it was not retained in the final regression models.

To generate the data to be used in the regression analysis (1) the total AA (TAA) concentration was calculated by adding Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Trp + Val + Ala + Asn + Asp + Cit + Cys + Gln + Glu + Gly + Orn + Pro + Ser + Tau + Tyr; (2) the plasma concentration (μM basis) of any individual AA in response to the infusion of Lys or fed RP-Lys that was significantly different from the plasma concentration of the same AA in the control treatment was removed from the TAA calculation for all treatments (i.e., control, infused, and fed); and (3) the plasma Lys data, expressed as a proportion of TAA subtracted from Lys, were then reanalyzed to generate the LSM that were used in the regression models (Equation 1):

$$\text{Plasma Lys, \% of TAA} - \text{Lys} = \frac{\{\text{Lys } (\mu\text{M}) / [\text{TAA } (\mu\text{M}) - \text{Lys } (\mu\text{M})]\} \times 100. \quad [1]$$

The LSM for all levels of infused Lys or fed RP-Lys were subjected to the REG procedure of SAS to generate intercepts, slopes, and coefficient of determination (R^2) using the slope ratio assay for studies 1 to 5 (Finney, 1978) and the 3-point assay (a derivative of the slope ratio assay; Littell et al., 1995) for studies 6 to 13.

In studies 5 to 7, 12, and 13, LSM were used to calculate RBV for RP-Lys supplements as (Equation 2)

$$\text{RBV} = \frac{\text{slope of the RP-Lys fed}}{\text{slope of Lys infusion}} \times 100. \quad [2]$$

We assumed that the increase in plasma Lys concentrations with the infused or fed treatments was due to

treatment effects, as all cows received the same basal diet within each study. We further assumed that the contribution of Lys from RUP, microbial protein, and endogenous protein was constant within each study. Confidence intervals (CI) for RBV were calculated by multiplying standard error of the mean (SEM) $\times$ 1.96 $\pm$ the slope for both Lys infusion or RP-Lys treatments. The upper limit CI was obtained by dividing the upper limit of RP-Lys by the upper limit of the Lys infusion treatment, whereas the lower limit CI was obtained by dividing the lower limit of RP-Lys by the lower limit of the Lys infusion treatment.

Variables such as plasma concentration of Lys expressed as a proportion of TAA in cows assigned to the control treatment (0 g/d of infused Lys), plasma concentration of Lys expressed as a proportion of TAA in cows assigned to the greatest Lys infusion amount, NE_L and MP balances, MP supply, DIM, milk yield, milk concentrations of fat, true protein, and lactose, milk true protein yield, and DMI were regressed against the plasma concentration of Lys to assess their effects on the regression slopes.

In studies 2, 5, and 7 to 13, data were analyzed using the MIXED procedure of SAS with and without the covariate term to determine whether the covariate should be retained in the final model. These data were also analyzed using the MIXED procedure of SAS to determine if there were any significant differences between the plasma concentration of Lys during the covariate period and the treatment with 0 g/d of infused Lys or fed RP-Lys.

RESULTS AND DISCUSSION

Determination of Lys Linearity in Plasma

For the plasma free AA dose-response technique to be a widely accepted method for determining RBV of Lys from RP-Lys supplements, a positive linear response between increasing amounts of infused or fed Lys and plasma Lys concentrations must be unequivocally established. In fact, the plasma concentrations of Lys increased in a linear fashion in all 13 studies regardless of the number of infused doses of Lys used (Table 1), which agrees with previous research (Rulquin and Kowalczyk, 2003; Borucki Castro et al., 2008; Hanigan et al., 2009). No quadratic or cubic effects were observed for plasma Lys concentration as a result of multiple amounts of infused Lys or fed RP-Lys (Supplemental Tables S7–S11; <https://doi.org/10.3168/jds.2017-12695>). The threshold-plateau model also indicated a linear response (data not shown). The effects of infused Lys on plasma AA concentration are shown in

Supplemental Tables S7–S20 (<https://doi.org/10.3168/jds.2017-12695>).

Period Length and Carryover Effects

Period lengths of 14 and 21 d were used in studies 1 and 2, respectively (Table 1). Similar period lengths were used in previous Latin square experiments with Lys infusion (King et al., 1991; Schwab et al., 1992; Varvikko et al., 1999). Period length of 4 d was used in studies 3 and 4, which is of similar length to that reported by Borucki Castro et al. (2008). Period length of 7 d was used in the remaining 9 studies (Table 1). Benfield et al. (2009) investigated whether length of experimental periods (i.e., 7 and 14 d) used in a Latin square design would affect DMI, milk yield, and concentrations and yields of milk components when dairy cows were fed different amounts of 2 sources of RP-Met supplements. They concluded that there were no carryover effects with feeding RP-Met supplements for most production variables tested when using 7- or 14-d periods (Benfield et al., 2009). The TMR/RP-Lys supplement mixes used in the current study were readily consumed by the cows in most feeding events, indicating minimal effect on animal acceptance. At the few occasions when a cow did not consume all of the TMR/RP-Lys supplement mix within 30 min, it coincided with the first 2 feedings at the beginning of the experimental period. In those cases, less than 0.75 kg of the TMR/RP-Lys supplement mix was left and it was placed directly in the rumen via the cannula. The effects of infused Lys on DMI, milk yield, and milk components are presented in Supplemental Tables S1 to S6 (<https://doi.org/10.3168/jds.2017-12695>).

We compared the plasma concentration of Lys obtained during d 5 to 7 in cows assigned to the control treatment versus abomasal infusion of Lys or fed RP-Lys (Table 4). In 7 out of 9 studies presented in Table 4, no effect of day of sampling on plasma Lys concentration was observed, indicating that 7-d periods appeared to be adequate. It is important to note that a quadratic effect was observed for plasma Lys concentrations in both control and Lys infusion treatments in study 7, as well as in the Lys infusion treatment in study 9. Because the methodology used in studies 7 and 9 was similar to that used in the remaining 7 studies, we cannot explain these quadratic effects associated with day of sampling. However, it seems that at least 3 consecutive days of blood samples should be taken to obtain a representative sample. Overall, our inferences about period length should be interpreted cautiously, as studies reported in Table 4 were not originally designed to test period length.

We also investigated for potential carryover effects when using a 7-d period by comparing the plasma concentration of Lys obtained during the covariate period with that obtained from the control treatment during the sampling collection period (Table 5). Linear effects (decrease or increase) in plasma Lys concentration could have occurred in the event of carryover effects

when cows were changed from the 60 g/d of infused Lys or fed RP-Lys treatment to the control treatment or vice-versa. No difference in plasma Lys concentration was noted between covariate and sampling collection periods for the control treatment, suggesting no carryover effects when cows were switched from treatment to treatment.

Table 4. Effect of day of sampling on plasma Lys concentration [% of plasma total AA (TAA) minus Lys]¹ during the last 3 d of the sampling period in lactating dairy cows receiving all Lys treatments (studies 5–7, 12, and 13) or only the control treatment (0 g/d Lys) and the abomasally infused Lys treatments (studies 8–10)

Item ²	Plasma Lys			SEM	Day	P-value ³	
	d 5	d 6	d 7			Linear	Quadratic
Study 5							
0 g/d	3.74	3.62	3.67	0.25	0.77	0.67	0.58
30 g/d Infusion	4.49	4.36	4.62	0.21	0.65	0.65	0.43
60 g/d Infusion	4.99	5.02	4.62	0.34	0.29	0.21	0.34
30 g/d AjiPro-L ⁴	3.93	3.95	3.93	0.23	0.96	0.98	0.79
45 g/d AjiPro-L	4.01	4.11	4.20	0.20	0.23	0.19	0.91
60 g/d AjiPro-L	4.18	4.18	4.00	0.27	0.18	0.12	0.35
Study 6							
0 g/d	3.34	3.42	3.50	0.25	0.53	0.27	0.99
60 g/d Infusion	4.91	4.94	5.12	0.52	0.78	0.53	0.79
60 g/d Smartamine M ⁵	4.74	4.78	4.89	0.25	0.69	0.41	0.79
60 g/d New AminoShure L ⁶	3.51	3.66	3.70	0.26	0.15	0.13	0.33
Study 7							
0 g/d	3.66	3.96	3.74	0.15	0.10	0.55	0.04
60 g/d Infusion	5.25	4.86	5.83	0.31	0.05	0.13	0.04
60 g/d AjiPro-L	4.31	4.31	4.26	0.08	0.80	0.56	0.78
60 g/d New AminoShure L	3.98	3.88	4.03	0.10	0.39	0.66	0.19
Study 8							
0 g/d	3.69	3.61	3.60	0.25	0.73	0.50	0.67
60 g/d Infusion	5.69	6.06	5.86	0.42	0.53	0.61	0.33
Study 9							
0 g/d	3.61	3.57	3.63	0.20	0.81	0.85	0.55
60 g/d Infusion	5.32	5.93	5.25	0.29	0.47	0.81	0.02
Study 10							
0 g/d	3.56	3.65	3.64	0.18	0.32	0.22	0.38
60 g/d Infusion	5.75	5.80	5.64	0.24	0.71	0.60	0.55
Study 11							
0 g/d	3.88	3.85	3.76	0.11	0.39	0.21	0.65
60 g/d Infusion	5.52	5.36	5.55	0.34	0.47	0.85	0.24
Study 12 ⁷							
0 g/d	3.58	3.52	3.61	0.18	0.62	0.70	0.38
60 g/d Infusion	5.14	5.10	5.20	0.18	0.92	0.81	0.74
60 g/d RP-Lys ⁸ supplement A	3.79	3.87	3.85	0.20	0.66	0.54	0.51
60 g/d RP-Lys supplement B	4.00	3.93	3.90	0.20	0.23	0.10	0.71
60 g/d RP-Lys supplement C	3.96	4.02	4.09	0.18	0.55	0.28	0.91
Study 13 ⁷							
0 g/d	4.05	4.04	4.10	0.16	0.85	0.65	0.73
60 g/d Infusion	5.10	5.46	5.37	0.33	0.69	0.53	0.55
60 g/d RP-Lys supplement 1	4.76	4.62	4.73	0.17	0.83	0.87	0.56
60 g/d RP-Lys supplement 2	4.60	4.49	4.60	0.17	0.39	0.96	0.17
60 g/d RP-Lys supplement 3	4.64	4.53	4.64	0.13	0.21	0.98	0.18

¹Plasma Lys, % of TAA – Lys = {Lys (μM)/[TAA (μM) – Lys (μM)]} × 100 (Equation 1).

²Only listed studies with 3 d of sampling were used; studies were conducted in 2011 (studies 5 and 6), 2012 (study 7), 2013 (studies 8 to 11), and 2014 (studies 12 and 13).

³Significance was declared at $P \leq 0.05$.

⁴AjiPro-L (Ajinomoto North America, Fort Lee, NJ).

⁵Smartamine M (Adisseo USA Inc., Alpharetta, GA).

⁶New AminoShure L (Balchem Corp., New Hampton, NY).

⁷Supplements provided by QualiTech Inc. (Chaska, MN).

⁸RP-Lys = rumen-protected lysine.

Table 5. Plasma concentration of Lys [% of plasma total AA (TAA) minus Lys]¹ during the covariate period (3 d before the start of study) and sampling collection period (d 5 to 7) in lactating dairy cows assigned to the control treatment (0 g/d of Lys) across 8 studies with tested rumen-protected Lys supplements

Item ²	Covariate period			Sampling period			SEM	P-value ³ Day
	d -3	d -2	d -1	d 5	d 6	d 7		
Study 5	3.48	3.50	3.73	3.74	3.62	3.67	0.25	0.35
Study 7	3.56	3.56	3.66	3.66	3.96	3.74	0.12	0.13
Study 8	3.56	3.70	3.52	3.68	3.62	3.60	0.21	0.43
Study 9	3.42	3.42	3.47	3.63	3.57	3.64	0.17	0.12
Study 10	3.50	3.50	3.56	3.56	3.65	3.64	0.18	0.30
Study 11	3.70	3.81	3.76	3.88	3.85	3.75	0.10	0.24
Study 12	3.51	3.55	3.62	3.57	3.52	3.61	0.20	0.84
Study 13	4.29	4.20	4.04	4.05	4.04	4.10	0.16	0.45

¹Plasma Lys, % of TAA - Lys = {Lys (μ M)/[TAA (μ M) - Lys (μ M)]} $\times$ 100 (Equation 1).

²Only listed studies where 3 d of sampling were used; studies were conducted in 2011 (studies 5 and 6), 2012 (study 7), 2013 (studies 8 to 11), and 2014 (studies 12 and 13).

³Significance was declared at $P \leq 0.05$.

Effect of Experimental Conditions on Regression Slopes, Intercepts, and Variation

The regression intercepts between infused Lys and plasma Lys concentrations were significantly lower in cows fed Lys-deficient diets (studies 1 and 2) than in those fed Lys-adequate diets (studies 3 to 13; Table 1), with dietary Lys supply relative to animal requirements determined by the NRC (2001), as shown in Table 2. Within the 11 studies where cows were fed Lys-adequate diets, the intercepts of the regressions obtained in studies 3 and 4 were greater than those obtained in studies 5 to 13 (Table 1). Cows in studies 3 and 4 received the greatest amount of infused Lys and were late in lactation, producing the least amount of milk across all studies (Table 1), which may help explain the difference in intercept values between these 2 studies and the remaining 9 Lys-adequate experiments.

The R^2 of the regression between infused Lys and plasma Lys concentration in the studies where more than 2 doses of Lys were infused or fed ranged from 0.84 (study 2) to 0.97 (studies 1 and 5; Table 1). We observed a positive linear increase in the plasma concentration of 3-methylhistidine as the amount of infused Lys increased in study 2 (Supplemental Table S8; <https://doi.org/10.3168/jds.2017-12695>). Blum et al. (1985) suggested that an increase in plasma concentration of 3-methylhistidine could be used as an indicator of muscle protein degradation. Because cows in study 2 were in early lactation, they may have mobilized AA, including Lys, from muscle tissues, possibly affecting the relationship between infused and plasma Lys concentration. That AA mobilization may have occurred is supported by the observation that the predicted concentration of Lys in MP averaged 6.55% in the treatment with the greatest amount of infused Lys (i.e., 32 g/d), lower than the 7.1 to 7.2% MP-Lys recommended

by the NRC (2001) to ensure Lys adequacy. Although diets in study 1 were also formulated to be Lys-deficient, the dietary concentration of Lys in MP averaged 6.76 and 7.15% in cows infused with 49.5 or 66 g/d of Lys, respectively, which is closer to meeting the NRC (2001) recommendation. According to Lapierre et al. (2006), as protein supply increases in protein-deficient diets there is more gut catabolism of Lys and no change in its hepatic removal, but an increase in the uptake of Lys by the mammary gland occurs. This suggests that Lys-deficient diets are not recommended when using the plasma free AA dose-response technique to calculate RBV for RP-Lys supplements.

Assessing the Need for Including all Treatments in the Same Latin Square

We selected studies 8, 9, and 10 that were conducted in the same year and used cows fed forage sources harvested on similar dates to assess whether or not all treatments should be included in the same Latin square. Our data showed that the slope of the regression line was greatest in study 8, intermediate in study 10, and lowest in study 9 (0.037, 0.035, and 0.032, respectively; Table 1). In addition, the mean ($\pm$ SD) of the individual slopes from the 86 cows that received incremental amounts of infused Lys across all 13 studies averaged 0.0275 ± 0.0137 , thereby resulting in 16 and 14% of the cows having a slope below or above the 95% CI, respectively (data not shown). Thus, the difference in slopes among studies 8, 9, and 10, as well as the proportion of cows that were below or above the 95% CI, reinforce our recommendation that all treatments should be included in the same Latin square to minimize the likelihood to over- or underestimate the RBV of RP-Lys supplements due to animal variation. Further justification for having all treatments in the

Table 6. Relative bioavailability (RBV), CI, and CV values for 3 rumen protected-Lys supplements tested in study 13 as determined for each of the 10 cows that were used¹

Cow	Treatment	RBV, %	CI, %	CV, %
1	Supplement 1	58.1	46.9–69.3	30.8
	Supplement 2	45.7	31.7–59.5	36.1
	Supplement 3	38.3	22.1–54.5	39.0
2	Supplement 1	20.7	14.4–27.1	20.3
	Supplement 2	12.7	6.8–18.6	15.8
	Supplement 3	9.2	2.8–15.6	15.7
3	Supplement 1	36.4	28.6–44.2	14.6
	Supplement 2	23.5	14.9–32.1	14.5
	Supplement 3	27.9	19.2–36.6	13.3
4	Supplement 1	31.9	26.5–37.3	12.6
	Supplement 2	15.8	7.7–23.9	16.9
	Supplement 3	19.5	11.9–27.1	17.8
5	Supplement 1	38.9	34.1–43.7	14.0
	Supplement 2	25.3	16.4–34.2	17.7
	Supplement 3	16.5	6.7–26.3	20.9
6	Supplement 1	30.7	20.8–40.6	18.9
	Supplement 2	24.1	16.1–32.1	13.5
	Supplement 3	23.1	14.1–32.1	16.3
7	Supplement 1	60.9	55.1–66.7	11.4
	Supplement 2	46.7	39.8–53.6	16.4
	Supplement 3	46.0	35.2–56.8	18.8
8	Supplement 1	27.0	18.8–35.2	16.5
	Supplement 2	21.4	13.1–29.7	13.0
	Supplement 3	29.6	22.9–36.0	12.6
9	Supplement 1	21.0	13.7–28.3	15.5
	Supplement 2	25.4	16.2–34.6	16.6
	Supplement 3	32.0	26.4–37.6	13.1
10	Supplement 1	45.3	40.0–50.6	11.3
	Supplement 2	25.4	19.4–31.4	12.1
	Supplement 3	29.5	23.2–35.8	11.9

¹The rumen protected-Lys supplements used in study 13 were supplied by QualiTech Inc. (Chaska, MN).

same Latin square is the inherent cow-to-cow variation. We selected study 13, where 3 RP-Lys supplements were tested in a replicated 5×5 Latin square, to demonstrate how cow-to-cow variation affects RBV (Table 6). Overall, calculated RBV for all 3 RP-Lys supplements in study 13 were consistently greater in cows 1 and 7 and consistently lower in cow 2 compared with the remaining animals (Table 6).

We also used our data to further demonstrate that RBV could be seriously over- or underestimated if 1 Latin square is used for infused Lys and another for fed RP-Lys. For example, using the mean slope (0.01579; data not shown) for infused Lys from square 1 in study 13 and the mean slope (0.00904; data not shown) for fed RP-Lys from square 2 in the same study, the calculated RBV would be 57.3% compared with the previously determined RBV of 37.2% (Figure 1D). Likewise, using the mean slope (0.02820; data not shown) from square 2 for infused Lys and the mean slope (0.00602; data not shown) from square 1 for fed RP-Lys would lead to a calculated RBV of 21.3% compared with the previously determined RBV of 37.2% (Figure 1D). Thus, we recommend that all treatments (infused Lys and fed RP-Lys) are included in the same Latin square.

Effect of Different Animal Variables on Regression Coefficients of Determination and Slopes

The mean, range, SEM, and R^2 generated by regressing the LSM of different variables against the slopes for the 13 studies are presented in Table 7. The plasma Lys concentration at the greatest infusion amount regressed against the slope resulted in the greatest R^2 (0.32), whereas the R^2 for the remaining variables were low (i.e., ≤ 0.088). These low R^2 indicate that variables such as the plasma concentration of Lys in cows assigned to the control treatment, DIM, milk yield, DMI, NE_L and MP balances, MP supply, and milk true protein concentration and yield had minimal effect on changing the slope of the regression between incremental amounts of infused Lys and plasma Lys concentration.

Assessing the Need for Including a Covariate Collection Period

Studies 2, 5, and 7 to 13 were used to determine whether the covariate term should be retained in the final statistical model (Table 8). The covariate terms of studies 2, 5, and 11 were not significant, and the SEM of the regression slope between incremental amounts of

Figure 1. Effects of incremental amounts of infused Lys-HCl and fed rumen-protected Lys supplements on plasma concentration of Lys. Panel A shows the relationship between infused Lys-HCl (◆) and fed AjiPro-L (Ajinomoto North America, Fort Lee, NJ; ■) in lactating dairy cows offered a Lys-adequate diet (study 5). Infused Lys: $Y = 3.79 + 0.0183x$; $P < 0.001$, slope SE = 0.003, intercept SE = 0.12, $R^2 = 0.97$. Fed AjiPro-L: $Y = 3.79 + 0.0068x$; $P = 0.001$, slope SE = 0.002, intercept SE = 0.06, $R^2 = 0.93$; relative bioavailability = $(0.0068 \div 0.0183) \times 100 = 37.2\%$. Panel B shows the relationship between infused Lys-HCl (◆) and fed Smartamine ML (Adisseo USA Inc., Alpharetta, GA; ●) or New AminoShure-L (Bachem Corp., New Hampton, NY; ▲) in lactating dairy cows offered a Lys-adequate diet (study 6). Infused Lys: $Y = 3.40 + 0.0265x$; $P < 0.001$, slope SE = 0.002, intercept SE = 0.07. Fed Smartamine ML: $Y = 3.40 + 0.0231x$; $P < 0.001$, slope SE = 0.001, intercept SE = 0.05; relative bioavailability = $(0.0231 \div 0.0265) \times 100 = 87.2\%$. Fed New AminoShure-L: $Y = 3.40 + 0.0033x$; $P = 0.05$, slope SE = 0.001, intercept SE = 0.06; relative bioavailability = $(0.0033 \div 0.0265) \times 100 = 12.5\%$. Panel C shows the relationship between infused Lys-HCl (◆) and fed AjiPro-L (■) or New AminoShure-L (▲) in lactating dairy cows offered a Lys-adequate diet (study 7). Infused Lys: $Y = 3.79 + 0.0264x$; $P < 0.001$, slope SE = 0.004, intercept SE = 0.20. Fed AjiPro-L: $Y = 3.79 + 0.0094x$; $P = 0.002$, slope SE = 0.001, intercept SE = 0.06; relative bioavailability = $(0.0094 \div 0.0264) \times 100 = 35.6\%$. Fed New AminoShure-L: $Y = 3.79 + 0.0041x$; $P = 0.004$, slope SE = 0.001, intercept SE = 0.06; relative bioavailability = $(0.0041 \div 0.0264) \times 100 = 15.5\%$. Panel D shows the relationship between infused Lys-HCl (◆) and fed supplement 1 (□), supplement 2 (Δ), or supplement 3 (○; QualiTech Inc., Chaska, MN) in lactating dairy cows offered a Lys-adequate diet (study 13). Infused Lys: $Y = 4.22 + 0.0223x$; $P < 0.001$, slope SE = 0.002, intercept SE = 0.06. Fed supplement 1: $Y = 4.22 + 0.0083x$; $P = 0.001$, slope SE = 0.0004, intercept SE = 0.02; relative bioavailability = $(0.0083 \div 0.0223) \times 100 = 37.2\%$. Fed supplement 2: $Y = 4.22 + 0.0060x$; $P = 0.001$, slope SE = 0.0004, intercept SE = 0.02; relative bioavailability = $(0.0060 \div 0.0223) \times 100 = 26.9\%$. Fed supplement 3: $Y = 4.22 + 0.0061x$; $P = 0.001$, slope SE = 0.0007, intercept SE = 0.03; relative bioavailability = $(0.0061 \div 0.0223) \times 100 = 27.4\%$.

infused Lys and plasma concentration of Lys (% TAA – Lys) in studies 5 and 11 were not reduced by retaining the covariate term in the model. With the exception of study 9, all remaining studies with significant covariate terms (i.e., studies 7, 8, 10, and 13) were conducted as replicated Latin squares. In all these replicated studies, the SEM of the regression slope was reduced by keeping the covariate term in the statistical model. Therefore, we recommend the collection of covariate data followed

by statistical analysis to determine whether or not the covariate term should be maintained in the final model.

Units to Express Plasma Lys Concentration and Calculation of Relative Bioavailability

Equation 1 was used to generate values of plasma Lys that were run in the regression models presented in Table 9. This equation decreased the SEM of the

Table 7. Mean, range, and coefficient of determination of measured animal variables that were regressed against the slopes of the regressions between incremental amounts of infused Lys and plasma concentration of Lys

Variable	Mean	Range	SD	R ²
Difference between plasma [Lys] in the control treatment vs. treatment with greatest infusion amount	1.68	0.06–3.15	0.76	0.802
Plasma [Lys] at greatest infusion amount, % TAA – Lys ¹	5.28	2.53–7.51	0.99	0.315
Plasma [Lys] in the control treatment, % TAA – Lys ¹	3.64	1.83–5.21	0.62	0.088
NE _L balance, Mcal/d	–1.25	–8.60–9.40	3.52	0.014
MP supply, g/d	2,646	1,613–3,356	344	0.001
MP balance, g/d	30.7	–378–720	229	0.003
DIM	180	60–335	65.8	0.003
Milk yield, kg/d	39.0	11.9–61.9	9.65	0.002
Milk true protein, %	3.09	2.54–3.72	0.26	0.003
Milk true protein yield, kg/d	1.2	0.40–1.70	0.23	0.001
Milk fat, %	3.78	2.54–4.74	0.50	0.0002
Milk lactose, %	4.75	4.16–5.20	0.17	0.029
DMI, kg/d	23.1	15.6–28.8	2.69	0.003
BW, kg	700	571–846	6.50	0.0001
BCS	3.18	2.08–4.25	0.44	0.017

¹Plasma Lys, % of total AA (TAA) – Lys = {Lys (μM)/[TAA (μM) – Lys (μM)]} × 100 (Equation 1).

slopes and intercepts, as well as CI and coefficient of variation in most regression models, except in studies 11 and 12, where the SEM were the same when plasma Lys concentration was expressed according to Equation 1 or as a proportion of TAA concentration (Table 9). Therefore, the RBV of different RP-Lys supplements were calculated by expressing plasma Lys concentration according to Equation 1. Furthermore, in study 12, the RBV (calculated by expressing plasma Lys

concentration on a micromolar basis) did not differ significantly across 3 RP-Lys supplements (Table 10). However, when plasma Lys concentration was expressed as proposed in Equation 1, the RBV for supplements B and C were not different from each other but were greater than the RBV for supplement A, thereby indicating that decreased variation (i.e., SE, CI, and CV) improved accuracy to detect RBV differences among supplements.

Table 8. Significance of including the covariate term in the statistical model and SE for the regression slope between incremental amounts of infused Lys and plasma concentration of Lys [% of plasma total AA (TAA) minus Lys]¹ when including or not the covariate term in the model across 9 studies with tested rumen-protected Lys supplements

Item ²	Slope	Covariate	P-value ³	SE
Study 2	0.0366	Yes	0.33	0.25
	0.0369	No		0.27
Study 5	0.0181	Yes	0.27	0.16
	0.0180	No		0.16
Study 7	0.0264	Yes	0.05	0.15
	0.0265	No		0.16
Study 8	0.0374	Yes	<0.001	0.11
	0.0377	No		0.32
Study 9	0.0321	Yes	0.05	0.17
	0.0320	No		0.21
Study 10	0.0352	Yes	<0.001	0.09
	0.0353	No		0.17
Study 11	0.0271	Yes	0.92	0.24
	0.0271	No		0.20
Study 12	0.0232	Yes	0.09	0.09
	0.0231	No		0.10
Study 13	0.0221	Yes	0.03	0.13
	0.0221	No		0.18

¹Plasma Lys, % of TAA – Lys = {Lys (μM)/[TAA (μM) – Lys (μM)]} × 100 (Equation 1).

²Only listed studies where a covariate period sampling was used; studies were conducted in 2011 (study 5), 2012 (study 7), 2013 (studies 8 to 11), and 2014 (studies 12 and 13).

³Significance was declared at $P \leq 0.05$.

Evaluation of RP-Lys Supplements Using the Plasma Free AA Dose-Response Technique

The RBV for several RP-Lys supplements were calculated using Equation 2 and are presented in Figure 1 (A, B, C, and D). The RBV for AjiPro-L 1G (Ajinomoto North America Inc., Fort Lee, NJ), which is no longer on the market, averaged 37.2% (Figure 1A; study 5) and is close to the 40% bioavailability claimed by Ajinomoto (I. Shinzato, Ajinomoto Heartland Inc., Chicago, IL; personal communication) using the highly protected L-Arg procedure, as described by Swanepoel et al. (2010) and Robinson et al. (2011). Figure 1B (study 6) shows the evaluation of Lys in Smartamine ML (Adisseo France SAS, Antony, France) and New AminoShure-L (Balchem Corp., New Hampton, NY), both supplements which are no longer on the market. The RBV for Lys in Smartamine ML and New AminoShure-L averaged 87.2 and 12.5%, respectively. We reevaluated AjiPro-L 1G and New AminoShure-L (Figure 1C; Study 7), which were initially evaluated in studies 5 and 6, respectively. The RBV averaged 35.6% for AjiPro-L 1G and 15.5% for New AminoShure-L and were very similar to their original RBV calculations, thereby showing that the plasma free AA dose-response

Table 9. Confidence intervals (between parentheses), SE, and CV for the slopes and intercepts of regression models for infused Lys across 13 studies with tested rumen-protected Lys supplements, and plasma concentration of Lys expressed as a proportion of total AA (TAA) or as proportion of TAA minus plasma Lys

Item ¹	Plasma Lys (% TAA)					Plasma Lys (% TAA – Lys) ²				
	Slope	SE	Intercept	SE	CV, ³ %	Slope	SE	Intercept	SE	CV, ³ %
Study 1	0.025 (0.018–0.032)	0.003	2.56 (2.36–2.75)	0.10	3.96	0.024 (0.019–0.028)	0.002	2.49 (2.31–2.68)	0.10	3.73
Study 2	0.034 (0.013–0.056)	0.011	2.40 (2.00–2.80)	0.20	8.60	0.037 (0.018–0.055)	0.009	2.45 (2.10–2.81)	0.18	7.78
Study 3	0.020 (0.010–0.030)	0.005	4.35 (3.97–4.73)	0.20	3.36	0.024 (0.017–0.031)	0.003	4.33 (3.99–4.67)	0.18	3.07
Study 4	0.025 (0.013–0.037)	0.006	4.56 (3.98–5.14)	0.29	5.35	0.027 (0.016–0.037)	0.005	4.40 (3.97–4.83)	0.22	5.04
Study 5	0.016 (0.008–0.026)	0.005	3.70 (3.43–3.96)	0.14	3.96	0.018 (0.012–0.025)	0.003	3.79 (3.55–4.03)	0.12	3.09
Study 6	0.024 (0.018–0.030)	0.003	3.30 (3.08–3.51)	0.11	3.81	0.026 (0.022–0.031)	0.002	3.40 (3.21–3.61)	0.10	3.47
Study 7	0.023 (0.012–0.034)	0.006	3.65 (3.26–4.03)	0.20	6.07	0.025 (0.017–0.033)	0.004	3.79 (3.45–4.12)	0.17	5.35
Study 8	0.034 (0.030–0.037)	0.002	3.49 (3.33–3.65)	0.08	3.78	0.037 (0.033–0.041)	0.002	3.65 (3.49–3.80)	0.08	2.85
Study 9	0.029 (0.021–0.037)	0.004	3.48 (3.18–3.77)	0.15	6.30	0.032 (0.025–0.039)	0.003	3.60 (3.31–3.89)	0.15	5.62
Study 10	0.032 (0.028–0.036)	0.002	3.48 (3.37–3.60)	0.06	2.19	0.035 (0.033–0.038)	0.001	3.61 (3.45–3.77)	0.05	1.99
Study 11	0.025 (0.022–0.028)	0.001	3.68 (3.59–3.78)	0.05	1.97	0.027 (0.025–0.029)	0.001	3.83 (3.74–3.91)	0.04	1.65
Study 12	0.024 (0.023–0.025)	0.001	3.45 (3.40–3.50)	0.03	1.20	0.026 (0.025–0.07)	0.001	3.58 (3.53–3.62)	0.02	0.93
Study 13	0.021 (0.015–0.027)	0.003	4.06 (3.87–4.24)	0.10	3.81	0.022 (0.017–0.026)	0.002	4.22 (4.04–4.39)	0.09	3.21

¹Studies were conducted in 2001 (study 1), 2008 (study 2), 2010 (studies 3 and 4), 2011 (studies 5 and 6), 2012 (study 7), 2013 (studies 8 to 11), and 2014 (studies 12 and 13).

²Plasma Lys, % of TAA – Lys = {Lys (μM)/[TAA (μM) – Lys (μM)]} $\times$ 100 (Equation 1).

³CV are for the regression model.

technique generates repeatable RBV calculations. Three RP-Lys supplements (Figure 1D; study 13) from QualiTech Inc. (Chaska, MN) were also evaluated, and 1 product is commercially available. The RBV averaged 37.2, 26.9, and 27.4% for supplements 1, 2, and 3, respectively. As QualiTech was testing different encapsulation technologies (J. E. Garnet; QualiTech Inc., per-

sonal communication), these results indicated that the plasma free AA dose-response technique is sensitive to changes in encapsulation matrices. Overall, we reported RBV ranging from 5.1 to 87.2% for the RP-Lys supplements evaluated in these studies, which reinforces the sensitivity of the plasma free AA dose-response technique to changes in encapsulation technologies adopted

Table 10. Plasma Lys concentration, expressed on an absolute basis (μM) or as a proportion of total AA minus Lys (% TAA – Lys), regression slopes, and relative bioavailability (RBV) obtained from cows infused with Lys-HCl or fed 3 rumen-protected Lys supplements in study 12¹

Item	Plasma Lys, μM				Plasma Lys, %TAA – Lys ²			
	Slope ³	SE	RBV, % (CI)	CV, ⁴ %	Slope ⁵	SE	RBV, % (CI)	CV, ⁴ %
Infusion	0.626	0.022	—	1.80	0.023	0.001	—	0.93
Supplement A	0.108	0.026	17.1 (9.7–23.7)	2.47	0.004	0.0005	17.1 ^b (14.0–19.7)	1.17
Supplement B	0.142	0.044	23.3 (9.6–34.2)	3.03	0.006	0.0006	24.6 ^a (21.3–27.4)	1.23
Supplement C	0.170	0.042	27.4 (15.0–37.7)	3.76	0.007	0.0004	28.8 ^a (27.8–29.7)	1.34

^{a,b}Means in the same column differed at $P < 0.05$ according to the Tukey test.

¹Conducted in 2014.

²Plasma Lys, % of TAA – Lys = {Lys (μM)/[TAA (μM) – Lys (μM)]} $\times$ 100 (Equation 1).

³Slope differs from 0 at $P < 0.01$.

⁴CV are for the regression model.

⁵Slope differs from 0 at $P = 0.02$.

Figure 2. Relative bioavailability of 27 rumen-protected Lys (RP-Lys) supplements across 13 studies tested using the plasma free AA dose-response technique.

Table 11. Recommendations for determining relative bioavailability (RBV) of rumen-protected Lys (RP-Lys) supplements using the plasma free AA dose-response technique

Experimental design	Replicated Latin square with all treatments in the same square to minimize variation.
Animals	Ruminally cannulated lactating dairy cows, preferably at similar DMI and DIM. Avoid cows in early lactation due to negative energy and protein balance.
Basal diet	A TMR balanced to meet animal nutritional requirements fed 3 times daily at 8-h intervals.
Covariate period	Collect milk and blood samples during 3 d of the covariate period to obtain baseline data.
Treatment	It is recommended that 3 amounts (e.g., 0, 30, and 60 g/d) of abomasally or duodenally infused Lys and the same amounts of fed Lys from RP-Lys supplements be used.
Infusion	Mix and dissolve Lys-HCl in a minimum of 4 L of warm tap water, and infuse continuously into the abomasum or duodenum. Pump infusion rates should be monitored regularly to ensure uniform and complete daily administration of the infusate.
Feeding RP-Lys	Mix RP-Lys with 1.5 kg of TMR, place in plastic container with lids, and store at 4°C for 8 h before feeding to expose the tested supplements to TMR conditions. Place the TMR/RP-Lys mix in rubber tubs and offer to the cows 30 min before the actual feeding time. If the mix is not completely consumed within 15 min, place leftovers directly in the rumen via cannula.
Period length	Minimum of 7 d.
Blood sampling	Collect 4 blood samples (coccygeal or jugular veins) at 2-h intervals following the morning feeding during the last 3 d of each sampling period.
Outlier analysis	Conduct a univariate analysis to determine outliers in the plasma concentration of AA, including Lys.
Calculation of RBV	Divide the slope of the RP-Lys fed supplement by the slope of the infused Lys and multiply by 100.

by different companies in the dairy industry (Figure 2). Our recommendations for determining RBV of RP-Lys supplements using the plasma free AA dose-response technique are outlined in Table 11.

CONCLUSIONS

The results from the studies presented herein support our hypothesis that a positive linear relationship exists between plasma Lys concentration and increasing amounts of Lys continuously infused into the abomasum or duodenum or fed as RP-Lys supplements. The refinements and improvements we propose helped to reduce experimental variation through decreased SEM, CI, and coefficient of variation, as well as increased R^2 , thereby improving the accuracy of the plasma free AA dose-response technique for determining the RBV of Lys in RP-Lys supplements.

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