Predicting omasal flow of nonammonia N and milk protein yield from in vitro-determined utilizable crude protein at the duodenum

H. Gidlund,* M. Vaga,* S. Ahvenjärvi,† M. Rinne,† M. Ramin,* and P. Huhtanen*¹

*Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden

†Natural Resources Institute Finland (Luke), Green Technology, FI-31600 Jokioinen, Finland

ABSTRACT

This study evaluated the relationship between utilizable crude protein (uCP) at the duodenum estimated in vitro and omasal flow of crude protein (CP; omasal flow of nonammonia N $\times$ 6.25) measured in lactating dairy cows. In vivo data were obtained from previous studies estimating omasal digesta flow using a triple-marker method and ¹⁵N as microbial marker. A total of 34 different diets based on grass and red clover silages were incubated with buffered rumen fluid previously preincubated with carbohydrates for 3 h. The buffer solution was modified to contain 38 g of NaHCO₃ and 1 g of (NH₄)HCO₃ in 1,000 mL of distilled water. Continuous sampling of the liquid phase for determination of ammonia-N was performed at 0.5, 4, 8, 12, 24, and 30 h after the start of incubation. The ammonia N concentrations after incubation were used to calculate uCP. The natural logarithm of uCP [g/kg of dry matter (DM)] at time points 0.5, 4, 8, 12, 24, and 30 h of incubation was plotted against time to estimate the concentration of uCP (g/kg of DM) at time points 16, 20, and 24 h using an exponential function. Fixed model regression analysis and mixed model regression analysis with random study effect were used to evaluate the relationships between predicted uCP (supply and concentration) and observed omasal CP flow and milk protein yield. Residual analysis was also conducted to evaluate whether any dietary factors influenced the relationships. The in vitro uCP method ranked the diets accurately in terms of total omasal CP flow (kg/d) or omasal CP flow per kilogram of DM intake. We also noted a close relationship between estimated uCP supply and adjusted omasal CP flow, as demonstrated by a coefficient of determination of 0.87, although the slope of 0.77 indicated that estimated uCP supply (kg/d) was greater than the value determined in vivo. The linear bias with mixed model analysis indicated that

uCP supply overestimated the difference in omasal CP flow between the diets within a study, an error most likely related to study differences in feed intake, animals, and methodology. Predicting milk protein yield from uCP supply showed a positive relationship using a mixed model (coefficient of determination = 0.79), and we observed no difference in model fit between the time points of incubation (16, 20, or 24 h). The results of this study indicate that the in vitro method can be a useful tool in evaluating protein value of ruminant diets.

Key words: protein evaluation, protein degradation, metabolizable protein, dairy cow

INTRODUCTION

Protein evaluation systems aim to optimize the feeding of protein, and accurate prediction of the protein requirement of the animal is a prerequisite of a functioning evaluation system (Schwab et al., 2005). Since the 1970s, when the MP systems were established, they have become commonly used and increasingly accurate in predicting production responses and particularly milk protein yield (MPY). Metabolizable protein is defined as AA absorbed in the small intestine and it consists of microbial protein, RUP, and a small fraction of endogenous AA. Several systems for calculating MP have evolved (GfE, 2001; NRC, 2001; Volden, 2011). Microbial (bacterial) MP is calculated as a function of digestible OM, with or without discounting substrates that provide little or no energy for microbes.

The procedures to determine ruminal CP degradability, and hence supply of RUP to the small intestine, are most often based on the in situ bag procedure (NRC, 2001; Volden, 2011). Several problems exist with the use of the in situ technique when measuring ruminal protein degradability (Michalet-Doreau and Ould-Bah, 1992; Broderick and Cochran, 2000), and the technique has proven difficult to standardize, as indicated by large between-laboratory differences in ruminal protein degradability values (Madsen and Hvelplund, 1994). The major problems causing bias in the procedure

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¹Corresponding author: pekka.huhtanen@slu.se

are the assumption that the soluble protein fraction is completely degradable, poor bag porosity preventing microbes from entering the bag, and microbes colonizing the bag residues (Broderick et al., 2010).

Broderick et al. (2010) found a strong relationship between NAN flow observed at the omasal canal and total CP flow from the rumen predicted by the NRC (2001) model. However, evaluation of the NRC model indicated a 26% slope bias in the RUP supply, suggesting that the system overestimated the range of RUP supply (Broderick et al., 2010). Those authors also speculated that the overestimation derives from problems with the in situ procedure. Santos et al. (1998) performed a meta-analysis studying the effects of replacing soybean meal with high-RUP sources and found that milk yield increased only in 17% of the comparisons. Similarly, Ipharraguerre and Clark (2005) found that mean milk production responses to RUP supplements were negligible compared with the response to solvent-extracted soybean meal. In both analyses (Santos et al., 1998; Ipharraguerre and Clark, 2005), there were indications of reduced microbial protein synthesis with RUP supplementation. These previous studies indicate what was stated by Lebzién and Voigt (1999), that the current protein evaluation systems may overemphasize the importance of RUP supply to the dairy cow and that calculated microbial CP and RUP may not be completely additive.

The modified Hohenheim gas test (Steingäß et al., 2001; Steingäß and Südekum, 2013) offers an in vitro method that simplifies the aim and can eliminate some methodological inaccuracies of modern protein evaluation systems. The method involves incubation of feeds with rumen fluid, after which $\text{NH}_3\text{-N}$ is measured. The NAN content is used to calculate utilizable crude protein (**uCP**) at the duodenum, which corresponds to ruminal microbial CP and RUP flowing to the duodenum. Edmunds et al. (2012) used the modified Hohenheim gas test to validate forage protein values against the German feed protein evaluation system (GfE, 2001) and reported that the method has high potential for estimating uCP. Theoretically, the problems of the in situ method (particle loss, soluble N, microbial contamination) should be smaller in the uCP method, which also takes into account possible effects on microbial N synthesis.

Our hypothesis was that the in vitro-determined uCP would predict omasal flow of CP (omasal flow of NAN $\times$ 6.25) determined in dairy cows fed different diets. By incubating the whole diets instead of single feeds, possible interactions between diet components and effects on microbial synthesis could be better taken into account. Our objective was to evaluate the relationship between uCP estimated in vitro and omasal CP flow

measured in cannulated cows fed a wide range of diets. An additional objective was to determine the relationship between in vitro uCP concentration and MPY.

MATERIALS AND METHODS

Animal studies conducted in Sweden were registered and conducted according to guidelines approved by the Swedish University of Agricultural Sciences Animal Care and Use Committee and the National Animal Research Authority. Animal studies conducted in Finland were managed according to legislation documented within the Finnish Animal Welfare Act (247/96) and the Order on Using Vertebrate Animals for Scientific Purposes (1076/85; Ministry of Agriculture and Forestry, 1996).

Experimental Design and Animals

Thirty-four diets were evaluated in vitro using rumen fluid inoculum. Each diet was randomly distributed within and between 4 runs, resulting in 4 observations per diet. In each run, 2 blanks were incubated.

Rumen fluid for in vitro inoculum was collected from 3 lactating Nordic Red cows fitted with 10-cm ruminal cannulas (Bar Diamond Inc., Parma, ID). The cows were fed diets consisting of grass silage, crimped barley, canola meal, and minerals (55, 34, 10, and 1% on DM basis, respectively). Milking was performed 2 times daily, at 0600 and 1500 h.

Origin of Samples

Thirty-four diets from 8 different in vivo omasal flow studies (Table 1) conducted at the MTT Agrifood Research Finland (now Natural Resources Institute Finland, Luke, Helsinki, Finland) and the Swedish University of Agricultural Sciences were evaluated in vitro. Dietary ingredients within a study were pooled over experimental periods, and TMR samples for in vitro runs were prepared in the same proportions as fed to the animals.

All production studies used ruminally cannulated lactating dairy cows in complete and incomplete Latin square designs. Experimental diets were typical for northern Europe, based on grass and red clover silages from different harvest regimens, supplemented with a variety of grains and protein feeds (Table 1). Descriptive data on the in vivo studies (Table 2) showed mean values of 19.6 kg of DMI/d and 27.7 kg of ECM/d. In all studies, omasal digesta was collected according to the sampling technique by Huhtanen et al. (1997) with the modification of Ahvenjärvi et al. (2000), placing a collection tube through the rumen cannula by the

Table 1. Overview of feed components in 8 in vivo studies used in the data set for predicting omasal flow of CP

Study	Reference	Feeds
1	Korhonen, 2003	Primary growth grass silage, regrowth grass silage, 2.1 kg of canola meal, 6 or 10 kg of barley
2	Kuoppala et al., 2010	Early or late primary growth grass silage, early or late regrowth grass silage, 8 kg of concentrate
3	Vanhatalo et al., 2004	Grass silage, barley, canola meal, dry peas, ensiled peas
4	Vanhatalo et al., 2009	Early or late primary growth grass silage, early or late primary growth red clover silage, 9 kg of concentrate
5	Rinne et al., 2015	1:1 grass silage/red clover silage, canola expeller, soybean expeller, 1:1 barley/oats
6	K. Kuoppala (National Resource Institute Finland, Green Technology, Animale, Jokioinen, Finland, personal communication)	Grass silage, standard concentrate, canola meal, fava bean, blue lupin
7	Krizsan et al., 2017	Grass silage, crimped barley, canola meal
8	Cabezas-Garcia et al., 2018	Early cut primary growth grass silage, late cut primary growth grass silage, rolled barley, canola expeller, standard concentrate

omasal orifice and connecting the tube to vacuum. Digesta flow was estimated using a triple-marker method (France and Siddons, 1986) and ^{15}N was used as a microbial marker.

The in vitro substrates consisted of 500 mg of silage and concentrate ingredient samples in the same proportions as fed to the animals. All feed samples had been previously dried, milled through a 1.0-mm screen, and analyzed for nutritional content. A current DM analysis was performed for all samples at 105°C in a forced-air oven for 24 h.

In Vitro Procedure

The in vitro procedure followed the scheme of Hetta et al. (2003), with some modifications (Karlsson et al., 2009) that permitted collection of samples from the same bottle at different time points. Rumen fluid was collected after morning milking and filtered through 2 layers of cheesecloth into prewarmed thermos flasks flushed with CO_2 . Within 45 min of collection, the fluid arrived at the analytical laboratory, where the fluid pH from each cow was recorded and 0.4 mL were sampled

Table 2. Descriptive data on the 8 in vivo studies used in the data set for predicting omasal flow of CP

Item	N	Mean	SD	Minimum	Maximum
Intake					
DM, kg/d	34	19.6	2.00	15.8	23.6
Silage DM, kg/d	34	11.4	1.69	7.5	14.0
CP, kg/d	34	3.23	0.664	2.10	3.98
Concentrate proportion, kg/kg	34	0.41	0.079	0.12	0.61
Diet composition, g/kg of DM					
CP	34	164	22.7	133	257
RDP	34	118	16.1	81	149
RUP	34	46	21.8	25	122
NDF	34	399	33.7	308	464
Omasal CP flow, ¹ kg/d					
Total	34	3.14	0.598	2.31	4.41
Feed ²	30	1.09	0.256	0.52	1.66
Microbial	30	2.10	0.461	1.46	3.13
Digestibility, g/kg					
Total apparent OM	34	726	24.1	677	773
Rumen NDF	30	553	86.2	397	692
Total NDF	30	610	49.5	484	686
rNDFD/tNDFD ³	30	0.90	0.088	0.73	1.03
Total apparent N	30	676	31.8	616	732
Rumen NH_3 , mmol/L	30	5.0	1.77	1.9	7.5
Production data					
ECM, kg/d	34	27.7	4.22	21.6	34.6
Milk protein, g/d	34	898	116.0	702	1136
MUN, mg/dL	34	9.0	2.35	4.9	13.5
BW, kg	34	629	29.6	591	681

¹Calculated as $\text{NAN} \times 6.25$.

²Total omasal CP flow – microbial omasal CP flow, includes endogenous CP.

³Rumen NDF digestibility (g/kg)/total NDF digestibility (g/kg).

into 0.016 mL of H_2SO_4 and kept frozen for later $\text{NH}_3\text{-N}$ analysis.

A mix of rumen fluid (800 mL) from all cows was filtered through 4 layers of cheesecloth into a flask kept at 39°C in a water bath under constant flushing of CO_2 . This filtered rumen fluid was preincubated with a carbohydrate mixture to stimulate microbial activity and reduce the background NH_3 concentration. The mixture consisted of 1.6 g of pectin, 3.2 g of maltose, 1.6 g of starch, and 1.6 g of xylose dissolved in 100 mL of buffer (Menke and Steingäß, 1988) at 39°C . The rumen fluid and carbohydrate mixture was stirred for a few minutes, 0.4 mL was sampled as described for rumen fluid, pH was measured, and then the stirrer was turned off. After 30 min, the buoyant layer of feed particles was removed with a tube connected to vacuum via the faucet. The stirrer was started and the remaining fluid was left to incubate under a constant CO_2 stream for another 2.5 h. Every hour during preincubation, the pH was measured and 0.4 mL of fluid was sampled as described for rumen fluid.

After the preincubation step, the rumen fluid was mixed with a buffered mineral solution (Menke and Steingäß, 1988; 20:80 vol/vol) under constant stirring and flushing with CO_2 . The buffer solution was modified to contain 38 g of NaHCO_3 and 1 g of $(\text{NH}_4)\text{HCO}_3$ in 1,000 mL of purified water (Milli-Q, Millipore Academic, Millipore Corporation, Billerica, MA). The aim of modification was to decrease the addition of $\text{NH}_3\text{-N}$ from the buffer. Buffered mineral solution and buffered rumen fluid were sampled in the same manner as described for rumen fluid. Samples of 500 mg of experimental diets comprised dietary ingredients in the same proportions as fed to the animals and were weighed separately into 250-mL screw-cap bottles (Schott, Mainz, Germany). These bottles containing substrate were filled with CO_2 followed by addition of buffered rumen fluid (60 mL) and placed in a water bath (39°C) with constant agitation for 30 h. The incubations were repeated in 4 consecutive runs. Each run included 2 blank bottles containing only buffered rumen fluid without substrate.

$\text{NH}_3\text{-N}$ Measurement

The liquid phase was sampled for $\text{NH}_3\text{-N}$ with measurement at 0.5, 4, 8, 12, 24, and 30 h after the first bottle was placed in the water bath. Sampling of liquid phase from the bottles was performed according to Karlsson et al. (2009), with tubes connected to the bottles. A 0.4-mL sample from each bottle was taken with a separate syringe and transferred to a test-tube filled with 0.016 mL of H_2SO_4 (96%) for preservation. These samples were kept on ice to stop further fermentation before being stored at -20°C until further analysis.

The sample tubes were thawed to room temperature in lukewarm water, which was performed in a sequence to keep the samples unfrozen for the shortest time possible. The samples were centrifuged at $12,500 \times g$ for 7 min at room temperature and 0.15 mL of the supernatant were pipetted into vials containing 0.85 mL of purified water. The $\text{NH}_3\text{-N}$ in the dilution was analyzed with a continuous flow analyzer (AutoAnalyzer 3 HR, SEAL Analytical Ltd., Southampton, UK) following the instructions provided by the manufacturer [Method No. G-102-93 Rev 7 (multitest MT7)].

Calculations and Statistical Analysis

Utilizable CP was calculated according to the equation by Edmunds et al. (2012):

$$\begin{aligned} \text{uCP (g/kg of DM)} = & (\text{NH}_3\text{-N}_{\text{blank}} + \text{N}_{\text{sample}} \\ & - \text{NH}_3\text{-N}_{\text{sample}}) / \text{sample weight (mg of DM)} \\ & \times 6.25 \times 1,000, \end{aligned} \quad [1]$$

where $\text{NH}_3\text{-N}_{\text{blank}}$ is the average amount (mg) of $\text{NH}_3\text{-N}$ in the blanks at each time point, N_{sample} is the amount (mg) of N in the initial substrate, and $\text{NH}_3\text{-N}_{\text{sample}}$ is amount (mg) of $\text{NH}_3\text{-N}$ in the liquid phase in the bottle at each time point.

The data were analyzed using the mixed procedure in SAS (release 9.3, SAS Institute Inc., Cary, NC). Least squares means of the uCP concentration (g/kg of DM) between runs were estimated using the statistical model

$$Y_i = \mu + D_i + R_j + B_k + e_{ijk},$$

where Y_i is the dependent variable, μ is the mean of all observations, D_i is the effect of diet i , R_j is the effect of run j , B_k is the effect of bottle k , and $e_{ijk} \sim N(0, \sigma_e^2)$ is the random residual error where σ is the standard deviation.

To estimate the concentration of uCP at time points 16, 20, and 24 h (uCP_{16} , uCP_{20} , and uCP_{24} , respectively), the natural logarithm of uCP (g/kg of DM) at time points 0.5, 4, 8, 12, 24, and 30 h of incubation was plotted against time. An exponential function was used to calculate uCP concentration at estimated time points 16, 20, and 24 h:

$$\begin{aligned} \text{Effective uCP (g/kg of DM)} = & \text{Exp}(\text{intercept} \\ & + \text{slope} \times \text{time point}), \end{aligned} \quad [2]$$

where time point is the assumed rumen retention time of 16, 20, or 24 h. In situ methods commonly use 16 h of

incubation to estimate RUP (Varvikko and Vanhatalo, 1991).

Estimated supply of uCP₁₆, uCP₂₀, and uCP₂₄ was calculated as

$$\text{uCP}_x \text{ (kg/d)} = \text{uCP (g/kg of DM)} \times 1,000 \times \text{DMI (kg/d)}. \quad [3]$$

Some of the variation in the in vivo data are related to differences between studies, and this variation must be excluded to establish a correct relationship with the in vitro data. Therefore, the relationship between the in vitro and in vivo data was evaluated with the mixed procedure of SAS as

$$Y = \mu + B_1X_{1ij} + b_0 + b_1X_{ij} + B_2X_{2ij} + e_{ij},$$

where Y is the dependent variable; μ , B_1X_{1ij} , and B_2X_{2ij} are the fixed part of the model; $b_0 + b_1X_{ij}$ and e_{ij} are the random parts of the model where $i = 1 \dots 8$ studies and $j = 1 \dots n_i$ values within a study.

The fit of the model was compared using Akaike's information criterion (AIC). The model with the smallest AIC value is assumed to be the most accurate. The relationship between in vitro and in vivo data was also evaluated with fixed regression:

$$Y_i = \mu + B_1X_{1ij} + B_2X_{2ij} + e_{ij},$$

where Y is the dependent variable; μ , B_1X_{1ij} , and B_2X_{2ij} are the fixed part of the model; and e_{ij} are the random parts of the model, where $i = 1 \dots 8$ studies and $j = 1 \dots n_i$ values.

Figure 1. Least squares means of $\text{NH}_3\text{-N}$ concentration (mg/L) for blanks and diets (blank-corrected) and utilizable CP (uCP) concentration (g/kg of DM) for diets at 0.5, 4, 8, 12, 24, and 30 h after start of in vitro incubation.

Residual analysis was conducted to evaluate whether any dietary factor influenced the relationship between uCP and omasal CP flow. Estimated uCP supply was evaluated by fixed and mixed model regression of residuals as described by St-Pierre (2003). Fixed model residuals were observed omasal CP flow – predicted uCP₁₆ flow, and mixed model residuals were observed adjusted omasal CP flow (adjusted by the mixed model procedure) – predicted uCP₁₆ flow. Predicted values ($X = \text{uCP supply}$) were centered by subtracting the mean of all predicted values from individual predictions. This made the slope and intercept estimates from the regression orthogonal, and therefore independent. From the regression equations, the mean bias (in vivo CP flow – uCP supply) was taken as the intercept and the linear bias was taken as the slope.

RESULTS

Data

The uCP concentrations at 16, 20, and 24 h were strongly correlated ($R^2 = 0.94$ to 0.99). Figure 1 shows the least squares means of $\text{NH}_3\text{-N}$ concentration (mg/L) for blanks and diets (blank-corrected) and uCP concentration (g/kg of DM) for the diets at different time points after the start of in vitro incubation. The concentration of $\text{NH}_3\text{-N}$ declined at early stages of incubation, but started to increase immediately in blank incubations.

The mean $\text{NH}_3\text{-N}$ concentration in the incubation fluid (buffered rumen fluid) was 39.1 (SD = 2.46) mg/L. Comparison of the uCP supply and omasal CP flow (Figure 2) showed a positive relationship for all studies except for study 8.

Figure 2. Indirect comparison of utilizable CP (uCP) supply (kg/d) estimated at 16 h of incubation and omasal CP flow (kg/d) between 8 in vivo omasal flow studies.

Table 3. Regressions to predict the omasal CP flow from utilizable CP (uCP) supply estimated at 16, 20, and 24 h of incubation

X ₁	Intercept	SE	P-value	Slope ₁	SE	P-value	RMSE ¹	AIC ²
Y = omasal CP flow (kg/d), fixed regression								
uCP ₁₆	-0.56	0.426	0.20	1.04	0.119	<0.01	0.329	
uCP ₂₀	-0.63	0.438	0.16	1.10	0.127	<0.01	0.331	
uCP ₂₄	-0.43	0.487	0.38	1.14	0.154	<0.01	0.368	
Y = omasal CP flow (kg/d), mixed model regression								
uCP ₁₆	0.40	0.193	0.05	0.77	0.054	<0.01	0.149	4.8
uCP ₂₀	0.44	0.206	0.04	0.79	0.060	<0.01	0.156	7.2
uCP ₂₄	1.00	0.233	<0.01	0.68	0.074	<0.01	0.177	15.4

¹Residual mean squared error; adjusted for the random study effect.²Akaike's information criteria.

Prediction of Omasal CP Flow from uCP at the Duodenum

We found a close relationship between estimated uCP supply and omasal CP flow (Figure 3). The fixed model from regressing omasal CP flow and uCP₁₆ had a coefficient of determination (**R**²) of 0.71 and a slope of 1.04. The mixed model from regressing adjusted omasal CP flow and uCP₁₆ had an adjusted R² of 0.87 and a slope of 0.77.

Relationships between omasal CP flow and uCP (at 16, 20, or 24 h) are presented in Table 3. With fixed regression, the slope was not different from 1.0 for all time points and the intercept was not significant ($P > 0.10$). Regression of CP flow on uCP₁₆ gave the best fit of fixed models due to the smallest residual mean square error (**RMSE**; 0.329) compared with uCP₂₀, and uCP₂₄. With the mixed model regression describing the relationships within a study, the slope was smaller than 1.0 for all time points ($P < 0.01$) and the lowest estimate (0.68) was associated with uCP₂₄. The inter-

Figure 3. Fixed regression between utilizable CP (uCP) supply estimated at 16 h of incubation and omasal CP flow (○, dotted line; intercept $P = 0.20$) and mixed regression between uCP supply estimated at 16 h of incubation and adjusted omasal CP flow (●, solid line; intercept $P = 0.05$).

cept was significant ($P < 0.01$) for all time points. As with the fixed model, uCP₁₆ resulted in the best mixed model fit, as displayed by the smallest adjusted RMSE and AIC.

For residual analysis, a fixed model (residual = omasal CP flow – uCP₁₆) and a mixed model (residual = adjusted omasal CP flow – uCP₁₆) were plotted against uCP₁₆ (in Figure 4). The fixed model gave negative ($P < 0.01$) mean bias of 0.42 kg/d, but the linear bias 0.04 was not significant ($P = 0.75$). In the mixed model analysis, mean bias of –0.42 kg/d and linear bias of –0.23 were significant ($P < 0.01$). Furthermore, the residual (difference between CP flow and calculated uCP intake) was negatively related to organic matter digestibility (**OMD**; $P = 0.04$) and the ratio of rumen NDF digestibility to total NDF digestibility ($P < 0.01$), and tended to be influenced by dietary CP concentration ($P = 0.09$; Table 4) when analyzed with the fixed regression model. Analysis with mixed model regression indicated that the residual was negatively related to DMI ($P < 0.01$) but not to the other factors ($P \geq 0.31$).

The results of residual analysis of fixed model with omasal CP flow (g/kg of DMI) – utilizable CP flow (g/kg of DM) estimated at 16 h of incubation, and mixed model regression with adjusted omasal CP flow (g/kg of DMI) – utilizable CP flow (g/kg of DM) estimated at 16 h of incubation are shown in Table 5. With the fixed regression model, the residual was positively ($P = 0.05$) related to DMI and negatively ($P \leq 0.05$) to OMD and the ratio of rumen NDF digestibility to total NDF digestibility, and tended ($P = 0.09$) to be positively related to dietary CP concentration. No factor influenced the residual analyzed with the mixed model regression ($P \geq 0.21$).

Prediction of MPY from uCP at the Duodenum

The results of fixed and mixed model regression predicting MPY from uCP_x or from omasal CP flow can be found in Table 6. With fixed regression, MPY was positively ($P < 0.01$) related to uCP_x at all time points,

Table 4. Residual analysis with fixed regression of omasal CP flow (kg/d) – utilizable CP supply (kg/d) estimated at 16 h of incubation, and mixed model regression of adjusted omasal CP flow (kg/d) – utilizable CP supply (kg/d) estimated at 16 h of incubation

Item	Linear estimate		
	Slope	SE	P-value
Fixed regression			
DMI, kg/d	0.04	0.028	0.13
OMD, kg/kg	−4.7	2.24	0.04
NDF, kg/kg	1.08	1.693	0.53
CP, kg/kg	4.27	2.413	0.09
Concentrate proportion, kg/kg	0.34	0.726	0.64
rNDFD (g/kg)/tNDFD (g/kg) ¹	−1.97	0.585	<0.01
Rumen NH ₃ , mmol/L	−0.058	0.0213	0.01
Mixed model regression			
DMI, kg/d	−0.04	0.014	<0.01
OMD, kg/kg	−1.1	1.34	0.41
NDF, kg/kg	−0.01	0.964	0.99
CP, kg/kg	−0.60	1.428	0.68
Concentrate proportion, kg/kg	0.08	0.412	0.85
rNDFD (g/kg)/tNDFD (g/kg) ¹	−0.41	0.390	0.31
Rumen NH ₃ , mmol/L	−0.016	0.0198	0.43

¹Rumen NDF digestibility (g/kg)/total NDF digestibility.

but addition of uCP_x as a quadratic factor did not improve the model. A similar positive relationship was found for all linear uCP_x with mixed model regression, and quadratic uCP_x further improved the model fit, as indicated by reduced RMSE and AIC. We observed no clear difference in model fit between expressing MPY from uCP₁₆, uCP₂₀, or uCP₂₄.
Milk protein yield was positively associated with omasal CP flow using both fixed and mixed model regression. When estimated by fixed models, omasal

Table 5. Residual analysis with fixed regression of omasal CP flow (g/kg of DMI) – utilizable CP concentration (g/kg of DM) estimated at 16 h of incubation, and mixed model regression of adjusted omasal CP flow (g/kg of DMI) – utilizable CP concentration (g/kg of DM) estimated at 16 h of incubation

Item	Linear bias		
	Slope	SE	P-value
Fixed regression			
DMI, kg/d	2.7	1.32	0.05
OMD, g/kg	−0.23	0.110	0.05
NDF, g/kg	0.07	0.083	0.41
CP, g/kg	0.21	0.118	0.09
Concentrate proportion, kg/kg	8.5	35.67	0.81
rNDFD (g/kg)/tNDFD (g/kg) ¹	−88	30.0	0.01
Rumen NH ₃ , mmol/L	−2.96	1.040	0.01
Mixed model regression			
DMI, kg/d	−0.31	0.757	0.68
OMD, g/kg	−0.05	0.063	0.44
NDF, g/kg	0.01	0.045	0.77
CP, g/kg	0.01	0.067	0.88
Concentrate proportion, kg/kg	−7.1	19.24	0.71
rNDFD (g/kg)/tNDFD (g/kg) ¹	−24	18.3	0.21
Rumen NH ₃ , mmol/L	−0.85	0.907	0.35

¹Rumen NDF digestibility (g/kg)/total NDF digestibility.

Figure 4. Plot of residuals versus estimated utilizable CP (uCP) supply at 16 h of incubation and y = residuals. Left axis and black markers (●) = mixed regression (adjusted omasal CP flow – utilizable CP supply estimated at 16 h of incubation; intercept P < 0.001); right axis and white markers (○) = fixed regression (omasal CP flow – utilizable CP supply estimated at 16 h of incubation; intercept P < 0.001). Predicted values were centered by subtracting the mean of utilizable CP supply from each value of utilizable CP flow.

CP flow predicted MPY better than uCP, as indicated by smaller RMSE. When evaluated according to AIC, quadratic uCP models were likely to be better than quadratic models based on omasal CP flow. Adjusted RMSE was smaller for the quadratic uCP₁₆ models than for the quadratic omasal CP model (30 compared with 40 g/d).

DISCUSSION

In Vitro Methodology

Protein evaluation systems require correct estimation of the feed protein value MP, which is composed of absorbed AA from microbial protein and RUP. The methods used for estimation of protein value have been refined over the years, but they all have different problems and contribute different types of methodological error and variation. Most current protein evaluation systems are based on in vivo (microbial MP) and in situ (feed MP) data. In the past, measurement of nutrient flows within the digestive tract and microbial protein synthesis in the rumen have relied on sampling through simple T-cannulas fitted in the abomasum or proximal duodenum (Harmon and Richards, 1997; Titgemeyer, 1997) or from the omasal canal (Huhtanen et al., 1997; Ahvenjärvi et al., 2000). Digesta flow studies are accurate with the correct choice of marker system and sampling site (Ahvenjärvi et al., 2003), but too laborious and expensive for routine analysis. The in situ technique has long been the most common way to

Table 6. Fixed and mixed model regressions for predicting milk protein yield from utilizable CP supply (uCP; kg/d) estimated at 16, 20, and 24 h or the omasal CP flow

Linear	Quadratic	Intercept	SE	<i>P</i> -value	Slope ₁	SE	<i>P</i> -value	Slope ₂	SE	<i>P</i> -value	RMSE ¹	AIC ²
Y = Milk protein yield (g/d), fixed regression												
uCP ₁₆		228	94.5	0.02	188	26.3	<0.01				73.1	
uCP ₁₆	uCP ₁₆	−240	572.0	0.68	449	315.7	0.16	−35.8	43.10	0.41	73.4	
uCP ₂₀		213	96.5	0.03	200	27.9	<0.01				73.0	
uCP ₂₀	uCP ₂₀	−154	568.1	0.79	413	325.9	0.21	−30.3	46.29	0.52	73.7	
uCP ₂₄		232	100.8	0.03	213	32.0	<0.01				76.3	
uCP ₂₄	uCP ₂₄	146	531.1	0.78	268	335.0	0.43	−8.7	52.43	0.87	77.4	
CP flow		369	54.4	<0.01	169	17.0	<0.01				58.4	
CP flow	CP flow	613	377.4	0.11	18	230.9	0.94	22.4	34.25	0.52	59.0	
Y = Milk protein yield (g/d), mixed model regression												
uCP ₁₆		421	43.4	<0.01	134	12.1	<0.01				33.6	351
uCP ₁₆	uCP ₁₆	−457	235.3	0.06	618	129.9	<0.01	−65.3	17.73	<0.01	30.2	336
uCP ₂₀		421	44.5	<0.01	139	12.9	<0.01				33.7	351
uCP ₂₀	uCP ₂₀	−478	232.1	0.05	653	133.1	<0.01	−72.0	18.91	<0.01	30.1	336
uCP ₂₄		484	43.9	<0.01	132	13.9	<0.01				33.2	352
uCP ₂₄	uCP ₂₄	−250	204.1	0.23	596	128.7	<0.01	−72.0	20.15	<0.01	29.8	337
CP flow		447	36.0	<0.01	144	11.3	<0.01				38.7	352
CP flow	CP flow	487	252.6	0.06	118	154.5	0.45	4.0	22.92	0.86	39.5	343

¹Residual mean squared error; adjusted for the random study effect.²Akaike's information criterion.

determine rumen CP degradability and RUP, but the method is associated with several problems (Broderick and Cochran, 2000; Nozière and Michalet-Doreau, 2000).

Some of the assumptions made when estimating RUP with the in situ method may not be correct; for example, the assumption that soluble protein is completely degraded in the rumen, although some can clearly escape to the small intestine (Nozière and Michalet-Doreau, 2000). Furthermore, the fact that small particles that escape from the bag without being degraded are assumed to be degraded and that microbial colonization of the undegraded protein residues in the bag may occur means that rumen degradability is misestimated using the in situ method. Madsen and Hvelplund (1985) demonstrated a good relationship between protein solubility and in situ degradability, but the relationship was much weaker for in vivo degradability.

Tilley and Terry (1963) developed the first in vitro procedure for incubating feed samples in rumen fluid to imitate the rumen. Menke et al. (1979) continued the development and determined protein degradation based on measurements of NH₃ concentration and gas production. Raab et al. (1983) refined the system based on the relationship between fermentation of carbohydrates and microbial CP. Microbial CP was calculated based on extrapolation of the linear regression between NH₃-N and gas production at different time points. Later, Broderick (1987) investigated the addition of chloramphenicol with hydrazine to inhibit microbial synthesis.

In vitro methods determining protein degradation are closed systems with no new feed intake and no removal of digesta though passage, as from the rumen,

and the time required to reach a given digestibility or degradability can be shorter than in vivo (Varvikko and Vanhatalo, 1991). Lebzién and Voigt (1999) and Edmunds et al. (2012) simplified the method to determine protein degradability and predicted the MCP and RUP as 1 value (uCP). An advantage of the in vitro method of estimating uCP is that measurements of RUP and microbial protein are performed simultaneously, but this could also be a disadvantage, as microbial and feed N cannot be separated. However, Steingäß and Südekum (2013) devised a procedure to distinguish between RUP and microbial CP using the modified Hohenheim gas test.

Another risk with the in vitro method using rumen fluid is that the microbial community might be stressed and might not achieve optimal function during the incubation. Therefore, an attempt at stimulation of microbial activity and reduction of background NH₃ concentration through preincubation of the filtered rumen fluid with carbohydrates was performed in our study, similar to Broderick et al. (2004) and Karlsson et al. (2009).

Apart from being more efficient for routine analysis and requiring less use of rumen-cannulated cows, in vitro methods could overcome the problems discussed with the in situ method. The in vitro method accounts for the effect of fermentable energy in the diet on microbial growth and could also possibly take into account the effects of protein quality on microbial protein synthesis. Soluble NAN components stimulate microbial N synthesis compared with NH₃-N (Walker et al., 2005; Ahvenjärvi et al., 2018), whereas RUP sources decrease the flow of microbial N (Ipharraguerre

and Clark, 2005). For example, Broderick and Reynal (2009) replaced solvent-extracted soybean meal and urea with lignosulfonate-treated soybean meal and urea and RDP and RUP concentrations were the same. Both microbial and total NAN flow to the omasum decreased with increased proportion of treated soybean meal and urea.

A fixed retention time (**RT**; 16 h) was used for calculating uCP. However, RT required to reach effective uCP in batch culture systems depends on digestion rate of the substrate; the faster the digestion rate the shorter RT is required to reach effective uCP with a given passage rate. In the present study, mean RT (**MRT**) for the average diet was 36.8 h when estimated by a model derived from rumen evacuation data (Krizsan et al., 2010). Estimated MRT for forage and concentrate particles were 40 and 25 h, respectively. When used in 2-compartment rumen model (Allen and Mertens, 1988) with ratios of 0.4:0.6 (forages) and 0.2:0.8 (concentrates) for MRT in the nonescapable and escapable compartments, respectively, required RT to reach effective uCP in batch system ranged from 17 to 23 h for digestion rates of 0.05 to 0.12/h. However, no single RT is correct for all diets because that time also varies with digestion rate. High correlations between uCP values estimated using different RT suggest that this is not a major issue for ranking the diets.

Prediction of Omasal CP Flow

Estimated supply (kg/d) and concentration (g/kg of DM) of uCP were greater than the corresponding *in vivo* values. This could be associated with immediate uptake of $\text{NH}_3\text{-N}$ from the incubation medium to intracellular pools. During the first hours of incubation $\text{NH}_3\text{-N}$ concentration decreased, in contrast to typical increases in ruminal $\text{NH}_3\text{-N}$ concentrations after feeding. It could be speculated that the observed slow increase in $\text{NH}_3\text{-N}$ concentration in the bottle may be due to microbes being deprived of intracellular $\text{NH}_3\text{-N}$ during the preincubation, resulting in a faster uptake than production of $\text{NH}_3\text{-N}$ in the early stage of incubation or even synthesis of microbial N directly from peptides or AA without degrading them to the extracellular ammonia N pool (Wallace and Lahlou-Kassi, 1995). During the preincubation, the $\text{NH}_3\text{-N}$ concentration fell below 50 mg/L, which could cause microbes to starve and, consequently, adsorb high amounts of $\text{NH}_3\text{-N}$ to fill up the intracellular pool when new substrate becomes available. The uptake of $\text{NH}_3\text{-N}$ by microbes can be very rapid according to Blake et al. (1983), who reported a rapid increase in ^{15}N enrichment in the intracellular ammonia N pool just 2 min after administration of $^{15}\text{NH}_4\text{Cl}$ to the rumen. In recent studies, M. Vaga and

P. Huhtanen (unpublished data) observed more than 50% disappearance of labeled $^{15}\text{NH}_3\text{-N}$ during the first 15 min of *in vitro* incubation.

It is also possible that $\text{NH}_3\text{-N}$ production from the blank components (microbial lysis, nutrients in rumen fluid) begins earlier and is greater without than with substrates. When no fermentable substrate is available, ATP production can be insufficient to meet the maintenance requirements of microbes. If the blank $\text{NH}_3\text{-N}$ production is overestimated, then uCP will also be overestimated. However, both immediate uptake of $\text{NH}_3\text{-N}$ and overestimation of blank correction lead only to systematic mean bias, and do not influence the ranking of treatments.

We found no linear bias with fixed model regression analysis, but the mixed model analysis indicated that uCP supply overestimated the difference in omasal CP flow between the diets within a study. Figure 5 shows examples of relationships between *in vitro* uCP and *in vivo* omasal CP from 4 studies in which wide ranges in omasal CP flow were observed. *In vitro* uCP ranked the diets precisely in terms of the total omasal CP flow or CP flow per kilogram of DMI, but the slope varied between studies. In study 4, red clover increased *in vitro* uCP similarly to omasal CP flow observed *in vivo* (Vanhatalo et al., 2009). Greater N flow to the duodenum (Dewhurst et al., 2003) or omasal canal (Vanhatalo et al., 2009) consisted of both microbial and feed N. Huhtanen et al. (2014) reported much greater feed N in soluble fractions and particles $<38\text{ }\mu\text{m}$ for diets based on red clover silages than for diets based on grass silages. Losses of solubles and fine particles from the bags, for which the *in situ* method is criticized, were avoided in the uCP method based on measurements of ammonia N production.

In study 5 (Rinne et al., 2015), the barley-oats mixture was replaced with 2 isonitrogenous levels of heat-treated canola or soybean expeller, whereas in study 7 crimped barley was replaced with graded levels of solvent-extracted, heat-moisture-treated canola meal (Krizsan et al., 2017). In both studies, the *in vitro* uCP method ranked the diets correctly according to the *in vivo* data, but the slope was considerably below 1.0. This indicates that the uCP method overpredicted the differences observed *in vivo*. Interestingly, in 2 other studies [study 3 (Vanhatalo et al., 2004) and study 6 (K. Kuoppala, National Resource Institute Finland, Green Technology, Animale, Jokioinen, Finland, personal communication)] where the grain mixture was replaced with 1 level of untreated canola meal, the observed increase in CP flow to the omasal canal was 89 and 111% of the increase in uCP supply. No differences between untreated and treated canola meal in terms of protein flow from the rumen were observed by Khorashani et

al. (1993) or Ahvenjärvi et al. (1999) in dairy cows, despite substantially different in situ degradability. Ipharraguerre and Clark (2005) reported a significant decrease (7%) in the flow of microbial N to the small intestine (34 comparisons) with RUP supplements com-

pared with solvent-extracted soybean meal. In a study by Krizsan et al. (2017), the efficiency of microbial N synthesis tended to decrease with increased inclusion of solvent-extracted, heat-moisture-treated canola meal in the diet. Further, the recovery of increased feed N

Figure 5. Relationships between calculated utilizable CP (uCP) supply (kg/d) and omasal CP flow (kg/d; bottom panel), and uCP concentration (g/kg of DM) and omasal CP flow (g/kg of DMI; top panel) in 4 studies. In study 4, grass silage harvested at 2 stages of maturity was replaced partly or completely with red clover silage (Vanhatalo et al., 2009); in study 5 grain was replaced with 2 isonitrogenous levels of heat-treated canola or soybean expeller (Rinne et al., 2015); in study 6 barley and oats were replaced with rapeseed meal, fava bean seeds or blue lupin seeds; and in study 7 crimped barley was replaced with graded levels of solvent-extracted, heat-moisture-treated canola meal (Krizsan et al., 2017).

flow as NAN flow to the omasal canal was only 60%, which means that increased feed NAN flow entering the omasal canal decreased microbial NAN flow (Krizsan et al., 2017). In the study by Rinne et al. (2015), the efficiency of microbial N synthesis also decreased with increased levels of heat-treated protein supplements.

The reasons for the overprediction of in vivo omasal CP flow by the uCP method are not clear, but it could be speculated that the adverse effects of heat-treated protein supplements on the efficiency of microbial synthesis may not be detected in short in vitro incubations, or that the proteolytic activity to degrade heat-treated proteins is lower in vitro than in vivo. Therefore, care should be exercised when assuming that quantitative improvements in uCP will be directly translated into similar increases in protein supply to the small intestine with heat-treated protein supplements. It also seems that different protein feeds require different conditions to optimize uCP without increasing ADIN (Vaga et al., 2016).

Residual Analysis

Residual analysis was conducted to evaluate whether any dietary factor influenced the relationship between uCP and omasal CP. Most of the significant effects were observed with the simple regression model. This may indicate systematic differences between the studies in feed intake, animals, and analytical methods rather than real biological effects.

The positive relationship between the residual [omasal CP – uCP (g/kg of DM)] and DMI is probably due to the effect of increased microbial efficiency with increased DMI (Volden, 1999; Broderick et al., 2010). With increased DMI, the digesta passage rate increases the flow of both microbial and feed N from the rumen. These factors affect the omasal CP flow based on in vivo data but not uCP supply, as it is calculated with a fixed MRT; the opposite effect was found with mixed model analysis. The reasons for the linear effect on the residual associated with OMD are not clear. One reason could be that digestion is more rapid for highly digestible diets and the substrate becomes exhausted and microbial lysis begins earlier in the in vitro system.

With simple model regression, the residual was strongly related to the ratio of rumen NDF digestibility to total NDF digestibility. However, the effect was not significant when one particular study (Study 6, K. Kuoppala, National Resource Institute Finland, Green Technology, Animale, Jokioinen, Finland, personal communication) with markedly lower ratio (0.720) compared with the mean from 7 studies (0.925) was excluded from the analysis. According to a meta-analysis of omasal sampling data (Huhtanen et al., 2010), the

mean proportion of ruminal NDF digestion in total NDF digestion was 0.95. The much lower ratio in this particular study, despite similar diet composition, suggests overestimation of digesta flow rather than underestimation of uCP. None of the variables were significantly related to the residual when analyzed with mixed model regression. Therefore, it can be concluded that the linear relationships between DMI, some diet variables, and ruminal $\text{NH}_3\text{-N}$ concentration were more likely to be related to systematic differences between studies in DMI, animals, or methodology, rather than these factors influencing the relationship between uCP and omasal CP flow.

Predicting MPY

Responses measured in production studies are the final test of the nutritive value of the diet. In flow studies, the number of animals is usually small (4–5 in this data set), and intensive sampling procedures can interfere with production responses; the cows can also be at different stages of lactation and have different production potential. Therefore, the relationship between X and Y can be biased when estimated by a simple regression model (St-Pierre, 2001), which is why our analysis was also conducted with mixed model regression using study as random effect.

The uCP method predicted MPY well using the mixed model, and adjusted RMSE and AIC were smaller than for omasal CP models. The adjusted RMSE with quadratic regression (30.2 g/d) was comparable to the RMSE (31 g/d) of predicting MPY from omasal CP flow obtained by Huhtanen et al. (2010). Firkins et al. (2006) reported higher RMSE (47 g/d) with a model including DMI and concentration of RUP and RDP. In contrast to uCP, the in situ method predicts only RUP and, consequently, feed MP. In a study by Tuori et al. (1998), MP calculated using a constant CP degradability for all diets predicted MPY more precisely than MP calculated using in situ degradability to calculate feed MP. Similarly, Schwab et al. (2005) found that prediction of MPY using a constant value proved to be as accurate as using individual RUP values determined in situ.

CONCLUSIONS

Estimation of uCP ranked diets accurately in terms of omasal CP flow. We observed a relationship using mixed model regression between estimated uCP supply and adjusted omasal CP flow ($R^2 = 0.87$), although the slope of 0.77 indicated that the estimated uCP supply (kg/d) was greater than the corresponding in vivo value. This may be associated with immediate uptake

of $\text{NH}_3\text{-N}$ or overestimation of blank correction or both, which would lead to systematic mean bias but would not influence the ranking of treatments. Predicting MPY from uCP supply showed a positive relationship, with no difference in model fit for different time points of incubation (16, 20, or 24 h). The uCP method predicted MPY well using a mixed model ($R^2 = 0.79$), and adjusted RMSE and AIC were smaller than for omasal CP models. The uCP method and other flow estimates, such as omasal CP flow, only indicate the potential supply of protein to the small intestine, which must be discounted using the undigested fraction (in the small intestine) of microbial protein and RUP. Although the intestinal digestibility of microbial protein may be high and relatively constant, intestinal digestibility of RUP may be more variable.

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