Estimation of correction factors to determine the true amino acid concentration of protein after a 24-hour hydrolysis

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ABSTRACT

Although it has been acknowledged for a long time that a single period of hydrolysis, normally 21 to 24 h, is not the optimal time for most of the AA, a single period is routinely used due to time and cost constraints. As models to balance dairy rations for proteins are evolving toward balancing for AA, it becomes critical to improve the predictions of AA supply from digested proteins. Our objective was to develop correction factors that could systematically be applied to AA concentrations obtained after a 24-h hydrolysis of proteins to account for incomplete recovery and therefore determine their true AA composition. Thirteen substrates were selected to represent different types of proteins commonly used to estimate the supply of AA in ration formulation models: feed ingredients (grass silage, corn silage, soybean meal, canola meal, high-protein corn dried distillers grains, and wheat dried distillers grains plus solubles), 16-h rumen residues (soybean meal and canola meal), digesta (duodenal digesta and feces), and rumen microorganisms (fluid-associated bacteria, particle-associated bacteria and protozoa). Each protein was hydrolyzed in 6 N HCl for multiple hydrolysis times: 13 (2, 4, 8, 12, 18, 21, 24, 30, 48, 72, 96, 120, and 168 h) for feed ingredients, rumen residues, and digesta, and 9 (2, 4, 8, 18, 24, 30, 48, 96, and 168 h) for rumen microorganisms; all analyses were conducted in triplicate. Using nonlinear regression, the AA composition in the protein before the hydrolysis (A_0) was derived for each AA in each protein. Two ratios were calculated as potential correction factors: A_0 /24-h concentration (A_0 /24h) and the maximal concentration/24-h concentration (max/24h). Both ratios were tested to determine if the type of proteins was affecting them. The ratios A_0 /24h were not affected by the type of proteins, whereas the ratios max/24h were also not affected by the type of proteins except for 3 nonessential AA (Ala,

Glu, and Gly). In an attempt to propose correction factors, our results were combined with results from the literature reporting ratios A_0 /24h, ratios max/24h, or the ratio of the AA composition calculated from gene structure/24 h. The correction factors proposed for individual AA varied from 1.02 (Asp) to 1.12 (Thr). For the essential AA, the highest ratios were obtained, as expected, for the branched-chain AA and Thr. Formulation programs balancing dairy rations for essential AA would need to acknowledge the incomplete recovery of AA when obtained from 24-h hydrolysis and include correction factors, specific for each AA, but the same across different types of proteins, to correctly estimate the true AA supply to dairy cows.

Key words: amino acid, hydrolysis, protein, correction factor

INTRODUCTION

Most of the values available on AA composition of proteins, either for feed ingredients or digesta samples, are concentrations obtained after a 21- to 24-h hydrolysis, in acidic conditions for all AA except for Trp, which requires an alkaline hydrolysis. However, it has been known for a long time that acid-labile AA such as Ser and Thr are partially destroyed after their release from the protein during a 24-h hydrolysis (Rees, 1946). On the other hand, because peptide bonds involving the branched-chain AA (BCAA: Ile, Leu, and Val) are difficult to cleave, a 24-h hydrolysis is also recognized to be insufficient to release all the BCAA (Blackburn, 1968). Therefore, hydrolyzing a protein for 21 to 24 h to determine its AA composition is a compromise to reduce laboratory time and cost. Thus, concentrations obtained after a 24-h hydrolysis are useful to rank or compare feed ingredients, but concentrations obtained after 24 h of hydrolysis might underestimate the true concentrations of some AA. Therefore, when these concentrations are used as inputs in formulation models to balance dairy ration, the true supply of some AA might be underestimated. Indeed, estimated digestible flows of EAA in dairy cows obtained using AA com-

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position of feed ingredients and duodenal digesta after 24-h hydrolysis (NRC, 2001) were systematically lower than measured net portal absorption of free AA into blood circulation (Pacheco et al., 2006). Part of this systematic discrepancy might be due to the lower than true concentrations of some AA obtained after a 24-h hydrolysis of proteins, used to predict the digestible flows of EAA.

In addition, in ration formulation models used to balance rations for EAA using a factorial approach, the AA composition of all net protein requirements is obtained from 24-h hydrolysis of the proteins except that of milk, which is obtained from theoretical calculation based on the fractions of protein in milk and the AA composition of each milk protein fraction based on its primary structure [e.g., Swaisgood, 1995, Cornell Net Carbohydrate and Protein System (Fox et al., 2004)]. So, when setting up a factorial approach to balance AA supply and requirement, it is not coherent to use, in the same equation, AA composition obtained after a 24-h hydrolysis, underestimating some AA, for the supply and all the net requirements except milk protein for which a theoretical approach is used.

To determine the true AA composition of a protein, a method of extrapolation with 5 times of hydrolyses, ranging from 4 to 141 h, has been initially proposed (Robel and Crane, 1972) and further explored with up to 19 times of hydrolysis, ranging from 2 to 141 h (e.g., Rutherford et al., 2008). The multiple times allow the determination of the simultaneous release of AA from the protein and degradation of the free AA in the hydrolysate once the peptide bonds have been cleaved. Other alternatives to estimate the true AA composition of a protein have also been used: (1) the maximal AA concentration obtained after different times of hydrolysis (4 to 10) ranging from 2 to 72 h has been compared with the 24-h value (e.g., Rowan et al., 1992); or (2) the theoretical value of a simple protein like lysozyme, calculated based on its known primary structure, has also been compared with the 24-h value (e.g., Darragh et al., 1996). The latter proposed that a nonlinear regression including 10 hydrolysis intervals (from 2 to 141 h) would provide an acceptable compromise between accuracy and the cost of analysis. Although that might be an ideal situation, this solution cannot be applied routinely to laboratory analyses of AA, due to cost and time constraints.

Therefore, we hypothesized that correction factors could systematically be applied to AA concentrations obtained after a 24-h hydrolysis of proteins, accounting for their incomplete recovery, to determine their true composition. Therefore, our first objective was to determine if correction factors differ between different types of proteins involved in the estimation of

AA supply in dairy cows. The second objective was to propose correction factors based on the results obtained in objective 1 and results from literature.

MATERIALS AND METHODS

To achieve the first objective, multiple hydrolysis times were conducted on 6 feed ingredients, rumen residues of 2 protein supplements, rumen bacteria and protozoa, duodenal digesta, and feces. Sub-samples of the different proteins were weighed to yield 10 mg of N. The hydrolyses were performed using the weighed sub-samples of 10 mg of N into 50 mL of 6 *N* HCl containing 0.1% phenol (AOAC International, 2000) at 110°C in a block heater. Thirteen times of hydrolysis were performed (2, 4, 8, 12, 18, 21, 24, 30, 48, 72, 96, 120, and 168 h) on all samples except for rumen bacteria and protozoa: due to a lack of material these were hydrolyzed at 2, 4, 8, 18, 24, 30, 48, 96, and 168 h. These times were selected based on results obtained with the other types of proteins. Each time was performed in triplicate. Feed ingredients were grass silage, corn silage, soybean meal (**SBM**), canola meal (**CM**), high-protein corn dried distillers grains (**HP-DDG**), and wheat dried distillers grains plus solubles. Residues of RUP of SBM and CM were obtained after 16 h-rumen incubation as described in Maxin et al. (2013). Microorganisms and digesta were all collected during 3 consecutive days from 4 lactating dairy cows. Rumen fluid-associated bacteria (**FAB**) and particle-associated bacteria (**PAB**) were isolated according to Reynal and Broderick (2005), except that 2 layers of cheese cloth were used initially to separate the FAB and PAB and that the low speed centrifugations were at $500 \times g$ rather than 1,000. Protozoa were extracted according to Fessenden et al. (2017). Formalin was not used on bacteria or protozoa samples. Each type of microorganism was pooled into one sample after collection and separation. Duodenal digesta were collected by the mean of a closed-T duodenal cannula and feces were a sub-sample of a total collection. The experimental protocol was approved by the Institutional Committee for Animal Care of the Sherbrooke Research Centre. Care of cows followed the recommended guidelines of the Canadian Council on Animal Care (2009).

Concentrations of all AA, except Trp, were determined in hydrolysates obtained after acid hydrolysis by isotopic dilution using a GC-MS (Hewlett-Packard Model GC6890-MS5973, Agilent Technologies, Wilmington, DE) and a mass selective detector (Hewlett-Packard, Palo Alto, CA) as described by Calder et al. (1999) and adapted by Borucki Castro et al. (2007). The time-course of Cys and Met concentrations with performic oxidation before the hydrolysis to protect

their sulfur group is not reported: the extended times of hydrolysis resulted in explosion of the hydrolysis tubes, a crucial technical problem to share, and we are currently working on alternative to this procedure. Alternatively, dithiothreitol (**DTT**) was included to the hydrolysate (1.54 mg of DTT in 100 μ L of water was added to 1 mL of hydrolysate) and 0.31 mg (in 20 μ L of water) was added to 80 μ L of 2 *N* NH_4OH to reconstitute the samples before the derivatization, to reduce the oxidation of Met and Cys during analysis process.

The estimation of the AA concentration in the protein before hydrolysis was estimated based on the compartmental analysis proposed by Robel and Crane (1972). In this model, state A represents the AA concentration of AA bound in the protein matrix; state B represents the AA hydrolyzed from the protein and measureable in the hydrolysate; state C represents the AA from the hydrolysate that are degraded into nonmeasured compounds. Based on previous measurements (data not shown), it was assumed that the free AA concentration at time 0 was equal to 0 in the tested proteins. Therefore, to estimate the true concentration of AA in the protein, the following nonlinear regression was used:

$$B(t) = A_0 \times h (e^{-lt} - e^{-ht}) / (h - l),$$

where $B(t)$ = the AA concentration measured in the hydrolysate at time t ; A_0 = the concentration of AA bound to protein before hydrolysis at time 0; h = the hydrolysis rate at which the AA are released as measurable AA into the hydrolysate, expressed as a proportion of the protein-bound AA (state A) per unit of time; l = the loss rate at which free AA are degraded in the hydrolysate to nonmeasured compounds, expressed as a proportion of the free AA in the hydrolysate (state B), per unit of time.

The model assumes that h and l are constant. The means of the triplicate analyses for each time were used to derive A_0 , h , and l for each AA in each protein using least squares nonlinear regression (Proc NLIN, SAS version 9.4, SAS Institute Inc., Cary, NC). As discussed in the Results and discussion section, after inspection of the initial results obtained with this equation, the constraint that the loss rate must be greater than or equal to 0 has been added.

In addition to the determination of the A_0 value, the maximal concentration (using the mean of the triplicate analyses for each time) was statistically determined and recorded for each AA. Then the ratio of $A_0/24\text{h}$ concentration (**$A_0/24\text{h}$**) and the ratio of the maximal/24h concentration (**$\text{max}/24\text{h}$**) were calculated for each AA for each protein. These ratios represent by how much

we would need to multiply the values obtained after a 24-h hydrolysis to obtain the true AA concentration for each protein: these will be referred to as the correction factors. Each type of ratio for each AA was then submitted to an ANOVA using Proc GLM of SAS (SAS Institute Inc.). The type of proteins was tested comparing feed ingredient (grass silage, corn silage, SBM, CM, HPDDG, and wheat dried distillers grains plus solubles), RUP fractions (SBM and CM), microorganisms (FAB, PAB, and protozoa), and digesta (duodenal digesta and feces) to determine if the type of proteins affects the correction factors of each AA. Means were separated using adjusted Tukey test when the effect "type of proteins" was significant (SAS Institute Inc.).

RESULTS AND DISCUSSION

The DTT added during the analysis process gave some degree of protection to Met, but still high coefficients of variation (**CV**) were observed for the triplicate analyses. However, despite high CV, average Met concentrations were in a similar range of reported values when performic acid oxidation is performed before the hydrolysis with Met sulfone being measured, as recommended. Therefore, because the objective is to determine ratios and not absolute values, Met results were kept. However, the DTT offered very limited protection to Cys and very low concentrations were obtained; therefore, Cys results were discarded. For comparison purposes with other references, the AA concentrations obtained after 24-h hydrolysis are presented in Table 1. The sum of AA represented between 82 to 110% of CP, indicating an adequate but incomplete recovery of AA after 24 h of hydrolysis. Indeed, because the hydrolysis process is adding 1 molecule of water to AA for each cleaved peptide bond, the hydrolysis of 100 g of pure protein should yield slightly more than 115 g of free AA, depending of the AA composition of the protein.

The time-course concentrations of 3 AA is presented in Figure 1: Asp represents an AA for which the 24-h value is fairly close to the true concentration; Ser represents a labile AA, which is liberated quickly during the hydrolysis and for which the concentration begins to decline before 24 h; and Val represents those tightly bound AA not totally released after a 24-h hydrolysis. The CV of these triplicate analyses averaged 1.3, 1.6, and 1.5% for Asp, Ser, and Val, respectively. These are the classical patterns reported, for example, in goat milk by Rutherford et al. (2008) and in rumen microorganisms by Fessenden et al. (2017). During examination of the results obtained from the equation proposed by Robel and Crane (1972) and used by others (e.g., Darragh et al., 1996; Rutherford et al., 2008;

Table 1. Amino acid concentrations (g of AA/kg of CP) of different proteins measured after a 24-h acid hydrolysis

AA	Grass silage	Corn silage	Soybean meal (SBM)	Canola meal (CM)	Wheat distillers grain	Corn distillers grain	RUP from SBM	RUP from CM	Duodenal digesta	Feces	Rumen FAB ¹	Rumen PAB ¹	Rumen proto ¹
Ala	57.7	76.1	43.9	45.1	42.8	81.1	52.2	49.1	63.4	68.2	65.4	62.4	46.7
Arg	40.8	26.2	78.6	65.1	48.8	51.6	83.0	61.2	40.2	38.2	48.5	45.7	45.4
Asp	117.3	85.7	132.3	84.0	61.2	86.4	148.8	95.8	97.6	103.7	126.0	118.8	126.7
Gln	86.0	107.8	178.6	158.3	236.8	190.1	174.7	137.7	101.8	109.5	117.6	110.2	129.9
Gly	47.0	44.0	42.8	50.9	41.4	44.7	49.7	52.7	47.0	53.0	52.1	49.1	44.1
His	19.6	21.3	30.7	28.9	23.8	34.9	32.8	28.1	19.3	19.8	17.7	16.5	17.8
Ile	42.6	34.7	45.7	40.3	36.6	42.2	54.8	47.7	44.2	47.0	53.1	49.9	58.5
Leu	73.6	100.0	77.9	71.3	71.6	130.8	91.7	76.2	71.2	74.6	72.1	67.2	75.5
Lys	48.0	32.3	64.5	58.3	26.8	34.4	67.1	59.6	55.9	53.3	72.3	66.9	89.7
Met	13.1	20.3	11.8	22.2	19.0	27.7	20.1	19.5	22.8	22.7	26.7	25.5	25.5
Phe	47.4	46.8	52.8	41.8	47.1	54.2	59.2	47.1	42.7	46.6	46.7	43.4	51.9
Pro	52.9	72.3	52.7	62.4	92.5	90.9	57.6	70.6	43.0	43.3	33.9	31.1	37.5
Ser	43.7	43.7	52.7	43.4	46.1	54.6	58.2	51.9	40.6	41.7	40.2	37.7	39.9
Thr	41.8	39.4	40.1	43.8	31.9	42.5	47.8	55.8	45.3	48.5	49.9	46.8	45.3
Tyr	35.7	34.8	39.4	33.2	34.8	51.5	46.6	43.3	37.6	37.6	48.5	46.6	50.6
Val	54.4	49.6	47.3	51.1	45.5	55.8	59.8	62.6	49.1	53.4	55.5	52.2	46.4
Total AA	821	835	992	900	907	1,073	1,104	959	822	861	926	870	931

¹FAB = fluid-associated bacteria; PAB = particle-associated bacteria; proto = protozoa.

Figure 1. Effect of time of hydrolysis on the concentrations of Asp, Ser, and Val in the hydrolysate of soybean meal (panel a, b, and c, respectively). Each time point is the mean of triplicate analyses.

Fessenden et al., 2017), we observed that the loss rate from the hydrolysate was negative for the AA, still showing an increment of their concentration after 24 h of hydrolysis. Although this negative loss rate gives a good mathematical fit to the data, it does not apply to its initial representation in the model, which should be the proportion of state B (i.e., in the pool of AA in the hydrolysate) being degraded. A negative value indicates an entry rate into the pool of the AA in the hydrolysate rather than an outflow. Therefore, we forced the loss rate to be equal or greater than 0. The equation had a poorer fit to the data than the equation with the negative loss rate, but the solutions for A_0 were slightly higher than when the constraint that the loss rate must be to be equal or greater than 0 was not applied. Therefore, we considered that this estimation better fits with the concept of an estimated A_0 larger than the 24-h value (see example of corn silage in Table 2; comparing with 24-h values from Table 1).

The ratios of $A_0/24h$ and $max/24h$ are reported for each AA and each type of protein in Table 3. Numerically, the $A_0/24h$ ratios were lower than the $max/24h$ ratios for all AA except Thr and Ser for which they were higher. These 2 AA consistently had a loss rate greater than 0, whereas for most of the other AA, the constraint that the loss rate must be greater than 0 applied (results not shown). The fact that the $max/24h$ ratios were lower than the $A_0/24h$ ratios for these 2 AA indicates that the loss of the released AA in the hydrolysate began early before 24 h, and when a maximal value was obtained, there was already a significant

loss of these AA. Indeed, maximal concentrations of Ser and Thr were observed after 9 ± 3.4 h and 18 ± 5.5 h of hydrolysis, respectively (Table 4). As previously mentioned, it has been known for a long time that acid-labile AA like Ser and Thr are partially destroyed after their release from the protein during a 24-h hydrolysis (Rees, 1946). Accordingly, when multiple time hydrolyses have been conducted, Ser and Thr are the AA with a positive and high loss rate (e.g., Darragh et al., 1996; Fessenden et al., 2017). Besides Ser and Thr, only Met had a positive loss rate across all the proteins with an average of 24 h to reach its maximal concentration. Rutherford et al. (2008) also reported a positive loss rate for Met, either measured as Met or as Met sulfone. For the other AA, Arg also had a positive loss rate for SBM, HPDDG, duodenal digesta, FAB, and PAB and Tyr for SBM and HPDDG (results not shown). Therefore, for all other AA except Ser and Thr, the average hydrolysis time to reach maximal concentrations was, on average, equal or greater than 24 h (Table 4).

The $A_0/24h$ ratios were not affected ($P > 0.10$) by the type of proteins. The $max/24h$ ratios were also not affected ($P > 0.10$) by the type of proteins except for 3 NEAA, Ala, Glu, and Gly ($P \leq 0.05$) and a tendency ($P = 0.08$) for Pro. Overall these data indicate a systematic underprediction of AA concentration with 24-h hydrolysis, which is different between AA. These data also suggest that the same correction factor can be used across the different proteins for each AA, at least for the EAA and for the types of proteins analyzed in the current study.

Table 2. Effect of including the constraint that the loss rate of the AA from the hydrolysate must be greater or equal to 1 to estimate the AA composition of corn silage using nonlinear regression from multiple time hydrolyses

AA	Constraint: loss rate ≥ 0			No constraint on loss rate			
	A_0^1	h^2	l^3	A_0^1	h^2	l^3	SE of 1
Ala	76.0	0.93	0	75.0	0.98	-0.0002	0.00009
Arg	26.3	0.35	0	25.6	0.38	-0.0004	0.00020
Asp	86.1	0.87	0	84.9	0.91	-0.0003	0.00011
Glu	107.6	0.55	0	105.5	0.59	-0.0004	0.00017
Gly	44.3	0.85	0	43.1	0.95	-0.0005	0.00015
His	21.5	0.40	0	20.6	0.46	-0.0008	0.00022
Ile	37.9	0.19	0	35.0	0.24	-0.0011	0.00033
Leu	101.3	0.50	0	98.8	0.54	-0.0004	0.00017
Lys	32.7	0.54	0	31.9	0.59	-0.0004	0.00026
Met	22.5	0.59	0.0044	22.5	0.59	0.0044	0.00066
Phe	47.4	0.46	0	45.7	0.51	-0.0006	0.00020
Pro	72.1	0.66	0	71.7	0.67	-0.0001	0.00008
Ser	46.2	0.81	0.0035	46.2	0.81	0.0035	0.00018
Thr	39.7	0.48	0.0011	39.7	0.48	0.0011	0.00016
Tyr	34.6	0.74	0	34.5	0.75	0.0000	0.00015
Val	52.6	0.27	0	49.2	0.33	-0.0011	0.00032

¹Amino acid concentration (g of AA/kg of CP) determined by nonlinear regression; see text for the equation.

² h = hydrolysis rate.

³ l = loss rate.

Table 3. Ratios of the AA concentrations determined by nonlinear regression (A_0) or the maximal (Max) concentration relative to the 24-h value from multiple time hydrolyses of different types of proteins¹

AA	A ₀ /24h					Max/24h						
	Type of protein					Type of protein						
	Feed	RUP	Digesta ²	Rumen org ³	SEM	P-value	Feed	RUP	Digesta ²	Rumen org ³	SEM	P-value
Ala	1.01	1.01	1.00	1.00	0.058	0.58	1.03 ^a	1.02 ^{ab}	1.01 ^{ab}	1.01 ^b	0.006	0.05
Arg	1.01	0.98	1.01	0.99	0.018	0.34	1.05	1.01	1.03	1.00	0.019	0.10
Asp	1.01	1.00	1.01	1.00	0.007	0.40	1.03	1.01	1.03	1.01	0.009	0.17
Glu	1.00	0.99	0.99	1.00	0.005	0.29	1.05 ^a	1.01 ^b	1.01 ^b	1.02 ^b	0.007	0.002
Gly	1.01	1.01	1.02	1.02	0.006	0.29	1.05 ^a	1.02 ^a	1.08 ^{ab}	1.12 ^b	0.015	0.003
His	1.02	1.00	1.02	1.01	0.019	0.89	1.07	1.03	1.06	1.03	0.033	0.51
Ile	1.07	1.05	1.10	1.05	0.016	0.12	1.12	1.09	1.15	1.09	0.019	0.13
Leu	1.01	1.01	1.01	1.00	0.006	0.68	1.03	1.02	1.03	1.02	0.009	0.22
Lys	1.01	1.01	1.00	1.00	0.009	0.87	1.04	1.03	1.01	1.02	0.013	0.30
Met	1.05	1.08	1.05	1.01	0.035	0.46	1.08	1.10	1.04	1.04	0.036	0.43
Phe	1.00	1.00	1.02	1.00	0.009	0.44	1.04	1.01	1.05	1.02	0.012	0.23
Pro	1.00	1.00	1.00	1.00	0.005	0.95	1.02	1.01	1.01	1.00	0.006	0.08
Ser	1.09	1.11	1.07	1.10	0.014	0.14	1.05	1.07	1.03	1.06	0.012	0.16
Thr	1.03	1.05	1.02	1.05	0.010	0.27	1.02	1.01	1.00	1.00	0.009	0.21
Tyr	1.00	1.00	1.02	1.00	0.012	0.72	1.02	1.02	1.03	1.01	0.014	0.74
Val	1.06	1.05	1.09	1.05	0.018	0.37	1.12	1.10	1.15	1.10	0.019	0.22

^{a,b}Within a ratio, values with a different superscript differ between types of proteins at $P \leq 0.05$.

¹See text for description of the ratios; LSM with the highest SEM reported.

²Duodenal digesta and feces.

³Rumen microorganisms: fluid- and particle-associated bacteria and protozoa.

Table 4. Time (h) needed to reach the maximal AA concentrations across all proteins analyzed

AA	Mean	SD	Minimum	Maximum
Ala	96	46.7	21	168
Arg	37	27.2	18	120
Asp	75	48.0	24	168
Glu	82	51.5	30	168
Gly	140	33.7	72	168
His	116	43.6	48	168
Ile	120	31.0	72	168
Leu	105	45.9	21	168
Lys	109	36.3	30	168
Met	24	22.8	8	96
Phe	106	40.1	30	168
Pro	66	48.8	21	168
Ser	9	3.4	4	18
Thr	18	5.5	8	24
Tyr	63	39.8	12	120
Val	135	28.6	96	168

To meet the second objective, to determine the best correction factors to use to estimate the true AA concentration from concentrations obtained after 24-h hydrolysis, the results of other studies were combined to our results and 3 types of ratios were used. First, the 2 ratios presented in Table 3 and calculated in the current study (i.e., the $A_0/24h$ and $max/24h$ ratios) were used. A third ratio reported in the literature was also used: the ratio of the true AA concentrations deter-

mined based on the DNA sequence of a purified protein on the concentrations measured after a 24-h hydrolysis (gene/24h). In addition to the original work of Robel and Crane (1972) and the current study, the $A_0/24h$ ratios were estimated in egg white lysozyme (Darragh et al., 1996), goat milk formulation (Rutherford et al., 2008), and rumen microorganisms (Fessenden et al., 2017). The $max/24h$ ratios were determined in wheat (Kohler and Palter, 1967), pig diet and digesta (Rowan et al., 1992), and different soybean products (Albin et al., 2000). Finally, the gene/24h ratios were determined in egg white lysozyme (Robel and Crane, 1972; Darragh et al., 1996) and BSA (H. Lapierre and D. R. Ouellet, unpublished, Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada). The ratios were averaged within each study for each AA and the mean calculated for each type of ratio (Table 5). The reported ratios below 1 and above 1.23 were excluded from the means of each publication. In the mean of the ratios for each AA, used as the proposed correction factors for each AA, we did not include the ratio $max/24h$ for Ser, and Thr and the ratio $A_0/24h$ for the BCAA. We excluded the $max/24h$ ratio of Ser and Thr because, as discussed above, these 2 AA presented a significant loss before the 24-h measurements. The very low ratio of $A_0/24h$ compared with $max/24h$ for the BCAA indicates that the current nonlinear model does not apply well to these AA

Table 5. Ratios of AA composition of proteins determined by nonlinear regression (A_0) or maximal (Max) concentration during multiple time hydrolyses or theoretically derived from gene composition relative to the 24-h values¹

AA	$A_0/24h^2$	$Max/24h^3$	Gene/24h ⁴	Mean	CV
Ala	1.03	1.04	1.06	1.04	1.4
Arg	1.04	1.06	1.08	1.06	1.8
Asp	1.03	1.03	1.01 ⁵	1.02	1.0
Cys	1.12	1.09	1.23	1.15	6.4
Glu	1.05	1.06	1.04 ⁵	1.05	0.8
Gly	1.09	1.08	1.13	1.10	1.1
His	1.06	1.07	1.10	1.07	1.9
Ile	1.10 ⁶	1.12	1.12	1.12	0.2
Leu	1.02 ⁶	1.07	1.06	1.06	1.2
Lys	1.04	1.06	1.10	1.07	3.1
Met	1.03	1.05	1.07	1.05	2.0
Phe	1.02	1.07	1.09	1.06	3.5
Pro	1.02	1.03	1.09	1.04	3.7
Ser	1.09	1.05 ⁶	1.15	1.12	3.8
Thr	1.05	1.04 ⁶	1.09	1.07	2.5
Trp ⁷	1.06	—	—	1.06	—
Tyr	1.05	1.03	1.07	1.05	1.7
Val	1.03 ⁶	1.13	1.08	1.10	3.4

¹See text for complete description of the ratios.

²Average of the current study, Darragh et al. (1996), Rutherford et al. (2008), and Fessenden et al. (2017).

³Average of the current study, Kohler and Palter (1967), Rowan et al. (1992), and Albin et al. (2000).

⁴Average of H. Lapierre and D. R. Ouellet (unpublished), Robel and Crane (1972), and Darragh et al. (1996).

⁵Asp is the sum of Asp + Asn and Glu is the sum of Glu + Gln.

⁶These ratios were not included in the mean: see text for detail.

⁷Only the ratio $A_0/24h$ has been reported for Trp.

with a known resistance to hydrolysis. The mean of the 3 types of ratios, including the exclusions mentioned above, was calculated for each AA (Table 5) and is proposed as the current best estimation of the correction factors which should be applied to the concentrations obtained after a 24-h hydrolysis to account for incomplete recovery and assess the true AA concentrations of a protein. Although the correction factors for Cys were retained only when Cys was measured as cysteic acid after performic acid oxidation of the protein before hydrolysis, it showed the largest variation between the types of ratios. Only the $A_0/24h$ ratio was reported for Trp, after an alkaline hydrolysis. The labile AA, Thr and Ser, known to be partially destroyed before the 24-h measurement as well as the BCAA showed the typical high correction factors. However, although smaller for other AA, correction factors would need to be applied to all AA in order for the sum of all the AA to yield the expected 1.15 ratio (sum of the weight of free AA/weight of the protein).

In conclusion, AA concentrations obtained after 24-h hydrolysis of a protein should be multiplied by an average correction factor, specific to each AA. Currently, there is no indication that these factors should be different across different types of proteins. Inclusion of these factors into models used to balance AA for dairy rations will improve the estimations of the AA truly available to the dairy cows.

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REFERENCES

- Albin, D. M., J. E. Wubben, and V. M. Gabert. 2000. Effect of hydrolysis time on the determination of amino acids in samples of soybean products with ion-exchange chromatography or precolumn derivatization with phenyl isothiocyanate. *J. Agric. Food Chem.* 48:1684–1691.
- AOAC International. 2000. Official Methods of Analysis. 17th ed. AOAC International, Arlington, VA.
- Blackburn, S. 1968. Amino Acid Determination; Methods and Techniques. M. Dekker Inc., New York, NY.
- Borucki Castro, S. I., L. E. Phillip, H. Lapierre, P. W. Jardon, and R. Berthiaume. 2007. Ruminal degradability and intestinal digestibility of protein and amino acids in treated soybean meal products. *J. Dairy Sci.* 90:810–822.
- Calder, A. G., K. E. Garden, S. E. Anderson, and G. E. Lobley. 1999. Quantitation of blood and plasma amino acids using isotope dilution electron impact gas chromatography/mass spectrometry with U- $(^{13})C$ amino acids as internal standards. *Rapid Commun. Mass Spectrom.* 13:2080–2083.
- Canadian Council on Animal Care. 2009. Guide to the Care and Use of Experimental Animals. 2nd ed. Vol. 1. E. D. Rolfert, B. M. Cross, and A. A. McWilliam, ed. Canadian Council Anim. Care, Ottawa, ON, Canada.
- Darragh, A. J., D. J. Garrick, P. J. Moughan, and W. H. Hendricks. 1996. Correction for amino acid loss during acid hydrolysis of a purified protein. *Anal. Biochem.* 236:199–207.
- Fessenden, S. W., T. J. Hackmann, D. A. Ross, A. Foskolos, and M. E. Van Amburgh. 2017. Ruminal bacteria and protozoa composition, digestibility, and amino acid profile determined by multiple hydrolysis times. *J. Dairy Sci.* 100:7211–7226.
- Fox, D. G., L. O. Tedeschi, T. P. Tylutki, J. B. Russell, M. E. Van Amburgh, L. E. Chase, A. N. Pell, and T. R. Overton. 2004. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. *Anim. Feed Sci. Technol.* 112:29–78.
- Kohler, G. O., and R. Palter. 1967. Studies on methods for amino acid analysis of wheat products. *Cereal Chem.* 44:512–520.
- Maxin, G., D. R. Ouellet, and H. Lapierre. 2013. Ruminal degradability of dry matter, crude protein, and amino acids in soybean meal, canola meal, corn, and wheat dried distillers grains. *J. Dairy Sci.* 96:5151–5160.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Pacheco, D., C. G. Schwab, R. Berthlaume, G. Raggio, and H. Lapierre. 2006. Comparison of net portal absorption with predicted flow of digestible amino acids: Scope for improving current models? *J. Dairy Sci.* 89:4747–4757.
- Rees, M. W. 1946. The estimation of threonine and serine in proteins. *Biochem. J.* 40:632–640.
- Reynal, S. M., and G. A. Broderick. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. *J. Dairy Sci.* 88:4045–4064.
- Robel, E. J., and A. B. Crane. 1972. An accurate method of correcting unknown amino acid losses from protein hydrolyzates. *Anal. Biochem.* 48:233–246.
- Rowan, A. M., P. J. Moughan, and M. N. Wilson. 1992. Effect of hydrolysis time on the determination of the amino acid composition of diet, heal digesta, and feces samples and on the determination of dietary amino acid digestibility coefficients. *J. Agric. Food Chem.* 40:981–985.
- Rutherford, S. M., P. J. Moughan, D. Lowry, and C. G. Prosser. 2008. Amino acid composition determined using multiple hydrolysis times for three goat milk formulations. *Int. J. Food Sci. Nutr.* 59:679–690.
- Swaigood, H. E. 1995. Protein and amino acid composition of bovine milk. Pages 464–472 in *Handbook of Milk Composition*. R. G. Jensen, ed. Academic Press, London, UK.