Development of an *in vitro* Assay to Measure Intestinal Protein Digestion and Why

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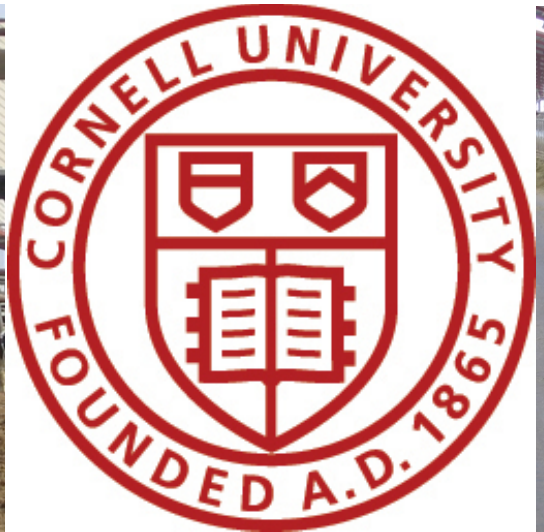

Feed Library Updates to Pool Descriptions

- CNCPS feed library has a set digestibility for the Protein B1 and B2 fractions (formerly B2 and B3 fractions)
- Some changes in nomenclature have occurred: we abandon the use of Trichloroacetic acid or Tungstic acid precipitation for describing NPN and Soluble true protein in the feed library.
- The CNCPS will now use Ammonia for the soluble A1 fraction (on a CPE)
- Any soluble protein not ammonia will be soluble true protein A2
- Renamed the B1 to A2 to be consistent with soluble pools (A) whereas B pools are insoluble, thus B2 becomes B1 and B3 becomes B2

Feed Library Updates to Pool Descriptions

- In the CNCPS the B1 and B2 proteins have 100% and 80% intestinal digestibility for concentrates
- Forages have B2 protein ID of 20%
- We know from many other data sources this is not correct and varies considerably by source, treatment and processing
- CNCPSv6.1 is more accurate and precise for MP and rumen N balance predictions/evaluations
- With the updates to the model Intestinal Dig. (ID) of protein and AA sources becomes more important

ID of RUP from Alfalfa by various methods

Feed	RUP dig, % of RUP	Ruminal Incubation	Method	Animal	Reference
Alfalfa hay	66.7	Yes	Mobile bag	lactating cow	de Boer et al., 1987
Alfalfa hay	66.0	Yes	Mobile bag	lactating cow	Erasmus et. al., 1994
Alfalfa hay	29.5	Yes	Mobile bag	Angus steers	Kononoff et al., 2007
Alfalfa hay	36.5	Yes	Mobile bag	Angus steers	Kononoff et al., 2007
Alfalfa haylage	17.2	Yes	Mobile bag	Angus steers	Kononoff et al., 2007
Alfalfa haylage	13.8	Yes	Mobile bag	Angus steers	Kononoff et al., 2007
Alfalfa meal	79.3	No	Mobile bag (i)	Holstein bulls	Todorov et al 1991
Alfalfa meal	80.7	No	Mobile bag	Holstein bulls	Todorov and Girginov, 1991

RUP ID of Blood Meal by 3 Step or Modified 3 Step (Howie et al., 1996)

Feed	RUP dig, % of RUP	Ruminal Incubation	Method	Animal
Blood meal, ring-dried	82.4	Yes	3 step	In vitro
Blood meal, ring-dried	72.1	Yes	3 step	In vitro
Blood meal, ring-dried	72.0	Yes	3 step	In vitro
Blood meal, ring-dried	82.5	Yes	3 step	In vitro
Blood meal, ring-dried	77.2	Yes	3 step	In vitro
Blood meal, ring-dried	80.9	Yes	3 step	In vitro
Blood meal, ring-dried	90.3	Yes	3 step	In vitro

RUP ID of Blood Meal by 3 Step or Modified 3 Step (Boucher, 2008)

Feed	RUP dig, % of RUP	Ruminal Incubation	Method
Blood meal, ring-dried bovine	68.7	Yes	Modified 3 step
Blood meal, ring-dried bovine	70.0	Yes	Modified 3 step
Blood meal, ring-dried porcine	88.7	Yes	Modified 3 step
Blood meal, ring-dried bovine	73.2	Yes	Original 3 step
Blood meal, ring-dried bovine	60.1	Yes	Original 3 step
Blood meal, ring-dried porcine	70.6	Yes	Original 3 step
Blood meal, ring-dried bovine	79.3	No	Modified3 step
Blood meal, ring-dried bovine	76.8	No	Modified 3 step
Blood meal, ring-dried porcine	89.0	No	Modified 3 step

Beginning of Three Step Procedure

- **Stern et al**. (1997) stated : ‘It was evident that an in vitro technique to estimate protein digestion should include enzymes with activity and specificity similar to those found in the digestive tract of the animal’.

Three Step

Calsamiglia and Stern, 1995. J. Anim. Sci

•16-h rumen incubation: 1.5 g ground (2mm) feed in 6 x 10 cm bag (50 um) *in situ;* rinse and dry residue

15 mg of residual N

•Abomasal: in 10 ml 0.1 N HCl (pH 1.9) + 1 g pepsin (porcine) per L, 1-h at 39°C. Neutralize with 0.5 ml 1 N NaOH

•Intestinal: add 13.5 ml buffer containing 3 mg/ml pancreatin, 24-h at 39°C. Terminate with TCA, centrifuge and analyze supernatant for soluble N

and Modified Three Step (MTS)

Gargallo et al. 2006: Modified the 3 step by using Ankom System for intestinal digestion

- 12-h Ruminal incubation: 5 g ground feed in 5 x 10 cm bag (50 um); rinse in washer. For AA determination of RUP use methyl cellulose to detach microbes in low protein feeds
- Abomasal & Intestinal: assay conditions identical to original except place up to 30 bags (5 x 10 cm; 50 um) containing 0.5 - 5 g of RUP in Ankom jar containing 2 L respective reagent. Analyze residual N left in bag.

Identified issues with procedure

- Ran several hundred MTS with varying success
- Became concerned with
  - enzyme activity (Pancreatin not standardized)
  - bag loss
  - Labs grinding samples – especially commercial products
  - Lack of standardization of procedure and steps

- Started to run modifications and decided to re-investigate/redo the entire assay to improve the feed library

Approach – Standardize Procedures and Reduce Particle Loss

- Depending on feed, measured up to 30% particle loss out of the bags – needed a better filtration step to improve recoveries
- Enzymes were not standardized – no Trypsin activity in the Pancreatin we evaluated (some protease, but not trypsin) – required standardization and species specific as much as possible
- Needed correction for microbial contamination to estimate RUP
- Need positive and negative controls for both fermentation and intestinal digestibility steps

Assay conditions

- In vitro fermentation in glass Erlenmeyer flasks under anaerobic conditions (CO_2) for 18-h (12-18 h OK)

- pH buffer to 6.8 w/ conc. HCl; 39°C water bath (tightly controlled)

- Rumen fluid from 2 cows – approx. 4 to 6 hrs after major meal filtered through 4 layers cheesecloth + 50 um nylon cloth + glass wool

- Use corn silage ND residue washed with ammonium sulfate to remove detergent as the control for microbial attachment: +/- bugs

- Include positive and negative controls for fermentation (CS ND dig) and intestinal digestion (blood meal)

PROCEDURE

Weigh

- 0.5 g sample into 4 - 6 flasks (for most conc. unground)
  - 2 RUP
  - other 2- 4 continue on for intestinal digestion of RUP and amino acids

16 h FERMENTATION – (12 to 18 h same answer for RUP digestibility)

- For RUP determination
  - Quantitatively filter flask contents on tared filters (9 cm) (Whatman 934-AH (1.5 µm) with boiling water
  - Dry residue; hot weigh for DM disappearance and perform Kjeldahl to determine N content of residue
- For Intestinal digestion - continue with flask, as is

RUP determination -this was not one of our objectives but appears to be a necessary step for acceptance

- To correct for microbial contamination of RUP

  -corn silage ND with and without rumen fluid

  - mostly an issue with lower CP feeds (<~12%CP)

  - for higher protein feeds the contamination is lost in the variation of measurement

Procedure (con't)

Intestinal digestion step - 2 to 4 Flasks

Place flasks in shaking water bath

- Add 3 M HCl to decrease pH < 2 (~1.9) and shake for 1 minute and then:

- Add 0.013 M HCl (~ pH 2) containing pepsin (~282 U/ml of assay mix; ~0.6 g); incubate 1-h at 39°C

- Stop reaction & neutralize acid to pH ~5 with 2 M NaOH (shake vigorously)

INTESTINAL dig

- For amino acid disappearance, freeze dry residues to avoid heat damage

INTESTINAL dig: incubate 24-h at 39°C

- Enzymes added in 10 ml 1.8 M KH_2PO_4 buffer to yield this quantity in total of 70 ml

- Enzyme mix: U per ml: 168 trypsin; 140 chymotrypsin; 705 amylase and 28 lipase to yield 24, 20, 50 and 4, U per ml, respectively
- Pancreatin: 12.04 mg per ml to yield 1.72 mg/ml (Gargello et al., 2006)

- Quantitatively filter (Whatman 934-AH, 90 mm – greater surface area for easier filtration) and hot weigh as done for RUP

Pancreatin Spec Sheet

Sold as USP (8) specifications (Sigma). Mix of :

- Amylase: solubilize not less than 25 times its weight of potato starch in 5 min
- Protease: digest not less than 25 times its weight of casein in 60 min (tested for trypsin activity – none detected)
- Lipase: release not less than 2 uEq of acid/min/mg

- 'Contains many enzymes, including amylase, trypsin, lipase, ribonuclease and protease. The National Formulary (N.F.) and the U.S. Pharmacopeia (USP) specify amylase, protease and lipase only (Sigma)'

~ Enzyme activity in assay contains

	Pancreatin	Enzyme Mix
Amylase	25.8 mg maltose/3 min	50 mg maltose/3 min
Protease	0.49 mg casein/min	0.42 mg casein/ min

			Feed N RUP digested by			ADIN	RUP ADIN digested by	
	TN	ADIN, TN	RUP frac	enzyme mix	Pancr.	IV RUP frac	enzyme mix	Pancr.
anchovy	0.115	0.013	0.837	0.695	0.760	1	0.000	0.000
			0.011	0.039	0.020			
alfalfa silage	0.038	0.061	0.517	0.751	0.765	1	0.240	0.403
			0.044	0.010	0.018		0.001	0.002
bloodmeal	0.162	0.047	0.948	0.758	0.915	0.143	0.821	0.969
			0.003	0.005	0.002	0.020	0.000	0.044
canola	0.065	0.063	0.447	0.639	0.721	0.917	0.011	0.140
			0.031	0.007	0.029			
corn silage	0.014	0.092	0.402	0.679	0.723	0.920	0.000	0.037
			0.073	0.014	0.009	0.052		0.001

	Feed N		RUP digested by ADIN				RUP ADIN digested by	
	TN	ADIN, TN	RUP frac	enzyme mix	Pancr.	IV RUP frac	E mix	Pancr.
ddg	0.064	0.327	0.944	0.705	0.856	0.719	0.963	0.988
			0.004	0.012	0.016	0.028	0.012	0.002
HDBM	0.161	0.018	0.995	0.034	0.025	0.877	0.000	0.000
			0.004	0.002	0.007	0.173		
SBM	0.076	0.067	0.329	0.764	0.768	0.086	0.324	0.582
			0.050	0.066	0.021	0.056	0.002	0.132
protected soy protein	0.077	0.065	0.934	0.904	0.954	0.977	0.991	0.998
			0.014	0.014	0.002	0.972	0.001	0.003
SoyPlus	0.073	0.079	0.698	0.840	0.906	0.715	0.797	0.881
			0.026	0.004	0.080			

Percent of feed nitrogen, estimated RUP and RUP intestinal digestion and percent nitrogen digested by compartment. RUP and nitrogen results were corrected for microbial contamination. Results are means (SD), except for calculated.

	Total feed N % DM	Estimated RUP % N	RUP intestinal digestion, % N
Blend	7.0 (0.2)	67.5 (7.1)	74.7 (0.1)

Estimated percent nitrogen digested by compartment

	Rumen	Intestine*	R + SI**
Blend	32.5 (7.1)	50.4	82.9 (0.1)

***calculated**

****R and SI: digestion following rumen fermentation, abomasal digestion with pepsin in HCl and small intestinal digestion with trypsin, chymotrypsin, lipase and amylase.**

Table 2. Feed amino acid content as percent of dry matter and amino acid nitrogen as percent of total nitrogen.

	AA, % DM	AAN, % TN
	Amino Max	Amino Max
THR	1.91	3.21
VAL	2.19	3.73
CYS	1.01	1.69
MET	1.11	1.49
ILE	1.90	2.90
LEU	3.27	4.98
TYR	1.09	1.21
PHE	1.68	2.03
TRP	0.72	1.41
LYS	2.25	6.15
HIS	1.01	3.90
ARG	2.73	12.56
TOTAL	20.66	76.09

AA	Rumen digestibility (not corrected)	Rumen + SI digestibility*
THR	0.205	0.772
VAL	0.203	0.760
CYS	0.577	0.862
MET	0.615	0.904
ILE	0.141	0.773
LEU	0.245	0.770
TYR	0.077	0.810
PHE	0.280	0.791
TRP	0.518	0.836
LYS	0.275	0.833
HIS	0.403	0.845
ARG	0.518	0.899
mean±sd	0.371± 0.207	0.823±0.053

*Rumen – SI digestibility after ruminal fermentation, abomasal digestion with pepsin in HCl and intestinal digestion with trypsin, chymotrypsin, lipase and amylase

Enzyme standardization characteristics

	Enzyme units
Pepsin	ΔA_{280nm} of 0.001 per min at pH 2.0,37°C measured as TCA-soluble products using hemoglobin.
Trypsin	ΔA_{253nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-arginine ethyl ester (BAEE).
Chymotrypsin	ΔA_{256nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-tyrosine ethyl ester (BTEE).
Amylase	One unit will liberate 1.0 mg maltose in 3 min at pH 6.9, 37°C.
Lipase	One unit releases 1 uEq of acid from olive oil per min.

Summary

- Assay appears to provide reasonable and repeatable results

- Sample loss is minimized with in vitro and updated filtration procedures

- Enzymes are standardized and evaluated if re-evaluation is required, new enzymes are purchased

- RUP estimation was not in our objectives but appears to be necessary for commercial acceptance – use of corn silage ND residue for bacterial contamination

- Assay is compatible with commercial laboratory procedures – lab will need to know basic chemistry/makeup of the sample

- Expect final procedure by end of summer

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Assay Results Using Both Enzyme Systems

	RUP	RUP digested enzyme mix	RUP digested pancreatin
Blood meal	0.954 ± 0.006	0.890 ± 0.031	0.935 ± 0.001
heat dam. blood meal	0.973 ± …	0.029 ± …	0.024 ± …
VM1	0.911 ± 0.006	0.811 ± 0.018	0.860 ± 0.000
VM2	0.954 ± 0.002	0.774 ± 0.097	0.930 ± 0.012
VM5	0.928 ± 0.004	0.730 ± 0.011	0.814 ± 0.018
VM7	0.637 ± 0.060	0.784 ± 0.010	0.841 ± 0.012
VM10	0.708 ± 0.011	0.841 ± 0.013	0.850 ± 0.007
VM11	0.660 ± 0.015	0.856 ± 0.003	0.874 ± 0.023
VM13	0.688 ± 0.018	0.841 ± 0.027	0.891 ± 0.005
VM18	0.896 ± 0.007	0.675 ± 0.004	0.829 ± 0.010
VM19	0.794 ± 0.004	0.403 ± 0.051	0.537 ± 0.035

Assay Results Using Both Enzyme Systems

	RUP	RUP digested enzyme mix	RUP digested pancreatin
SoyPlus	0.776 ± 0.006	0.824 ± 0.034	0.809 ± 0.003
heat dam. blood meal	0.979 ± 0.007	0.016 ± …	0.020 ± …
VM20	0.421 ± 0.008	0.817 ± 0.006	0.883 ± 0.009
VM21	0.658 ± 0.006	0.764 ± 0.000	0.851 ± 0.000
VM22	0.798 ± 0.027	0.818 ± 0.011	0.878 ± 0.018
VM23	0.471 ± 0.003	0.698 ± 0.002	0.734 ± 0.003
VM24	0.968 ± 0.001	0.885 ± 0.033	0.943 ± 0.006
VM25	0.957 ± 0.003	0.865 ± 0.066	0.955 ± 0.012
VM26	0.960 ± 0.004	0.835 ± 0.003	0.951 ± 0.003
VM27	0.950 ± 0.003	0.858 ± 0.053	0.942 ± 0.016