Technical note: A new high-performance liquid chromatography purine assay for quantifying microbial flow¹

S. M. Reynal^{*2} and G. A. Broderick^{†3}

^{*}Department of Dairy Science, University of Wisconsin, Madison 53706

[†]Agricultural Research Service, USDA US Dairy Forage Research Center, 1925 Linden Drive West, Madison, Wisconsin 53706

ABSTRACT

An HPLC method was developed to quantify the purines adenine and guanine and their metabolites xanthine and hypoxanthine in hydrolysates of isolated bacteria and omasal digesta and to assess the effect of using either purines only or purines plus metabolites as microbial markers for estimating microbial flow from the rumen. Individual purines and their metabolites were completely resolved on a C18 column using gradient elution with 2 mobile phases. Intraassay coefficient of variation ranged from 0.6 to 3.1%. Hydrolytic recovery of the 4 purine bases from their corresponding nucleosides averaged 101% (control), 103% (when added to bacterial isolates), and 104% (when added to omasal digesta). Mean concentrations of adenine, guanine, xanthine, and hypoxanthine were, respectively, 53, 58, 2.8, and 3.5 $\mu\text{mol/g}$ of dry matter in omasal bacteria and 10, 12, 7.5, and 7.5 $\mu\text{mol/g}$ of dry matter in omasal digesta, indicating that xanthine plus hypoxanthine represented 5% of total purines in bacterial hydrolysates but 41% of total purines in digesta hydrolysates. A significant negative relationship ($R^2 = 0.53$) between the sum of adenine and guanine and the sum of xanthine and hypoxanthine in digesta samples (but not bacterial isolates) indicated that 89% of the adenine and guanine originally present in ruminal microbes were recovered as xanthine and hypoxanthine. These results suggested that, when total purines are used as the microbial marker, both purines and their metabolites should be quantified and used to compute microbial nonammonia N and organic matter flows.

Key words: microbial flow, purines, high-performance liquid chromatography

The purines adenine plus guanine are commonly used as markers to quantify microbial protein and organic matter flow from the rumen (Zinn and Owens, 1986; Broderick and Merchen, 1992). The introduction of HPLC methodology (Balcells et al., 1992) improved the precision of purine determination compared with assays based on precipitation and spectrophotometric quantitation. However, current HPLC methods are unable to resolve guanine from the purine metabolites xanthine and hypoxanthine, which may be present in some samples of bacteria and digesta (Reynal et al., 2005). Moreover, if xanthine and hypoxanthine originate from incomplete degradation of feed purines, or from purines released with intraruminal turnover of microbial cells, then these metabolites should not be included as part of total purine marker. Alternatively, the metabolites should be included in total purines when estimating microbial flow if they originate from intact microbial cells, for example, during sample storage. It has been reported that freezing does not completely inactivate microbial enzymes involved in purine degradation (Lou, 1998); thus, xanthine and hypoxanthine could originate from enzymatic degradation of microbial adenine and guanine after digesta collection. The objective of this study was to develop an HPLC method that would completely resolve adenine, guanine, xanthine, and hypoxanthine in hydrolysates of omasal digesta and bacteria.

Nucleic acid hydrolysis was accomplished using a modification of published methods (Balcells et al., 1992; Makkar and Becker, 1999; Reynal et al., 2005). Specifically, duplicate samples of bacteria (37.5 mg) and omasal digesta (100 mg) were prepared as described by Reynal et al. (2005). Both types of samples were stored at -20°C for an average of 6 mo before being freeze-dried. Freeze-dried samples were placed in 25-mL screw-cap Pyrex tubes, and 1 mL of 2 M HClO_4 plus 0.25 mL of 6 mM allopurinol dissolved in 2 M HClO_4 (internal standard) were added. Standard solutions of purines (adenine, guanine, xanthine, and hypoxanthine) and their corresponding nucleosides (adenosine, guanosine, xanthosine, and inosine) were prepared at concentrations of 1, 2, 4, and 8 mM by dissolving the

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³Corresponding author: glen.broderick@ars.usda.gov

Figure 1. Chromatograms of a) purine base standards, b) omasal bacteria, and c) omasal digesta.

pure compounds (Sigma-Aldrich, St. Louis, MO) in 2 *M* HClO₄. Incremental amounts of nucleosides were added to triplicate samples of bacteria and omasal digesta to quantify recovery of hydrolyzed purines. Yeast RNA (10 mg) was carried through the hydrolysis system to determine purine recoveries when added to bacterial isolates and digesta samples and to serve as the blank for estimating the detection limits for xanthine and hypoxanthine. All tubes containing 2 *M* HClO₄ plus sample, or sample plus recovery standard, were incubated in a water bath at 95°C for 1 h. After cooling, 3.75 mL of 0.3 *M* KH₂PO₄ buffer solution was added and tubes were returned to the 95°C water bath for 15 min (Zinn and Owens, 1986; Makkar and Becker, 1999). After cooling again, tube contents were transferred to centrifuge tubes and centrifuged at 3,000 × *g* at 5°C for 10 min. Supernatants were transferred to a second set of centrifuge tubes, placed in an ice bath, and then pH was adjusted to 4.0 using 8 *M* KOH. Tubes were then recentrifuged at 3,000 × *g* at 5°C for 10 min, and the supernatant was finally filtered through a 0.45-μm filter membrane.

The HPLC system was a Shimadzu class-VP, version 5.03 (Shimadzu Scientific Instruments Inc., Columbia, MD) consisting of an SIL-10ADvp autosampler, 2 LC-10ADvp pumps, an SCL-10Avp system controller, an SPD-M10Avp photodiode array detector, and a 250 × 4.6-mm Inertsil ODS-3 column (MetaChem Technologies Inc., Torrance, CA) that was held isothermal at 28°C. The analytical column was protected by a Meta-Guard 4.6-mm Inertsil ODS-3 guard column (Varian Inc., Palo Alto, CA). Absorbance was monitored at 254 nm. A 10-μL aliquot of standard or sample was injected onto the column and separation performed using variable flow of a gradient mixture of 2 mobile phases. Flow rate was increased linearly from 1.0 to 1.4 mL/min over the first 6 min, and then flow rate was returned linearly to 1.0 mL/min over the next 6 min, and then held at 1.0 mL/min from 12 to 45 min. Solvent A was 0.3 *M* KH₂PO₄ and similar to that used in the single solvent system of Piñeiro-Sotelo et al. (2002); solvent B was 80% (vol/vol) 0.3 *M* KH₂PO₄ plus 20% (vol/vol) acetonitrile. Both solvents were adjusted to pH 4.0, filtered through 0.45-μm filters, and degassed using ultra-sonication while applying vacuum. Elution was achieved using a series of linear gradients: 100% solvent A (0% solvent B) was used for the first 7 min; solvent B was increased from 0 to 50% from 7 to 20 min; solvent B was increased from 50 to 100% from 20 to 25 min; solvent B was held at 100% from 25 to 30 min; solvent B was reduced from 100 to 0% (from 0 to 100% solvent A) from 30 to 35 min; and solvent A was held at 100% from 35 to 45 min to re-equilibrate the column to the starting conditions before injecting

the next sample. Guanine, hypoxanthine, xanthine, adenine, and allopurinol (internal standard) eluted at, respectively, about 9.5, 10.8, 12.0, and 13.3, and 20 min in the chromatogram. Chromatograms of purine standards, a bacterial sample, and an omasal digesta sample are in Figure 1.

The background interference from yeast RNA (which was assumed to contain essentially zero xanthine and hypoxanthine) at the elution times for xanthine and hypoxanthine was used to estimate the average gross blank signal (*S_b*) and the standard deviation of the gross blank signal (*σ_b*). The following formula (American Chemical Society, 1980) was used to estimate the limits of detection (*S_t*) for xanthine and hypoxanthine:

$$S_t = S_b + 3\sigma_b.$$

Peaks were identified by comparing their retention times with those of known standards and by spiking samples of bacterial isolates and omasal digesta before hydrolysis with known amounts of adenine, guanine, hypoxanthine, or xanthine. Hydrolytic efficiency was assessed by comparing chromatographic responses obtained after hydrolyzing incremental amounts (0.25 to 2 μmol) of adenosine, guanosine, inosine, and xanthosine to those from, respectively, equimolar amounts of adenine, guanine, hypoxanthine, and xanthine. The effect of sample matrix on hydrolysis and recovery of purines was assessed by hydrolyzing nucleosides and yeast RNA alone, and after addition to bacteria and omasal digesta samples, and comparing the chromatographic responses to those of the corresponding purines. The interassay and intraassay errors were determined (Sheps and Munson, 1956) by subjecting aliquots of bacterial and omasal samples (duplicate aliquots of each sample in each of 2 separate assays) to the complete assay procedure.

Analytical precision within assay was high for all purines as indicated by intraassay CV ranging from 1.2 to 3.1% for isolated ruminal bacteria and from 0.6 to 1.0% for omasal digesta (Table 1). Interassay variation was greater but interassay CV were still <10% except for xanthine and hypoxanthine determination in bacterial samples. Lower precision for the purine metabolites in bacterial hydrolysates resulted from their very low concentrations: 0.11 μmol/mL for xanthine and 0.08 μmol/mL for hypoxanthine, which were close to the detection limits of 0.07 and 0.08 μmol/mL. Efficiency of hydrolytic release of purines from corresponding nucleosides ranged from 95 to 108% (Table 2). Similarly, recoveries of purines from nucleosides ranged from 97 to 112% (Table 2) when added to isolated bacteria and from 96 to 116% (Table 2) when added to omasal digesta samples. We speculate that the tendency to recover in

Table 1. Intra- and interassay coefficients of variation (%) for bacterial and omasal digesta samples

Purine	Bacteria		Omasal true digesta	
	Intraassay	Interassay	Intraassay	Interassay
Adenine	1.2	2.8	1.0	7.2
Guanine	3.1	5.5	1.1	3.3
Xanthine	1.7	15.8	0.6	1.2
Hypoxanthine	1.3	13.2	0.6	6.2

excess of 100% of hypoxanthine from inosine hydrolysis may be due to hypoxanthine contamination of that nucleoside. However, purine recoveries from RNA added to bacterial and omasal digesta also averaged >100%, ranging from 103 to 113%.

Lim et al. (2006) recently showed that artifact xanthine and hypoxanthine could be formed from guanine and adenine during DNA hydrolysis with 60% formic acid at high temperatures (140°C for 45 min). However, xanthine and hypoxanthine were not detected in yeast RNA samples, or in adenine, guanine, adenosine and guanosine standards; thus, the hydrolytic conditions used in the present study did not result in formation of artifact xanthine and hypoxanthine. Alternatively, xanthine and hypoxanthine may have originated from enzymatic degradation of adenine and guanine after sample collection, as previously suggested by the significant negative correlations between adenine and xanthine in bacterial and protozoal isolates (Reynal et al., 2005). It was reported that storing samples at -18°C did not completely inactivate the enzymes involved in purine degradation, which led to gradual change in the proportions of purine bases over time (Lou, 1998; Piñeiro-Sotelo et al., 2002). A significant negative correlation ($P < 0.001$) in omasal digesta samples between the sum of adenine plus guanine (Y) and the sum of xanthine plus hypoxanthine (X), which yielded the regression: $Y = 35.8$ (SE = 2.4; $P < 0.001$) - 0.89 (SE = 0.16; $P < 0.001$) X ($R^2 = 0.52$); moreover, the slope of this regression was not significant ($P = 0.20$) for bacterial samples. We were not able to determine whether the purine metabolites present in omasal digesta derived from microbial, dietary, or endogenous origin. However, the negative relationship implied that adenine and guanine in digesta samples had been converted to xanthine and hypoxanthine with an average recovery of 89%. This suggested that it would be more appropriate to define the “total purines” marker for estimating flows of microbial NAN and organic matter as the sum of all 4 compounds. This also implies that microbial flow quantified from adenine and guanine only would be underestimated.

Conversion of adenine and guanine to xanthine and hypoxanthine during storage of digesta samples may

explain some experimental observations. We used the current method and approach in a recent study to measure microbial organic matter and NAN flows at the omasal canal of dairy cows fed different levels of cornstarch and sucrose (Broderick et al., 2008). Using only adenine plus guanine as the microbial markers resulted in lower estimates of microbial NAN flow from the rumen relative to those obtained by including xanthine and hypoxanthine as part of the total purines or as computed using the NRC (2001) model (Table 3). Proportions of dietary CP estimated to be RDP and RUP also were, respectively, lower and higher using only adenine plus guanine compared with using total purines or those computed with the NRC (2001) model. However, this does not confirm that catabolism of adenine and guanine accounts for all of the xanthine and hypoxanthine in digesta samples. Moreover, the estimates of microbial efficiency and organic matter truly digested in the rumen (Table 3) were within acceptable ranges (Titgemeyer, 1997) using either marker approach. Because hypoxanthine is precipitated by silver nitrate at pH 2 (Kerr and Seraidarian, 1945) and has an extinction coefficient at 254 nm that is comparable to that of guanine (Reynal et al., 2005), this metabolite (and probably xanthine) would be detected by the Zinn and Owens (1986) spectrophotometric assay for total purines. Thus, use of the Zinn and Owens approach likely includes xanthine and hypoxanthine as part of the estimate of total purines, regardless of their origin.

We suggest that, in application of the proposed HPLC purine assay, microbial flows from the rumen be quantified using total purines, defined as the sum of adenine, guanine, xanthine, and hypoxanthine. How-

Table 2. Recovery (%) of purines from hydrolyzed nucleosides alone (control) or when added to ruminal bacteria or omasal true digesta (OTD)

Purine	Purine recovery from nucleoside added to:		
	Control	Bacteria	OTD
Adenine	95	97	100
Guanine	100	99	96
Xanthine	100	102	105
Hypoxanthine	108	112	116

Table 3. Effect of using adenine plus guanine only, or total purines (adenine, guanine, xanthine, plus hypoxanthine), on estimates of ruminal N metabolism and organic matter truly digested in the rumen

Item	Purine marker				NRC (2001) ¹
	Adenine + guanine only		Total purines		
	Mean	SE	Mean	SE	
RDP, % of dietary CP	32	3	56	2	70
RUP, % of dietary CP	68	3	44	2	30
Microbial NAN, % of total NAN flow	38	2	60	2	62
Dietary NAN, % of total NAN flow	62	2	40	2	38
Microbial efficiency ²	22	2	30	2	—
OM truly digested in the rumen, % of intake	54	2	62	2	—

¹Computed using NRC (2001) model.²Grams of microbial NAN/kg of OM truly digested in the rumen.

ever, additional research is needed to confirm the origin of xanthine and hypoxanthine present in stored digesta samples and to identify the conditions under which adenine and guanine would remain stable.

REFERENCES

- American Chemical Society. 1980. Guidelines for data acquisition and data quality evaluation in environmental chemistry. *Anal. Chem.* 52:2242–2249.
- Balcells, J., J. A. Guada, J. M. Peiro, and D. S. Parker. 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. *J. Chromatogr. A* 575:153–157.
- Broderick, G. A., N. D. Luchini, S. M. Reynal, G. A. Varga, and V. A. Ishler. 2008. Effect on production of replacing dietary starch with sucrose in lactating dairy cows. *J. Dairy Sci.* 91:4801–4810.
- Broderick, G. A., and N. R. Merchen. 1992. Markers for quantifying microbial protein synthesis in the rumen. *J. Dairy Sci.* 75:2618–2632.
- Kerr, S. E., and K. Seraidarian. 1945. The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. *J. Biol. Chem.* 159:211–225.
- Lim, K. S., S. H. Huang, A. Jenner, H. Wang, S. Y. Tang, and B. Halliwell. 2006. Potential artifacts in the measurement of DNA deamination. *Free Radic. Biol. Med.* 40:1939–1948.
- Lou, S. 1998. Purine content in grass shrimp during storage as related to freshness. *J. Food Sci.* 63:442–444.
- Makkar, H. P. S., and K. Becker. 1999. Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods. *Br. J. Nutr.* 81:107–112.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Piñeiro-Sotelo, M., A. Rodríguez-Bernaldo de Quirós, J. López-Hernández, and J. Simal-Lozano. 2002. Determination of purine bases in sea urchin (*Paracentrotus lividus*) gonads by high-performance liquid chromatography. *Food Chem.* 79:113–117.
- Reynal, S. M., G. A. Broderick, and C. Bearzi. 2005. Comparison of four markers for quantifying microbial protein flow from the rumen of lactating dairy cows. *J. Dairy Sci.* 88:4065–4082.
- Sheps, M. C., and P. L. Munson. 1956. The role of between-assay error in biological assays. *Biometrics* 12:534–551.
- Titgemeyer, E. C. 1997. Design and interpretation of nutrient digestion studies. *J. Anim. Sci.* 75:2235–2247.
- Zinn, R. A., and F. N. Owens. 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. *Can. J. Anim. Sci.* 66:157–166.