Technical report

Purine quantification in digesta from ruminants by spectrophotometric and HPLC methods

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The method of Zinn & Owens (1986; *Canadian Journal of Animal Science* **66**, 157–166), based on release of purine bases by $HClO_4$ followed by their precipitation with $AgNO_3$, was used to study recovery of purines from lyophilized rumen microbial or *Escherichia coli* preparations added to matrices such as cellulose, starch and neutral-detergent fibre. The recovery of purines was poor (approximately 50 %). Under the hydrolysis conditions (12 M-$HClO_4$, 90–95° for 1 h) used in the method of Zinn & Owens (1986), the recovery of purines from the rumen microbial preparations added to matrices measured using an HPLC method was 95–102 %, suggesting that the lower recovery of purines in the method of Zinn & Owens (1986) was not due to incomplete hydrolysis of nucleic acids. Using the HPLC method, adenine and allopurinol (an internal standard) were found to be heat-labile as substantial destruction was observed on heating at 121°. On the other hand, another commonly used internal standard, caffeine, was stable at 121°. A complete hydrolysis of nucleic acids from the rumen microbial preparation was observed with 2·5 ml 0·6 M-$HClO_4$ in a total volume of 3 ml (0·5 M-$HClO_4$ during hydrolysis) at 90–95° for 1 h, and under these conditions adenine, guanine, allopurinol and caffeine were stable. Moreover, under these milder hydrolysis conditions, the recovery of purine bases from the rumen microbial or *E. coli* preparations added to matrices ranged from 92 to 108 % using the method of Zinn & Owens (1986). Based on the results, changes in hydrolysis conditions have been proposed for accurate determination of purine bases using spectrophotometric or HPLC methods.

Purine bases: Micro-organisms: HPLC method: Spectrophotometry

The rapid degradation of dietary nucleic acids in the rumen (McAllan & Smith, 1973) has led to the wide use of nucleic acids or their constituent purine or pyrimidine bases as markers for determination of microbial protein synthesis in the rumen. The method of Zinn & Owens (1986) for quantification of purines has been used by several workers because it is simple and inexpensive. The present study originated from our *in vitro* rumen fermentation studies wherein we were interested in measuring microbial mass as a function of incubation time from substrates such as cellulose, isolated neutral-detergent fibre (NDF) and starch. A prerequisite for using the method was to study the recovery of purine bases from a lyophilized rumen microbial preparation when present as a mixture with other organic material. Ushida *et al.* (1985) have shown good recovery of added purine bases and of the purine bases in yeast RNA added to bacterial samples using the method of Zinn & Owens (1982). Similarly, recovery from yeast RNA averaged 98·6 % when it was hydrolysed alone or together with casein, maize starch or Solka floc (cellulose) (Zinn & Owens, 1986). However, studies on the recovery of purines from the isolated rumen microbial fraction added to matrices such as cellulose, NDF and starch using the method of Zinn & Owens (1986) are lacking. The HPLC method of Balcells *et al.* (1992) was also used to monitor adenine and guanine released in the recovery studies. The stability of adenine, guanine and internal standards such as allopurinol and caffeine to heat is also reported.

Materials and methods

Preparation of lyophilized rumen microbial fraction

About 1 litre of rumen contents was collected 2 h after the morning feed from a cow fed on a roughage-based diet. The material was passed through two layers of muslin cloth and

Abbreviation: NDF, neutral-detergent fibre.

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then kept at 4° for 30 min in a CO_2-flushed cylinder of 1 litre capacity. Rumen fluid devoid of heavy and light particles was separated by pipetting from the cylinder the fraction between the heavy particles which settle down and the light particles which float on the top. This method for collection of rumen fluid was essentially adapted from Yang & Russell (1992). Several portions (each 35 ml) of this fluid were centrifuged at about 20 000 ***g*** for 20 min at 4°. The pellets were washed with distilled water followed by centrifugation (20 000 ***g*** for 20 min). This washing step was repeated two more times. The pellets were lyophilized and pooled. Sub-samples from one rumen sample were used for all assays. The N content of this fraction was 77 g/kg, which is in close agreement with that reported by Ørskov (1982).

Preparation of neutral-detergent fibre

NDF was prepared from hay using the procedure of Van Soest *et al.* (1991). The NDF was exhaustively washed with distilled water (about 1·5 litres per crucible containing 300–400 mg NDF) to remove sodium dodecylsulfate.

Preparation of apparent undigested residue

Hay (500 mg) was incubated in an *in vitro* rumen fermentation system (Makkar *et al.* 1995). After 24 h fermentation, the contents were centrifuged at 20 000 ***g*** for 30 min and the supernatant fraction was discarded. The pellet was washed with distilled water followed by centrifugation (20 000 ***g*** for 30 min). The pellet, consisting of undigested substrate and microbial mass, was lyophilized. The lyophilized residue has been termed 'apparent undigested residue'. Sub-samples from this one preparation were used for all assays.

Spectrophotometric method for determination of purines

The method of Zinn & Owens (1986) was used. In brief, the lyophilized microbial fraction (25–75 mg) alone or mixed with cellulose, NDF, starch or apparent undigested residue (75–200 mg) was placed into a 25 ml screw-cap tube and 2·5 ml 12 M-$HClO_4$ was added. The mixture was incubated in a water bath at 90–95° for 1 h or was autoclaved at 121° for 2 h. After cooling, 7·5 ml 28·5 mM-$NH_4H_2PO_4$ was added and the tube was returned to the water bath (90–95°) for 15 min. After cooling, the contents were centrifuged at 3000 ***g*** for 10 min. A portion (0·25 ml) of the supernatant fraction was added to 4·5 ml 0·2 M-$NH_4H_2PO_4$ and then the pH was adjusted to between 2 and 3 (generally to 2·5) using 10 M-NaOH. After the pH adjustment, 0·25 ml $AgNO_3$ (0·4 M) was added and the mixture was kept overnight at 5° in the dark. The contents were centrifuged at about 12 000 ***g*** for 10 min and the supernatant fraction was discarded. Care was taken not to disturb the pellet. The pellet was washed with 4·5 ml distilled water adjusted to pH 2·0 (with H_2SO_4) followed by centrifugation. The pellet was suspended in 5 ml 0·5 M-HCl, vortex-mixed thoroughly and transferred to the 90–95° water-bath for 30 min after covering the tube with a marble. The tubes were centrifuged at about 12 000 ***g*** for 10 min and absorbance of the supernatant fraction was recorded at 260 nm against 0·5 M-HCl. For studies with RNA used in the range of 25–75 mg, the absorbance was read at 260 nm after 1:10 dilution of the supernatant fraction. Without adjustment of the pH (which was generally 1·6) to between 2 and 3 before addition of the $AgNO_3$ solution, the recovery of purine bases from yeast RNA (Sigma, Steinheim, Germany) was lower (80–90 % *v.* 94–99 %), suggesting the importance of the pH-adjustment step in obtaining satisfactory recoveries. Addition of the $AgNO_3$ solution did not change the pH. Hydrolysis of the lyophilized microbial preparation alone or mixed with the matrices was also conducted using 2·5 ml 0·6 or 2·0 M-$HClO_4$, and before addition of the $AgNO_3$ reagent the pH was adjusted to 2·7. The pH values before the adjustment were 3·4 and 2·7 respectively. H_3PO_4 was used for adjustment of pH to 2·7 when 0·6 M-$HClO_4$ was used.

HPLC method for determination of adenine and guanine

The method was essentially according to Balcells *et al.* (1992). The HPLC equipment used consisted of a Merck Hitachi L-7100 HPLC pump, an L-7450 photo diode array detector, an L-7200 autosampler, a D-700 interphase module and an LC organizer. The analytical column was reverse phase C18 (LiChrospher 100, endcapped 5 μm) 250 × 4 mm i.d. (Lichrocart; Merck, Darmstadt, Germany) protected by a guard column containing the material as in the main column. The solvents used were: (A) 10 mM-$NH_4H_2PO_4$ adjusted to pH 6 with 2·86 M-NH_4OH, and (B) acetonitrile (150 ml) added to 600 ml 12·5 mM-$NH_4H_2PO_4$ and the pH adjusted to 6 with 2·86 M-NH_4OH. All solvents were filtered through a 0·45 μm filter and degassed by ultrasonication and application of vacuum. The gradient used was a 30 min linear gradient from 0 to 100 % solvent B. After 40 min, solvent A was increased to 100 % in the following 5 min and the column was equilibrated to the starting condition (100 % A) in the next 15 min before injecting the next sample. Separation was performed at room temperature (approximately 22°) and the flow rate was 0·8 ml/min. The effluent was monitored at 254 nm with a full scale deflection set at 0·2 absorbance. Guanine and adenine appeared at about 11 and 15·5 min respectively. Allopurinol and caffeine were used as internal standards and appeared at about 13·5 and 29·5 min respectively.

Recovery of purines from the microbial fraction in the presence of matrices using the HPLC method. The lyophilized microbial fraction (50 mg) alone or mixed with cellulose, NDF or starch (200 mg each) or apparent undigested residue (75 mg) was placed into a 25 ml screw-cap tube and 2·5 ml 12 M-$HClO_4$ was added. The mixture was incubated in a water bath at 90–95° for 1 h or was autoclaved at 121° for 2 h. After cooling, the pH was adjusted to between 6·6 and 6·9 with KOH (approximately 8 M) and then the volume was adjusted to 10 ml with buffer A of the HPLC system. A portion (15 μl) was injected into the HPLC after centrifugation (3000 ***g***) and filtration (45 μm).

Recovery of adenine, guanine and allopurinol, subjected to different heat treatments, using the HPLC method. The recovery was studied when the substances were present as solution in water and in perchloric acid. For the solution in water the following method was used. To 2·50 ml of a solution of adenine + guanine + allopurinol (1000 μM each) in distilled water was added 47·5 ml distilled water. This

solution was distributed into five aliquots of 10 ml each and subjected to heat treatments (90–95° for 60 min; 121° for 30 min; 121° for 60 min and 121° for 120 min). A portion (50 μl) was injected into the HPLC. For the solution in perchloric acid, the following method was used. To 0·50 ml of a solution of adenine + guanine + allopurinol (1000 μM each) in distilled water was added 2·5 ml 0·6 M-$HClO_4$. This was subjected to the heat treatments described earlier for solution in water. After the heat treatment, the pH was adjusted to between 6·6 and 6·9 with KOH (approximately 8 M) and the volume was made up to 10 ml with buffer A of the HPLC system. A portion (50 μl) was injected into the HPLC after centrifugation (3000 ***g***) and filtration (45 μm).

Standard procedure for sample hydrolysis. The procedure which resulted from this study and is suggested for purine analysis is as follows. Weigh 25–100 mg sample into a 25 ml screw-cap tube and add 2·5 ml 0·6 M-$HClO_4$ and 0·5 ml of an internal standard (3 mM-allopurinol or 8 mM-caffeine). Incubate the mixture in a water bath at 90–95° for 1 h. After cooling, add 7·5 ml buffer A of the HPLC system, adjust the pH to between 6·6 and 6·9 using concentrated KOH (approximately 8 M) and centrifuge (3000 ***g***) to remove the precipitate formed. Filter through a 0·45 μm filter and inject an appropriate volume (15–50 μl) into the HPLC.

Statistical analysis

The results are expressed as means and standard deviations. The difference between means was tested for significance using the least significant difference test after ANOVA for one-way classified data. A level of $P < 0·05$ was chosen as the minimum for significance.

Results

Table 1 shows recovery of purine bases from the rumen microbial preparation when added to matrices such as cellulose, NDF or starch, using the method of Zinn & Owens (1986). The recovery was approximately 50 %. Similar results were obtained when apparent undegraded residue (undigested hay + microbial mass) was added to the rumen microbial preparation or when *Escherichia coli* K-12 (lyophilized) was added to cellulose (Table 1). The possibility of incomplete hydrolysis of nucleic acids in the rumen microbial preparation, under the conditions of the assay (12 M-$HClO_4$ and 90–95° for 1 h), when present together with the matrices was considered. Therefore, a drastic heat treatment (121° for 2 h) was used in the next experiment. Under these conditions as well, the recovery of purine bases was very low (Table 1). Absorbance at 260 nm was higher when the microbial preparation was hydrolysed at 121° for 2 h compared with 90–95° for 1 h. Under both these heat treatment conditions, the relationship between absorbance and amount of the microbial preparation was linear (Table 1).

The recovery of purines from yeast RNA added to cellulose, NDF or starch when hydrolysed at 90–95° for 1 h using 12 M-$HClO_4$ was 92–98 % (results not shown). These results are similar to those of Zinn & Owens (1986).

On hydrolysis of the rumen microbial preparation at 90–95° for 1 h using 0·6, 2 or 12 M-$HClO_4$, the absorbance at 260 nm using the method of Zinn & Owens (1986) was statistically similar for 0·6 M and 2 M while the absorbance using 12 M was significantly higher (Tables 1 and 2).

Table 3 shows levels of adenine and guanine in the rumen microbial preparation measured using the HPLC procedure. The recoveries of adenine and guanine added to cellulose,

Table 1. Recovery of purines from a rumen microbial preparation by the method of Zinn & Owens (1986)*
(Mean values and standard deviations for three samples)

Treatment …	90–95° for 1 h			121° for 2 h		
	Absorbance at 260 nm (A260 nm)†		Recovery of added LRM (%)	Absorbance at 260 nm (A260 nm)‡		Recovery of added LRM (%)
	Mean	SD		Mean	SD	
25 mg LRM	0·215	0·006	–	0·373	0·01	–
50 mg LRM	0·456	0·005	–	0·755	0·005	–
75 mg LRM	0·675	0·005	–	1·141	0·06	–
50 mg LRM + 200 mg cellulose	0·217	0·003	52·4	0·362	0·007	52·1
50 mg LRM + 200 mg NDF	0·214	0·003	53·0	0·478	0·023	36·7
50 mg LRM + 200 mg starch	0·209	0·003	54·2	0·408	0·006	45·9
25 mg LRM + 175 mg cellulose	0·101	0·005	53·0	0·131	0·003	65·1
50 mg LRM + 150 mg cellulose	0·200	0·008	56·1	0·301	0·02	60·0
75 mg LRM + 125 mg cellulose	0·331	0·013	51·0	0·554	0·009	51·5
75 mg apparent undigested residue	0·152	0·002	–	ND		ND
75 mg apparent undigested residue + 25 mg LRM	0·268	0·004	54·0	ND		ND
75 mg apparent undigested residue + 50 mg LRM	0·388	0·011	51·8	ND		ND
50 mg *E. coli*	0·443	0·002	–	ND		ND
50 mg *E. coli* + 200 mg cellulose	0·217	0·001	49·0	ND		ND

LRM, lyophilized rumen microbes; NDF, neutral-detergent fibre; ND, not determined.
* 12 M-$HClO_4$ was used as suggested by Zinn & Owens (1986).
† A260 nm = 0·009207 × mg LRM − 0·01178 (r^2 0·99; n 3).
‡ A260 nm = 0·01537 × mg LRM − 0·01222 (r^2 0·99; n 3).

Table 2. Values for absorbance at 260 nm of samples of lyophilized rumen microbes (LRM) hydrolysed alone or in the presence of cellulose, neutral-detergent fibre (NDF) or starch, using modifications of the method of Zinn & Owens (1986)*

(Mean values and standard deviations for three samples. Values for percentage recovery are given in parentheses)

	Absorbance at 260 nm			
	0·6 M-$HClO_4$		2 M-$HClO_4$	
	Mean	SD	Mean	SD
25 mg LRM	0·165	0·002	0·166	0·001
50 mg LRM	0·331	0·004	0·330	0·003
75 mg LRM	0·469	0·005	0·465	0·002
25 mg LRM + 100 mg cellulose	0·175 (106·0)	0·003	0·172 (103·6)	0·003
50 mg LRM + 100 mg cellulose	0·323 (97·6)	0·004	0·307 (93·0)	0·004
75 mg LRM + 100 mg celluose	0·471 (100·4)	0·006	0·465 (100·0)	0·007
25 mg LRM + 200 mg cellulose	0·171 (103·6)	0·004	0·180 (108·0)	0·003
50 mg LRM + 200 mg cellulose	0·318 (96·0)	0·003	0·329 (99·7)	0·004
75 mg LRM + 200 mg celluose	0·465 (99·1)	0·003	0·470 (101·1)	0·004
25 mg LRM + 175 mg cellulose	0·165 (100·0)	0·001	0·170 (102·4)	0·002
50 mg LRM + 150 mg cellulose	0·328 (99·1)	0·002	0·318 (96·4)	0·003
75 mg LRM + 125 mg celluose	0·475 (101·2)	0·003	0·460 (98·9)	0·004
50 mg LRM + 100 mg NDF	0·318 (96·1)	0·004	0·309 (93·6)	0·004
50 mg LRM + 100 mg starch	0·322 (97·3)	0·004	0·322 (97·6)	0·003
50 mg *E. coli*	0·410	0·003	0·405	0·002
50 mg *E. coli* + 200 mg cellulose	0·400 (97·6)	0·004	0·408 (100·7)	0·003
100 mg wheat straw†	0·044		0·030	
50 mg LRM + 100 mg wheat straw†	0·348 (91·8)		0·347 (96·1)	
100 mg hay†	0·094		0·089	
50 mg LRM + 100 mg hay†	0·422 (99·1)		0·412 (97·9)	
25 mg RNA†	0·210		0·196	
50 mg RNA†	0·414		0·396	
25 mg RNA + 200 mg cellulose†	0·210 (100·0)		0·188 (95·9)	
50 mg RNA + 200 mg cellulose†	0·408 (98·6)		0·399 (100·8)	

* Hydrolysis was performed at 90–95° for 1 h using 0·6 or 2 M-$HClO_4$; the original method uses 12 M-$HClO_4$.
† Mean of two values.

starch and NDF varied from 91 to 102 % when hydrolysed using 12 M-$HClO_4$ at 90–95°C for 1 h or at 121° for 2 h. However, the absolute amount of adenine was much lower when the rumen microbial preparation was hydrolysed at 121° for 2 h, suggesting destruction of adenine (Table 3). This was confirmed by subjecting a mixture of adenine, guanine and allopurinol to different heat treatments (Table 4). The recovery of adenine was lower when it was subjected to 121° in an autoclave for 30 min or longer in the presence of $HClO_4$. In the absence of the acid, the heat treatment did not affect the recovery of adenine. The recovery of guanine was not affected by any of the heat treatments studied (Table 4). Another interesting point to note is the higher susceptibility of allopurinol to heat. The recovery of allopurinol was lower both in the absence and in the presence of $HClO_4$ (Table 4). Caffeine was also used as an internal standard. Its recovery was 95–99 % under the conditions of the heat treatments mentioned in Table 4 (results not shown).

From the results obtained using HPLC (Table 3), it is evident that hydrolysis of nucleic acids in the rumen microbial preparation was complete under the hydrolysing conditions: 12 M-$HClO_4$ at 90–95° for 1 h. The results obtained using Zinn & Owens' (1986) method indicate complete hydrolysis on using 0·6 M-$HClO_4$ (Tables 1 and 2). In another experiment, the hydrolysis was conducted using 0·6, 0·8, 1 or 2 M-$HClO_4$ at 90–95° for 1 h. Adenine and guanine levels (n 3) of 2·50 (SD 0·03) μmol and 3·11 (SD 0·04) μmol in 50 mg of the rumen microbial preparation were observed when 0·6 M-$HClO_4$ was used for the hydrolysis using the standard method (see p. 109). These values did not differ statistically when the concentration of $HClO_4$ used was 0·6 M or higher (results not shown).

Discussion

The method of Zinn & Owens (1986) based on oxidative hydrolysis of nucleic acids and then precipitation of the released bases with $AgNO_3$ at a pH between 2 and 3 offers a convenient method for quantification of purines in microbial preparations. However, the presence of undigested feed produces errors in the determination of purines (Table 1). The HPLC studies showed that the lower recovery of purines in the presence of various matrices using the method of Zinn & Owens (1986) was not due to incomplete hydrolysis of nucleic acids. The method of Zinn & Owens (1986) uses 12 M-$HClO_4$ at 90–95° for 1 h for the hydrolysis

Table 3. Recovery of purines from a lyophilized rumen microbe (LRM) preparation in the presence of different matrices using an HPLC method*
(Mean values and standard deviations for three samples)

Treatment …	90–95° for 1 h						121° for 2 h					
	Adenine (μmol)		Guanine (μmol)		Recovery of adenine (%)	Recovery of guanine (%)	Adenine (μmol)		Guanine (μmol)		Recovery of adenine (%)	Recovery of guanine (%)
	Mean	SD	Mean	SD			Mean	SD	Mean	SD		
50 mg LRM	2·35	0·03	2·95	0·02	–	–	1·65	0·02	2·84	0·04	–	–
50 mg LRM + 200 mg cellulose	2·25	0·04	2·85	0·03	95·7	96·6	1·55	0·01	2·90	0·03	93·9	102·1
50 mg LRM + 200 mg NDF	2·40	0·05	2·80	0·04	102·1	94·9	1·50	0·02	2·79	0·02	90·9	98·2
50 mg LRM + 200 mg starch	2·27	0·03	2·90	0·03	96·6	98·3	1·53	0·02	2·80	0·04	92·7	98·6
75 mg apparent undigested residue	1·01	0·04	1·15	0·01	–	–	ND	–	ND	–	–	–
50 mg LRM + 75 mg apparent undigested residue	3·30	0·02	4·05	0·05	97·4	98·3	ND	–	ND	–	–	–

ND, not determined; NDF, neutral-detergent fibre.
* For details of procedures, see pp. 107–109.

although the hydrolysis can be complete at much lower levels of $HClO_4$, as was evident from the recovery studies using HPLC. The higher absorbance at 260 nm on hydrolysis of the lyophilized microbial preparation alone at 121° for 2 h compared with 90–95° for 1 h when 12 M-$HClO_4$ was used (Table 1), and the higher absorbance on using 12 M-$HClO_4$ compared with 0·6 M or 2 M-$HClO_4$ at 90–95° for 1 h (Tables 1 and 2) suggest that the method of Zinn & Owens (1986) is not specific for purines and that substances other than purines are measured at 260 nm. The lower absorbance at 260 nm observed on hydrolysis of the lyophilized microbial preparation in the presence of matrices appears to be due to binding of substances other than purines (released from the lyophilized rumen microbial preparation on hydrolysis) to matrices, since the recovery of purines from yeast RNA added to matrices was satisfactory. These interfering substances are removed during the centrifugation step along with the matrices before purines are precipitated using $AgNO_3$. Another observation which supports the hypothesis that matrices remove substances other than purines was that the 260 nm : 280 nm values for the lyophilized rumen microbial preparation using the method of Zinn & Owens (1986) were 1·45 (SD 0·01, *n* 3) and 2·03 (SD 0·02, *n* 6) in the absence and presence of cellulose or NDF (values for cellulose and NDF were statistically similar) respectively. Similar results were obtained for *E. coli* (1·62 *v.* 1·98 respectively). The values observed in the presence of cellulose or NDF were higher than in its absence, and moreover the values in the presence of matrices were closer to that obtained using yeast RNA (260 nm : 280 nm for yeast RNA was 1·94 (SD 0·12, *n* 4). If the matrices bind to interfering substances, then the recovery of purines from the lyophilized microbial preparation added to matrices should be higher when the hydrolysis is performed using 0·6 M or 2 M-$HClO_4$, as the extent of release of interfering substances, if any, is expected to be lower under these conditions compared with at 12 M-$HClO_4$ (Tables 1 and 2). This was found to be the case; the recoveries using 0·6 M or 2 M-$HClO_4$ were of the order of 92–108 % (Table 2). The interference due to the presence of matrices along with microbes can be eliminated by using mild hydrolysis conditions (0·6 or 2 M-$HClO_4$ at 90–95° for 1 h).

Allopurinol and caffeine are generally used as internal standards in the HPLC method. Allopurinol is sensitive to heat. Its destruction at 121° was higher in the presence of the acid than in its absence (Table 4). Similarly, adenine is sensitive to heat at 121°. At 90–95°, both adenine and allopurinol are stable (Tables 3 and 4). On the other hand, guanine and caffeine are stable even at 121°. As the hydrolysis is complete when the sample is hydrolysed at 90–95° using 0·6 M-$HClO_4$, allopurinol or caffeine can be used as an internal standard. In another study (G Getachew, HPS Makkar and K Becker, unpublished results), it was found that caffeine should not be used as an internal standard in studies where tannin-rich feeds are incubated in *in vitro* systems or fed to livestock, as the recovery of caffeine was significantly lower due to binding with tannins.

Table 4. Recovery of adenine, guanine and allopurinol, subjected to different heat treatments, using an HPLC method*
(Mean values and standard deviations for three samples)

Treatment	Solution in water (μmol measured/10 ml)						Solution in perchloric acid (μmol measured/10 ml)					
	Adenine		Guanine		Allopurinol		Adenine		Guanine		Allopurinol	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control (no heating)	0·498	0·006	0·508	0·003	0·494[a]	0·004	0·505[a]	0·003	0·510	0·005	0·496[a]	0·006
90–95° for 1 h	0·495	0·003	0·510	0·007	0·484[a]	0·007	0·502[a]	0·005	0·511	0·007	0·482[a]	0·006
121° for 30 min	0·485	0·009	0·500	0·004	0·403[b]	0·012	0·482[b]	0·003	0·502	0·002	0·353[b]	0·005
121° for 60 min	0·490	0·010	0·505	0·009	0·272[c]	0·003	0·470[c]	0·002	0·500	0·003	0·254[c]	0·004
121° for 120 min	0·499	0·011	0·510	0·010	0·260[d]	0·003	0·433[d]	0·004	0·505	0·010	0·150[d]	0·002

[a,b,c,d] Mean values within a column with unlike superscript letters were significantly different, $P < 0·05$.
* For details of procedures, see pp. 107–109.

Before using a Merck column for determination of adenine and guanine, we used a reverse phase Bondapack C18 column (10 μm, 250 × 46 mm, Crom, Herrenberg, Germany). Using this column, the retention times of guanine, allopurinol and adenine were 6·8, 7·6 and 10·4 min respectively. On hydrolysis of cellulose, starch or NDF, a peak appeared whose retention time was quite near (10 min) to that of adenine and therefore interfered with its determination. The absorption maxima of this peak from all the three matrices, cellulose, starch and NDF, were 280 nm and 230 nm suggesting the same nature of the interfering substance. However, efforts were not made to identify this component. The use of the Bondapack column was discontinued.

Using the standard procedure for hydrolysis (see p. 109), adenine and guanine values (*n* 15) in the lyophilized microbial preparation were found to be 4·98 (SD 0·14) and 6·04 (SD 0·24) μmol/100 mg microbes respectively, or 11·02 (SD 0·09) μmol adenine + guanine/100 mg microbes, giving 1·43 (SD 0·02) μmol purines/mg N (the N content of the microbial preparation was 77 g/kg). This value is in accordance with those reported by Pérez *et al.* (1996). They reported purine bases: N (μmol/mg N) values for bacterial samples to be from 1·21 to 1·70 depending on the supplement used. For bacterial samples isolated from the liquid or particulate rumen material, these values were 1·9 and 1·6 respectively (Pérez *et al.* 1997). Similarly, 4·98 μmol adenine + 6·04 μmol guanine/100 mg microbes observed in the present study translates to 0·35 + 0·42 mg N respectively/100 mg microbes, giving a value of 0·77 mg purine N/100 mg microbes or a purine N : microbial N value of 0·1, which is also close to the values (0·078 to 0·089) reported by Calsamiglia *et al.* (1996) for bacterial fractions.

In conclusion, the method of Zinn & Owens (1986) was not specific for purines. Also, the presence of matrices such as cellulose, starch, NDF or undigested feed interfered with this method. The complete hydrolysis of nucleic acids was achieved at 90–95° for 1 h using 0·6 M-$HClO_4$, and therefore the use of higher concentrations of the acid can be avoided. This will make the hydrolysis procedure safer and cheaper. Both caffeine and allopurinol, commonly used as internal standards, are stable under the hydrolysis conditions (90–95° for 1 h, 0·6 M-$HClO_4$). Caffeine should not be used as an internal standard in studies where tannin-rich feeds are used as a substrate. The reverse phase Bondapack C18 column (10 μm, 250 × 46 mm) is not appropriate for quantification of adenine by the HPLC procedure outlined in the present study. The HPLC procedure reported is simple and specific. The method of Zinn & Owens (1986) has some advantages over HPLC. It is simple, cheaper and does not require sophisticated equipment. It is suggested that 0·6 M instead of 12 M-$HClO_4$ should be used for the hydrolysis in this procedure as well; 2 M-$HClO_4$ can also be used instead of 12 M (Table 2). The advantage of using 2 M-$HClO_4$ is that the pH adjustment step (to between 2 and 3) before addition of the $AgNO_3$ solution can be avoided, as a pH of 2·7 was found for samples investigated in this study. This study, in common with previous ones, has not investigated the specificity of the Zinn & Owens (1986) method in depth. It is likely that substances other than purines are also measured by the method even when 0·6 M-$HClO_4$ is used. However, absence of interference by matrices and a linear relationship between the amount of the rumen microbial preparation and the absorbances at 260 nm suggest that even in the event of absence of absolute specificity, the absorbance values or the absorbance values after conversion into yeast RNA equivalent can be taken as an index of microbial mass.

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