DETERMINATION OF PURINE AND PYRIMIDINE BASES IN RUMEN MICRO-ORGANISMS BY REVERSED PHASE HPLC AFTER HYDROLYTIC DIGESTION UNDER PRESSURE

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Summary

A rapid and accurate method is described for the determination of nucleo-bases in rumen micro-organisms. A procedure to satisfactorily hydrolyse the micro-organisms involving reaction with a mixture of readily volatile organic acids (acetic and formic acids) under high pressure, is proposed, and optimal conditions for an analytical procedure with reversed phase HPLC is described. The following nucleobases contents (mmol/kg DM) of rumen micro-organisms were found: Adenine (Ade), 82.62; Guanine (Gua), 61.34; Cytosine (Cyt), 84.61; Thymine (Thy), 35.74; Uracil (Ura), 68.62; Hypoxanthine (Hxn), 13.06; Xanthine (Xn), 8.35. Total purine-N content (g/kg N) of rumen micro-organisms were 99.60. The nucleic acid N content (g/kg N) of microbial isolates were: RNA-N, 109.9; DNA-N, 50.9.

(**Key Words**: Nucleobases, Purine-N, High Pressure Hydrolysis, HPLC)

Introduction

Nucleic acids and their bases have been investigated as a potential marker which could serve to measure microbial protein synthesis in the rumen (Koenig et al., 1980; Smith and McAllan, 1970). The use of the excretion of allantoin in urine for estimating microbial protein synthesis in the rumen (Rys et al., 1975) and is based on the relationship between microbial purine bases and urinary allantoin excretion.

While a considerable amount of work has been conducted in this area, the progress has probably been limited due to the laborious and relatively inaccurate and unspecified quantitative methods for determining the polynucleic acids.

The development of high-performance liquid chromatography (HPLC) has facilitated the isolation and quantification of the nucleic acid constituents in rumen micro-organisms (Koenig et al., 1980). The HPLC-analyses are accomplished with high sensitivity, resolution and precision. Thus the extraction procedures, which are necessary prior to chromatography, must be rapid and reliable with a minimum loss of compounds during the extraction process.

The analytical method described in this report enables rapid and accurate measurement of Ade, Gua, Cyt, Thy, Ura, Hxn and Xn in rumen micro-organisms using reversed phase HPLC after hydrolytic digestion under pressure.

Materials and Methods

Sample preparation

Rumen micro-organisms

Four "white alpine sheep", 3-6 years old, and weighing 76-81 kg, were fitted permanent cannulas in the rumen and abomasum four months before the experiment. The hay ration, containing 59 g crude protein (N × 6.25)/kg was offered in equal portions (370 g DM) at 8 h intervals (7:00, 15:00 and 23:00 hours) together with a mineral-vitamin mixture. The rumen fluid was taken four times daily (9:00, 11:00, 17:00 and 19:00 hours) from each sheep and strained through double layers of gauze. The strained rumen fluid was centrifuged at 19500 × g for 5 min. Supernatants were discarded, cells obtained were washed with 0.9% (w/v) NaCl solution and centrifuged as before. The sediments were immediately frozen at −20 ℃ and freeze-dried.

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Received June 20, 1990
Accepted March 4, 1991

Hydrolysis

Each sample weighing about 500 mg of rumen micro-organisms was inserted in a 25 ml teflon vessel and mixed with 10 ml Trifluoro acetic acid-Formic acid mixture (1:1). The vessel was put in the pressure-digestion-system and the system was closed with torque of 12.5 Nm (Newton meter) and heated to 240 ℃ in a drying oven. After 90 min. the System was immediately cooled with water and opened carefully. The sample was evaporated to dryness in a rotary evaporator, and diluted with double distilled (deionized) water. The sample was then filtered through a 0.20 μm Milipore filter disk.

Recovery experiments were carried out by adding known amounts of standard to a sample. These solutions were evaporated according to the procedure outlined above.

Instrumentation

The HPLC system consisted of a programmable UV detector (UVIS 203; Linear Inst.), a serial double piston pump (Model 2200, Bischoff) and an integrator (CR-6A; Gynkotek). A Reodyne model 7125 injector was used and fitted with a 20 μl loop. The column pressures ranges between 17.5-18.4 mpa.

Pressure-digestion-system DAB II (Forschungsinstitut Berghof GmbH, Abt. Labortechnik, D-7400 Tübingen 1, FRG).

Separation procedures

The separation was performed on Hypersil ODS, 3μm (250 × 4.6 mm I.D.) and coupled with a precolumn (Hypersil ODS 5 μm, 20 × 4.6 mm I.D.). Potassium dihydrogen phosphate (0.01 M., adjusted to pH 6.25 for adenine, cytosine, thymine, uracil and xanthine to pH 3.45 for guanine and hypoxanthine, respectively) mixed with methanol (90:10) was used as the mobile phase, and the flow rate was set at 0.8 ml/min. and maintained at ambient temperature. The eluating peaks were detected at 255 nm and 0.06 a.u.f.s.

Peak identification

With cell extracts, initial peak identification was made on the basis of retention times. The standard addition method was also used to determine peak identities. A known quantity of a standard compound was added to the cell extract and a quantitative increase in the area of a specified peak was taken as further identification of the peak. Standard solutions were run daily before and after each sample injection to monitor reproducibility of retention times.

Results and Discussion

There was a highly linear relationship between peak areas and concentration of compounds in standard solution ($r > 0.99$). The separation of seven nucleobases (in order of elution) cytosine, uracil, guanine + hypoxanthine, xanthine, thymine and adenine is shown in figure 1a. Because under conditions used above (pH = 6.25) guanine and

TABLE 1. THE MEAN COMPOSITION OF NUCLEOBASES WITH THEIR STANDARD ERRORS OF RUMEN MICRO ORGANISMS BETWEEN SAMPLES OF THREE PERIODS

Moisture (g/kg DM)	56.5	Nitrogen (g/kg DM)	93.10
RNA N (g/kg N)*	109.90	DNA-N (g/kg N)*	50.9
Nucleobases (mmol/kg DM)			
Adenine (Ade)	82.62 ± 2.81	Uracil (Ura)	68.62 ± 2.38
Guanine (Gua)	61.34 ± 3.44	Hypoxanthine (Hxn)	13.06 ± 1.18
Cytosine (Cyt)	84.61 ± 4.34	Xanthine (Xn)	8.35 ± 1.25
Thymine (Thy)	35.74 ± 1.16		
Total purine-N (g/kg N)[a]	99.60		

* Estimated by computer programm (Herbel and Montag, 1987), depending on the purine and pyrimidine base composition.

Nitrogen content of nucleic acid : RNA, 16.34%; DNA, 17%.

[a] Nitrogen content of purine bases (g/g) : Ade, 0.415; Gua, 0.371; Hxn, 0.412; Xn, 0.368.

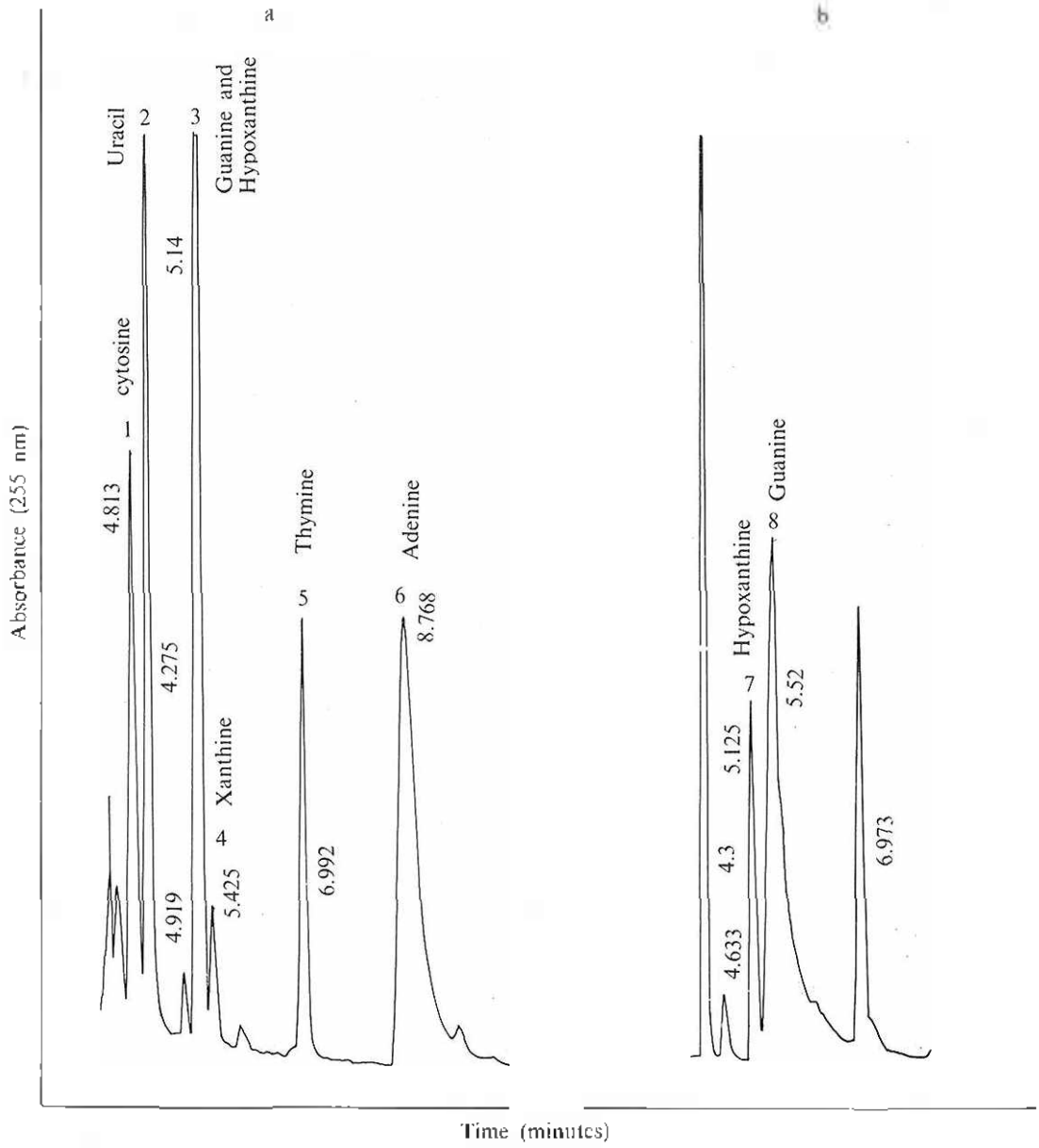

Figure 1. Separation of nucleobases in rumen micro-organisms.
1) Cytosine 2) Uracil 3) Guanine and Hypoxanthine 4) Xanthine 5) Thymine 6) Adenine 7) Hypoxanthine 8) Guanine. Mobile phase at pH 6.25 (a) and 3.45 (b). Extraction and chromatographic conditions are described in the text.

hypoxanthine are not resolved, the resolution was achived using changed pH 3.45 (figure 1b).

Both recovery and peak shapes of compounds were reproducible, and remained constant for sample sizes up to 200 nmol. Variations in retention times over a month' time averaged 2.45%.

The percentage of recovery and the coefficient of variation between hydrolysis (n = 9) for Ade, Gua, Cyt, Thy, Ura, Hxn and Xn were calculated to average 98.41%, 98.00%, 95.72%, 98.34%, 97.96%, 99.00% and 101. 75%; 5.89%, 9.71%, 8.88%, 5.62%, 6.01%, 15.65% and 25.93%, respectively.

The content of purine and pyrimidine bases

in rumen micro-organisms are listed in table 1.

Our estimates of both RNA-N and DNA-N in total N agree well with other estimates of Storm and Ørskov (1983) and survey of literature (Storm, 1982).

Total purine-N contents represented 62% of the total nucleic acid nitrogen in rumen micro-organisms.

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