Acta Univ. Sapientiae, Alimentaria, **1** (2008) 99–108

Rapid method for the determination of diaminopimelic acid using ion exchange chromatography

J. Csapó[1,2]
email: csapo.janos@ke.hu

Cs. Albert[2]
email:
albertcsilla@sapientia.siculorum.ro

G. Pohn[1]
email: pohn.gabriella@ke.hu

Zs. Csapó-Kiss[1]
email: csapo.janosne@ke.hu

[1]University of Kaposvár,
Faculty of Animal Science,
Guba S. u. 40, 7400 Kaposvár, Hungary;

[2]Sapientia–Hungarian University of Transylvania,
Csíkszereda Campus, RO-530104, Libertatii 1., Miercurea-Ciuc

Abstract. A new method for the determination diaminopimelic acid (DAPA) from rumen fluid was developed. The concentration of DAPA is used as an indicator for the estimation of protein content of bacterial origin. Due to the performic acid oxidation preceding hydrolysis of proteins, the neighbouring amino acids do not interfere in the determination of DAPA. As a result, even trace concentrations of DAPA may be accurately determined. Since, following the performic acid oxidation the sample does not contain methionine, the buffers developed for rapid determination of methionine may be used to advantage. As a result of this, DAPA may be determined by ion exchange column chromatography in ca. 18 minutes.

Key words and phrases: diaminopimelic acid, DAPA, fast method, oxidation with performic acid, bacterial protein

Following the development of the analytical method, it was applied to the determination of DAPA in the rumen fluid and the bacterial proteins prepared from the rumen fluid of cattle, goats and sheep. Based on the results, a method for evaluation of protein content of bacterial origin, based on the DAPA content, was derived.

1 Intoduction

In the case of ruminants, a large proportion of the protein content of feeds decomposes in the rumen. Microorganisms of the rumen use the ammonia derived from decomposition to build up new proteins. As a consequence, a sizeable proportion of the protein content of feeds is converted into microbial protein. This conversion may be advantageous if starting from low grade proteins or from NPN materials, because the synthesised bacterial proteins have higher biological value. In contrast to this, in many cases the decomposition of feed proteins of high biological value in the rumen is undesirable. In the future it will become more important to be able to ascertain the proportion of the feed proteins which is decomposed in the rumen. It will also be desirable to estimate the proportion of proteins arriving into the duodenum which originate from the feed and from bacteria of the rumen.

In recent years, several methods were developed for estimation of the proportion of microbial origin nitrogenous materials which enters the small intestine. The determination of nucleic acids, and the tracing of B_{12} and S^{35} isotopes have been proposed as methods for evaluation of the proportion of nitrogenous compounds of microbial origin. Stern and Hoower [12] compiled a critical review of these methods. Schleifer and Kandler [11] have shown that DAPA and D-alanine may be found only in the peptidoglycan of the cell walls.

Both of the above mentioned compounds proved to be adequate for the marking and quantitative determination of the protein of bacterial origin. Czerkawski [2] estimated the proportion of proteins of bacterial origin by measuring the DAPA content of protein and the determination of 2-amino-ethylphosphonic acid was used for the calculation of protozoa nitrogen. Garrett et al. [4] reported that D-alanine was a better indicator of bacterial-origin protein than was DAPA. They attributed this difference in accuracy to the fact that the DAPA position in the peptidoglycan of the cell wall may often be occupied by lysine or ornithine [11]. Garrett et al. [5] also reported that prior to analysis of DAPA, it was necessary to pretreat samples with performic acid to convert methionine to methionine sulfone. Ibrahim et al. [9] reported that the peaks for both DAPA and methionine occurred at 134 minutes while

the peak for methionine sulfone occurred at 50 minutes.

Besides those described above, other methods have been developed for the determination of DAPA. Hutton et al. [8] used an automatic amino acid analyser with photometric detection at 420 nm, Czerkawski [2] used acid ninhydrin and Pongor and Baintner [10] combined a video densitometer with thin layer ion-exchange chromatography for the determination of DAPA. Edols [3] used an automatic amino acid analyser combined with a dual column technique for the determination of DAPA. Following the optimisation of the composition of buffers, it was possible to obtain a sharp and well defined DAPA peak which was located between the methionine and isoleucine peaks, and was distinctly separated from these. This latter method ensures a good separation of DAPA as long as it's concentration is of the same order of magnitude as that of the amino acids. Another requirement is that the concentration of the two amino acids that show chromatographic peaks adjacent to that of DAPA, methionine and isoleucine, should not exceed more then tenfold the concentration level of DAPA. Should the concentration of the above mentioned amino acids exceed the specified limit, the DAPA chromatographic peak will appear as a shoulder on the methionine and isoleucine peaks. Such a situation makes uncertain or impossible the evaluation of the chromatogram. In order to avoid the problem described above, an improved method [1] for the determination of DAPA was developed. The method is based on oxidation with performic acid preceding the hydrolysis of proteins. During the oxidation process, methionine is converted into methionine-sulfone. During the ion-exchange column chromatographic separation, methionine-sulfone elutes between aspartic acid and threonine, making available the space between valine and isoleucine for the elution of DAPA.

Following oxidation with performic acid, the sample does not contain free methionine and by a judicious change in the composition of buffers, one may achieve a situation in which the DAPA peak appears on the chromatogram in the place that is normally reserved for methionine. Since the ion-exchange column chromatographic properties of DAPA are very similar to those of methionine, the methods developed for the determination of methionine content of foods and feeds were used as the basis for developing a rapid method for the determination of DAPA in samples of biological origin.

2 Material and methods

Materials tested The DAPA contents of rumen fluid and rumen bacteria (the latter being separated by centrifugation) were determined on samples from three Holstein-Friesian cows fitted with fistulae, 12 Hungarian improved white goats and 9 Hungarian combing merino ewes. The rumen contents of the goats and ewes were taken from the rumen immediately after the animals were slaughtered at the experimental slaughter house. During the experimental period, the cows consumed maize silage, good quality lucerne and meadow hay ad libitum, and about 0.5–1.0 kg/cow/day of a mixture of farm grains. Before slaughter, the goats consumed wheat-, barley-, pea- and vetch straw and good quality maize silage ad libitum, occasionally supplemented with fodder cabbage or meadow and leguminous hay. The sheep were on pasture and also consumed about 0.4 kg/ewe/day of a mixture of farm grains and plus wheat and barley straw ad libitum.

The traditional method [3] was compared with the rapid, new method involving oxidation with performic acid [1]. Edols [3] used a TSM amino acid analyser equipped with 8% cross linked cation exchanged resin. Two columns were used: column A for the basic and column B for the acidic and neutral amino acids, with resin bed sizes of 9.5×0.4 cm I.D. and 23.5×0.5 cm I.D., respectively. Three different buffers were used. An aliquot (50 μl or 100 μl) of the hydrolysate sample was applied to both columns. Column A was run first with buffer 1 (pH = 5.25; sodium ion concentration = 0.405 M) for 36 min. Fractionation on column B was then effected for 24 min with buffer 2 (3.25 pH and 0.2 M) and 24 min with buffer 3 (4.10 pH and 0.2 M). Both columns were run at 60 °C with a flow rate of 30 ml/h. With each sample, 50 nmol of norleucine were used as an internal standard.

The rumen fluid and the rumen bacteria were prepared for analysis by filtering through three layers of gauze in order to eliminate the larger feed particles from the rumen fluid. Separation from the protozoa was achieved by centrifugation for 20 minutes at 500 g. The DAPA content of the resulting fluid was determined. The rumen bacteria were separated from the same fluid by centrifugation for 30 minutes at 15.000 g. After discarding the supernatant of the centrifuged fluid, the residue was washed thrice with acetone, and thrice with distilled water. The remaining bacterial mass was dried at 40 °C.

Hydrolysis and processing of the hydrolysate Rumen fluid, 0.1 ml, was pipetted into a Pyrex hydrolysis tube with I.D. 8 mm. The fluid was brought to dryness in a lyophilising installation. The residue left after lyophilization

of rumen fluid and a separate sample of 10 mg of air dried rumen bacteria were oxidised with performic acid, according to the method described by Hirs [6]). Following oxidation, the tubes were dried in the lyophilising installation at −55 °C. The residue was subjected to hydrolysis for 24 hours at 110 °C in the presence of 6 M HCl. The remaining HCl was eliminated by lyophilization. The residue was dissolved in a buffer with pH = 2.2 and $[Na^+] = 0.2$, the solution was filtered and DAPA was determined on the material after the necessary dilution.

Determination of DAPA Following the oxidation with performic acid, the DAPA content of the hydrolysate was determined with an LKB 4101 Amino Acid Analyser. The analysis conditions were:

Ion exchange column:	length	10 cm	
	diameter	6 mm	
	temperature	55 °C	
Ion exchange resin	CHROMEX UA-8		
Buffer A:	pH = 3.28	0.2 M $[Na^+]$	5 min
Buffer B:	pH = 4.25	0.2 M $[Na^+]$	20 min
NaOH:		0.4 M	3 min
Equilibration:	Buffer A	10 min	

The flow rates of the buffers were 50 ml/h, and the flow rate of ninhydrin was 25 ml/h. The column pressure did not exceed 3.5×10^6 Pa for buffers and the ninhydrin pressure was 5×10^5 Pa after the column. The other parameters were those recommended by the manufacturer of the amino acid analyser for amino acid analysis.

Statistical analysis Twenty seven samples (two from each of three fistulated cows, 12 from goats and nine from sheep) of rumen fluid and corresponding samples of rumen bacteria were analysed for DAPA contents. Each of the 54 samples were analysed by the rapid method, by the traditional method of Edols [3] without oxidation and by the traditional method with oxidation [1]. Preliminary analysis of the data showed that variation among samples within a cow was as great as that among cows. Therefore, the cow samples were treated as six samples.

The data were subjected to analysis of variance within main effects of species (cow, goat, sheep), type of sample (fluid, bacteria), and method of analysis

(rapid, traditional and traditional-oxidation) in the statistical model. All interactions were also in the model.

3 Results

In *Fig. 1,* a typical chromatogram obtained by adding 25 nmol of a DAPA standard is shown. This chromatogram was developed using the experimental conditions described above. The peaks of some acidic and neutral amino acids overlap in the initial part of the chromatogram. However, after valine, the DAPA peak appears well separated and distinct from both valine and isoleucine. In the chromatographic conditions used, the chromatogram may be also used for the evaluation of valine, lysine and histidine. If the purpose of the analysis is only the determination of DAPA, then after 18 minutes, following the appearance of the DAPA peak, the residual amino acids left in the column may be washed away with 0.4 M sodium hydroxide. After subsequent equilibration with Buffer A, the analysis of new samples may be started.

Figure 1: Amino acid composition of rumen fluid + 25 nmol DAPA

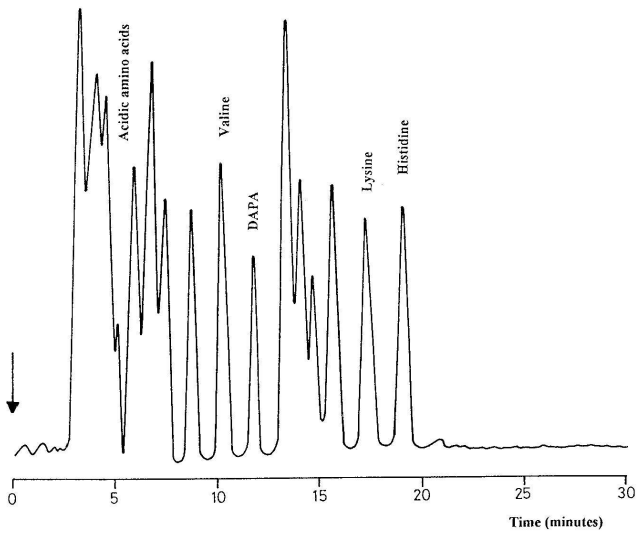
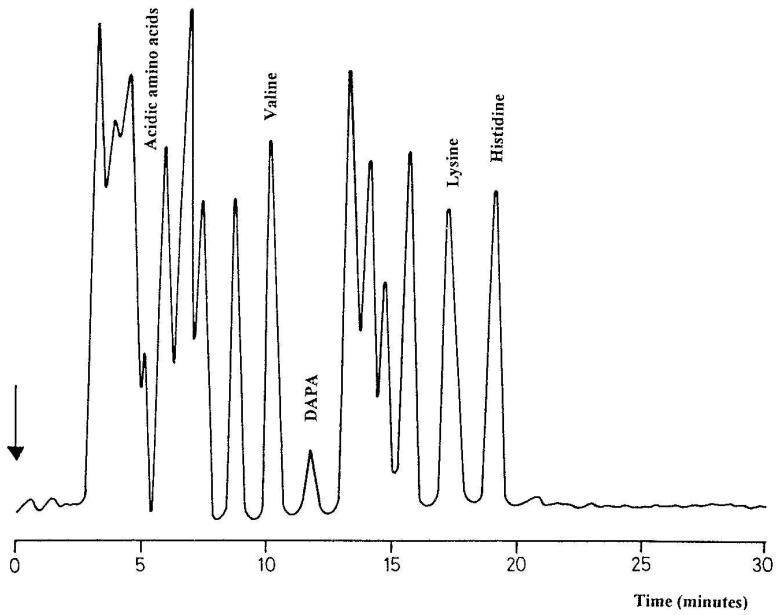


Fig. 2 shows a typical chromatogram of the determination of DAPA in rumen fluid. Comparing this to the previous chromatogram, where a DAPA standard was added to the sample, it may be concluded that low concentrations of DAPA may be quantified adequately. High concomitant concentrations of valine and isoleucine do not interfere with the determination.

Figure 2: Amino acid composition of rumen fluid



The analysis of variance revealed that the only significant effect ($P < 0.01$) was the difference between DAPA contents of rumen fluid and rumen bacteria. Since there were no significant interactions, the means can be pooled across species and/or analytical methods.

Means and standard errors for DAPA contents of rumen bacteria are shown in *Table 1.* Means, both within a species and pooled across species, clearly show that the three analytical methods were equivalent. The variance among samples within species were lowest when the rapid method was used. However, the test for homogeneity of variances did not indicate ($0.10 < P_{\chi^2} < 0.20$) lack of homogeneity. The small apparent improvement of precision could be

attributed to oxidation and consequent elimination of methionine. The overall average of 0.711 g DAPA/100 g protein provided a basis for estimating bacterial protein. The factor (100 / 0711 = 140.6) can be used to convert g DAPA/100 g protein in unknown samples to g bacterial protein/100 g protein.

Table 1: Means ± standard errors for DAPA contents (g AA/100 g protein) of rumen bacteria samples

		Analytical methods			
Species	**No.**	**Rapid**	**Trad-ox**	**Trad**	**Pooled**
Cows	6	0.697	0.712	0.710	0.706
		± 0.018	± 0.026	± 0.048	± 0.019
Goats	12	0.708	0.709	0.712	0.710
		± 0.014	± 0.021	± 0.026	± 0.012
Sheep	9	0.724	0.709	0.717	0.717
		± 0.023	± 0.023	± 0.031	± 0.015
Pooled	27	0.711	0.710	0.713	0.711
		± 0.011	± 0.013	± 0.019	± 0.008

Trad = traditional or Edols [3]
Trad-ox = traditional following oxidation with performic acid

DAPA contents of the corresponding rumen fluid samples are shown in *Table 2.* Since neither species nor analytical method influenced DAPA content of samples, the pooled value of 0.604 g DAPA/100 g protein, when multiplied by 140.6, yields a value of 84.9 g bacterial protein/100 g protein in the rumen fluid.

It is well known from university text-books that, if the diet of the ruminant animal changes, the quality and the quantity of the bacteria will also change [4, 7]. Hungate [7] stated that, if cattle are fed with only forage diets, the gram-negative bacteria will be predominant in the rumen. However if cattle consume more concentrate, the proportion of gram-positive bacteria will increase. The different bacteria have different peptidoglycans in the cell wall, which would lead to higher or lower DAPA concentration. For example the gram-positive bacteria contain 30–70% peptidoglycan in the cell wall, and the gram-negative bacteria contain only 10%. Moreover the amino acid composition (involving DAPA) of the peptidoglycan is almost identical among gram-negative bacteria, independent of species. The comparable composition is highly variable among gram-positive bacteria [4, 7].

Table 2: Means ± standard errors for DAPA contents (g AA/100 g protein) of rumen fluid samples

		Analytical methods			
Species	**No.**	**Rapid**	**Trad-ox**	**Trad**	**Pooled**
Cows	6	0.588	0.598	0.590	0.592
		± 0.051	± 0.033	± 0.047	± 0.026
Goats	12	0.603	0.600	0.601	0.601
		± 0.030	± 0.032	± 0.034	± 0.018
Sheep	9	0.610	0.622	0.612	0.615
		± 0.015	± 0.026	± 0.029	± 0.014
Pooled	27	0.601	0.607	0.602	0.604
		± 0.018	± 0.018	± 0.021	± 0.011

Trad = traditional or Edols [3]
Trad-ox = traditional following oxidation with performic acid

For this reason when DAPA is used as an indicator of rumen micro flora, one must consider all factors which might change the bacterial population after the indicator is used. If the forage is changed, this may cause a change of bacterial species, and therefore peptidoglycan content, and DAPA content. The factors for calculating the bacterial protein synthesis must be determined after major changes in diet.

Diets for cattle, goats and sheep were not identical in this study, in fact, had little overlap in dietary ingredients. Diet for all three species were, however, primarily composed of forage ingredients. Since there were no significant species differences in DAPA contents of bacteria, these data indicate that a single conversion factor can be used to estimate microbial protein derived from forages.

4 Acknowledgement

The financial support of Hungarian Research Fund (OTKA T 6653) is gratefully acknowledged.

References

[1] J. Csapó, S. Gombos, Z. Henics, L.-né Tóth, Modified method of diaminopimelic acid determination in samples of biological origin, *Acta Alimentaria,* 2 (1988) 159–167.

[2] W.J. Czerkawski, Methods for determining 2-6-diaminopimelic acid and 2-aminoethylphosphonic acid in gut contents, *J. Sci. Food. Agric.,* 25 (1974) 45.

[3] R.W. Edols, Simple method for the determination of diaminopimelic acid in rumen liquor hydrolysates, *J. Chrom.,* 329 (1985) 199.

[4] J.E. Garrett, R.D. Godrich, J.C. Meiske, *Protein requirements for cattle,* Symposium. Oklahoma State University, 1982. 109.

[5] J.E. Garrett, R.D. Godrich, J.C. Meiske, Measurement and use of D-alanine as a bacterial marker, *Can. J. Anim. Sci.,* 67 (1987) 735–743.

[6] C.H.W. Hirs, The oxidation of ribonuclease with performic acid, *J.Biol. Chem.,* 219 (1956) 611.

[7] R.E. Hungate, *The rumen and its microbes,* Academic Press, New York, 1966.

[8] K. Hutton, F.J. Bailey, E.F. Annison, Measurement of the bacterial nitrogen entering the duodenum of the ruminant using diaminopimelic acid as a marker, *Br. J. Nutr.,* 25 (1971) 165.

[9] E.A. Ibrahim, J.R. Ingalls, D.B. Bragg, Separation and identification of amino acids present in rumen microorganisms, *Canadian J. Anim. Sci.,* 50 (1970) 397.

[10] S. Pongor, K. Baintner, Quantitative evaluation of ion-exchange thin-layer chromatograms by video-densitometry (IV.) Determination of diaminopimelic acid, *Acta Biochem. et Biophys. Acad. Sci. Hung.,* 15. (1980) 1.

[11] K.H. Schleifer, O. Kandler, Peptidoglycan types of bacterial cell walls and their taxonomic implications, *Bacteriol. Rev.,* 36 (1972) 407.

[12] M.D. Stern, W.H. Hoower, Methods for determining and factors affecting rumen microbial orotein synthesis, *J. Anim. Sci.,* 49 (1979) 1590.

Received: August, 2008