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Quantitative determination of the protein of bacterial origin based on D-amino acid contents

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Abstract. In recent years, several methods have been developed for determination of the proportion which is of microbial origin of the nitrogen-containing substances passed from the rumen into the abomasum or small intestine. These methods are based on analysis of components which were believed could only be of microbial origin, or which were known to be very closely related to protein of microbial origin. Assays have also been developed to use nucleic acids and adenosine triphosphate as indicators. Radioisotopes ^{35}S, ^{15}N, ^{32}P and ^{33}P have been incorporated into bacterial protein and phospholipids to estimate bacterial protein content. Duodenal amino acid composition, amino-ethylphosphonic acid (AEP), diaminopimelic acid (DAPA) and D-alanine (D-Ala) contents of the duodenum have been investigated as indicators.

Key words and phrases: D-aspartic acid, D-Asp, D-glutamic acid, D-Glu, diaminopimelic acid, DAPA, bacterial protein synthesis, protein of bacterial origin

Recently, the D-amino acid content of foodstuffs, particularly milk and milk products, has been determined and it has been observed that, in addition to D-alanine (D-Ala), D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) were also detected in products which are associated with bacterial activity. This suggested the possibility of using D-Glu and D-Asp content of bacteria extracted from the rumen of cattle and of chyme from the same cattle to estimate protein of bacterial origin. The efficiency of these indicators would need to be compared with that of an established indicator such as 2,6-diaminopimelic acid (DAPA).

DAPA, D-Asp and D-Glu content of duodenal chyme from five growing bulls and of ruminal bacteria from the same bulls was determined by means of amino acid analyser and high performance liquid chromatography. Based on linear regression, coefficients of correlation among these indicators were 0.778 and 0.703 respectively for DAPA and D-Asp and for DAPA and D-Glu in chyme. Corresponding values in ruminal bacteria were 0.758 and 0.808. The r values between the crude protein content of ruminal bacteria and the markers were: DAPA, 0.737; D-Asp, 0.725; D-Glu, 0.614. In the model experiment performed to estimate percentage of protein derived from bacteria the values determined on the basis of D-Asp and D-Glu content were approximately 8% lower than the value based on DAPA. It is recommended that, in addition to DAPA, these two amino acids be considered to be markers for estimating bacterial protein.

1 Introduction

A common characteristic of new protein evaluation systems introduced in cattle nutrition in the past decade is that protein content of diets is evaluated on the basis of quantity of amino acids absorbed in the small intestine. In addition to endogenous amino acids, which are only a small proportion, there are two substantial sources of absorbed amino acids. These are microbial protein synthesised in the rumen and dietary protein not broken down in the rumen (by-pass protein). Approximately 70% of the protein content of the diet is broken down into amino acids in the rumen, which may be utilised for synthesis of amino acids or microbial proteins, or broken down still further to provide ammonia for the construction of microbial body proteins. Ammonia not utilised for synthesis of bacterial protein is absorbed from the rumen and converted into urea via the ornithine cycle in the liver. The quantity of microbial protein produced in the rumen must be established if we are to determine the proportion of by-pass protein in the diet.

In order to define the quantity of microbial protein synthesised in the rumen, it is essential that microbial protein in duodenal chyme be separable from the

dietary by-pass protein and protein of endogenous origin. This is possible only if components characteristic solely of microbial protein can be identified in the total protein.

Recently several methods have been developed for determination of the proportion which is of microbial origin of nitrogen-containing substances passed from the rumen into the abomasum. Attempts to estimate the proportion of microbial origin nitrogen-containing substances have been based on nucleic acid, vitamin B_{12} and sulphur 35 isotope. A critical evaluation and summary of these methods was given by Stern and Hoover [19].

Czerkawski [2] was successful in estimating protozoan nitrogen by means of the measurement of 2-amino-ethylphosphonic acid (AEP) and estimating nitrogen content of bacterial origin by measuring 2,6-diaminopimelic acid (DAPA). The AEP was found to occur almost exclusively in protozoa, while DAPA occurs exclusively in peptidoglycans in the bacterial cell wall. Despite the fact that the quantity of DAPA in the cell wall is strongly dependent on the species of bacterium, the ratio of DAPA to total bacterial protein does not vary under constant dietary conditions. Therefore, in comparative experiments, DAPA can be used effectively to estimate the proportion of protein of bacterial origin found in the contents of the intestine.

Schleifer and Kandler [17] discovered that, like DAPA, D-alanine (D-Ala) also occurs only in peptidoglycans in the bacterial cell wall. This compound was also suitable for use as a marker of proteins of bacterial origin and their quantitative determination. Garrett et al. [12] succeeded in determining nitrogen of bacterial origin by measuring the quantity of D-Ala in ruminal fluid. Garrett et al. [13] performed comparative experiments using DAPA and D-Ala to investigate the precision of estimating nitrogen of bacterial origin. They established that D-Ala was a better indicator of nitrogen of bacterial origin, since the coefficient of variation resulting from the data based on D-Ala was substantially lower than that for data based on DAPA. In addition, greater accuracy was achieved in determinations based on D-Ala than in those based on DAPA. In a series of experiments the authors [7] also established that both DAPA and D-Ala are suitable for use in the estimation of the quantity of protein of bacterial origin. No difference was observed between the two substances with respect to error either in analytical method (ion exchange column chromatography for DAPA and high performance liquid chromatography for D-Ala) or in determination of protein of bacterial origin.

Edols [10] determined DAPA in a hydrolysate from the ruminal fluid by the application of a two-column method using an automated amino acid analyser. By the optimization of the composition of buffers DAPA appeared between

methionine and isoleucine in the chromatogram, well separated from these two amino acids, in the form of a sharp, easily quantifiable peak. Csapó et al. [6]. oxidized samples with performic acid prior to protein hydrolysis, as a consequence of which – the disturbance effects of neighbouring amino acids having been eliminated – DAPA, even if present only in trace quantities, could be determined accurately. Subsequently to this, exploiting the similar representation on chromatograms of methionine and DAPA, the authors developed a fast method for the determination of DAPA alone by means of ion exchange column chromatography [4].

Analysis of data in the literature suggests that solutions have been found with respect to analytical methods for DAPA, allowing even trace quantities of the compound to be determined. The investigation of D-amino acid content of foodstuffs, particularly milk and milk products [3, 8], revealed that, in addition to D-Ala, D-glutamic acid (D-Glu) and D-aspartic acid (D-Asp) could be detected in similar quantities. This was primarily true for products which were produced through bacterial activity. These findings suggested that D-Asp and D-Glu content of ruminal bacteria and duodenal chyme could provide additional markers for estimating the percentage of protein derived from bacteria.

2 Material and method

2.1 Methodology for the animal experiment.

Chyme and ruminal bacteria samples were taken from two experiments using five growing bulls (crossbred Hungarian Simmental × Holstein Friesian) weighing 480–500 kg. Each was fitted with ruminal and duodenal fistulae. The main objectives of these experiments were to determine ruminal protein degradability of various diets, and to establish the effect of various types of feed additives on ruminal degradability of proteins. After a ten-day adaptation period duodenal chyme samples were taken through the duodenal fistula and ruminal fluid was taken through the ruminal fistula on several occasions during the same experimental period; the average chyme and ruminal fluid samples were analyzed for DAPA, D-Asp and D-Glu.

2.2 Preparation of samples for chemical analysis.

Ruminal fluid was centrifuged at 3000 g to separate the feed particles from infusorians. The bacterial mass was then separated by centrifugation of the

fluid phase at 10 000 g. The bacterial mass was dried by lyophilization. Aliquot parts of the chyme samples taken from the duodenum were also lyophilised.

2.3 Chemical analysis of samples.

DAPA content was determined, by means of the procedure developed by Csapó et al. [9], using Aminochrom-II (Labor MIM, Hungary) type amino acid analyser. Protein was oxidized with performic acid followed by 24 hour hydrolysis with 6 M hydrochloric acid containing 0.1% phenol.

Prior to determination of D-Asp and D-Glu, the protein was hydrolysed with 6 M hydrochloric acid for 30 minutes at 170 °C in order to minimize the degree of racemization [5]. Separation and determination of the enantiomers was performed by means of high performance liquid chromatography in accordance with the method described by Einarsson et al. [11]. Derivatization and analysis were carried out with a MERCK-Hitachi (Darmstadt, Germany) HPLC containing the following modules: L-7250 programmable autosampler, L-7100 pump, L-7350 column thermostat, L-7480 fluorescence detector, AIA data conversion utility for D-7000 HPLC system manager.

For derivative formation, *o*-phthaldialdehyde (OPA) and 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG) were purchased from Sigma (St. Louis, Mo). Enantiomer separation was performed on a reversed phase (250 $\times$ 4.6 mm internal diameter (i.d.), 5 μm particle size, Kromasil octyl (C-8)) analytical column. In order to extend the life of the column a guard column (RP-8, Newguard, 25 $\times$ 3.2 mm i.d., 7 μm particle size, Brownlee) was connected between the injector and the analytical column and a cleaning column (C-18, 36 x 4.5 mm i.d., 20 μm particle size, Rsil) was fitted between the pump and the injector. A gradient system consisting of two components was used for enantiomer separation, the composition of this system being the following: A = in 40% methanol phosphate buffer (9.5 mM, pH = 7.05); B = acetonitrile. The rate of flow used was 1 ml per minute. The derivatives were detected with a fluorescence detector (ex.: 325 nm, em.: 420 nm).

Since, in this series of experiments, only the quantities of D-Asp and D-Glu were of interest to the authors, determination was restricted to the enantiomers of these two amino acids. By this method D-Asp and D-Glu present in very small quantities can be detected and determined alongside L-amino acid present in large quantities.

3 Results

Table 1 presents the associations between DAPA and D-Asp, DAPA and D-Glu and D-Asp and D-Glu in both chyme and ruminal bacteria samples. Analysis of the data established that there were very high correlations between DAPA and D-Asp and between DAPA and D-Glu content. In both bacterial protein and chyme the value observed between DAPA and D-Glu content of the chyme was the lowest, at 0.703, while the same correlation in ruminal bacteria was the highest, at 0.808. The highest correlation was that between D-Asp and D-Glu for chyme (r = 0.949) and for ruminal bacteria (r = 0.843).

Table 1: Linear regression parameters and correlations among DAPA, D-Asp, and D-Glu contents of chyme and ruminal bacteria[a]

Parameter	Chyme			Ruminal bacteria		
statistical characteristic	DAPA-D-Asp mg/kg	DAPA-D-Glu mg/kg	D-Asp-D-Glu mg/kg	DAPA-D-Asp mg/kg	DAPA-D-Glu mg/kg	D-Asp-D-Glu mg/kg
A	0.313	0.440	-0.168	1.121	2.686	2.346
sd	0.089	0.142	0.074	0.560	0.436	0.437
B	0.655	0.830	1.331	0.840	0.771	0.729
sd	0.094	0.148	0.078	0.186	0.145	0.120
S.D.	0.118	0.188	0.083	0.238	0.185	0.169
n	34	34	34	17	17	17
r_{xy}	**0.78**	**0.70**	**0.95**	**0.76**	**0.81**	**0.84**
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]Derived from regression equation Y=A+B×X, where Y is dependent and X is independent variable. In each column, first variable listed is Y and second is X.
A = The intercept of the value of Y when X = 0.
B = The slope of the line of the units change in Y per one unit change in X.
sd = Standard deviation of A and B.
S.D. = Standard deviation
n = number of observations
r_{xy} = correlation coefficient
P = Probability

Figure 1 illustrates the relationships of D-Glu and D-Asp to DAPA content of ruminal bacteria, and *Figure 2* shows the same relationships for chyme. *Figure 3* demonstrates regression of D-Glu on D-Asp content of the chyme.

Figure 1: Linear regressions of D-Asp and D-Glu on DAPA in ruminal bacteria

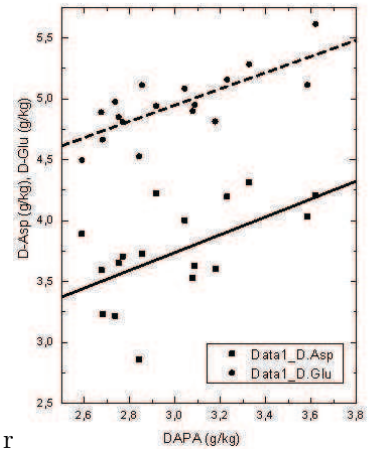

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Figure 2: Linear regressions of D-Asp and D-Glu on DAPA in chymus

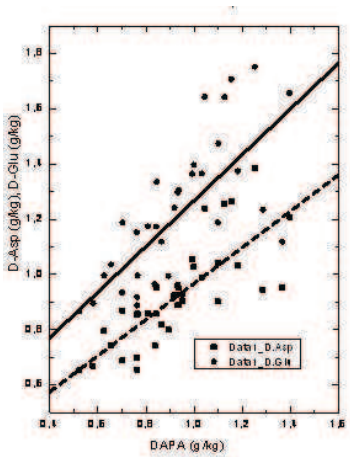


Figure 3: Linear regressions of D-Glu on D-Asp in chyme

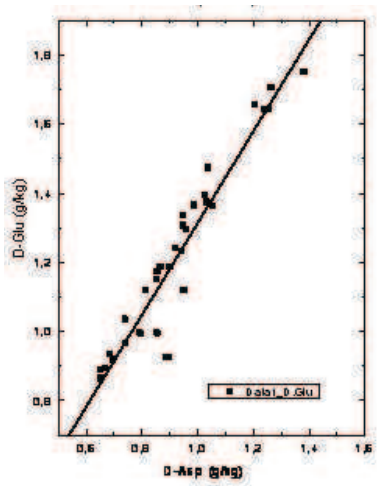


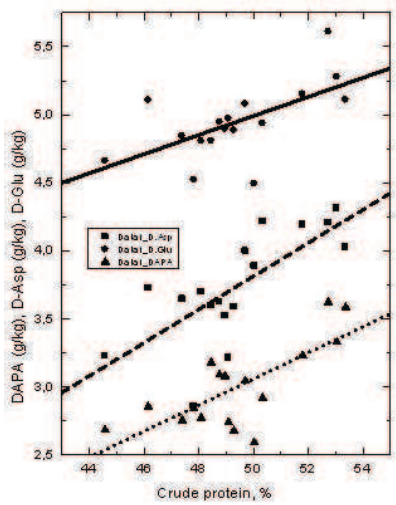
Table 2: Linear regression parameters for crude protein regressed on DAPA, D-Asp and D-Glu for ruminal bacteria and chyme[a]

	Crude protein %					
Parameter	**Chyme**			**Ruminal bacteria**		
statistical characteristic	**D-Asp mg/kg**	**D-Glu mg/kg**	**D-Glu mg/kg**	**D-Asp mg/kg**	**D-Glu mg/kg**	**D-Glu mg/kg**
A	−0.698	−1.746	1.485	1.073	1.267	1.309
sd	1.704	1.125	1.152	0.322	0.379	0.453
B	0.088	0.096	0.070	−0.006	−0.014	−0.004
sd	0.035	0.023	0.023	0.013	0.015	0.018
S.D.	0.329	0.217	0.222	0.187	0.221	0.263
n	17	17	17	34	34	34
r_{xy}	**0.73**	**0.74**	**0.61**	**−0.08**	**−0.16**	**−0.04**
P	<0.01	<0.001	<0.01	0.644	0.381	0.832

[a]Derived from regression equation Y=A+B×X. where Y is dependent and X is independent variable. In each column, first variable listed is Y and second is X.
Abbreviations: Identical as at *Table 1.*

Table 2 shows relationships between crude protein content of the ruminal bacteria and chyme and D-Asp, DAPA and D-Glu for the same samples. *Figure 4* displays the relationships of DAPA, D-Asp and D-Glu with crude protein content of the ruminal bacteria.

Figure 4: Linear regression of DAPA, D-Asp and D-Glu content on crude protein content of ruminal bacteria



It was established that, for ruminal bacteria, the closest correlation was between DAPA and crude protein content ($r = 0.737$); the r value between D-Asp and crude protein content was only very slightly lower ($r = 0.725$), while the relationship between D-Glu and crude protein content was somewhat lower ($r = 0.614$). However, the same analysis of data on chyme samples did not produce a close correlation between the markers and crude protein content. In all three examinations, linear regression analysis demonstrated a very weak negative correlation (r values varying between -0.038 and -0.155). This lack of correlation could be explained by the fact that only part of the protein present in the chyme is derived from bacteria, the rest being comprised of dietary proteins which do not undergo bacterial degradation in the rumen, plus small amounts of endogenous protein. Ruminal degradability of dietary proteins is on average 70%. However, there is a very wide range of reported values. Results from experiments performed under *in vivo* and *in vitro* con-

ditions have demonstrated very great differences among individual proteins with respect to ruminal degradability [15]. There are feedstuffs whose protein content undergoes almost total degradation in the rumen, while the ruminal degradability of other dietary proteins is only 15–20%. The great deviations are attributed to protein structure, primarily with the number of disulphide bonds present [16]. The quantity and ratio of protein fractions influence ruminal degradability [17] along with protein amino acid composition [18] and the chemical and heat treatment of protein [19, 20]. This implies that variations in the proportions of microbial protein in the chyme and non-degradable dietary protein in the rumen will be dependent on the dietary proteins. In the experiment from which the chyme and ruminal bacteria samples were taken the animals were fed diets of different ruminal degradability, which resulted in different ratios of microbial protein to by-pass dietary protein in the chyme for individual experimental periods. In the opinion of the authors, this is the fundamental reason for low associations of protein in the chyme with levels of DAPA and the two D-amino acids.

In the next step the average crude protein content and the average DAPA, D-Asp and D-Glu content were calculated from the analysis data for 17 ruminal bacteria produced by the method described in the section Material and method. The composition of the lyophilized ruminal bacteria (100% dry matter) was: 49.50% crude protein, 0.300% DAPA, 0.366% D-Asp and 0.494% D-Glu. By means of these results and with the use of crude protein content of the lyophilised bacteria, the DAPA, D-Asp and D-Glu content of the bacterial protein was calculated. The protein of the ruminal bacteria contained on average 0.606% DAPA, 0.739% D-Asp and 0.998% D-Glu. No possibility exists to compare values for D-Asp and D-Glu with literature values because, as far as the authors are aware, no other researchers have investigated these components of bacterial protein. The value of 0.606% obtained for DAPA is lower than the value of 1.0 $\pm$ 0.25% reported for bacterial protein by Orskov [21]. Estimates in the literature vary from 0.6 to 1.4%, and may be influenced by differences in the quality of diets fed to experimental livestock. Since the objective of these investigations was to evaluate new markers for bacteria, it was necessary to establish that the deviation of DAPA content from data in the literature did not influence the results obtained for D-Asp and D-Glu in this study.

After analysis of ruminal bacteria for DAPA, D-Asp, D-Glu and crude protein content, multiplying factors were calculated to enable the proportion of protein of bacterial origin in an unknown sample to be estimated on the basis of DAPA, D-Asp and D-Glu content. The multiplying factor used for DAPA was

100/0.606 = 165; for D-Asp, 100/0.739 = 135; and for D-Glu, 100/0.998 = 100. By the application of the multiplying factors the quantity of the protein of bacterial origin can be calculated very easily. In order to establish the applicability in practice of the multiplying factors calculated in this study, two experiments were performed. In the first, these multiplying factors were applied to various chyme samples. The results obtained are presented in *Table 3.* It can be seen that values estimated on the basis of DAPA content (the average of the 10 estimates being 12.774%) were on average 8% higher than the microbial protein quantities based on D-Glu (11.705%) or D-Asp (11.800%) content. The explanation for this may be that the DAPA content of ruminal bacteria was measured at a slightly lower value when the multiplying factors were calculated. When protein content determined on the basis of D-Glu was compared with that based on D-Asp, the level of concordance was obvious, and in most cases, the values obtained concurred almost exactly.

Table 3: Examples of the application of multiplying factors in the determination of protein of bacterial origin content in chyme samples

Chyme sample	Analysis results*			Protein of bacterial origin, % calculated on the basis of		
	D-Asp %	DAPA %	D-Glu %	D-Asp	DAPA	D-Glu
1.	0.08546	0.07630	0.11513	11.54	12.59	11.67
2.	0.06681	0.05814	0.08935	9.03	9.59	9.06
3.	0.12546	0.11276	0.16415	16.96	18.61	16.64
4.	0.07402	0.06560	0.10342	10.01	10.82	10.49
5.	0.06519	0.05249	0.08644	8.81	8.66	8.77
6.	0.10546	0.09933	0.13637	14.26	16.38	13.83
7.	0.08565	0.07666	0.09952	11.58	12.64	10.09
8.	0.08671	0.07041	0.11865	11.72	11.61	12.03
9.	0.08591	0.08090	0.11730	11.61	13.34	11.89
10.	0.09230	0.08189	0.12402	12.48	13.50	12.58
			Mean	11.800	12.774	11.705

*Different chyme samples were analysed for D-Asp, DAPA and D-Glu, and the protein of bacterial origin was calculated by applying multiplying factors.

After the multiplying factors were tested for chyme samples, a mean sample

was produced from 17 lyophilized ruminal bacteria samples, and the multiplying factors were also tested for ruminal bacteria. The crude protein content of this mean sample was 49.5%, its DAPA content was 0.325%, its D-Asp content was 0.364% and its D-Glu content was 0.492%. By application of the multiplying factors crude protein content values of 53.62% (0.325% × 165), 49.14% (0.364% × 135) and 49.20% (0.492 × 100) respectively were estimated.

Subsequently, the authors lyophilized beef to produce a meat meal of zero DAPA content. The D-Asp and D-Glu content was below 0.01% after hydrolysis of its protein. Racemization testing was performed during protein hydrolysis. A mixture of 1 g meat meal and 1 g bacterial sample was analyzed for DAPA, D-Asp and D-Glu content using 5 parallel analyses. This was then repeated using a mixture of 9 g meat meal and 1 g bacterial sample. The results are presented in *Table 4*. From the data given in *Table 4* it can be established that the S.D.% for the first sample, containing a higher quantity of bacterial protein, are in each case below 5; thus, the standard deviation of the results meets the requirements for a reliable analytical method.

The second sample contained only 20% of the quantity of bacterial protein contained by the first sample. S.D.% for D-Asp and D-Glu was below 5, but that calculated for DAPA slightly exceeded 5. When compared with the values calculated for bacterial origin protein content (24.76% and 4.95%), the only values to deviate significantly ($P<0.05$) were three individual values and the two mean values based on DAPA content *(Table 4).* DAPA estimates (26.58% and 5.54% protein of bacterial origin) averaged 9.5 and 13.5% respectively, higher than those for D-Asp (24.63 and 4.89%) and D-Glu (24.82 and 4.87%). DAPA estimates averaged 7.3 and 11.9%, higher than the percentages calculated (24.76 and 4.95%) for samples one and two.

Table 4: Model experiment to investigate the accuracy of determination of protein of bacterial origin

1:1 mixture of meat meal and bacterium with 24.76% calculated protein of bacterial origin						
Parallel analysis	**Analysis results**			**Protein of bacterial origin, % calculated on the basis of**		
	D-Asp %	**DAPA %**	**D-Glu %**	**D-Asp**	**DAPA**	**D-Glu**
1.	0.182	0.162	0.247	24.60	26.71	25.05
2.	0.181	0.159	0.239	24.47	26.22	24.24
3.	0.179	0.155	0.251	24.20	25.56	25.45
4.	0.184	0.164	0.241	24.87	27.04*	24.44
5.	0.185	0.166	0.246	25.01	27.37	24.94
Mean	0.1822	0.1612	0.2448	24.63	26.58	24.82
SE $\overline{X}$				0.144	0.318	0.217

9:1 mixture of meat meal and bacterium with 4,952% calculated protein of bacterial origin						
Parallel analysis	**Analysis results**			**Protein of bacterial origin, % calculated on the basis of**		
	D-Asp %	**DAPA %**	**D-Glu %**	**D-Asp**	**DAPA**	**D-Glu**
1.	0.037	0.037	0.047	5.001	6.101*	4.766
2.	0.035	0.035	0.049	4.731	5.772	4.969
3.	0.038	0.032	0.051	5.136	5.277	5.171
4.	0.034	0.031	0.046	4.596	5.112	4.664
5.	0.037	0.033	0.047	5.001	5.442	4.766
Mean	0.0362	0.0336	0.0480	4.893	5.541*	4.867
SE $\overline{X}$				0.099	0.118	0.091

SE = Standard error of mean.
*Differs from calculated protein of bacterial origin ($P<0.05$).

4 Conclusions

These investigations have provided evidence that both D-Asp and D-Glu may be used to estimate percentage of protein of bacterial origin. The results obtained using these two markers proved to be approximately 8% lower than those obtained using DAPA. This deviation was not attributable to error associated with the new markers, but rather to unreliability of determination using DAPA. The analyses performed on samples of known bacterial protein content indicate that D-Asp and D-Glu produced practically identical values for bacterial protein content, which were very close to the theoretical (calculated) values.

The preparation process for DAPA analysis is lengthy due to the treatment with performic acid required. However, without performic acid treatment, the determination of DAPA present in small quantities is unreliable, due to interference of other amino acids in concentrations which may, in some cases, be higher by several orders of magnitude. In addition to the large amount of work required for the process, determination using DAPA also requires more time and more chemical agents, and is therefore considerably more expensive. An amino acid analyser operating on the principle of ion exchange column chromatography is suitable for analysis with DAPA, but separation of the D-amino acids cannot be performed with an amino acid analyser operating on the traditional principle.

Determination using D-Asp and D-Glu can be carried out with precision by means of high performance liquid chromatography. By the application of the fast procedure developed by the authors, determination of the quantity of D-Asp and D-Glu can be performed in 25% the amount of time required for ion exchange column chromatography analysis used for DAPA.

When determining D-amino acids, racemization may occur during protein hydrolysis, which can falsify the results obtained. Due to racemization, quantities of D-Asp and D-Glu greater than those actually present may be measured and lead to overestimation of the amount of protein of bacterial origin present. Two methods are recommended for the elimination of this source of error. One is the application of a hydrolysis procedure which allows only a very low degree of racemization to take place (for example, the protein hydrolysis method developed by the authors, performed at high temperature and for a short period, i.e. 160–170 °C for 30–45 minutes; [5]. The other method is to determine, by means of the method to be applied, the D-Asp and D-Glu content of bacteria obtained from the rumen, and developing multiplying factors for use in estimating the quantity of protein of bacterial origin (this being our new method).

In the latter case, racemization occurring during protein hydrolysis is regarded as a constant error, in both calculation of multiplying factors and analysis of real samples. As a result, it exerts no substantial influence on the accuracy of the determination procedure. Thus, the use of D-Asp or D-Glu is associated with minimum error if a protein hydrolysis procedure involving a low degree of racemization is used, and if multiplying factors are determined and applied.

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