

F. M. Muller, J. G. Dijkhuis and S. Heida

*Institute for Storage and Processing of Agricultural Produce (IBVL),  
Wageningen*

On the relationship between chemical  
composition and digestibility  
in vivo of roughage 2



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## Abstract

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Treatment of ether-extracted roughage with a solution of Pronase (a proteinase from *Streptomyces griseus*) at pH 7.4, instead of hot-water extraction, in preparation of roughage samples for lignin determination did not improve the correlation between lignin content and digestibility of organic matter.

Data from comprehensive analysis of roughage and corresponding faeces from metabolic trials support results given in the authors' earlier paper (Muller et al., 1970) about the digestibility of the components.

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## 1 Introduction

In our earlier paper (Muller et al., 1970) we discussed the role of lignin as a determinant of digestibility of organic matter in vivo. We inferred that the indigestible part of the lignin from our estimates (the ash-free residue after successive extraction with ether and hot water and finally digestion in 72 % sulphuric acid) governs digestibility in vivo. This points to the need for a simple and reliable method of estimating of the indigestible lignin and suggested that a microbial proteinase active at neutrality be used in preparation of the material.

We gave a comprehensive analysis of some roughages and of corresponding faeces from cattle and sheep and calculated digestibility of the constituents.

We report now on estimates of lignin in samples of roughage from the Institute for Livestock Feeding and Nutrition Research 'Hoorn' after preparation of the roughage at a pH of 7.4 with a proteinase produced by *Streptomyces griseus* (Pronase from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England). Besides having its pH optimum near neutrality, this proteinase has the advantage of splitting most of the peptide linkages occurring in proteins. We also continued our comprehensive analysis of roughages and corresponding faeces from metabolic trials at the Department of Animal Physiology of the Agricultural University at Wageningen and calculated the digestibility of their constituents.

## 2 Materials and methods

As the samples from Hoorn used in earlier work were exhausted, we used other samples from that institute to estimate Pronase lignin. Unfortunately this new set of samples was less varied than the older one: 41 of the 49 samples were of grass hay and the range of digestibility was much less, 59 – 75 % against 49 – 84 %.

Corresponding samples of faeces from wethers were available for 18 of these 49 samples. From the Department of Animal Physiology, we received 17 samples of fodder (7 of which had been used in compound rations) from 11 metabolic trials with sheep, together with some samples of fodder residues and corresponding faecal samples.

In treating the roughage with Pronase, we used the method of Nomoto et al. (1960). The roughage was first extracted with ether. In a preliminary trial, we treated 25 g of a hay sample (air-dry) with 3, 5, 10, 15, 20 or 25 mg Pronase in 0.5 litre phosphate buffer 0.03 M of pH 7.4 with 5 % ethanol, for 48 h at 38 – 40 °C. The lignin in the Pronase-treated hay was estimated in the usual way with 72 % sulphuric acid. The results are given in Table 1.

Table 1. % N in lignin and % lignin of hay treated with various amounts of Pronase.

mg Pronase per 25 g hay	% of oven-dry hay		N in lignin (%)
	extract	lignin	
none	—	6.9	4.25
3	38.1	6.0	4.05
5	39.2	6.0	4.14
10	40.3	5.9	3.91
15	40.9	5.9	3.74
20	41.3	5.6	3.61
25	41.8	5.6	3.48

On the basis of these results, we chose a treatment with 20 mg Pronase per 25 g of air-dry roughage. The Pronase treatment replaced the hot-water extraction used previously, lignin thus being estimated in the residue of ether extraction plus Pronase treatment.

Samples were analysed comprehensively as earlier, lignine being estimated by sulphuric acid digestion after ether and hot-water extraction.

### 3 Results

#### *3.1 Content of Pronase lignin in samples and correlation between Pronase lignin content and digestibility of organic matter*

Table 2 shows digestibilities, contents of Pronase lignin and N contents of the lignin of the 49 samples from Hoorn. All Pronase lignins still contained an appreciable amount of N. Table 3 gives contents of Pronase lignin in the 18 faecal samples, together with the calculated digestibilities of Pronase lignin, which had a wide range from — 7 to + 40 %<sup>1</sup>.

Correlation and regression coefficients and standard errors for the 41 grass hay samples and for all 49 samples are shown in the first two rows of Table 4, in which the fourth row gives corresponding values for hot-water lignin in 85 samples from our earlier paper. Correlation coefficients for Pronase lignin are lower than those for hot-water lignin, but standard errors are also much lower. This suggests that Pronase lignin might be a better parameter of digestibility of organic matter *in vivo* than hot-water lignin. However the two sets of samples in our present and former papers are not comparable in proportions of the various types of roughage and range of digestibilities (Section 2). For better comparison, we selected 17 samples from both sets with closely matched digestibilities.

1. An error crept into the formula for lignin digestibility on p. 11 of our earlier paper. The factor:  $100/(100-\text{DOM})$  should be:  $(100-\text{DOM})/100$ .

Table 2. Digestibility of organic matter (DOM) and content of Pronase lignin in roughage samples from Hoorn.

Sample No.	Type	DOM <sup>1</sup> (%)	Lignin(% of OM) <sup>2</sup>	N in lignin(%)
V 644	Fresh grass	74.7	8.9	3.80
V 646		74.0	8.5	2.44
V 791		74.8	7.6	3.58
V 633	Grass hay	63.5	11.1	1.94
V 634		64.6	11.6	2.39
V 637		64.5	11.4	2.48
V 639		65.2	11.1	2.57
V 642		68.6	10.7	2.69
V 645		71.5	10.7	2.69
V 649		70.4	10.0	3.00
V 651		65.9	11.8	2.41
V 652		68.0	10.9	2.75
V 658		65.6	10.7	2.45
V 660		69.6	11.0	2.75
V 661		71.6	10.1	3.24
V 664		72.6	9.4	3.30
V 668		67.0	10.7	2.66
V 669		67.8	10.4	2.82
V 672		72.8	9.3	3.50
V 682		64.4	10.2	3.34
V 692		74.6	8.1	2.51
V 698		64.6	11.4	2.09
V 706		68.1	9.6	2.03
V 712		66.0	10.9	2.10
V 728		69.5	10.4	3.16
V 729		66.5	10.5	2.87
V 737		64.7	12.3	2.83
V 738		66.4	10.6	3.00
V 739		68.1	11.4	3.14
V 749		64.0	11.5	2.03
V 753		64.5	11.2	2.47
V 755		68.9	9.8	3.12
V 758		62.6	11.8	1.79
V 761		62.2	11.8	2.22
V 762		71.0	9.8	2.75
V 766		65.1	11.1	2.01
V 777		63.6	11.7	2.24
V 778		63.2	11.0	1.94
V 780		73.0	9.4	2.77
V 784		64.0	12.6	2.75
V 787		61.1	11.6	1.78
V 789		62.9	11.2	2.85
V 792		64.1	10.0	2.24
V 795		63.0	10.8	2.49
V 631	Grass silage	65.2	11.8	3.73
V 665		74.5	8.1	2.31
V 743		74.5	8.3	3.08
V 771		67.7	8.4	2.36
V 670	Lucerne hay	59.4	13.8	2.50

1. Data supplied by the Institute for Livestock Feeding and Nutrition Research 'Hoorn'.
2. Calculated with total ash data from the 'Hoorn' Institute.

Table 3. Digestibility of organic matter (DOM), content of Pronase lignin in roughage and faeces, and digestibility (D) of Pronase lignin in 18 samples.

Sample No.	Type	DOM (%)	Lignin in roughage (% of OM)	Lignin in faeces (% of OM)	D lignin
V 791	Fresh grass	74.8	7.6	29.0	4
V 749	Grass hay	64.0	11.5	26.0	18
V 753		64.5	11.2	26.5	16
V 755		68.9	9.8	26.3	16
V 758		62.6	11.8	27.0	14
V 761		62.2	11.8	26.9	14
V 762		71.0	9.8	26.6	21
V 766		65.1	11.1	25.0	22
V 777		63.6	11.7	27.7 <sup>1</sup>	14
V 778		63.2	11.0	26.5	11
V 780		73.0	9.4	20.9 <sup>1</sup>	40
V 784		64.0	12.6	29.3	17
V 787		61.1	11.6	25.4	15
V 789		62.9	11.2	27.5	9
V 792		64.1	10.0	27.0	3
V 795		63.0	10.8	27.0	8
V 743	Grass silage	74.5	8.3	29.7	9
V 771		67.7	8.4	27.8	-7

1. Mean of two estimates.

Matched samples are listed in Table 5 with digestibilities of organic matter, content of Pronase extract against hot-water extract, Pronase lignin against hot-water lignin and content of nitrogen in these lignins.

Content of Pronase extract is generally somewhat more than of hot-water extract, whereas content of pronase lignin is somewhat less than of hot-water lignin, the content of nitrogen in Pronase lignin being generally distinctly less than in hot-water lignin. But in all three columns, there are exceptions to these generalizations.

The third and fifth rows of Table 4 show the correlation and regression coefficients and standard errors for these two sets of 17 samples. Apart from a moderate difference in regression coefficient, these figures are practically identical. Hence Pronase treatment is probably no better than simple hotwater extraction as preparation of roughage for estimation of lignin.

### 3.2 Comprehensive analysis of roughage and faeces and calculation of their digestibility

Table 6 gives the composition of the roughages and fodder residues, and the calculated composition of the compound rations and where there were feed residues, of the ingested fodder. Most samples were hay, but some were lucerne, dried grass and

Table 4. Correlation and regression coefficients and standard errors of contents of Pronase lignin or hot-water lignin against digestibility of organic matter by sheep wethers.

Type of roughage	Number of samples	Pronase lignin			Hot-water lignin		
		correlation coefficient	regression coefficient	standard error	correlation coefficient	regression coefficient	standard error
Grass hay	41	-0.770	-2.94	2.2			
All types	49	-0.841	-2.72	2.2			
All types	17	-0.896	-2.80	2.4			
All types	85				-0.888	-3.12	3.6
All types	17				-0.899	-2.40	2.4

Table 5. Contents of Pronase lignin or hot-water lignin in matched samples from Hoom.

Sample No.	Type	DOM (%)	Pronase/hot-water extract % of dry matter	Pronase/hot-water lignin % of organic matter	% N in Pronase/hot water lignin
V 692 / V 691	Grass hay	74.6 / 75.3	42.0 / 32.7	8.1 / 9.9	2.51 / 3.33
V 780 / V 693		73.0 / 75.0	33.4 / 36.9	9.4 / 9.0	2.77 / 3.19
V 672 / V 756		72.8 / 73.4	34.2 / 33.0	9.3 / 9.1	3.50 / 3.85
V 664 / V 675		72.6 / 73.0	35.1 / 28.4	9.4 / 11.1	3.30 / 3.94
V 661 / V 699		71.6 / 71.3	32.1 / 31.2	10.1 / 10.2	3.24 / 3.13
V 642 / V 641		68.6 / 68.4	28.6 / 22.6	10.7 / 11.4	2.69 / 3.62
V 729 / V 751		66.5 / 66.7	29.2 / 23.3	10.5 / 12.5	2.87 / 3.58
V 651 / V 643		65.9 / 65.9	25.6 / 24.3	11.8 / 11.3	2.41 / 2.76
V 777 / V 630		63.6 / 63.6	29.9 / 22.9	11.7 / 11.9	2.24 / 2.78
V 761 / V 734		62.2 / 62.3	27.2 / 22.1	11.8 / 13.6	2.22 / 3.57
V 787 / V 703	61.1 / 61.2	22.8 / 17.6	11.6 / 14.6	1.78 / 3.27	
V 644 / V 781	Fresh grass	74.7 / 74.9	40.2 / 36.6	8.9 / 8.2	3.80 / 3.38
V 646 / V 782		74.0 / 73.9	41.5 / 36.4	8.5 / 8.0	2.44 / 3.07
V 631 / V 801	Grass silage	65.2 / 65.2	19.4 / 23.2	11.8 / 10.9	3.73 / 2.21
V 665 / V 798		74.5 / 74.1	22.9 / 23.4	8.1 / 9.2	2.31 / 2.96
V 771 / V 635	Lucerne hay	67.7 / 67.7	30.0 / 25.1	8.4 / 11.9	2.36 / 3.79
V 670 / V 673		59.4 / 59.5	30.1 / 29.3	13.8 / 14.3	2.50 / 3.79
Averages of 17 samples			30.8 / 27.6	10.2 / 11.0	2.64 / 3.26

Table 6. Comprehensive analysis of roughages from metabolic trials with sheep.

No. of trial and type of roughage	% of dry matter			% of organic matter				lignin	pento- san	acetyl CO <sub>2</sub>	alpha- cellu- lose	unac- counted	N in lignin (%)
	total ash	soluble ash	organic matter in aqueous extract	ether extract	organic matter in aqueous extract	insol- uble protein	insol- uble protein						
F 23 1154 lucerne pellets	11.0	7.1	20.7	4.2	23.3	11.5	17.1	12.8	1.3	1.7	23.8	4.3	3.67
F 23 1156/7 crushed hay	8.2	6.7	19.0	3.4	20.6	7.4	13.2	21.0	1.4	1.1	26.0	5.9	3.37
F 23 lucerne/hay 4:1	-	-	-	4.0	22.8	10.7	16.3	14.4	1.3	1.6	24.2	4.7	-
F 24 1167 hay	8.2	6.0	19.6	3.1	21.4	7.2	12.9	21.5	1.3	1.1	25.3	6.2	3.11
F 24 1171 Ital. ryegrass pellets	11.6	9.1	26.5	5.1	30.0	7.6	11.5	17.3	1.2	0.9	22.5	3.9	4.36
F 24 Ital. ryegrass/hay 4:1	-	-	-	4.7	28.3	7.5	11.8	18.1	1.2	0.9	23.1	4.4	-
F 25 1196 hay	9.3	7.5	19.8	3.7	21.8	6.9	9.5	20.7	1.3	1.2	27.3	7.6	3.41
F 26 1271 dried grass	10.2	6.5	21.1	3.0	23.5	5.8	11.5	21.6	1.3	1.2	27.6	4.5	3.00
F 27 1285 3500 g late silage	13.9	8.1	8.4	6.3	9.8	4.1	18.5	23.8	1.5	1.2	32.9	1.9	2.39
F 27 1322 382 g fodder residue sheep J	23.1	11.7	2.6	4.8	3.4	3.8	20.4	25.9	1.7	1.0	35.9	3.1	2.04
F 27 ingested fodder sheep G	-	-	-	6.5	10.5	4.2	18.3	23.6	1.5	1.2	32.5	1.7	-
F 27 1324 702 g fodder residue sheep J	26.4	11.7	3.1	6.1	4.2	3.8	20.2	24.1	1.5	1.1	32.4	6.6	3.25
F 27 ingested fodder sheep J	-	-	-	6.4	11.3	4.1	18.0	23.8	1.5	1.3	33.0	0.6	-
F 27 1290 1000 g late hay	9.6	5.7	14.9	2.5	16.5	5.4	15.1	22.2	1.5	1.0	30.8	5.0	2.66
F 27 1323 261 g fodder residue sheep J	10.8	5.0	16.3	2.2	18.3	2.5	16.7	22.5	1.7	1.0	28.8	6.3	1.98
F 27 ingested fodder sheep J	-	-	-	2.6	15.9	6.4	14.5	22.1	1.5	0.9	31.6	4.5	-
F 28 1310 late hay	9.1	5.8	16.4	2.2	18.0	5.0	15.6	23.4	1.7	1.0	29.2	3.9	2.72
F 29 1337 hay	9.8	7.7	19.1	3.9	21.2	7.2	9.4	19.9	1.2	1.3	29.0	6.9	3.37
F 29 1339 French lucerne pellets	11.0	8.7	22.8	3.5	25.6	10.0	15.1	11.9	1.2	1.7	24.6	6.4	3.68
F 29 French lucerne/hay 4:1	-	-	-	3.6	24.7	9.4	13.9	13.5	1.2	1.6	25.5	6.6	-
F 29 1341 Amer. lucerne pellets	10.1	7.1	23.3	4.2	25.9	11.0	14.7	12.0	1.0	1.7	22.4	7.0	3.68
F 29 Amer. lucerne/hay 4:1	-	-	-	4.1	25.0	10.2	13.6	13.6	1.0	1.6	23.7	7.2	-
F 30 1392 early hay	11.6	7.0	19.9	3.9	22.5	11.1	11.1	20.4	1.1	1.1	24.0	4.8	3.89
F 31 1474 early hay	12.4	7.8	14.0	3.2	16.0	14.2	12.7	21.5	1.1	1.3	25.2	4.8	2.78
F 32 1521 burnt hay	9.7	6.1	15.3	4.6	16.9	14.1	11.6	16.8	0.8	1.2	25.0	9.0	4.39
F 32 1525 hay	9.2	6.9	19.5	4.1	21.5	6.9	9.4	20.1	1.2	1.2	26.9	8.7	3.25
F 33 1921 hay	10.9	7.4	15.4	3.3	17.3	9.2	12.5	23.0	1.3	1.2	27.9	4.3	3.89

silage. All constituents of organic matter of the ingested fodders varied widely. Unaccounted constituents ranged from 0.6 to 9.0 % of organic matter.

Values for faeces are collected in Table 7. Differences in contents expressed as percentages of organic matter between sheep in any one trial were usually slight, but the principal constituents (lignin, pentosan and alphacellulose) varied by a few per cent. Unaccounted constituents ranged from -0.3 to 7.0 %.

Table 8 shows the digestibilities of organic matter (DOM) as estimated by the Department of Animal Physiology and the calculated digestibilities of the constituents. The mean difference in DOM between sheep in any one trial was 2.0 percentage units, but in three was 3 - 4 percentage units. On the whole, the variation within trial for the constituents were below 10 percentage units, but higher values were sometimes found, especially among the figures for acetyl (up to 20 units), unaccounted constituents (up to 17 units) and N in lignin (up to 27 units). Digestibilities of organic matter ranged from 46 - 77 %, a considerably wider range than in the 10 metabolic trials mentioned in our earlier paper. The ranges in values for digestibility of most constituents were fairly continuous:

- ether solubles: 36 - 63 %
- organic hot-water solubles: 65 - 90 %
- insoluble protein: 40 and 44 % for late silage, otherwise 53 - 83 %
- hot-water lignin: 8 - 30 %
- pentosan: 44 - 82 %
- acetyl: 31 and 38 % for one of the lucerne rations and 40 % for one sheep on a late hay ration, otherwise 47 - 83 %
- uronic CO<sub>2</sub>: 44 - 83 %
- alphacellulose: 50 - 83 %
- unaccounted constituents: negative for late silage, otherwise 63 - 102 %
- N in lignin: negative for one sheep on dried grass, late silage and late hay, otherwise 6 - 43 %.

Comparison of the results of the various rations shows that there are no marked differences in the digestibility of constituents between hay and dried grass. The lucerne rations differ from the hay rations especially in the lower digestibilities of lignin, pentosan, acetyl groups, alphacellulose and unaccounted constituents. In trial 27 the silage differs from the corresponding hay (both made from the same grass) in the higher digestibility of ether solubles (due to organic acids in the silage), the lower digestibility of insoluble protein and the negative digestibility of the unaccounted constituents. In Trial 32, burnt hay had lower digestibilities for ether and organic hot-water solubles and for acetyl groups, but higher digestibilities for insoluble protein, lignin and N in lignin than the corresponding normal hay. The general averages show again that the cell-wall components other than lignin (pentosan, acetyl, uronic CO<sub>2</sub> and alphacellulose) have essentially the same digestibilities, which in the present series of trials are somewhat lower than those of organic hot-water solubles and unaccounted constituents.

Table 8. Digestibility of the organic components of roughages.

No. of trial and type of roughage	Organic matter	Ether extract	Organic matter in aqueous extract	Insoluble protein	Lignin	Pentosan	Acetyl	Uronic CO <sub>2</sub>	Alpha-cellulose	Uncounted	N in lignin
F 23 1173 hay sheep G	63.9	49	75	76	27	64	57	73	67	93	43
F 23 1175 hay sheep I	62.9	50	78	78	11	63	57	73	66	93	31
F 23 1174 hay/lucerne 1:4 sheep H	57.3	55	67	79	13	48	31	69	55	66	28
F 23 1176 hay/lucerne 1:4 sheep J	58.9	58	84	80	16	51	38	69	55	77	28
F 24 1209 hay/Ital.ryegrass 1:4 sheep H	68.9	72	84	73	14	70	67	69	72	89	25
F 24 1210 hay/Ital.ryegrass 1:4 sheep I	65.6	70	81	71	18	65	67	67	66	84	26
F 25 1278 hay sheep J	77.3	62	85	75	27	81	77	83	80	97	34
F 26 1291 dried grass sheep G	66.4	40	80	53	11	70	62	75	77	76	-2
F 26 1293 dried grass sheep I	64.8	48	80	57	20	65	54	67	70	89	17
F 27 1325 late silage sheep G	45.8	78	70	40	17	47	47	50	50	-6	6
F 27 1328 late silage sheep J	47.9	78	72	44	18	50	47	54	52	-12	-17
F 27 1326 late hay sheep H	48.2	44	70	63	9	44	40	50	52	82	24
F 27 1327 late hay sheep I	52.0	42	71	67	12	50	53	44	56	80	-3
F 28 1345 late hay sheep I <sup>1</sup>	52.9	36	78	56	22	51	53	60	54	67	30
F 28 1346 late hay sheep J <sup>1</sup>	55.5	45	89	64	25	54	53	60	56	67	37
F 29 1403 hay/Fr.Luc. 1:4 sheep G	59.2	47	89	75	9	52	75	69	59	53	27
F 29 1405 hay/Fr.luc. 1:4 sheep I	60.3	44	90	77	9	52	67	63	57	70	23
F 29 1404 hay/Am.luc. 1:4 sheep H	62.0	56	86	74	12	57	70	69	64	63	16
F 29 1406 hay/Am.luc. 1:4 sheep J	61.1	56	87	78	8	54	50	69	58	76	15
F 30 1478 800 g early hay sheep G <sup>1</sup>	73.2	54	75	80	30	82	73	82	80	90	30
F 30 1481 800 g early hay sheep J <sup>1</sup>	74.5	59	80	81	29	82	73	82	81	90	29
F 30 1479 1400 g early hay sheep H <sup>1</sup>	66.6	44	72	73	10	77	73	73	72	102	9
F 30 1480 1400 g early hay sheep I <sup>1</sup>	68.6	49	72	75	16	78	73	73	76	94	22
F 31 1544 early hay sheep G <sup>1</sup>	70.0	42	66	81	27	81	64	77	80	81	38
F 31 1545 early hay sheep H <sup>1</sup>	69.3	41	65	80	28	79	73	77	76	98	40

F 32 1641 burnt hay sheep G	69.2	48	70	81	19	75	63	75	71	95	31
F 32 1643 burnt hay sheep I	72.5	59	72	83	30	78	75	75	78	92	41
F 32 1644 normal hay sheep J	75.6	63	83	71	15	81	83	75	83	94	16
F 33 1966 hay sheep R	69.4	52	68	77	25	78	77	75	78	98	34
General average	63.4	53	77	71	18	65	62	69	67	74 <sup>2</sup>	23
Av. of trials with hay <sup>3</sup>	66.6	51	76	73	21	72	68	72	72	90	28
Av. of trials 24 and 26 (dried grass)	66.5	58	82	64	16	68	63	70	72	85	17
Av. of trials with lucerne pellets	59.8	53	84	77	11	53	55	68	58	68	23

1. Single estimate.
2. By excluding the negative values for late silage, this figure becomes 80 %.
3. Excluding abnormal burnt hay.

#### 4 Discussion

The Pronase extraction reduced the nitrogen content of the lignin in the roughage only moderately (Table 5, fourth column). Perhaps the digestibility of lignin in roughage as observed in our earlier and present work was due only partly to removal of any protein attached to it. Porter & Singleton (1971) showed that lignin is demethoxylated in the sheep's abomasum. Hence, perhaps Pronase treatment seems no improvement over the hot-water extraction in preparation of roughage for lignin estimation.

The compositional data for roughage and faeces from metabolic trials confirm and extend those reported in our earlier paper. Digestibility of cell-wall carbohydrates approached digestibility of total organic matter, whereas that of ether solubles was lower, unless these contained much volatile fatty acids. The organic hot-water extract and unaccounted constituents were generally more digestible than was total organic matter, whereas insoluble protein (which does not include the nitrogen attached to the lignin) tended to be more digestible in roughage with high DOM, than in roughage with low DOM, except in lucerne where it was much more digestible than total organic matter.

#### Summary

Treatment of ether-extracted roughage with a solution of Pronase (a proteinase from *Streptomyces griseus*) at pH 7.4, instead of hot-water extraction, in preparation of roughage samples for lignin determination did not improve the correlation between lignin content and digestibility of organic matter.

Data from comprehensive analysis of roughage and corresponding faeces from metabolic trials support results given in the authors' earlier paper (Muller et al., 1970), about the digestibility of the components.

#### References

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