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

Institute for Storage and Processing of Agricultural Produce, Wageningen

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

1970 Centre for Agricultural Publishing and Documentation

Wageningen

ISBN 90 220 0201 2

1 Introduction

1.1 The role of lignin in digestibility

Among animal nutritionists common opinion is that the classical method of analysis of roughage is a poor parameter of the nutritive value or digestibility of roughage, particularly when roughages of a different nature are compared like fresh grass, grass hay, grass silage and the corresponding products from leguminous plants. Most workers think that lignin in the cell-wall is the determinant of digestibility by ruminants.

However, there is no agreement on the best way of estimating lignin in roughage. The classical Klason method (developed for the nitrogen-poor raw materials of the paper-pulp industry like wood and straw) is generally considered to yield erroneous results after simple extraction with ethanol-benzene or ether and hot water because of artefacts from reactions under influence of 72 % sulphuric acid between lignin and protein or other nitrogen compounds which may represent up to 20 % of the dry matter of roughage. Therefore many workers prefer more drastic pretreatment than extraction with organic solvents and water, e.g. with hot dilute mineral acids, proteolytic enzymes or aqueous solutions of detergents.

According to Ellis et al. (1946) an estimate of lignin in roughage should be as low as possible and the lignin so estimated should be wholly indigestible. Among seven methods investigated, they chose one with successive pretreatments with ethanol-benzene, pepsin-HCl, and 5 % sulphuric acid under reflux. The actual analysis was the classical treatment with 72 % sulphuric acid at room temperature, followed by refluxing after dilution to 5 % acid. The amounts of lignin recovered from faeces varied from 95 to 106 % of that in the fodder.

Before the treatment with 72 % sulphuric acid, Armitage et al. (1948) extracted with ethanol-benzene, 5 % hydrochloric acid in reflux, and alkaline trypsin. In clover they found 4.8 % lignin with 2.02 % nitrogen; in oat straw 10 to 11 % with 2.3 % nitrogen. Thus, the drastic pretreatment with hot mineral acid and a proteinase still left an appreciable amount of nitrogen in the lignin.

Balch et al. (1954) quoted a paper by Ely et al. (1953) who used the method of Ellis et al. (1946) and found a digestibility for the crude lignin of 3.8 to 16.0 % and for the protein-free lignin of 7.5 to 19.8 %. These figures show that the Ellis lignin is by no means indigestible and that digestibility of lignin is not a function of its nitrogen content (as a matter of fact, the nitrogen had a negative digestibility). Balch et al. compared the lignin determinations of Gray (a relatively mild pre-

Table 1. Comparison of the lignin estimates by Gray (1947), Ellis et al. (1946), and Armitage et al. (1948).

Fodder	Gray			Ellis et al.			Armitage et al.		
	Crude lignin	N in lignin	Protein-free l.	Crude lignin	N in lignin	Protein-free l.	Crude lignin	N in lignin	Protein-free l.
Hay	11.3	2.89	9.3	10.4	2.19	9.0	8.3	1.70	7.4
Concentrate	7.1	3.21	5.7	6.2	2.14	5.4	5.4	1.77	4.8

treatment consisting of ethanol-benzene extraction and refluxing with 5 % H_2SO_4), Ellis et al. (1946) and Armitage et al. (1948).

The results of this comparison (Table 1) show that the more drastic pretreatments yield less lignin and less nitrogen in the lignin, but again that even after these drastic pretreatments the lignin is by no means free from nitrogen.

Digestion trials with cows yielded the following respective digestibilities for crude lignin and N-free lignin: Gray 7 to 13, 7 to 11; Ellis 2 to 7, 6 to 12; Armitage 2 to 3, 0 to 8.

Thus even the more drastic pretreatments did not always yield indigestible lignin.

Sullivan (1955) also used Ellis's method and obtained digestibilities of lignin between 3 and 12 %. Both more drastic pretreatment with acid and an extra extraction with a solution of Na_2CO_3 yielded higher digestibilities. Later he (Sullivan, 1959) used Ellis's method on 36 samples of grass and found nitrogen contents of 1.9 to 3.6 % N. The correlation coefficient between digestible energy and lignin content in these 36 grasses was —0.94 with a standard error of 2.3. However the regression equation for leguminous forage differed from that for the grasses.

Whitehead & Quicke (1964) compared six methods for lignin estimation:

A. Ethanol-benzene; pepsin-HCl; 72 % H_2SO_4.

B. Ethanol-benzene; refluxing with 5 % HCl; trypsin-Na_2CO_3; 72 % H_2SO_4; refluxing with dilute H_2SO_4.

C. Ethanol-benzene; pepsin-HCl; Na_2CO_3; 5 % H_2SO_4 in autoclave; 72 % H_2SO_4; 3 % H_2SO_4 in autoclave.

D. Ethanol-benzene; refluxing with 5 % H_2SO_4; 72 % H_2SO_4; refluxing with dilute H_2SO_4.

E. Ethanol-benzene; refluxing with water; refluxing with 1 % HCl; fuming HCl with HCl gas at 0°C.; refluxing with dilute HCl.

F. Maceration with ether-water; refluxing with 1 % HCl; ethanol-benzene; 72 % H_2SO_4; refluxing with dilute H_2SO_4.

The results of twenty replicates with a mixture of South African veld grasses are given in Table 2.

The six methods yielded widely divergent results. Methods D to F must be considered less suitable because of the high standard errors. Method B seems best

Table 2. Estimates of lignin by 6 methods in a mixture of South African veld grasses each value from 20 replicates (data from Whitehead & Quicke, 1964).

Method	Ash-free lignin	Ash in apparent lignin	Composition of ash-free lignin			
			C	H	N	Methoxyl
A	7.76 ± 0.51	38.4	57.0	6.30	2.91	5.30
B	7.16 ± 0.30	10.7	62.4	6.00	1.57	9.08
C	7.19 ± 0.22	19.8	59.8	6.15	1.82	7.54
D	9.51 ± 0.97	47.4	58.5	8.84	2.89	8.36
E	10.74 ± 2.65	10.0	57.5	6.40	1.90	6.22
F	14.64 ± 4.21	12.5	51.0	6.44	1.16	4.28

because of the lowness of ash and nitrogen and the highness of methoxyl, but the nitrogen content is still not negligible.

A different technique has been applied by van Soest (1963a) with the introduction of detergent solutions to separate proteins and other cell contents from the cell-wall. An advantage of this method is that a single pretreatment removes all constituents which might interfere in estimation of lignin. At pH values of 3.4 and more the anionic detergent sodium laurylsulphate dissolved more dry matter than the cationic cetyl-trimethyl-ammonium bromide, but less at pH values below 3.4. Extreme pH values entailed an excessive loss of dry matter. However, the residue of treatment with the cationic detergent at pH 2 was thought to be a suitable starting material for estimating lignin. This idea was elaborated in a further paper (van Soest, 1963b) which mentions a pretreatment with 2 % cetyl-trimethyl-ammonium bromide in 1N H_2SO_4 under reflux, before the conventional treatment with 72 % H_2SO_4.

In 1964 van Soest published some values for lignin by this method. Fig. 1 of his paper shows that legumes do not show the same relationship between digestibility of dry matter and detergent lignin content in percentage of dry matter as do grasses. The respective correlation coefficients were —0.74 (for 22 samples) and —0.82 (for 37 samples).

1.2 Comprehensive analysis of roughage

Some workers have not only estimated lignin but have tried to account for all the organic matter in roughage (and in faeces). This allows the digestibility of the various constituents of roughage to be estimated. The classical Weende method is already such an attempt but the proximate constituents crude fibre and nitrogen-free extract are so ill-defined that this method is hardly comprehensive.

A more recent attempt was by Ely & Moore (1955) who accounted for 97 to 104 % of the forage by the sum of ash, protein, lignin, ethanol-benzene extract, hot-water extract and holocellulose. Holocellulose is the residue of a delignifying

treatment applied after extractions with ethanol-benzene and hot water. Waite & Gorrod (1959a) showed that the delignifying treatment does not remove all the lignin, unless it is so drastic that it removes an appreciable amount of carbohydrate too. Anyway, Ely & Moore did not allow for the nitrogen content of lignin.

Van Soest et al. (1966) claimed that neutral detergent solution separates the cell contents from the cell-wall and that true digestibility of the organic matter in the extract was not lower than 98 %. This method seems therefore suitable in a comprehensive analysis but the separation may not always be clear-cut, especially in young cells whose walls are still growing actively.

Gaillard (1966) used the neutral detergent solution on 29 roughages (16 Gramineae and 13 Leguminosae) and analysed the residue for hemicellulose, cellulose, lignin and uronic acids. The sum of these four constituents was 0.4 to 13.9 % (average 4.3 %) less than the amount of neutral detergent residue. This difference may be at least partly due to mineral constituents like silica which are insoluble in the neutral detergent solution. Lignin was estimated by the 72 % sulphuric acid method after treatment of the neutral detergent residue with 1N H_2SO_4 under reflux. This drastic pretreatment led to quite low values.

Waite et al. (1959a, b, and 1964) developed an elaborate scheme for comprehensive analysis, comprising the following treatments:

a. extraction with ethanol-benzene, the extract being fractionated into an ether-soluble and a water-soluble portion
b. extraction with water at 60°C, the extract being combined with the water-soluble portion of the ethanol-benzene extract
c. extraction with ammonium oxalate at 80 to 85°C
d. treatment with pepsin-0.1N HCl at 46°C
e. treatment with sodium chlorite and acetic acid at 74°C, yielding a holocellulose with a few percent lignin with little loss of carbohydrate. The combined loss of carbohydrate in treatments d and e amounted to 1.6 %. The holocellulose is subsequently extracted with:
f. water at 85°C
g. 1 % KOH under nitrogen at 18-20°C
h. 1N H_2SO_4 under reflux
i. 72 % H_2SO_4 at 20°C.

In all extracts sugars were estimated by chromatography. The holocellulose was analysed also for acetyl groups, uronic acid residues, and lignin. The sum of the sugar residues in extracts g to h and the xylan in extract i is considered hemicellulose, the glucosan in extract i as cellulose.

Young cocksfoot grass contained the following: extract a 6.3 %, b 26.3 %, c 6.3 % and d 10.3 %; holocellulose residue 44.7 % of the starting material, consisting of 0.7 % galactan, 2 % glucosan, 2.2 % araban, 8.2 % xylan, 21.8 % cellulose glucosan, 0.8 % acetyl, 4.2 % aldobiuronic acid, 2.3 % lignin, 1.6 % crude protein and 0.9 % ash. Together with the analysis of the extracts a to e Waite et al. could account for 98 % of dry matter in young cocksfoot, and for

Table 3. Digestibility by sheep of organic matter and constituents of four consecutive cuts of ryegrass.

	Cut no. 1	2	3	4
Organic matter	86	83	79	62
Soluble carbohydrates	100	100	100	100
Organic acids	94	87	87	64
Ether extract	65	64	60	43
Protein N	82	78	71	50
Non-protein N	75	78	79	76
Pectin	72	61	47	35
Total hemicellulose	93	84	79	56
Cellulose	92	89	87	73
Lignin	23	23	18	0
Ash (excluding SiO_2)	64	62	61	52

97 and 98 % in two samples of ryegrass.

In 1964 they reported the use of the scheme on four consecutive cuts of ryegrass fed to sheep and on sheep's faeces. The digestibilities calculated of organic matter and the constituents are given in Table 3.

Although lignin was estimated after pretreatment with pepsin-HCl and with 1N H_2SO_4 under reflux, about 20 % was digested in cuts 1, 2 and 3; one value reached 42 %!

1.3 Programme of study

I have long been using the Klason method for estimating lignin after a mild extraction with ether, then hot water of cereal straw and similar materials. More recently I have successfully used it on the seed coat of pulses (Muller, 1967a) and on mushroom compost (Muller, 1967b). Like straw, seed coats consist of dead empty cells and thus almost entirely of cell-walls. Mushroom compost, however, contains much other matter, like cells and debris of bacteria and fungi besides straw in various stages of degradation; moreover the lignin changes during composting into a humus-like matter with a high nitrogen content called nitrogen-rich lignin-humus complex.

Not all the nitrogen in this complex could be an artefact from reaction of lignin and protein in 72 % sulphuric acid. Hence I doubted the formation of such artefacts in the Klason method after mild pretreatment of protein-rich materials like roughage. When various compounds were added to the straw, there was hardly any increase in nitrogen content of the lignin (Muller, 1967b). Much earlier Norman & Jenkins (1934) obtained marked increases in nitrogen content of lignin with much larger amounts of protein. Working with casein like Norman & Jenkins, I recently found that casein by itself was completely soluble in 72 % sulphuric acid.

My experience suggested that a high nitrogen content in Klason lignin might be

due to a linkage of native lignin with nitrogen compounds, especially in young cell-walls containing for instance enzymes involved in the synthesis of cell-wall compounds. Modern views on lignin biosynthesis (Freudenberg & Neish, 1968) show that nitrogen may be incorporated into lignin through reaction of amino groups with free radicals formed by dehydrogenation of *p*-hydroxycinnamyl, coniferyl and sinapyl alcohols (the three building blocks of lignin).

A linkage of nitrogen compounds with native lignin explains first why even drastic pretreatment does not yield a nitrogen-free Klason lignin and secondly it explains why lignin is sometimes fairly digestible: when peptide chains of some length are bound to the lignin macromolecule, part of any of them may be susceptible to attack by proteinases of the rumen microflora or of the intestine.

The question arose whether lignin estimates after mild pretreatment might not be better correlated with digestibility of organic matter than lignin estimates after more drastic pretreatment. In autumn 1966 many samples of roughage and of faeces became available from digestion trials with wethers at the Hoorn Research Institute for Livestock Nutrition. I estimated lignin in these samples and nitrogen in the lignin and related the results to digestibility coefficients of organic matter estimated by Hoorn.

During my work on straw I have also developed a comprehensive method of analysis which comprises the following values (Ritman et al., 1948; Smolders, 1955):

a. ether extract
b. hot-water extract
c. insoluble ash
d. insoluble protein
e. ash-free lignin
f. pentosan
g. acetyl
h. CO_2 from uronic acids
i. alphacellulose free from ash and pentosan.

Values c to i are estimated in the residue of the successive extractions a and b. A quarter of the uronic acid residue is accounted for by h, the other three quarters are included in the pentosan fraction f. The scheme does not include estimation of pectin which falls into both f and h. Asselman et al. (1956, Table 1) show that the sum of these nine constituents accounts for 98-100 % of dry matter in cereal straw. This range of $\pm$ 1 % may be due to hexose residues in the pectin-hemicellulose fraction which do not yield a furfural derivative in the pentosan estimate and are dissolved by the 17 % caustic soda solution applied in the alphacellulose determination.

When using this analytical scheme on nitrogen-rich materials such as mushroom compost and roughage, the nitrogen in lignin should be subtracted from insoluble nitrogen before multiplying by 6.25 to calculate the insoluble crude protein. Thus nitrogen must be estimated also in a separate batch of lignin.

Each scheme for comprehensive analysis of materials such as roughage must compromise the desire for as much information as possible and the available man-power. The elaborate scheme of Waite et al. (1959) is commendable for the amount of information it provides, but is time-consuming. The present scheme,

though much simpler, still requires four man-days per sample of a well-trained analyst.

Most samples of roughage and all samples of faeces from Hoorn were too small for comprehensive analysis. However the Department of Animal Physiology of the Agricultural University at Wageningen had some large samples of roughages and faeces from respiration trials with cows and sheep. From the analytical data I calculated digestibility coefficients for the various constituents of the roughage.

2 Results

2.1 Lignin content of samples of roughage and faeces and correlation between lignin content and digestibility of organic matter

Initially some idea was sought of lignin contents after various pretreatments. Values in a hay of medium quality are given in Table 4.

Without pretreatment the lignin content is evidently too high, because waxes and other lipids are insoluble in cold 72 % sulphuric acid and are thus included in the lignin. Ethanol-benzene in place of ether yields appreciably less lignin and detergent solutions yield slightly less again. Although ether takes more time (but not more work) than ethanol-benzene, I preferred ether as the first extraction, followed by extraction with water in a boiling water bath. The actual estimation of lignin was by treatment with 72 % sulphuric acid for 2 hours at 20°C and by refluxing with 3 % acid for 4 hours.

Table 5 shows values for lignin and nitrogen in lignin in the roughage samples from Hoorn. From nitrogen content of lignin in Column 5 the values were calculated for protein-free lignin in Column 6 (assuming that all this nitrogen is protein N). Nitrogen in lignin varies widely (between 5.18 and 1.16 %). The samples are grouped into six types:

1. fress grass
2. other fresh products
3. grass hay
4. other dried products
5. grass silages
6. other silages

Groups 1, 3 and 5 are uniform but the other groups are diverse. As a whole the samples span wide ranges of digestibility (from well over 80 to below 50 %) and lignin content (from 7.8 to 16.5 %).

Table 4. Percentage lignin in dry matter of a mediocre hay after different pretreatments.

Ether extract	Ethanol-benzene extract	Water extract	Detergent extract (Gaillard, 1966)	Detergent extract (van Soest, 1966)	Residue	Lignin
.	.	.	.	.	.	12.5
3.9	.	24.7	.	.	71.4	9.7
.	9.6	22.8	.	.	67.6	8.0
.	.	.	38.9	.	61.1	7.4
.	.	.	.	38.3	61.7	7.2

Table 5. Digestibility of organic matter (DOM), total and 'protein-free' lignin of roughage samples received from Hoorn.

Sample No.	Type	DOM [1]	Lignin (% of organic matter) [2]	N in lignin (%)	Protein-free lignin (% of organic matter)
V 705 II	Fresh grass	83.5	8.1	5.18	5.5
V 705 III		80.6	8.2	4.47	5.9
V 705 IV		78.9	9.8	3.92	7.4
V 708		68.3	10.7	3.25	8.5
V 779		76.8	7.8	4.32	5.7
V 781		74.9	8.2	3.38	6.5
V 782		73.9	8.0	3.07	6.5
V 783		65.7	9.8	2.45	8.3
V 785		64.4	11.3	2.27	9.7
V 790		72.4	9.8	4.94	6.8
V 793		72.5	11.8	4.86	8.2
V 811		77.6	8.9	4.44	6.5
V 812		75.4	9.6	4.06	7.1
V 814		74.6	10.3	3.31	8.2
V 815		68.8	11.1	3.00	9.1
V 816		64.5	12.0	2.72	10.0
V 818		78.7	9.0	4.09	6.7
V 820		57.4	13.3	1.84	11.8
V 825		59.8	13.2	2.39	11.2
V 827		60.8	14.8	2.33	12.7
V 829		72.3	11.0	3.38	8.7
V 831		67.4	10.5	2.51	8.9
V 624	Sugar beet shoots	88.0	2.0	—	—
V 648	Green barley	70.6	9.6	2.90	7.9
V 710	Green oats	52.9	14.6	1.58	13.2
V 788	Milk-ripe oats	56.2	12.4	1.50	11.2
V 626	Grass hay	55.2	15.1	2.61	12.6
V 630		63.6	11.9	2.78	9.8
V 641		68.4	11.4	3.62	8.8
V 643		65.9	11.3	2.76	9.3
V 675		73.0	11.1	3.94	8.4
V 691		75.3	9.9	3.33	7.8
V 693		75.0	9.0	3.19	7.2
V 699		71.3	10.2	3.13	8.2
V 703		61.2	14.6	3.27	11.6
V 723		57.3	13.3	3.25	10.6
V 725		56.6	13.3	2.91	10.9
V 732		61.1	14.0	3.77	10.7
V 734		62.3	13.6	3.57	10.6
V 735		56.2	13.3	3.11	10.7
V 751		66.7	12.5	3.58	9.7
V 756		73.4	9.1	3.85	6.9

Continued on next page.

Table 5. (Continued).

Sample No.	Type	DOM [1]	Lignin (% of organic matter) [2]	N in lignin (%)	Protein-free lignin (% of organic matter)
V 629 [3]	Grass hay pellets	49.9	16.5	2.52	13.9
V 636 [3]		58.3	12.7	2.71	10.6
V 640 [3]		57.0	12.6	2.58	10.6
V 654 [3]	Grassmeal	58.6	14.4	3.78	11.0
V 667 [3]	Grass hay pelets	60.6	11.8	3.65	9.1
V 673	Lucerne hay	59.5	14.3	2.99	12.6
V 678		55.0	16.3	3.19	13.0
V 679		59.6	14.6	3.11	11.8
V 683 [3]	Peavine meal	61.6	14.2	4.31	10.4
V 690	Dried peavines	64.9	12.7	4.32	9.3
V 748	Valerian straw	64.0	10.9	4.23	8.0
V 750 [3]	Grass pellets	59.8	12.0	4.78	8.4
V 773 [3]	Grassmeal	63.5	10.5	4.37	7.6
V 797 [3]	Grass pellets	64.5	11.9	5.03	8.2
V 627	Grass silage	76.7	8.8	3.23	7.0
V 628		69.0	13.5	4.47	9.7
V 632		76.4	8.7	3.10	7.0
V 635		67.7	11.9	3.79	9.1
V 662		71.3	10.3	2.66	8.6
V 663		77.1	10.7	4.46	7.7
V 666		79.6	8.9	4.07	6.6
V 713		78.8	7.8	5.01	5.4
V 715		76.4	8.3	4.02	6.2
V 716		69.1	9.2	3.23	7.3
V 718		75.6	8.6	3.14	6.9
V 745		73.6	8.5	3.34	6.7
V 765		69.8	9.9	3.08	8.0
V 767		68.2	10.6	3.43	8.3
V 769		66.9	9.5	3.12	7.6
V 794		71.9	9.5	3.29	7.5
V 796		78.1	8.0	3.81	6.1
V 798		74.1	9.2	2.96	7.5
V 800		70.6	9.1	2.56	7.6
V 801		65.2	10.9	2.21	9.4
V 803		64.3	12.2	2.12	10.6
V 838		76.5	10.1	3.92	7.6
V 840		76.1	9.1	3.50	7.1
V 841		71.8	11.6	3.14	9.3
V 842		72.1	10.5	2.29	9.0
V 844		64.3	12.7	2.41	10.8

Continued on next page.

Table 5. (Continued).

Sample No.	Type	DOM [1]	Lignin (% of organic matter) [2]	N in lignin (%)	Protein-free lignin (% of organic matter)
V 659	Barley silage	70.3	10.3	1.68	9.2
V 695		66.1	12.7	2.05	11.1
V 696	Peavine silage	65.5	12.9	3.39	10.2
V 697	Barley silage	65.1	12.4	2.30	10.6
V 714	Ripe oat silage	48.7	16.5	1.57	14.9
V 717	Oat silage	63.1	11.2	1.64	10.1
V 719	Peavine silage	63.3	13.3	3.83	10.1
V 720	Ripe oat silage	53.4	14.6	1.24	13.5
V 721	Peavine silage	66.4	11.8	3.49	9.2
V 722	Lucerne silage	55.7	14.8	2.65	12.3
V 724	Oat silage	54.5	13.7	1.16	12.7
V 741		59.1	14.0	1.27	12.9
V 754	Maize silage	69.0	10.0	2.17	8.6

1. Data supplied by the Hoorn Research Institute for Livestock Nutrition.
2. These values were calculated from data on total ash supplied by the Hoorn Research Institute for Livestock Nutrition. Each value is the mean of two estimates (standard error is 0.13, calculated from 121 estimates in duplicate).
3. Made from meal of dried forage.

The trials at Hoorn were with three wethers; values for lignin in faeces [1] are means of single estimates on the three samples (Table 6). Digestibility (D) of the lignin was calculated from the formula:

$$\text{D. lignin} = 100 \times \frac{\%\ \text{lignin in roughage} - \frac{100}{100 - \text{DOM}} \times \%\ \text{lignin in faeces}}{\%\ \text{lignin in roughage}}$$

where DOM is digestibility of organic matter. Lignin was up to 36 % digestible but negative values down to —16 % occurred, chiefly among the grass silages.

Except for 22 samples of faeces the samples were too small to allow estimation of nitrogen content. Thus in the 22 samples nitrogen-free lignin and its digestibility and digestibility of lignin nitrogen could be calculated. These data are collected in Table 7. These 22 samples are not representative of the 58 samples in Table 6 in that there are 10 samples of grass silage and only 2 of fresh grass and of other silages. On the whole protein-free lignin is slightly more digestible than total lignin

1. Faeces were available equivalent to 58 of the 95 forage samples listed in Table 5.

Table 6. Digestibility of organic matter (DOM), lignin content of roughage and faeces, and digestibility (D) of lignin in 58 samples.

Sample No.	Type	DOM	Lignin in roughage (% of organic matter)	Lignin in faeces (% of organic matter)	D lignin	Indigestible lignin (% of organic matter)
V 705 II	Fresh grass	83.5	8.1	31.5	36	5.2
V 705 III		80.6	8.2	33.2	22	6.4
V 705 IV		78.9	9.8	33.7	28	7.1
V 708		68.3	10.7	32.7	3	10.4
V 779		76.8	7.8	29.5	13	6.8
V 781		74.9	8.2	31.7	2	8.0
V 783		65.7	9.8	28.6	0	9.8
V 785		64.4	11.3	28.2	12	10.0
V 790		72.4	9.8	31.4	11	8.7
V 793		72.5	11.8	34.3	20	9.4
V 648	Green barley	70.7	9.6	26.8	18	7.9
V 710	Green oats	52.9	14.6	27.3	12	12.9
V 788	Milk-ripe oats	56.2	12.4	24.4	14	10.7
V 675	Grass hay	73.0	11.1	32.3	22	8.7
V 691		75.3	9.9	29.5	26	7.3
V 693		75.0	9.0	30.4	16	7.6
V 699		71.3	10.2	30.0	16	8.6
V 703		61.2	14.6	33.0	12	12.8
V 723		57.3	13.3	29.5	5	12.6
V 725		56.6	13.3	29.4	4	12.8
V 734		62.3	13.6	33.0	9	12.4
V 735		56.2	13.3	30.9	— 2	13.5
V 751		66.7	12.5	31.4	16	10.5
V 756		73.4	9.1	29.6	13	7.9
V 654	Grassmeal	58.6	14.4	29.1	17	12.0
V 667	Grass hay pellets	60.6	11.8	29.0	3	11.4
V 673	Lucerne hay	59.5	14.3	32.2	8	13.1
V 679		59.6	14.6	32.0	12	12.9
V 683	Peavine meal	61.6	14.2	33.6	9	12.9
V 690	Dried peavines	64.9	12.7	33.8	6	11.9
V 773	Grassmeal	63.5	10.5	28.4	1	10.4
V 662	Grass silage	71.3	10.3	31.9	11	9.2
V 666		79.6	8.9	36.7	16	7.5
V 713		78.8	7.8	36.2	1	7.7
V 715		76.4	8.3	35.7	— 1	8.4
V 716		69.1	9.2	32.6	— 10	10.1
V 718		75.6	8.6	35.0	1	8.5

Continued on next page.

Table 6. (Continued).

Sample No.	Type	DOM	Lignin in roughage (% of organic matter)	Lignin in faeces (% of organic matter)	D lignin	Indigestible lignin (% of organic matter)
V 745		73.6	8.5	32.6	— 1	8.6
V 765		69.8	9.9	32.6	1	9.8
V 767		68.2	10.6	34.1	— 3	10.9
V 769		66.9	9.5	33.3	— 16	11.0
V 794		71.9	9.5	33.5	1	9.4
V 796		78.1	8.0	35.2	4	7.7
V 798		74.1	9.2	33.4	5	8.7
V 800		70.6	9.1	31.7	— 2	9.3
V 659	Barley silage	70.3	10.3	30.0	14	8.9
V 695		66.1	12.7	33.1	12	11.2
V 696	Peavine silage	65.5	12.9	32.0	15	11.0
V 697	Barley silage	65.1	12.4	31.5	11	11.0
V 714	Ripe oat silage	48.7	16.5	29.0	10	14.9
V 717		63.1	11.2	30.6	3	11.3
V 719	Peavine silage	63.3	13.3	36.0	1	13.2
V 720	Ripe oat silage	53.4	14.6	27.8	11	13.0
V 721	Peavine silage	66.4	11.8	35.3	— 1	11.9
V 722	Lucerne silage	55.7	14.8	32.3	3	14.3
V 724	Oat silage	54.5	13.7	28.4	6	12.9
V 741		59.1	14.0	30.6	11	12.5
V 754	Maize silage	69.0	10.0	28.3	12	8.8

(mean values of +5.6 and +4.6, respectively) so that nitrogen in lignin usually has a negative digestibility (mean value —3.9, range +32 to —34).

From the values in Tables 5 and 6 correlation and regression coefficients and standard errors were calculated for total lignin, protein-free lignin and indigestible lignin with digestibility of organic matter (Table 8). The forages were grouped into fresh grass, grass hay, grass silages and other silages the groups of other fresh and of other dried products being too small to yield meaningful figures. Fresh grass, grass hay and grass silages were also combined to give values for all grasses. Table 5 listed 14 other dried products. Nine of these (meals and pellets) were finely ground products whose digestibility is depressed by rapid passage through the rumen; these 9 samples were omitted. Likewise sugar beet shoots V 624 were an aberrant material. There are less samples in the calculations for indigestible lignin than for total and protein-free lignin, but even so the various groups of roughage are fairly well represented for indigestible lignin.

Table 7. Digestibility (D) of lignin, 'protein-free' lignin and nitrogen in lignin in 22 samples by sheep.

Sample No.	Type	DOM	% lignin (in organic matter)		D lignin
			roughage	faeces	
V 785	Fresh grass	64.4	11.3	28.2	12
V 793		72.5	11.8	34.3	20
V 654	Fresh grass meal	58.6	14.4	29.1	17
V 693	Grass hay	75.0	9.0	30.4	16
V 703		61.2	14.6	33.0	12
V 723		57.3	13.3	29.5	5
V 725		56.6	13.3	29.4	4
V 735		56.2	13.3	30.9	— 2
V 756		73.4	9.1	29.6	13
V 667	Grass hay pellets	60.6	11.8	29.0	3
V 715	Grass silage	76.4	8.3	35.7	— 1
V 716		69.1	9.2	32.6	— 10
V 745		73.6	8.5	32.6	— 1
V 765		69.8	9.9	32.6	1
V 767		68.2	10.6	34.1	— 3
V 769		66.9	9.5	33.3	— 16
V 794		71.9	9.5	33.5	1
V 796		78.1	8.0	35.2	4
V 798		74.1	9.2	33.4	5
V 800		70.6	9.1	31.7	— 2
V 695	Barley silage	66.1	12.7	33.1	12
V 754	Maize silage	69.0	10.0	28.3	12

Table 8. Correlation and regression coefficients and standard error of lignin, protein-free lignin and indigestible lignin with digestibility of organic matter by sheep.

Type of roughage	Total lignin			
	Number of samples	Correlation coefficient	Regression coefficient	Standard error
Fresh grass	22	— 0.846	— 3.18	3.9
Grass hay	16	— 0.891	— 3.27	3.3
Grass silages	26	— 0.692	— 2.13	3.4
All grasses	64	— 0.853	— 2.98	3.5
Other silages	13	— 0.895	— 3.22	3.1
All roughage	85 [1]	— 0.888	— 3.12	3.6

1. Including 3 other fresh products and 5 other dried products.

% protein-free lignin (% in organic matter)		D protein-free lignin	% N (in organic matter)		D N in lignin
roughage	faeces		roughage	faeces	
9.7	23.2	14	0.257	0.79	— 9
8.2	25.3	15	0.573	1.44	31
11.0	23.5	12	0.544	0.89	32
7.2	23.8	17	0.287	1.06	6
11.6	26.9	10	0.477	0.99	19
10.6	23.8	4	0.432	0.92	9
10.9	24.1	4	0.387	0.91	— 2
10.7	25.3	— 4	0.414	0.89	6
6.9	22.9	12	0.350	1.07	19
9.1	22.8	1	0.431	0.99	10
6.2	26.9	— 3	0.334	1.42	— 0
7.3	24.8	— 5	0.297	1.25	—30
6.7	26.0	— 3	0.284	1.05	2
8.0	25.3	5	0.305	1.17	—16
8.3	26.5	— 1	0.364	1.23	— 7
7.6	25.9	—13	0.296	1.19	—29
7.5	25.9	4	0.312	1.21	— 9
6.1	26.0	7	0.305	1.47	— 6
7.5	25.9	11	0.272	1.21	—16
7.6	25.4	1	0.233	1.00	—25
11.1	26.6	19	0.260	1.03	—34
8.6	23.3	16	0.217	0.88	—26

Protein-free lignin			Indigestible lignin			
Correlation coefficient	Regression coefficient	Standard error	Number of samples	Correlation coefficient	Regression coefficient	Standard error
—0.921	—3.29	2.8	10	—0.950	—3.29	2.1
—0.931	—4.02	2.6	11	—0.900	—3.09	1.6
—0.810	—2.75	2.8	14	—0.971	—3.57	1.0
—0.910	—3.45	2.8	35	—0.983	—3.41	1.3
—0.944	—3.29	2.3	13	—0.877	—3.19	3.3
—0.931	—3.33	2.8	54 [2]	—0.940	—3.37	2.8

2. Including 3 other fresh products and 3 other dried products.

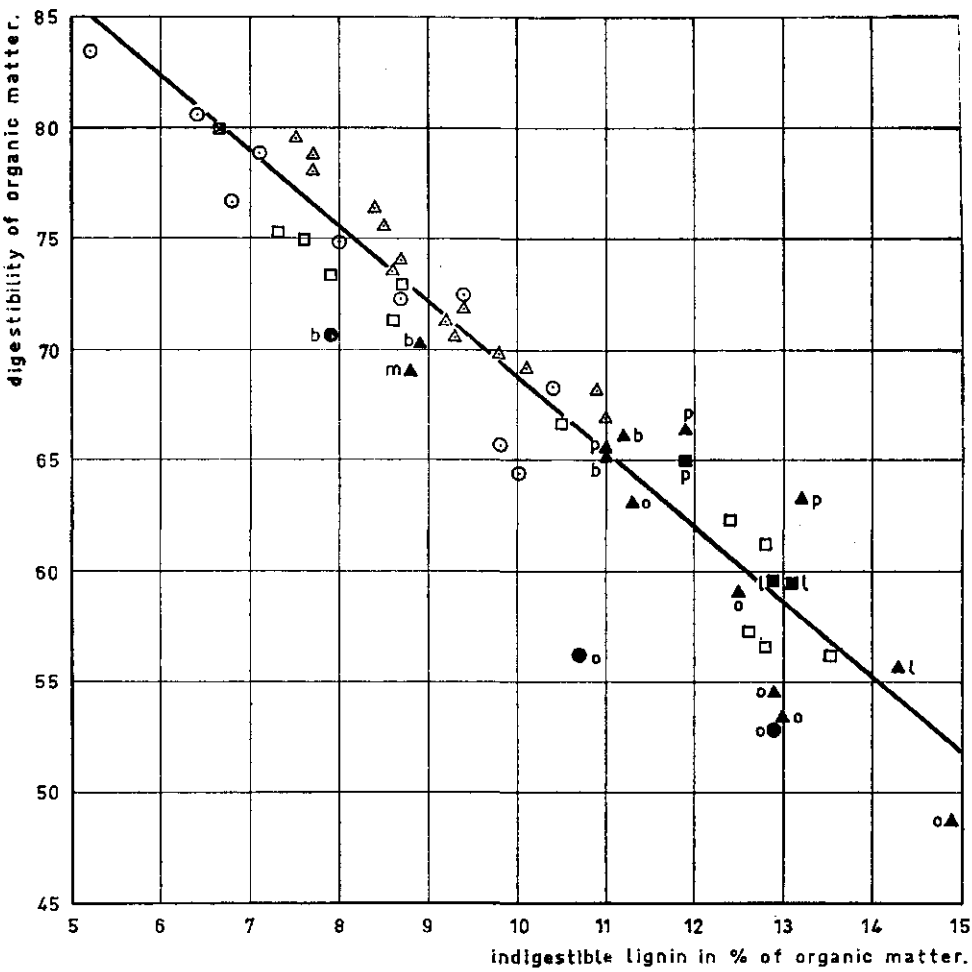

Fig. 1. Correlation between indigestible lignin and digestion coefficient of organic matter. The regression line was calculated for all grass samples.

For total lignin correlation coefficients are rather low and standard errors rather high, while regression coefficients vary from —2.1 to —3.3. Correlation coefficients and standard errors for protein-free lignin are markedly better, but the regression coefficients still show an appreciable variation (from —2.8 to 4.0). However, still better figures were obtained in the calculations for indigestible lignin, at least for the grasses; the diverse group of other silages had a lower correlation coefficient and a higher standard error than for protein-free lignin. Inclusion of other silages and of the three other fresh products made the figures for all roughage practically the same for indigestible and for protein-free lignin. The regression coefficients for indigestible lignin vary much less (from —3.1 to —3.6).

The correlation between indigestible lignin and digestibility of organic matter is illustrated in Fig. 1, in which the different groups of roughage are indicated by different signs. The regression line is that for all grasses. The regression line for all samples would hardly be different, as the constants for all grasses and for all samples respectively ware: $\bar{x}$ 9.2, 10.1, $\bar{y}$ 71.2, 67.7, and b —3.41, —3.37. It will be noted that the points of the grasses generally lie close to the regression line and that aberrant samples are chiefly of other fresh and dried products and of other silages; the oat samples are particularly aberrant. Unfortunately the material included only seven samples of leguminous roughage so that reliable coefficients could not be calculated for them; of these seven samples only two peavine silages lie away from the regression line.

2.2 Comprehensive analysis of roughage and faeces and calculation of digestibility of proximate constituents

Table 9 contains analytical data on twelve samples from Hoorn which were large enough (about 50 g being required). For the analysis of silages, amounts of volatile acids lost during drying were added to organic matter and to ether extract; together with the lactic acid they account for the high values of ether extract in silages.

The aberrant nature of sugar beet shoots is shown by the extremely high figure for aqueous extract (78.5 %). In the beet shoots the residue from aqueous extraction was too small for an estimate of insoluble protein, nitrogen in lignin, acetyl and CO_2 from uronic acids. Table 9 also lists analytical data on three samples from IBVL and on a sample of cow dung from the Institute for Biological and Chemical Research on Field Crops and Herbage.

The sum of the nine constituents accounts for 93 to 98 % of the dry matter of the roughage and for 99 % of the dung sample. As already pointed out in Section 1.3, the present scheme does not account for non-cellulosic hexosan. Waite & Gorrod (1959) mentioned 2.7 % of this constituent in young cocksfoot grass and Gaillard & Bailey (1968) found about 1 % galactose polysaccharide in red clover. Another unaccounted constituent is the lipid part of liproproteins, because it is not extracted by ether and is free from nitrogen. According to Dr W. B. Deys of the Institute for Biological and Chemical Research on Field Crops and

Table 9. Comprehensive analysis of roughages in percentage of dry matter (insoluble protein corrected for N in lignin).

Sample No.	Type	Ether extract	Aqueous extract	Insoluble ash	Insoluble protein
V 624	Sugar beet shoots	0.4	78.5	2.6	—
V 627	Grass silage	12.4	27.8	4.2	3.1
V 629	Hay pellets	1.6	15.3	3.2	4.7
V 636	Hay pellets	2.7	24.3	3.5	5.1
V 640	Hay pellets	2.4	24.4	3.2	4.7
V 654	Grassmeal	2.7	31.5	4.3	9.8
V 667	Hay pellets	2.3	24.8	3.8	7.0
V 690	Dried peavines	1.3	34.4	12.4	7.1
V 745	Grass silage	11.6	32.2	3.5	3.6
V 748	Valerian straw	4.9	29.6	29.6	6.8
V 773	Grassmeal	2.1	34.3	6.6	8.7
V 788	Milk-ripe oats	4.3	34.4	1.2	2.5
	Crushed hay 27 A	3.8	31.5	3.1	5.3
	Crushed hay 27 B	3.8	30.5	3.7	5.1
	Lucerne pellets	5.0	37.9	2.7	12.6
	Cow dung	5.0	18.4	8.6	4.6

Table 10. Comprehensive analysis of roughages and faeces from metabolic trials with cows and sheep.

	% of dry matter			% of organic matter	
	total ash	soluble ash	organic matter in aqueous extract	ether extract	organic matter in aqueous extract
Hay L1O	10.3	5.9	14.4	2.4	16.1
Hay L5W	9.3	7.2	15.0	2.7	16.5
Hay L4M	8.9/9.2	5.1/5.5	17.2/16.8	2.7/2.7	18.8/18.5
Silage R90	9.2	7.2	24.7	5.7	27.2
Hay R90/91	8.6	6.9	23.8	4.8	26.0
Faeces L1O Cow 6	22.7	3.9	8.5	4.2	11.0
L5W Cow 5	17.7	4.6	9.9	5.0	12.0
L4M Cow 6	19.4	3.7	8.5	4.8	10.5
L4M Cow 5	17.9	2.6	10.8	4.2	13.2
silage R90 sheep B	18.6	4.5	13.6	7.3	16.7
R90 sheep C	18.6	6.3	15.7	8.1	19.3
R90 sheep D	16.8	4.5	13.3	7.9	16.0
hay R90/91 sheep D	16.5	4.6	12.1	8.3	14.5
sheep A	14.7	5.5	15.4	8.4	18.1
sheep E	15.5	5.7	14.9	8.4	17.6

Lignin	N in lignin (%)	Pentosan	Acetyl	Uronic CO_2	Alpha-cellulose	Unaccounted
1.9	—	5.3	—	—	7.1	—
7.7	3.23	15.8	0.9	1.3	24.7	2.1
15.1	2.52	23.5	1.3	1.1	29.2	5.0
11.5	2.71	20.6	1.1	1.2	25.1	4.9
11.4	2.58	20.7	1.2	1.2	26.4	4.4
12.8	3.78	10.6	1.4	1.6	21.7	3.6
10.8	3.65	18.7	1.0	1.2	23.5	6.9
10.5	4.32	10.5	1.2	1.5	17.0	4.1
7.5	3.34	14.2	0.9	1.2	20.7	4.6
7.1	4.23	5.6	0.7	1.6	10.5	3.6
9.1	4.37	12.6	0.8	1.2	17.6	7.0
11.7	1.50	19.4	1.3	0.7	20.9	3.6
8.7	2.86	17.2	1.1	1.0	23.3	5.0
9.3	2.95	17.1	1.1	1.0	22.3	6.1
9.0	4.07	8.9	0.9	1.7	16.6	4.7
25.8	2.46	16.6	1.3	0.9	18.1	0.7

insoluble protein	lignin	pentosan	acetyl	uronic CO_2	alpha-cellulose	un-accounted	N in lignin (%)
5.6	12.4	22.9	1.31	1.28	31.9	6.1	2.80
5.7	11.9	22.4	1.01	1.30	31.6	6.9	3.02
4.4/4.4	11.0/11.1	22.3/22.3	1.27/1.27	1.28/1.29	31.0/31.1	7.2/7.3	2.51
9.3	8.7	17.2	0.94	1.11	23.5	6.4	4.23
9.9	8.5	17.4	0.96	1.11	25.8	5.5	4.39
7.7	35.3	17.0	0.77	1.16	22.9	—0.1	3.25
7.6	35.6	16.5	0.73	1.09	20.2	1.3	3.40
7.5	34.4	16.9	0.86	1.13	18.7	5.2	3.20
7.6	33.3	16.4	0.91	1.17	19.1	4.1	3.35
9.4	33.3	13.1	0.71	0.95	17.8	0.8	3.73
8.2	28.6	14.5	0.93	0.79	17.5	2.1	3.80
7.7	27.6	17.3	0.97	0.91	20.4	1.2	3.59
7.6	27.0	18.1	1.20	0.89	21.6	0.8	3.57
7.9	26.7	15.5	0.94	0.79	19.3	2.4	3.77
7.2	26.8	16.1	1.15	0.82	21.0	0.9	3.56

Table 11. Digestibility of the organic components of roughages.

Roughage	Animal	Organic matter	Ether extract	Organic matter in aqueous extract	Insoluble protein
Hay L1O	Cow 6	66.9	42	78	55
L5W	Cow 5	69.1	44	78	60
L4M	Cow 6	68.4	44	82	45
L4M	Cow 5	66.6	48	76	43
Silage R90	Sheep B	83.3	79	90	83
	Sheep C	77.3	68	84	80
	Sheep D	74.1	65	85	78
Hay R90/91	Sheep D	73.0	54	85	79
	Sheep A	75.3	56	83	80
	Sheep E	74.5	56	83	82
Mean		72.9	56	82	69

Herbage it may amount to 1.5 %. Table 10 contains analytical data on the samples of roughage and on corresponding faeces received from the Department of Animal Physiology. From the values for total ash supplied by the Department of Animal Physiology, the percentage water-soluble organic matter was calculated. The percentage unaccounted for is 5.5 tot 7.3 % for the roughage and 0 to 5.2 % for the faeces. Nitrogen in lignin varies from 2.5 tot 4.4 % in roughage and from 3.2 to 3.8 % in faeces.

In Table 11 is listed the digestibility of the different constituents calculated as in Section 2.1 and of organic matter as supplied by the Department of Animal Physiology. Digestibility coefficients were lower in the trials with cows than with sheep, perhaps because there was more lignin in the cows' roughage. This difference is most evident for lignin, being 0 to 8 % in the trials with cows and 14 to 36 % with sheep. But Table 6 shows that lignin may have a low or negative digestibility for sheep. The differences in digestibility are still more striking for nitrogen in lignin, in the cows being constantly negative and in the sheep distinctly positive.

An important feature is that the digestibilities of water-soluble organic matter are of the same magnitude as those of the cell-wall constituents other than lignin and that these constituents of the cell-wall show little variation among themselves (mean values at the bottom of Table 11). The digestibilities of unaccounted constituents are nearly always higher than those of aqueous extract and cell-wall carbohydrates. Although the values for unaccounted constituents are less accurate than for cell-wall carbohydrates, this is certainly remarkable. The digestibility of insoluble protein is generally a bit lower, and of ether extract distinctly lower than of aqueous extract and cell-wall carbohydrates. The higher digestibility of ether extract in silages will be due to the high proportion of organic acids.

Lignin	Pentosan	Acetyl	Uronic CO_2	Alpha-cellulose	Un-accounted	N in lignin
6	76	78	70	76	100	— 10
8	77	77	75	80	94	— 4
1	76	79	72	81	78	— 20
0	75	76	70	79	81	— 34
36	87	87	86	87	98	43
25	81	78	84	83	92	33
18	74	73	78	77	95	30
14	72	67	78	78	96	30
22	78	76	82	81	89	33
20	76	70	82	79	96	35
15	77	76	78	80	92	14

3 Discussion

Lignin estimated after mild extractions successively with ether and hot water may contain more than 5 % nitrogen and be up to 36 % digestible (Tables 5 and 6). However, there is no correlation between digestibility and nitrogen of lignin. As discussed in Section 1.1, lignin estimated after more drastic pretreatment generally has less nitrogen but may even so be appreciably digestible. The negative digestibility of lignin indicates that during the passage through the intestine there is a net synthesis of lignin; the preponderantly negative digestibility of nitrogen in lignin (Table 7) indicates that this net synthesis may be partly by attachment of nitrogen compounds to the lignin macromolecule. Whether this occurs in the rumen of further along the intestine is conjectural.

The correlation coefficients for total lignin shown in Table 8 are of the same magnitude as found by van Soest (1964) for 37 grasses and lower than found by Sullivan (1959) for 36 grasses. The coefficients for protein-free lignin approach that of Sullivan but those for indigestible lignin (at least for grasses) are distinctly better. It looks as though only the indigestible part of the lignin inhibits digestion of other constituents. A way is therefore needed of estimating this indigestible lignin. In my view drastic treatment like refluxing with dilute mineral acid, application of proteinases at extreme pH values (pepsin-HCl or trypsin-Na_2CO_3) or detergent solutions will not succeed. A microbial proteinase active at neutrality could yield better results and is now being tried.

Of course my scheme for comprehensive analysis could be extended by estimating for instance total nitrogen and by further analysis of ether extract and hot-water extract. It gives a fair idea of the digestibility of cell-wall carbohydrates but does not distinguish between hemicellulose and true cellulose [2] but such distinction is difficult. Pectin could also be separately extracted but its distinction from hemicellulose is equally blurred.

The incomplete digestion of the water-soluble organic matter (which represents the bulk of the cell contents) calls for further comment. Van Soest (1966) may be right in stating that the true digestibility of cell contents (organic matter soluble in neutral detergent solution) is at least 98 % but this does not seem true of apparent digestibility as found in trials with livestock in Wageningen. Faeces will contain

2. With true cellulose is meant a polysaccharide built up exclusively of at least 1000 glucose units by β-1-4 bonds.

some water-soluble material derived from the digestive juices of the intestine beyond the rumen and thus depress the apparent digestibility of the aqueous extract.

The nearly equal digestibility of pentosan, acetyl, CO_2 from uronic acids [3] and alphacellulose [4] seems to be a new feature (compare the figures of Waite et al., 1964 in Section 1.2 and the discussion in Gaillard's 1966 paper). The number of comprehensively analysed samples from digestion trials is still limited but I hope to extend such analysis to other types of roughage such as legumes to gain a wider picture.

3. The sum of these three constituents constitutes the bulk of the hemicellulose + pectin.
4. The alphacellulose free from ash and pentosan as estimated here is considered to be true cellulose.

Summary

Lignin in roughage is usually estimated in defatted material which has been pretreated more or less severely, for instance with proteolytic enzymes at extreme pH values, refluxing with dilute mineral acids or detergent solutions.

Mild aqueous extraction in a boiling water bath yielded more lignin with a higher content of nitrogen in many samples of roughage and faeces from digestion trials. From the digestibility of organic matter and the lignin contents of roughage and faeces, the percentage of indigestible lignin in roughage was calculated.

Correlation and regression coefficients and standard errors were calculated for total, protein-free and indigestible lignin against the digestibility of organic matter. For grasses (including fresh grass, grass hay and grass silage) the following values were obtained for total lignin, protein-free lignin and indigestible lignin, respectively: correlation coefficient —0.85, —0.91, —0.98; regression coefficient —3.0, —3.5, —3.4; standard error 3.5, 2.8, 1.3.

A scheme for comprehensive analysis of straw was used on some large samples of roughage and faeces. This scheme comprises ether extract, hot-water extract, insoluble ash, insoluble protein (corrected for N in lignin), ash-free lignin, pentosan, acetyl, CO_2 from uronic acids and alphacellulose free from ash and pentosan.

These nine constituents account for 93-98 % of the dry matter in roughage and for 95 to 100 % of that in faeces. The organic matter unaccounted for probably consists of non-cellulosic hexosan and lipid in lipoprotein.

From the digestibility of organic matter and from total ash in the samples, digestibility was calculated of organic constituents. Average digestibilities of water-soluble organic matter, pentosan, acetyl, CO_2 from uronic acids and alphacellulose were of the same magnitude (76 to 82 %) but that of unaccounted matter was distinctly higher (92 %). Digestibility was lower for insoluble protein, 69 % and ether extract, 56 %. Digestibility of lignin averaged 15 %, range 0 to 36 %.

Acknowledgements

The samples of roughages and faeces were from digestion trials with wethers at the Hoorn Research Institute for Livestock Nutrition and from metabolic trials with cows and sheep at the Department of Animal Physiology of the Agricultural University at Wageningen. Dr N. Dijkstra (Hoorn) and Dr A. H. J. van Es (Wageningen) provided me with data from these trials.

Miss M. S. M. Bosman (IBS Wageningen) suggested that I calculate correlation and regression for protein-free lignin.

References

Armitage, E. R., R. de B. Ashworth & W. S. Ferguson	1948	The determination of lignin in plant material of high protein content. J. Soc. Chem. Ind. 67: 241.
Asselman, T. H., W. van der Broek, J. G. Dijkhuis & F. M. Muller	1956	Über den Einfluss des Hemicellulose- und Ligningehaltes auf einige papiertechnischen Eigenschaften von Strohzellstoff. Treat. Neth. Exp. Stn Util. Straw 4.
Balch, D. A., C. C. Balch & S. J. Rowland	1954	The influence of the method of determination of lignin on the lignin-ratio technique for digestibility in the cow. J. Sci. Fd Agric. 5: 584.
Ellis, G. H., G. Matrone & L. A. Maynard	1946	A 72 percent H_2SO_4 method for the determination of lignin and its use in animal nutrition studies. J. Anim. Sci. 5: 285.
Ely, R. E. & L. A. Moore	1955	Holocellulose and the summative analysis of forages. J. Anim. Sci. 14: 718.
Freudenberg, K. & A. C. Neish	1968	Constitution and biosynthesis of lignin. Springer-Verlag, Berlin.
Gaillard, Blanche D. E.	1966	Calculation of the digestibility for ruminants of roughages from the content of cell-wall constituents. Neth. J. agric. Sci. 14: 215.
Gaillard, Blanche D. E. & R. W. Bailey	1968	The distribution of galactose and mannose in the cell-wall polysaccharides of red clover (Trifolium pratense) leaves and stems. Phytochemistry 7: 2037.
Muller, F. M.	1967a	Cooking quality of pulses. J. Sci. Fd Agric. 18: 292.
Muller, F. M.	1967b	Some thoughts about composting. Mushr. Sci. 6: 213.
Norman, A. G. & S. H. Jenkins	1934	The determination of lignin. II. Errors introduced by the presence of proteins. Biochem. J. 28: 2160.
Ritman, E. L., F. M. Muller & J. G. Dijkhuis	1948	Chemical composition of cereal straw. Paper Ind. 29: 1751.
Smolders, P. M.	1955	Digestions of straw and hardwood with magnesium monosulphite. Treat. Neth. Exp. Stn Util. Straw 3.
Soest, P. J. van	1963a	Use of detergents in the analysis of fibrous feeds. I. Preparation of fiber residues of low nitrogen content. J. Ass. off. agric. Chem. 46: 825.
Soest, P. J. van	1963b	Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fiber and lignin. J. Ass. off. agric. Chem. 46: 829.
Soest, P. J. van	1964	Symposium on nutrition and forage and pastures: new chemical procedures for evaluating forages. J. Anim. Sci. 23: 838.

Soest, P. J. van, R. H. Wine & L. A. Moore	1966	Estimation of the true digestibility of forages by the in vitro digestion of cell-walls. Proc. 10th Int. Grassld Congr., Helsinki, Section 2, p. 88.
Sullivan, J. T.	1955	Cellulose and lignin in forage grasses and their digestion coefficients. J. Anim. Sci. 14: 710.
Sullivan, J. T.	1959	A rapid method for the determination of acid-insoluble lignin in forages and its relation to digestibility. J. Anim. Sci. 18: 1292.
Waite, R. & A. R. N. Gorrod	1959a	The structural carbohydrates of grasses. J. Sci. Fd Agric. 10: 308.
Waite, R. & A. R. N. Gorrod	1959b	The conprehensive analysis of grasses. J. Sci. Fd Agric. 10: 317.
Waite, R. M. J. Johnston & D. G. Armstrong	1964	The evaluation of artificially dried grass as a source of energy for sheep. I. The effect of stage of maturity on the apparent digestibility of rye-grass, cocksfoot, and timothy. J. agric. Sci. 62: 391.
Whitehead, D. L. & G. V. Quicke	1964	A comparison of six methods of estimating lignin in grass hay. J. Sci. Fd Agric. 15: 417.