Dehydrated Alfalfa Composition. Correlation of Nonnutrient Components with Protein Content

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Eight samples of dehydrated alfalfa, ranging from 16 to 30% in protein content, were analyzed for nine major components. Nutritional and nonnutritional fractions for nonruminant animals were calculated. Fiber fractions were prepared by sequential extraction with benzene-alcohol and with dilute ammonium oxalate solution. An indirect method for cellulose was developed which avoids destructive effects of reagents commonly used. The sum of this cellulose, and the pentosans, lignin, ash, and anhydrouronic acid is considered to supply little or no energy for nonruminant animals, and shows a high degree of negative correlation with protein content.

The method of determining crude fiber in plant material as introduced by Henneberg and Stohman (Hansen *et al.*, 1958) over a hundred years ago is still used by animal nutritionists to predict the quality of a forage as a feedstuff. The original authors pointed out that crude fiber is not a chemical entity (Norman, 1935; Hallab and Epps, 1963; Hallsworth, 1950; Walker and Hepburn, 1955). In addition to having lost some of the cellulose and lignin, it still has varying proportions of the pentosans and protein.

Plant scientists have used newer methods for structural components of forages based on hot dilute ammonium oxalate extraction of defatted plant material (Henderson, 1928; Weihe and Phillips, 1947; Binger *et al.*, 1961; Sullivan, 1964). This extraction has the advantage of being done close to neutrality, and consequently avoids the destructive action of high or low pH. However, it produces a residue which still contains most of the protein of the original plant.

Another more recent scheme, designed to correlate with ruminant nutrition, is the acid detergent method (Van Soest, 1962, 1963, 1965). By this procedure, essentially all of the protein is removed from the residue, but much of the structural carbohydrate, especially pentosans, is destroyed.

Although the crude fiber and acid detergent fiber procedures produce an insoluble residue whose quantity can be more or less closely correlated with the feed value for ruminant animals, the authors were aware of no methods designed for use with nonruminant animals. While the rumen of cattle and sheep are large fermenters, where the action of bacteria converts much of the cellulose and hemicellulose to simpler carbohydrates and fatty acids, the nonruminant (chicken, pig) can make little use of those materials. As part of a large cooperative study to compare composition and nutritional values of different grades of dehydrated alfalfa, a method was developed for estimating the total lignin and carbohydrate fraction not digestible by monogastric animals.

Data accumulated during this study show the incompleteness of the distribution of the various chemical entities during ammonium oxalate and acid detergent extractions. Whenever dried plant material is treated with an extracting agent, some of nearly every chemical constituent goes into solution or is destroyed, and with the exception of the most simple compounds, none goes into solution completely within practical treatment limits. Consequently, the separations, by whatever solvent, of fatty materials, protein, cell wall constituents, and ash are only relatively complete.

MATERIALS AND METHODS

Each of four samples was a blend of 10 commercially dehydrated alfalfa meals chosen to give four different protein levels spaced through the normal range for commercial samples. These samples were prepared from materials gathered throughout the United States for analyses of vitamins, minerals, and amino acids (American Dehydrators Association, 1965). Four other samples were from a high protein, commercially dehydrated alfalfa meal. They included the original meal, a leaf fraction made from the original, and two blends of original and leaf meal to obtain intermediate protein values (Kohler *et al.*, 1966). Each of the eight samples was prepared in duplicate and all fractionations and analyses were performed in duplicate. Results reported are averages, and are on a moisture-free basis.

Doubly Extracted Residues (DER). The method of Henderson (1928), as modified by Binger *et al.* (1961), was used and consisted of a Soxhlet extraction with a benzene-alcohol mixture followed by a 4-hour and a 16-hour, 85° C. extraction with 0.5% ammonium oxalate solution. The difficulty of filtering a large volume of hot solution resulted in the use of centrifugation instead and transferral to a filter for the final washing and drying. Others who have used the benzene-alcohol extraction have not mentioned the point but, unless anhydrous conditions are rigorously maintained, the results will be meaningless. Any water present will put into solution significant quantities of material which should be removed later during the dilute oxalate extraction. The purpose of this work was not concerned with isolating the fats and waxes, so 95% alcohol-benzene and air-dried samples, whose moisture contents were determined separately, were used. The residue was not dried and weighed until completion of the ammonium oxalate extraction, this is reported as "doubly extracted residue."

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Acid Detergent Fiber (ADF). This was prepared by the method of Van Soest (1963) except that the sample weights and solution volumes were scaled up fivefold in order to have enough residue for subsequent analyses. Comparative tests at both levels gave comparable results.

Methods of Analysis. Methods of the Association of Official Agricultural Chemists (1960) were used for the determination of crude fat, crude fiber, nitrogen, and total and reducing sugars. Methods and conditions were as follows:

MOISTURE. 70° C. under vacuum for 16 hours.

CRUDE FAT. Soxhlet extraction with ethyl ether for 16 hours.

CRUDE FIBER. Digestion of the ethyl ether extracted residue with 0.255*N* H_2SO_4 followed by 0.313*N* NaOH, for 30 minutes each.

ASH. 550° C. for 16 hours.

NITROGEN. Improved Kjeldahl method.

TOTAL SUGARS. Determined by the Shaffer-Somoyi method after extracting with 80% ethanol, clearing with ion exchange resin, and inverting with invertase.

REDUCING SUGARS. Determined as for total sugars, but the invertase step was omitted.

PROTEIN. Per cent nitrogen × 6.25.

LIGNIN. The Ellis *et al.* method (1946) was used with slight modification as follows: Extraction thimbles with fritted disks were used instead of alundum crucibles. The hydrolyses in 5 and 3% sulfuric acid were carried out in an autoclave for 10 minutes at 17 pounds (Thacker, 1954) rather than by refluxing for 1 hour. Ashing was done at 500° C. for 3 hours to avoid fusing the fritted filters. Nitrogen determinations were made on a separate series of samples.

ANHYDROURONIC ACID. The residue remaining after extracting 5 grams of air-dried sample with 500 ml. of 80% ethanol for sugar determination was washed thoroughly with 80% ethanol, then extracted twice with 0.5% ammonium oxalate as for the preparation of DER. The solution was acidified by passing through a Dowex 50 (H^+) cation exchange column, and concentrated to 50 ml. under vacuum in a rotary evaporator at about 60° C. Five volumes of 95% ethanol were added, and the precipitate removed by centrifugation, washed with 95% ethanol, dried at 60° C. in vacuum overnight, and weighed. Anhydrouronic acid in that material was determined by the carbazole method of McComb and McCready (1952). Pectin, which is made up of the anhydrouronic acid plus an unknown fraction of the pentosans, cannot be easily determined.

PENTOSANS. Furfural produced by distillation from a suspension of the sample in 12% hydrochloric acid was determined by the method of Adams and Castagne (1948). The color produced by the reaction of furfural with aniline in acetic acid was measured in an Evelyn colorimeter with a 515-mμ filter. Furfural estimated from a standard curve × 1.55 (Binger *et al.*, 1961) equals xylose. A stock standard solution of freshly distilled furfural in absolute alcohol is stable for at least a year. Pure polygalacturonic acid also yields furfural equivalent to 30.6% xylose. Consequently, a correction of that amount of the anhydrouronic acid was subtracted from each pentosan value.

STARCH. The method of McCready *et al.* (1950) was applied to alfalfa except that the reagent and conditions for the anthrone color development were those used by Johnson *et al.* (1964).

RESULTS AND DISCUSSION

In Figure 1, the percentages of the original alfalfa meal appearing in the fractions prepared by both DER and ADF methods are compared with the protein content of the corresponding meals. Protein is the most commonly used indicator of forage quality, being highest in young, fast-growing leafy plants and lowest in the old or slow-growing or stemmy plants. Commercially dehydrated alfalfa meals usually fall in the 15 to 24% protein range, but highest quality leaf meal may contain over 30% protein (Kohler *et al.*, 1966). There are high negative correlations between the amount of DER and ADF and the protein content of the corresponding alfalfa samples (Figure 1).

The sodium chlorite holocellulose method of Wise *et al.* (1946) as modified by Whistler *et al.* (1948) and Binger *et al.* (1961) was applied to each of the residues. After four chlorite treatments, there was still 20 to 40% of the original lignin left in the doubly extracted residue and 10 to 25% in the acid detergent fiber, but an additional 5 to 10% of the pentosans and about half the remaining protein of either residue was removed. In view of these facts, no further use was made of the chlorite treatment.

Table I shows the analyses of each of these alfalfas for nine major components. The analyses of four of these same samples for 17 amino acids, 13 minerals, and 13 vitamins are listed in another report (American Dehydrators Association, 1965). Organic acids are being determined on the same samples and will be reported later. Table II shows the percentages of each component originally in the dehydrated alfalfa that remain in the DER and ADF residues. The material which has been removed may either remain in the extracting solution or in some cases may be destroyed by the extraction. From the three tables some generalizations can be made.

Crude Protein (N × 6.25). The use of the 6.25 protein factor is open to question. Calculations based on amino acid recovered indicates a factor of about 5.8. However, this factor is based on an amino acid recovery of only about 80% of the total nitrogen. When the composition

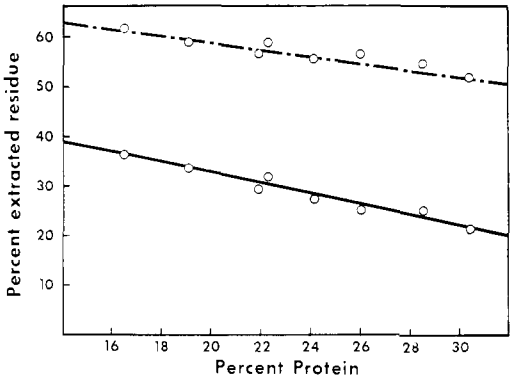

Figure 1. Comparison of residue yield with protein content

–·–·– Doubly extracted residue
$y = 71.1 - 0.61X \quad r = -0.971$
—— Acid detergent residue
$y = 53.6 - 1.05X \quad r = -0.981$

Table I. Components in Alfalfa as Per Cent of Original Meal[a]

Crude Protein	Crude Fiber	Ether Extractables	Ash	Pentosans	Lignin	Total Sugars	Reducing Sugars	Anhydro-uronic Acid	Starch
16.5	31.4	3.0	9.4	11.2	8.6	3.0	1.4	4.5	3.7
19.1	28.6	3.6	10.3	9.9	8.3	2.7	1.2	4.7	2.4
21.9	24.4	4.1	11.0	8.9	6.9	2.9	1.3	4.8	2.8
22.3	24.4	4.6	9.9	9.9	6.0	2.5	0.9	4.8	2.7
24.1	22.0	4.6	11.5	8.2	5.8	2.9	1.2	4.6	2.8
26.0	19.4	5.4	10.0	8.2	5.5	2.2	0.9	5.0	3.4
28.5	17.4	5.8	10.1	7.2	4.8	2.0	1.0	5.1	3.5
30.4	14.9	6.3	10.0	6.1	4.7	1.7	1.1	5.4	3.3

[a] Moisture-free basis.

Table II. Composition of Residues[a]

Protein of Original Meal, %	Residue of Original Meal, %	Original Component of Whole Alfalfa Remaining in Residues, %				
		Protein	Ether extractables	Ash	Pentosans	Lignin
		DOUBLY EXTRACTED RESIDUE				
16.5	62	73	3	32	84	100
19.1	59	75	2	37	76	89
21.9	57	73	2	37	72	90
22.3	59	76	4	45	81	102
24.1	56	79	0	43	72	106
26.0	56	75	5	46	75	94
28.5	54	76	3	51	74	103
30.4	53	76	3	52	64	104
		ACID DETERGENT FIBERS				
16.5	36	15	6	11	27	78
19.1	34	13	2	10	28	77
21.9	30	11	2	10	25	73
22.3	32	13	4	18	24	83
24.1	28	11	2	10	30	79
26.0	26	10	3	17	25	76
28.5	25	11	3	21	28	87
30.4	22	10	1	19	25	83

[a] Moisture-free basis.

of the remaining 20% of unidentified nitrogen fraction is established, a more precise figure for the protein conversion factor should be used in summative analysis. Regardless of the amount in the alfalfa, dilute ammonium oxalate solution removes only approximately 25% of it. This is apparently nonprotein nitrogen present largely as free amino acids. Acid detergent removes 85 to 90% of the crude protein, the higher percentage being extracted from those samples with the most protein.

Ether Extractables. About 3% of the original ether extractables remain in the residue obtained by either the DER or ADF method.

Ash. Although the ash content is relatively constant in the original samples regardless of protein content, after extraction by the DER method, the higher the original protein, the more ash there is in the fiber fraction.

Lignin. Lignin is reported on the ash- and protein-free basis. The pepsin treatment is not completely effective in reducing the nitrogen content of the lignin, and the results in Table III show that a nitrogen correction is necessary. Amino acid analyses of lignin protein indicates little difference between it and the protein of the whole plant but recovery values show that one quarter to one half the nitrogen is not recovered as amino acids by the standard analytical procedure used (Kohler and Palter, 1967). The 6.25 factor is possibly inaccurate for converting lignin nitrogen to protein, but is the best available at present. As is to be expected, lignin content decreases as crude protein increases. With alfalfa, the acid detergent removes 13 to 27% of the lignin present in the original sample. Norman (1935) in one experiment showed similar results when a variety of plant materials were extracted with 0.255*N* acid for 30 minutes, but surmised that the apparent loss of lignin was caused by the loss of lignin-connected protein. However, he failed to show the

Table III. Protein Remaining in Lignins as Per Cent of Each Lignin[a]

Protein in Original Meal %	Protein in Lignin from Original Meal	Protein in Lignin from ADF	Protein in Lignin from DER
16.5	18.7	15.8	18.5
19.1	19.3	13.2	17.9
21.9	21.9	19.0	22.3
22.3	19.8	14.4	22.9
24.1	23.2	20.3	23.2
26.0	25.2	19.6	28.6
28.5	28.5	22.4	33.1
30.4	31.1	23.6	36.3

[a] Moisture-free basis.

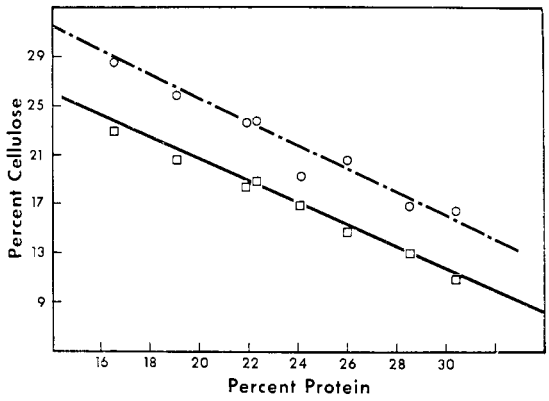

Figure 2. Comparison of "cellulose" with protein content

-·-·- "WRRL cellulose"
$y = 44.4 - 0.97X \quad r = -0.985$
—— "Cellulose" from acid detergent fiber
$y = 37.2 - 0.85X \quad r = -0.996$

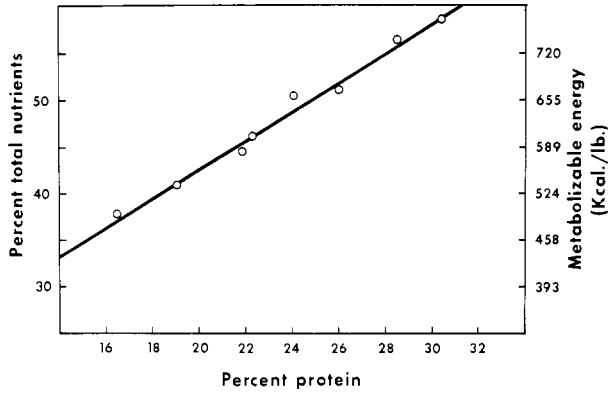

Figure 3. Total nutrients and derived metabolizable energies compared with protein content

Per cent "total nutrients" = 11.9 + 15.4 × per cent protein.
Metabolizable energy (kcal./lb.) = 156.4 + 20.16 × per cent protein

amount of nitrogen in the lignin before or after acid extraction. In the same paper, he showed that boiling 3% (0.612*N*) and 5% (1.02*N*) sulfuric acid removed little and no lignin from three different plant materials. Van Soest (1963) added a strong detergent to 1.0*N* acid and, quoting Norman, assumed no lignin was removed.

The dilute ammonium oxalate solution, because of its nearly neutral pH and 85° C. extraction temperature, removes little or none of the lignin.

Pentosans. The percentage of pentosans decreases in the alfalfa as the protein increases. Ammonium oxalate solution removes 16 to 36% of the pentosans, acid detergent removes 70 to 76%.

Anhydrouronic Acid (AUA). AUA percentages increase slightly as the quality of the forage increases (Aspinall and McGrath, 1966). This increase must come from pectin or some other uronic acid polymer soluble in dilute ammonium oxalate.

The summative accounting for the hemicellulose fraction of plants is extremely complex. By the present procedures, all noncellulosic, oxalate-insoluble hexosans and uronides (Hansen *et al.*, 1958; Myhre and Smith, 1960, 1962) are included with cellulose in the "WRRL Cellulose." Although pectin is not pure polygalacturonic acid, AUA represents a major part of it. Most of the nonuronic acid part of the pectin is included in the pentosan fraction.

Starch. No consistent pattern could be seen for starch. Although the material removed by perchloric acid was mostly starch, as was indicated by the strong clear blue color with iodine, possibly that material which was measured with anthrone may have included other carbohydrate materials insoluble in 80% ethanol and soluble in dilute perchloric acid.

Sugars. There is no increase in the amount of total sugars present as the quality of the alfalfa improves. Reducing sugars show no significant trend, and the quantities of nonreducing sugars (mostly sucrose) fluctuate widely.

Cellulose. A close approximation of the amount of cellulose present in the sample can be obtained from the

Table IV. Metabolizable Energy of Alfalfa Fed to Chicks[a]

Source	Reported Values, Whole Meal Basis: Protein %	Metabolizable Energy Kcal./lb.	Metabolizable Energy Kcal./g.	"Total Nutrients," %	Metabolizable Energy of "Total Nutrients" Kcal./G.
Potter & Matterson, 1960	16.65	322	0.7098	37.50	1.8928
	20.55	533	1.1750	43.50	2.7011
	21.1	566	1.2477	44.35	2.8133
Hill, 1960	18.9	688	1.5167	40.90	3.7083
	22.2	800	1.7636	46.10	3.8255
Matterson *et al.*, 1965	18.9	477	1.0515	40.90	2.5707
	22.2	566	1.2477	46.10	2.7065
Slinger, 1965–6	26.0	651	1.4351	51.90	2.7651
	28.5	562	1.2389	55.80	2.2202
	30.4	774	1.7063	58.70	2.9068
Slinger, 1966	16.5	499	1.1000	37.25	2.9530
	19.1	520	1.1463	41.25	2.7789
	21.9	798	1.7592	45.60	3.8578
	24.1	728	1.6049	48.95	3.2786
	16.38	345	0.7605	37.10	2.0498
Summers, 1967	16.4	531	1.1706	37.10	3.1552
Average					2.8864

[a] Moisture-free basis.

difference between the per cent DER and the sum of the percentages of all the known noncellulose materials (crude fat, protein, pentosans, lignin, and ash) remaining in the DER. All percentages are based on the original sample. This difference is defined as "WRRL Cellulose," WRRL standing for Western Regional Research Laboratory. The advantage of this means of determination of cellulose is that it is not subject to losses due to cellulose breakdown. The disadvantage is that it is subject to cumulative analytical errors. A "cellulose" figure can also be calculated from acid detergent fiber in the same way but, as can be seen in Figure 2, there is only about four fifths as much in the acid detergent fiber as in the corresponding doubly extracted residue. Norman (1935), working with several plants other than alfalfa, found that refluxing with 0.255*N* acid for 30 minutes removed as much as half of the cellulose and up to 17% of the lignin. Also, Harwood (1954) reported that 1.0*N* sulfuric acid at the temperature of boiling water for 1 hour appeared to degrade part of the cellulose to glucose and oligosaccharides.

A calculated value for "total nutrients" for nonruminant animals may be obtained as follows:

$$\%\text{ "total nutrients"} = 100\% - (\%\text{ WRRL cellulose} + \%\text{ lignin} + \%\text{ ash} + \%\text{ pentosans} + \%\text{ AUA})$$

All percentages are of the original meal. A metabolizable energy value for the total nutrients of alfalfa when fed to chicks was calculated from published metabolizable energy data. No results were used unless obtained from actual chick feeding experiments. "Total nutrients" values were obtained from Figure 3 using the protein content given with each metabolizable energy value. The metabolizable energy for the total nutrients of each sample was then calculated by dividing the reported metabolizable energy of the whole sample by its derived total nutrients. The average of the 16 values reported was 2.8864 kcal. per gram (Table IV).

Apparently, biologically determined metabolizable energy values for dehydrated alfalfa in the literature are few and extremely variable. Steam pelleting, as contrasted with conventional pelleting, increases metabolizable energy of alfalfa (Slinger, 1966) and wheat mill feeds (Cave *et al.*, 1965; Slinger, 1966). The biological data used for the above calculations were for unpelleted meal or for conventionally pelleted and reground meal only. Essentially all of the dehydrated alfalfa used at present is pelleted at the production plant and reground before use but is not steam-pelleted. Process variables in the plant may be responsible for some of the variability in metabolizable energy values. Also, the bioassay procedures used need re-evaluation as to their applicability to a product such as alfalfa which is ordinarily used at levels of less than 10% in poultry rations.

The calculation of total nutrients is based on certain assumptions which, though not completely valid, should not introduce major errors in predictive value. The doubly extracted residue, soluble pentosans, and pectin are assumed to be completely unavailable and the total protein is assumed to be available to nonruminants. However, the degree of digestibility of the various components is adjusted for by calculation of the metabolizable energy value per gram of WRRL digestible nutrients based on metabolizable energy determinations. This type of approach should be useful in determining the effects of process variables on alfalfa components which contribute to its metabolizable energy.

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