STUDIES ON LIGNIN AND RELATED COMPOUNDS IN FORAGE AND IN ANIMAL EXCRETA.

by

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A thesis

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Claims to Original Research.

A study has been made of the lignin of forage and feces with the following results.

A method has been developed for pre-extracting feces samples for the lignin determination without drying the sample. This results in a lower yield of lignin having a lower percentage of nitrogen.

The methoxyl and nitrogen contents of lignin from feces have been determined and compared with those of the lignin from the forage from which the feces is derived.

The Crampton & Maynard method of determining lignin has been compared with the 72% sulphuric acid method; the nature of the lignin isolated and the reproducibility of the results have been investigated.

The ultraviolet absorption spectra of lignin fraction from forage and feces have been determined. By this means an estimate of the purity of forage and feces lignin isolated by the Crampton & Maynard and by the 72% sulphuric acid methods has been obtained.

A preliminary investigation of the high pressure hydrogenation of feces and of young timothy has been made. No evidence of the presence of large amounts of known high pressure hydrogenation derivatives of lignin was found.

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INTRODUCTION

The present official system of feeding stuffs analysis has often been found unsatisfactory. This is especially true of the division of the carbohydrate portion of the feed into crude fibre and nitrogen-free extract. In pasture digestibility studies the crude fibre fraction is sometimes as digestible as the nitrogen-free extract. It is natural that, in seeking improvements in the methods, investigators should attempt to analyze the feeding stuff into chemical units. Of these, it has been held that lignin is one of the most important, since it is considered to be indigestible, and even to limit the digestibility of other nutrients.

The purpose of the present investigation has been to study the current methods for the determination of lignin with a view to improving their accuracy and to test their validity. To this end the nature of the lignin isolated has been investigated. A study has been made of the nitrogen and methoxyl content of forage and feces lignin. The lignins have been further characterized by their absorption spectra, and by the products formed by the high pressure hydrogenation of forage and feces.

PART A - STUDY ON METHODS OF LIGNIN DETERMINATION

HISTORICAL REVIEW

The Nature of Lignin:

The fundamental theories concerning the nature and structure of lignin have been reviewed by Freudenberg (1939), Hibbert (1942), Erdtman (1942), and others, and no attempt will be made to detail these theories here. These investigators are agreed that a phenylpropane group is the fundamental unit of the lignin molecule. These phenylpropane type compounds, such as propane derivatives of the syringyl or guaiacyl nuclei, may polymerize to form lignin which Freudenberg (1939) considered to exist in wood in different degrees of condensation, varying from simple units to complex aggregates. Erdtman (1942) pointed out that a wide variety of plant resins conform to a dimeric structure, the monomer being a propylphenol derivative closely related to coniferyl alcohol.

Hibbert (1942) concluded: "It seems probable that, in the case of the lower and higher forms of plant life, monomolecular polyphenols are synthesized in order that they may function as hydrogen transport respiratory catalysts. With the lower forms, these, or their more stable end products, or both, are isolable, as such, from the slightly acid medium; with the higher forms,

the catalysts presumably function in the same manner but, due to the strongly oxidizing, postmortal environment, readily undergo a complex series of dehydrogenation, condensation and polymerization reactions to yield protolignin." This theory, especially in the case of young forage grass where the catalysts would presumably be still active, is far removed from the picture of lignin as a large and relatively inert molecule.

Lignin is laid down during the thickening of the cell wall and may be found in the primary or secondary cell wall and in the middle lamella. Bailey (1938) stated that lignin and cellulose may form interpenetrating systems, the lignin being laid down in the intermolecular spaces of the cellulose. It is often assumed that there is a carbohydrate-lignin complex in plants. Percival (1942) pointed out that this union, if it does exist, is often weak, since it has been shown that the lignin of aspen and of poplar can be nearly all removed by aqueous butanol and sodium cymene-sulphonate, respectively. Norman (1937a) stated that modern theories of cellulose structure do not permit the combination of cellulose with a large molecule such as lignin, but that lignin is probably combined with hemicellulose in the plant.

Factors Affecting the Determination of Lignin:

The most satisfactory method for the determination of lignin has involved the removal of all non-lignin material in the substance to be analysed, leaving the lignin as a residue. This is accomplished by removing the more readily soluble plant components with mild reagents, then removing the cellulose and similar material by strong acid. The simpler carbohydrates and the nitrogenous material must be removed before treatment with the strong acid, since sugars and some nitrogenous compounds will condense in the presence of strong acid to form insoluble material. Norman & Jenkins (1934a, 1934b) have shown that pentoses produce furfuraldehyde which condenses with the lignin to produce an insoluble phenol-furfuran resin, resulting in high values for lignin. These materials may be removed by extraction with dilute acid. Protein interference may be reduced by the same means. In a later paper, Norman (1937b) stated that the protein interference is the result of condensation of lignin with large protein fragments, since amino acids do not interfere in the determination.

Goss & Phillips (1936) have recommended the use of 1% hydrochloric acid to remove interfering material and state that unless dilute acid hydrolysis is used erroneous values are obtained. In a later paper, Phillips (1939)

reported that the 1% hydrochloric acid extraction did not remove all the added nitrogen when protein materials were added to the material to be analysed. Fifty-six percent of the nitrogen left by the pretreatments appeared in the crude lignin. This cannot be corrected for by a nitrogen determination since the form of the nitrogen-containing compound that combined with the lignin varied.

The use of pretreatments, however, may not be free from error. Brauns (1939) isolated a material which he called "native lignin" by extracting spruce wood with ethanol: this material is partially soluble in 42% hydrochloric and 72% sulphuric acid. Perhaps of greater importance is the work of Cohen & Harris (1937) and Harris & Mitchell (1939). These investigators found that hot water and boiling dilute acids dissolve lignin from spruce and maple wood. A material settled out of the filtrate giving a characteristic lignin colour reaction with sodium sulphite and chlorine, and having 20.3% methoxyl. In spite of this, however, Norman (1937) thinks that the weak acid extraction is justified.

The question of the validity of a method for the determination of lignin cannot be finally settled in view of the uncertainty of the knowledge of the structure of lignin. Eastham, et al. (1944) stated that "The structural theories of Freudenberg and of Hibbert are still highly speculative in nature. The source of the ethanolysis products is still unknown. That they are derived from

oxyconiferyl and oxysyringyl alcohols now seems reasonably certain, but the actual form in which the latter are present in native lignin, whether as mono- or diglucosides, phenol ethers, ketals involving the carbonyl and phenol groups, or as readily reversible dimers of the truxillic acid type is still unknown."

MacDougall & DeLong (1942) have called attention to the fact that the temperature of drying a sample for analysis has a marked effect on the amount of lignin isolated. This is especially true of young and succulent tissue. A lignin percentage of 13.6 on rhubarb dried at 105° was reduced to a percentage of 1.73 when the tissue was air-dried after removing the soluble material with cold water and dilute alcohol. They ascribe the high percentages found in oven-dried material to the formation of artefacts and to a condensation of nitrogenous and carbohydrate material with the lignin.

Lignin in Digestibility Trials:

Norman (1935) has studied the determination of crude fibre and has found that this fraction contained most of the cellulose and a variable percentage of the lignin. Bondi & Meyer (1943) confirmed this finding and also noted that the lignin left in the crude fibre is less digestible and higher in methoxyl than the lignin dissolved by the dilute acid and alkali. They found that the percentage lignin in the crude fibre from feces is higher than the percentage in the crude fibre of grasses. The lignin molecule was apparently de-

methoxylated during the course of digestion, since the percentage of methoxyl of the feces lignin is lower than that of the feed lignin.

Crampton & Maynard (1938) have proposed that the carbohydrate part of the feed should be divided into cellulose, lignin and "other carbohydrates". Since this would divide the material in such a way that actual chemical units are determined, it should represent a real advance in feed analysis. To avoid the extraction with weak acid and its possible solution of lignin, they propose the use of pepsin digestion to remove nitrogen. The residue is dissolved in 72% sulphuric acid after treatment with formaldehyde and then precipitated into water. Manning & DeLong (1941) found it difficult to get accurately reproducible results by this method. They reported values of 14.03% with an average deviation for seven determinations of 1.48%, contrasting with 11.38% with an average deviation of 0.30% for six determinations by Norman's 72% sulphuric acid method.

Crampton & Whiting (1943) have proposed a difference method for the determination of lignin. The other constituents of the feed are determined and lignin is calculated as the difference. This can hardly be accurate since all the errors are thrown on the lignin fraction. For instance, the amount of protein may not be equal to the amount of nitrogen multiplied by 6.25.

Csonka and co-workers (1929) studied the digestibility of lignin by a dog and a cow. They isolated lignin from corn cobs by dissolving it in alkali and precipitating the dissolved lignin with acid. About 15% of this lignin was soluble in fuming hydrochloric acid. Because of the failure of the strong acid to remove all interfering substances from feces, they concluded that strong acid methods were not accurate enough for metabolism experiments on lignin. Since the amount of methoxyl can be determined accurately, they analysed the feed and feces for methoxyl, and estimated the digestion of lignin from the loss of methoxyl. The dog was fed a diet containing no methoxyl previous to the trial, and then lignin was added to the feed. They found a loss of 20% and 13% of the lignin methoxyl with the dog and 36.7% in the case of the cow. They also noted an increase in the hippuric acid content of the urine when lignin was fed. This presumably came from the benzene ring compounds of the lignin digested. In this connection, it is interesting to note that the urine of ruminants contains a large amount of hippuric acid. Thus it would seem possible that the ruminant may absorb a part of the lignin into its body and excrete the benzene ring containing compounds as hippuric acid. The aliphatic side chain present must, if this is true, be metabolized by the animal. However, experiments of the

type conducted by Phillips on the fate of isolated lignin added to the diet are not very conclusive, since it is known that the biological availability of lignin is affected by the process of isolation. Moreover, the lignin may be demethoxylated (Bartlett and Norman 1938) without the rest of the molecule being decomposed. Also, as noted later, hippuric acid is formed by protein metabolism and the increase in hippuric acid excretion that was noted may have been caused by a change in protein metabolism.

The contrasting results obtained by McAnally (1942) and by Ferguson (1942) on the digestibility of lignin in straw and straw pulp revealed the unsatisfactory nature of the present knowledge of lignin digestibility. Both workers used Norman's 72% sulphuric acid method for the lignin determination. In McAnally's experiments, 0.5 gm. samples of the feed were placed in small silk bags and suspended in a sheep's rumen through a fistula. These bags were removed after varying periods up to seven days, and the digestibility was determined by the amount of material that had been dissolved out of the bags. The materials were straw and straw pulped with 1.5% sodium hydroxide. A study of the digestibility of similar material was carried out by Ferguson using ordinary digestibility trial technique; that is, the feed and feces were analysed (in contrast to McAnally's work where the feces was not analysed). The results obtained by both workers for the digestibility of

cellulose and hemicellulose of straw and straw pulp were in fairly good agreement, but the digestibility of lignin determined by the two methods was different. McAnally reported a 30% digestibility of straw lignin and a 70% digestibility of the lignin of straw pulp. Ferguson reported that straw lignin is 4 - 10% digestible and straw pulp lignin 4 - 9% digestible. This discrepancy could hardly be the result of errors in the analysis of small samples, since the results for the digestion of cellulose and hemicellulose are in good agreement. It is suggested that the lignin is rendered soluble in the rumen so that it passes out of the silk bags, but is deposited again elsewhere in the alimentary tract without being digested. There is the third possibility that the material determined as lignin in the feces is merely a condensate of undigested residues.

Bondi & Meyer (1943) found that the lignin of various grasses was 35 - 64% digestible by sheep. They used a mixture of hydrochloric and sulphuric acids to determine the amount of lignin in the forage and feces materials.

Crampton & Maynard (1938) reported the recovery of 97% and 99% of dietary lignin in the feces of rabbits and steers. In later studies, Crampton, et al. (1939, 1940) reported lignin digestibilities ranging from 27% to 29% for rabbits, and from 13% to 38% for steers. In general, there was a tendency for the digestibility of lignin to be lowest in midsummer.

Jackson (1944) has shown a digestibility range of from 8.8% to 34% when the lignin is determined by the Crampton and Maynard procedures, and a range of 2.2% to 53.5% when the determination is made by the Crampton and Whiting method. Again, there was a general tendency for the digestibility to be lowest in midsummer. Steers were used as the test animals. Using sheep as the test animals and the Crampton and Maynard method of lignin determination, a range in digestibility of from 11% to 50% was found.

The foregoing discussion indicates the unsatisfactory state of present knowledge of the digestibility of lignin. The great variability in results reported by different investigators showed either that lignin from different sources is widely different in digestibility or that the various methods used isolated a different material. The work of Bondi & Meyer (1943) showed that lignin of plants can be divided into two parts by the action of 1.25% sodium hydroxide and 1.25% sulphuric acid. The lignin that was dissolved by these reagents was found to be more digestible than the insoluble part that remained as a residue in the crude fibre.

It has been shown (Csonka, et al. 1929) that when lignin is added to the feed of some animals there is an increase in the hippuric acid excretion. For this reason, Crampton (1940) claimed that the lignin serves no useful dietary purpose, the amount absorbed being re-excreted in

the urine. Since hippuric acid in the urine is also derived from protein metabolism, this theory is difficult either to prove or to disprove.

It is apparent that an investigation into lignin and related compounds in forage and feces must concern itself with the nature and chemical properties of the lignin isolated by the methods used. The effect, on the amount of lignin isolated, of various methods of preparing the material for analysis must also be studied. Since the factors affecting the determination of lignin of young plants were being investigated in this laboratory, this work, insofar as methods of preparing the samples are concerned, is confined to feces. The lignin isolated was characterized by its methoxyl and its nitrogen contents.

EXPERIMENTAL

Studies on 1942 Samples

The forage and feces used in this investigation were samples from material from the digestibility trials conducted by the Macdonald College Pasture Committee in 1942. It has been shown (Crampton, et al. 1939) that the digestibility of forage was often lower during the dry midsummer period. During this period, especially if rainfall is low, the grasses tend to mature, slow down in growth rate, and to increase in lignin content. It is often assumed that the decreased digestibility is due to the increase in lignin alone. For this reason it was decided to study forage and the corresponding feces samples therefrom for three periods. These were representative of the May and early June forage which is highly digestible, the less digestible midsummer forage, and the fall growth of forage which is usually more succulent and digestible than the midsummer forage.

In these digestibility trials the forage was cut twice daily and fed directly without drying to the steer used as the test animal.

The first samples were taken over the period June 8th to 12th. A sample of each day's feed was taken for analysis. These were combined, air-dried, and then ground in a Wiley mill. The cover on the plot from which the samples were taken was 25% couch grass and 73% timothy on May 14th. Weeds, clover and some other grasses were also present.

The second sample was taken and similarly treated over the period July 17th to 21st. On May 14th the cover of the area from which this sample was taken was about 30% legumes (alfalfa, alsike, red clover and white clover), 45% timothy, and the remainder, other grasses. The third sample was taken September 22nd to 26th from the same area as sample 1.

Some difficulty was experienced in handling the feces samples. Since it was known that drying at elevated temperatures increased the amount of apparent lignin in a sample of succulent plant tissue (MacDougall & DeLong, 1942), an attempt was made to dry the feces without heat. However, air drying was unsatisfactory; a hard film formed over the surface of the sample and made the drying process very slow. It was thought that during the period of drying enzyme and/or bacterial action might materially affect the composition of the sample. The procedure finally adopted was to stir the sample with an equal volume of cold alcohol and filter on a large Buchner funnel. The alcohol would remove some of the water and slow down bacterial action. It also removed the mucus-like material that acted to retain moisture. Thus the sample could then be air-dried fairly rapidly. The three feces samples, collected over the same period as the forage samples, were treated in this way.

The lignin determinations were made on these samples using the 72% sulphuric acid procedure described by Manning & DeLong (1941). This method of determination will be referred to throughout this paper as the 72% sulphuric acid method.

The lignin isolated for the methoxyl determination was filtered in sintered glass crucibles, naphthalene being used to facilitate filtration as suggested by Mueller & Herrmann(1926). The methoxyl determinations were carried out as described by Clarke (1932), the hydriodic acid being prepared as he recommended (1938). For the nitrogen determination, the lignin was isolated in weighed Gooch crucibles using a weighed amount of acid-treated diatomaceous earth as a filter aid. The lignin from an 0.5 gm. sample of extracted material was digested by the Kjeldahl method using mercury as a catalyst. The resulting solution was made up to 100 c.c. and 10 c.c. aliquots were distilled in an all-glass micro-Kjeldahl distillation apparatus. The nitrogen and methoxyl values were corrected for the amount of ash in the crude lignin. This correction was determined by isolating the lignin on weighed Gooch crucibles, drying, weighing, ashing and weighing again. There was considerable variation in this correction from sample to sample.

Lignin determinations on these samples were also made by the procedure described by Crampton & Maynard (1938). Methoxyl and nitrogen contents of the lignin isolated by the Crampton & Maynard procedure from these samples were determined in manner described above. Digestibility coefficients were calculated for the lignin as determined by both the 72% sulphuric acid and the Crampton & Maynard methods. The percentage figures for lignin, methoxyl and nitrogen are followed by the average deviations, and in brackets, the number of determinations made.

TABLE I

The lignin content of, and the nature and digestibility of the lignin isolated from, materials from the 1942 digestibility trials.

Type of Material	Results on moisture-free, ash-free basis						Ash in Crude Lignin	Digestibility
	Lignin		Methoxyl		Nitrogen			
	%	av.dev.	%	av.dev.	%	av.dev.	%	%
72% Sulphuric Acid Method								
Grass 1	6.10	0.03(3)	5.50	0.12(3)	2.77	0.04(3)	12	32.5
Feces 1	14.85	0.15(3)	4.84	0.17(3)	3.47	0.04(3)	25	
Grass 2	6.84	0.18(3)	4.10	0.04(3)	3.83	0.11(3)	8	23.4
Feces 2	16.18	0.07(2)	5.09	0.01(3)	4.19	0.02(3)	16	
Grass 3	5.77	0.07(3)	2.82	0.04(3)	5.31	0.06(3)	6	10.7
Feces 3	16.32	0.15(3)	3.63	0.04(4)	4.14	0.01(3)	17	
Crampton & Maynard Method								
Grass 1	11.14	0.32(2)	3.37	0.12(3)	5.38	0.16(2)	17	35.1
Feces 1	26.69	0.40(2)	3.74	0.06(3)	4.49	0.07(2)	33	
Grass 2	8.20	0.30(3)	3.62	0.01(3)	3.94	0.02(2)	18	13.4
Feces 2	23.92	0.66(2)	4.59	0.06(3)	4.74	0.08(2)	28	
Grass 3	11.10	0.11(2)	2.19	0.06(3)	4.42	0.03(2)	12	31.8
Feces 3	25.08	0.48(2)	3.23	0.09(3)	4.68	0.16(2)	23	

One feature of the above table is the difference in results secured by the Crampton & Maynard and the 72% sulphuric acid methods. The former isolates more apparent lignin than the latter. This lignin is lower in methoxyl and, in all but one case, higher in nitrogen than the 72% sulphuric acid lignin. This would indicate that the lignin isolated by the Crampton & Maynard method is less pure. The higher nitrogen percentages would indicate that the pepsin digestion is not so efficient in removing nitrogenous material as the 1% hydrochloric acid. The material that can be rendered soluble by pepsin hydrolysis has probably been removed from the feces during digestion. In the case of forage, the large pepsin molecule could not act on all the cell contents of the forage sample unless the cell wall was broken in the grinding of the sample. All the cells would hardly be broken, even by the finest grinding. The hydrogen ion, however, would penetrate the cell wall. The average deviations show that it is more difficult to secure good duplication by the Crampton & Maynard method. This is in accord with the results reported by Manning & DeLong (1941). The possible reasons for this will be discussed later.

With only one exception, the methoxyl content of the lignin isolated by either method is higher for feces lignin than for forage lignin. This is in direct contrast to the finding of Bondi & Meyer (1943) and would not support the theory that lignin is demethoxylated in its passage through the animal body. These contrasting results may be

due to a difference in the nature of the lignin isolated by the sulphuric - hydrochloric acid mixture used by Bondi & Meyer and that isolated by the 72% sulphuric acid method. Differences in the lignin might also be due to age or species differences in the plants used in the two investigations.

There is no constant difference between the amount of nitrogen in the lignin isolated from grass and from feces by either method. In four cases out of six the nitrogen content of forage lignin is less than that of feces lignin.

The percentage of ash in the crude lignin is very variable and it can be seen that the nitrogen and methoxyl percentages must be corrected for this impurity if they are to have any meaning.

The digestibility coefficients as determined by the two methods vary except in the case of the first sample. Since the chemical nature of lignin is not yet thoroughly understood, it is not possible to say definitely which method gives the more correct picture of the digestibility of lignin. However, the fact that the 72% sulphuric acid method gives results that are more accurately reproducible and isolates a lignin that is lower in nitrogen and higher in methoxyl, would indicate that it is a superior method.

Studies on the Crampton & Maynard Procedure:

As noted above, considerable difficulty is often experienced in securing satisfactory duplication of results

by the Crampton & Maynard method. It was thought that the length of time the material was boiled while driving off the chloroform and the temperature at which the filtration was carried out might materially affect the amount of apparent lignin isolated. The following experiment was designed to study these points. Eight 1 gm. samples of ether-extracted forage were taken and the lignin determination was carried out as described by Crampton & Maynard, up to the point where the chloroform was boiled off. Six of the samples were removed from the flame as soon as the chloroform was driven off. The last two were boiled for 5 minutes after the chloroform had disappeared. The results are tabulated below. Where speed of filtration varied, the fact is noted in the table.

TABLE 2

Variability in amount of lignin isolated by the Crampton & Maynard procedure.

Treatment	Weight of lignin isolated
1. Filtered at once, filtered rapidly.	.1132 gms.
2. Filtered at once, filtered very slowly.	.1403 "
3. Cooled before filtering.	.1372 "
4. " " "	.1117 "
5. Cooled 5 minutes, then filtered, slow filtering.	.1242 "
6. Cooled 5 minutes, then filtered.	.1117 "
7. Boiled 5 minutes after chloroform was driven off, cooled 5 minutes, then filtered.	.1006 "
8. Same as 7.	.1190 "

The results show the wide variation in the amount of apparent lignin isolated when the technique is changed slightly. The difference in amounts of lignin isolated by the same treatment make it difficult to draw definite conclusions. The results show, however, that if filtration of one sample is slower than that of the other so that the one sample becomes cold before filtration is complete, the amount of lignin isolated will be different. This cannot be prevented by keeping the solution hot, since, in that case, further hydrolysis goes on and the yield of lignin is low. The most satisfactory method is to let the suspension cool 5 minutes till the lignin settles, decant off the supernatant liquid, and then wash the lignin into the Gooch crucibles. With this method, filtration will often occur at the same rate for each sample, and good duplication will result. This is in accord with the experience of the writer (as an analyst for the Pasture Committee) when a large number of determinations were made in a routine way. In this case, too, good results could be secured only if the filtration time was uniform. A further difficulty with the method is failure to secure solution of the sample in the formaldehyde - sulphuric acid mixture. Some of the material dissolves very slowly. However, if the sample is left in contact with the strong acid for any considerable period of time, condensation products will undoubtedly form from the sugars, hemicellulose and protein fragments present. It was thought best to adhere closely to the 10-15 minute time of contact outlined by Crampton &

Maynard even if the sample was not entirely dissolved. The contamination of the isolated lignin by the slight amount of undissolved material would probably be less than that caused by reversion products formed by long contact with the acid.

The above results illustrate the highly empirical nature of the Crampton & Maynard procedure. Each of the treatments, after removing the pepsin, are for short periods of time and are not sufficiently long for any of the reactions to go to completion. The 72% sulphuric acid method, on the other hand, requires more time for the determination, but the duration of each operation is sufficiently long for the reactions to be complete. Therefore, slight and unavoidable variations in technique (speed of filtration, etc.) do not seriously affect the results. For this reason, and since the results in Table 1 showed that the method resulted in the isolation of a purer lignin (i.e., lower in nitrogen and higher in methoxyl) than the formaldehyde procedure, it was decided to confine the study to the 72% sulphuric acid method only. The effects of variations in the pretreatment and of the manner of preparation of the sample for analysis were to be studied.

Studies on Pretreatments and Methods of Preparing the Sample:

For the year 1943 the digestibility trials were continued using sheep as the experimental animals. It was felt that a more satisfactory method of preparing the feces

sample should be secured. The alcohol treatment used the previous year involved a relatively slow drying of the sample and, moreover, it had been shown that alcohol dissolved the more soluble lignin components (Brauns, 1939). Also, drying itself might favour condensation of soluble material with the lignin.

A small sample of fresh feces was secured each day of the 10-day trial period. These samples were kept frozen till the end of the test. The samples were then composited, mixed and the dry matter content determined.

Two 60 gm. portions of the sample were then extracted in a Waring Blendor with 400 c.c. of water and 40 c.c. of ether for ½ hour. The ether was used in the hope that it would remove the mucus-like material which prevented drying. The suspension was transferred to 250 c.c. centrifuge cups and centrifuged for 5 minutes. The supernatant liquid was then removed with a sintered glass filter stick using suction. The extraction in the Blendor and the separation of the suspended material by centrifuging were repeated twice. It was found that the water used to remove the sample from the centrifuge cups could not be filtered off. The substitution of alcohol for water overcame this difficulty. The samples were therefore washed into a Buchner funnel with alcohol and dried by sucking air through them with a water pump.

One of the samples (No. 1) was then extracted with alcohol-benzene 1:2 and then refluxed with 1% hydrochloric acid.

On the other sample (No. 2) the order of extraction with alcohol-benzene and 1% hydrochloric acid was reversed. Lignin determinations were made on the air-dried extracted material after passing it through a 40-mesh sieve. As a comparison, a lignin determination by the standard 72% sulphuric acid method was performed on a sample (No. 3) of oven-dried feces collected over the same period from the same sheep. On another oven-dried sample (No. 4) the usual order of alcohol-benzene and 1% hydrochloric acid extraction was reversed.

The ether-water extracted samples were found hard to dry unless they were treated with alcohol. For this reason, it was decided to try the effect of using a 10% calcium chloride solution as the extractant in place of ether-water. It was found that extraction in the Blendor with this solution in the same manner as described for the ether-water mixture at the rate of 10 cc. per gm. fresh weight of sample reduced the amount of nitrogen from 0.90% to 0.24% (on a fresh weight basis). Samples extracted with this solvent dried fairly readily.

A lignin determination was therefore carried out substituting this extractant for the ether-water, but otherwise the same procedure was used throughout (sample No. 5, Table 3). As a comparison, a lignin determination was made on the oven-dried feces substituting a Waring Blendor

extraction with 10% calcium chloride solution in the cold for the usual boiling water extraction of the standard procedure (No. 6).

A sample of frozen feces (No. 7 of Table 3) was pulverized in the Blendor for 5 minutes. It was then transferred to a round-bottomed flask and refluxed with distilled water (approximately 150 c.c. per gm. dry weight) for 3 hours. The water was removed with a filter stick and the sample was then refluxed with 150 c.c. per gm. dry weight of 1% hydrochloric acid for 3 hours, dried, and finally extracted with alcohol-benzene for 30 hours. After removing the alcohol-benzene and passing through a 40-mesh sieve, the extracted material was analysed for lignin. The nitrogen contents of the extracted material and of the lignin isolated therefrom were also determined. The results are tabulated in Table 3. The percentage figures for lignin and nitrogen are followed by the average deviations and, in brackets, the number of determinations made.

TABLE 3 Effect of pretreatment on the amount of lignin isolated and its nitrogen content. Results expressed on moisture-free, ash-free basis.

Treatment	Sample No.	Lignin		Nitrogen in Material (after extraction)		Nitrogen in Isolated Lignin		Nitrogen in Extracted Material Remaining in Lignin
		%	av.dev.	%	av.dev.	%	av.dev.	%
Ether-water, alc-benz., 1% HCl	1	10.53	0.01(2)	1.11	0.02(2)	2.32	0.04(2)	76
Ether-water, 1% HCl, alc-benzene	2	12.36	0.12(2)	1.59	0.01(2)	3.06	0.04(2)	75
Oven-dried Standard	3	15.66	0.03(2)	2.49	0.01(2)	4.27	0.03(2)	76
Oven-dried, 1% HCl, alc-benzene	4	16.32	0.15(2)	2.19	0.01(2)	3.82	0.01(2)	73
$CaCl_2$ - Frozen Sample	5	11.72	0.08(2)	1.14	0.00(2)	2.49	0.03(2)	77
$CaCl_2$ - Oven-dried Sample	6	15.33	0.12(3)	1.97	0.05(2)	3.29	0.01(2)	70
Boiling water, 1% HCl, alc-benzene	7	10.17	0.05(2)	1.25	0.02(2)	2.69	0.01(2)	76

From the above table it is evident that nitrogen must be removed insofar as possible prior to the treatment with 72% sulphuric acid since 70 - 76% of the nitrogen in the pre-extracted material appears in the isolated lignin. The order of extraction by alcohol-benzene and 1% hydrochloric acid had been reversed with the idea that the hydrochloric acid would hydrolyze any soaps formed in the digestion process and permit a more complete removal of fat (Holcomb, 1934). This should result in a lower yield of lignin. However, the amount of lignin isolated is higher in each case so there is obviously no advantage in reversing the order of extraction. It is also apparent that oven-drying increases the yield of lignin appreciably, but that there is no great difference whether the soluble material is extracted in the cold with ether-water or 10% calcium chloride solution or by boiling water. The boiling water treatment yielded slightly less apparent lignin though the isolated production was more contaminated with nitrogen than some other lignin preparations (e.g., that from the ether-water treated sample). The boiling water extraction was easier to use than the cold 10% calcium chloride extraction since it involved no centrifuging.

The reproducibility of the results for both the boiling water and cold 10% calcium chloride extractions was next investigated. Two samples (1 & 2) were extracted with boiling water (estimated 150 c.c per gm. dry weight), and two others (3 & 4) were prepared by the 10% calcium chloride solution treatment. Duplicate lignin determinations were made

on the extracted material. It was noted that the salt solution extraction resulted in a lower percentage of nitrogen in the lignin isolated than the boiling water extraction and it was thought that this might be the result of salt solubility effect on the protein and protein residues in the feces. Since 10% sodium chloride is used to dissolve the salt-soluble proteins, it was decided to compare the effect of refluxing for 3 hours with boiling salt solution with the solubility effects of boiling water. Therefore, 2 samples (5 & 6) were extracted with water, 1% hydrochloric acid and alcohol-benzene. Two others (7 & 8) were treated in the same way except that a 10% sodium chloride solution was used in place of the water. Different samples of feces were used for the last experiment (samples, 5, 6, 7 & 8 in Part B) than those used for previous work, so the percentages are not strictly comparable with those obtained earlier. The results are tabulated in Table 4. The percentage figures are followed by the average deviations and, in brackets, the number of determinations made.

TABLE 4

Table showing the effect of pretreatments and the reproducibility of results. Percentages expressed on a moisture-free, ash-free basis.

Treatment	Sample No.	Lignin		Nitrogen in Extracted Material		Nitrogen in Lignin	
		%	av.dev.	%	av.dev.	%	av.dev.
Part A							
Boiling water	1	11.49	0.03(2)	1.36	--(1)	3.03	0.02(2)
	2	11.37	0.10(2)	1.20	--(1)	2.89	0.01(2)
10% $CaCl_2$ Solution	3	12.18	0.09(2)	0.98	--(1)	2.28	0.02(2)
	4	12.23	0.04(2)	0.95	--(1)	2.19	0.03(2)
Part B							
Boiling water	5	12.34	0.04(2)	1.48	0.02(2)	3.04	0.02(2)
	6	12.73	0.06(2)	1.23	0.01(2)	3.13	0.02(2)
10% NaCl Boiling Solution	7	12.33	0.11(2)	1.27	0.01(2)	3.37	0.03(2)
	8	12.88	0.25(2)	1.58	0.00(2)	2.85	0.02(2)

It is to be noted that the extraction of the sample with 10% calcium chloride in the cold was the most efficient procedure for the removal of nitrogen. However, it resulted in the isolation of a higher percentage of lignin from the sample.

There was no significant difference between the effect of boiling 10% sodium chloride solution and boiling water either in the amount of lignin isolated or in its nitrogen content. The more efficient removal of nitrogen by

the 10% calcium chloride extraction was possibly due to the fact that the extraction was made in the cold. There was also little or no difference in the reproducibility of the results obtained by the different procedures.

It was decided that refluxing with boiling water and 1% hydrochloric acid, followed by extraction with alcohol-benzene was the most satisfactory procedure. This treatment gave as low a yield of lignin as the other methods, even though the amount of nitrogen contamination was slightly greater. This would indicate that it is satisfactory for removing interfering materials. The chief advantage of the method is its greater ease of manipulation. The boiling seems to coagulate or dissolve the mucus material so that it does not interfere with the removal of the water with a filter stick. Hence, centrifuging is unnecessary. Also, only one extraction is required instead of the three that were found necessary with the other methods. The above method was therefore used in the analysis of the 1943 feces samples.

Studies on Materials from the 1943 Digestibility Trials.

The 1943 digestibility trials were conducted using sheep as the test animals. The forage was cut at intervals of three weeks, dried in a drier at 100° F., and then cut and stored. This material was fed to the sheep soon after cutting.

Prior to the first cutting the contributions of various species to the bulk of herbage was estimated as follows: grasses - Kentucky Blue 2%, timothy 42%, couch 12%; clovers 30% and weeds 14%.

No estimate was made prior to the second cutting.

Prior to the third cutting, white clover had gained in prominence, and all clovers were in head and in flower. Little of the grass had headed out. Weeds added very little to the sward which was composed of 55% clovers and 40% grasses.

Before the fourth cutting the stand was estimated to be 65% clover, 30% grasses and 5% weeds. The entire area now had a grass - clover sward. The clovers, but not the the grasses, were in head.

No estimate was made before the fifth cutting.

Prior to the sixth cutting the clovers had decreased in amount, now comprising only 42% of the bulk of the forage. Grasses, chiefly timothy, had increased to 55%.

The species change was probably the most significant change that occurred in the forage from the test area. The season was unusual since there was no midsummer drought. Hence there was no tendency for the forage grasses and clovers to become dry and hard. The sward remained green all summer so that good yields of succulent forage were secured at each cutting.

MacDougall (1944) has shown that lignin of a lower nitrogen and higher methoxyl content is produced if the water soluble components of young cereal grass are removed before drying. Hence, it was decided to extract the forage samples with ether-water before drying. The method of preparing the

samples for the lignin determination was as follows: Forage samples were secured from each cutting, care being taken to make the sample representative. The sample taken for analysis was cut into short lengths, and the percentage of moisture determined. Sixty gram (fresh weight) samples were extracted in the Waring Blendor with 400 c.c. water and 40 c.c. of ether. The suspension was centrifuged, and the ether-water and dissolved materials were removed with a filter stick. The extraction and centrifuging were repeated twice. The residue was transferred to a Buchner filter and washed, then dried by sucking air through it overnight. The material was then allowed to air-dry. When dry, the loss in weight was determined. The extracted samples were combined, and were finally ground in a Wiley mill for analysis. The dried and ground samples were extracted with alcohol-benzene, then 1% hydrochloric acid. After air-drying, weighing to determine loss in weight, and passing through a 40-mesh sieve, the samples were ready for the lignin determination. For reasons of convenience mainly, the order of 1% hydrochloric acid and alcohol-benzene was the reverse of that used for feces samples. The feces samples were prepared and pre-extracted as described previously. The isolated lignins were analysed for nitrogen and methoxyl as indicated previously. The results are tabulated below, the percentage figures being followed by the average deviations and, in brackets, the number of determinations made.

TABLE 5 Nature of lignin isolated from material from the 1943 digestibility trials. Results on moisture-free, ash-free basis.

Period No.	Nature of Material	Lignin		Nitrogen in Lignin		Methoxyl in Lignin		Digestibility	
								Sulphuric Acid Method	Crampton & Maynard Method
		%	av.dev.	%	av.dev.	%	av.dev.	%	%
2	Forage	8.83	0.13(2)	5.47	0.06(2)	4.93	0.06(3)	48.2	11.6*
	Feces	14.75	0.11(3)	3.67	0.02(2)	6.60	0.12(3)		
3	Forage	8.23	0.01(2)	5.58	0.10(2)	4.07	0.08(3)	41.2	36.1*
	Feces	15.57	0.09(3)	3.61	0.07(2)	5.96	0.08(3)		
4	Forage	7.78	0.08(2)	6.64	0.09(2)	3.17	0.03(3)	54.6	46.5*
	Feces	12.39	0.10(3)	3.31	0.01(2)	6.10	0.08(3)		
5	Forage	7.81	0.01(2)	7.06	0.06(2)	3.01	0.09(3)	60.4	50.0*
	Feces	11.65	0.05(2)	3.63	0.05(2)	5.38	0.04(3)		
6	Forage	6.41	0.05(2)	7.80	0.25(2)	3.00	0.09(3)	65.2	48.8*
	Feces	8.74	0.01(2)	3.21	0.01(2)	5.34	0.02(3)		

* From unpublished Pasture Committee data.

From Table 5 it is apparent that the lignin isolated from feces is purer than that isolated from grass. It is very considerably lower in nitrogen and higher in methoxyl. This fact might go far to explain the high digestibility coefficients that are reported. The percentage of lignin decreases throughout the season, and the decrease is probably greater than would appear from the percentage lignin figures, since the isolated lignin became lower in methoxyl and increasingly contaminated with nitrogen as the season progressed. This is not true with the lignin isolated from the feces. The percentage nitrogen remains relatively constant, varying from 3.21% to 3.67%. The methoxyl content is somewhat more variable but not as variable as the percentage methoxyl in forage lignin. These results indicate the difficulty of obtaining reliable information on the digestibility of lignin with the methods in use. It is hardly possible that lignin could become methoxylated in its passage through the digestive tract, yet both Tables 5 and 1 show that the lignin isolated from feces is higher in methoxyl than that isolated from forage. It would seem that digestion removes some interfering materials which condense with the lignin isolated from the forage, or the animal digests the more soluble, less methoxylated lignin, leaving a lignin material higher in methoxyl in the feces.

It is thought that the change of species in the pasture sward may be partly responsible for the difficulty of securing a satisfactory lignin digestibility picture. There was a

gradual increase in the percentage clover in the sward from 30% at the first cutting (not reported in the table) to 65% in Period 4. There was some decline in the percentage of clover at the 6th cutting. Wild white clover was responsible for most of the increase, while the other clovers, especially alsike, actually decreased in amount. The effect of the change in the sward seemed equally or more significant in its effects on the amount of lignin isolated by the Crampton and Maynard method than in its effect on the amount isolated by the 72% acid procedure. Unpublished Pasture Committee data (Jackson, 1944), tabulated below, illustrate this point.

TABLE 6 Effect of species on lignin value.

Period No.	1	2	3	4	5	6
Percent clover in sward in 1942.	Not very large and counts not made, there being little species change.					
Percent lignin in forage in 1942.	9.25	9.58	10.47	10.60	11.65	11.14
Percent clover in sward in 1943.	30	-	55	65	-	42
Percent lignin in forage in 1943.	10.43	11.45	17.23	20.19	20.05	16.64

It is seen that in the 1942 season when the amount of clover in the sward remained relatively the same there was little change in the lignin values. In 1943, when the percentage of clover increased, there was also a marked increase in the percentage of lignin. Since each cutting was taken at approximately the same stage of growth and there was no hot dry

weather to promote rapid maturity, it would seem that the amount of clover had a decided effect on the forage lignin values determined by the Crampton & Maynard method.

The determination of lignin by the 72% sulphuric acid method was not affected in the same way. The percentage of lignin decreased rather than increased as the season progressed, but the isolated lignin was increasingly contaminated with nitrogen. The extraction procedure used in the 72% sulphuric acid method may be more efficient in removing certain types of interfering materials present in the clover.

Norman (1939) has called attention to the fact that there are great differences in the fructosan content of young rye grass and young orchard grass. There are probably differences in the carbohydrate fraction of clovers and grasses. Some of these carbohydrates might have a greater tendency to condense to form insoluble materials during the formaldehyde-sulphuric acid treatment that others. If easily hydrolyzed, they would be removed by the extractants used for the 72% sulphuric acid procedure.

The above shows that the lignin determination needs to be studied on pure species or, at least, on related species. In pasture studies a change in the species composition of the sward may obscure the effect of any change in degree of maturity.

Studies on Urine:

A preliminary study was made of the urine for possible degradation products of lignin.

Vanillin can be produced from lignin and is a possible degradation product. It has been shown by Sammons & Williams (1941) that about 83% of vanillin and vanillic acid fed to a rabbit can be accounted for by the material excreted in the urine. An attempt was made to study the urine of a sheep and a steer for the presence of conjugated vanillin. The method used was that described by Sammons & Williams (1941). No evidence of the presence of conjugated vanillin was found. Sammons & Williams determined free vanillic acid by determining the amount of methoxyl in an ether extract of urine. When this method was applied by the writer to the urine of a sheep, fairly high methoxyl values were obtained. The ether extract was resinous in nature and it may have been impossible to free it from all traces of ether. However, even after prolonged heating (several hours on a steam bath or heating in the oven at 105° C.), relatively the same methoxyl values were obtained. No methoxyl containing compound was isolated.

The urine was also examined spectroscopically over the range of 2510-3450 Å for possible lignin derivatives. Joubert (1939) found it possible to estimate the amount of benzoic and salicylic acids in urine by analysing the absorption curves. It was thought that it might be possible to identify lignin degradation products by the absorption curve of sheep urine. The absorption band at 2800 Å, that is characteristic of lignin derivatives, was not found. If compounds that show this absorption band were present, their

effect must have been masked by other compounds present.

If the lignin was degraded to benzoic acid and was conjugated to form hippuric acid, it would be impossible to distinguish between the hippuric acid formed from protein and that formed from lignin.

SUMMARY OF PART A

In this study the following points have been demonstrated:

1. The 72% sulphuric acid and the Crampton & Maynard lignins are partially digestible by a steer. The methoxyl content of feces lignin is higher than that of grass lignin in almost every case.
2. Extraction of feces samples by different extraction reagents before the samples are dried results in a lower percentage of purer lignin than if the extractions are made after oven-drying.
3. The 72% sulphuric acid lignin is partially digestible by a sheep (41- 65%), the feces lignin being higher in methoxyl and lower in nitrogen content than the forage lignin in every case.
4. The Crampton & Maynard method gives results that are less reproducible than those obtained by the 72% sulphuric acid method. The former method isolates more lignin from both forage and feces than does the latter.

5. Changes in the sward during the pasture season may have significant effects on the apparent digestibility of lignin.
6. No compounds that could have come only from lignin were found in the urine.

PART B

Spectrographic Studies

Introductory and Historical Theory of Absorption Spectra.

The theory and principles of absorption spectra are discussed thoroughly by Brode (1943). Only a very brief introductory summary of some of the important points is given here.

Resonating atoms or groups of atoms are responsible for the absorption of light by organic molecules. These resonating systems may be separated or coupled in the molecule. Unsaturation or an incomplete inner shell of electrons is usually the cause of the resonance. The resonance of systems of atoms is often affected by auxochromes though these latter may not, in themselves, produce resonance. It is not possible to analyse complicated resonating systems at the present time. However, as Brode (1943) has pointed out, "the absorption spectrum does act as an identification for very complicated structures, including the quantitative as well as the qualitative estimation of a substance." The chief characteristics of the chromophores or resonators that are of most interest to the study of lignin spectra are outlined below.

No absorption in the visible or in the near ultraviolet regions is shown by the carbon to carbon single bond.

The carbon to carbon double bond as in ethylene has an absorption band in the extreme ultraviolet.

The carbonyl bond in aldehydes and ketones shows an absorption band in the region of 2700 Å.

Benzene in the vapor state exhibits a band spectrum with remarkable resolution of the band components. Substitutions on the benzene ring exert a marked effect on the absorption bands.

The basis of quantitative absorption spectroscopy is the Lambert-Beer law, usually expressed mathematically as $I/I_o = 10^{-Kcd}$ or as $I/I_o = e^{-kcd}$, where d is the thickness of the cell through which the light must pass, c is the concentration in grams per litre, and k is the specific extinction. I/I_o is sometimes spoken of as the transmission or transmission factor T. Log I_o/I is called extinction E and is equal to Kcd.

Absorption spectra of lignins.

The ultraviolet absorption spectra of lignin and related compounds have been studied by a number of different investigators, e.g., Herzog & Hillmer (1927), Hagglund & Klingstedt (1931). These investigations have usually been carried out with a view to determining something of the structure of lignin or to secure information that would serve as a basis for speculation on the nature of the component

groups of the lignin molecule. Evidence has been secured to show that lignin is aromatic in nature. It has been demonstrated that lignins prepared by a wide variety of methods have a characteristic absorption band at about 2800 Å. There is some difference in the position of the maximum shown by softwood lignin and that exhibited by hardwood lignin.

Stamm, Semb & Harris (1932) chlorinated isolated lignin and dissolved it in 2% sodium sulphite for spectral analysis. The sodium sulphite solutions were satisfactory if they were a few days old, since the solution absorption changed for the first few days but was then constant. The solvent did not interfere with the determination of the absorption spectrum of the dissolved lignin, since the sodium sulphite solution itself showed no absorption above 2500 Å and transmitted light down to 2300 Å. They found that lignin prepared by a variety of modifications of the sulphuric acid method, by the Willstatter hydrochloric acid method and by Freudenberg's cupra-ammonium method gave absorption bands with extinction coefficients that differed only in the longer wave lengths. Differences in the methoxyl content only affected the results insofar as they shifted the extinction coefficients by changing the molecular weight. The presence of pentosans had no effect on the position of the absorption bands nor on the extinction coefficients when the concentration of the lignin solution was corrected for the pentosan impurity.

The thermal reaction product of cellulose gave an absorption curve somewhat similar to lignin but it had much lower extinction in the ultraviolet, especially in the 2800 Å region.

Glading (1940) has recently determined the absorption spectra of spruce 'native' lignin and some of its derivatives and of lignin isolated by the use of phenol, glycol, thiophenol, etc. He found that these preparations all show the characteristic absorption band at 2800 Å. He calculated the specific extinction k from the formula $T = e^{-clk}$, where T = transmittance (I/I_0), c = the concentration in gms. per litre, and l = the thickness of the cell in cms. The k values thus obtained (41.7 for native lignin) remained fairly constant even when the lignin molecule was altered by methylation, acetylation, or treatment with phenol, glycol, or thioglycolic acid, when a correction was made for the increase in molecular weight caused by the formation of the various derivatives. The value of k was high, however, if the group added to the lignin molecule absorbed light in the region of 2800 Å. Thus, for example, the absorption coefficients for phenol lignin and thiophenol lignin were high, but it is known that the added substituent also had a high absorption at 2800 Å.

Adams & Ledingham (1942) used a spectrographic method to estimate the amount of lignin in lignosulphonate

solutions. They used the method to determine the loss of lignin from solution as the result of biological decomposition. The general shape of the curve remained the same, indicating that the whole molecule had been decomposed and not merely some part of it. The method was not applicable to the present work and hence is not outlined here.

Nolan (1940) has claimed that spectrographic methods are sufficiently sensitive to detect 2 mgms. of lignin per litre and to distinguish between softwood and hardwood lignins. The latter point is disputed by some investigators.

Virasoro (1942) studied the absorption in the ultraviolet of lignin extracted from red willow and white quebracho by phenol and by ethyl acetoacetate. The absorption curves were similar to those of lignins extracted by other solvents and from other woods. The maxima were shown at 2800 Å for white quebracho and 2790 Å for red willow.

Patterson & Hibbert (1943a) have recently reported a study of the absorption spectra of ethanolysis products of wood, and of other pure substances closely related to them. These compounds represented four types of aromatic nuclei. In two of these types the position meta to the side chain is substituted, and in two others it is not. The compounds having "meta position freedom" showed an absorption band at about 2800 Å but the others did not. Hence it seemed likely that this band is the result of "meta position freedom". All the compounds showed a maximum at about 2300 Å. This band seems to be characteristic of the aromatic carbon to carbon linkage.

Compounds which had an unsaturated group (carbonyl or ethylene) in conjugation with a benzene ring showed absorption at 3100 Å, though with some substances there was a shift of this band toward the 2800 Å region. They have also reported (Patterson & Hibbert, 1943b) the absorption curves of the ether-insoluble, ether-soluble, and water-soluble lignins, and of the low boiling ethanolysis oils. The degree of molecular complexity decreased in the order the substances are named. These materials were obtained by extraction of spruce meal with a 2% solution of hydrogen chloride in absolute ethyl alcohol. The absorption curves for the ethanolysis fractions of maple wood meal are also reported. These fractions are similar to those of spruce except that the phenol fraction (Pyle et al., 1939) was substituted for the low boiling oils. All these products showed the characteristic absorption band at about 2800 Å, though the band shown by the water-insoluble maple wood lignin was not as distinct as that of the similar fraction from spruce lignin. A detailed description of these substances and the method of preparation for spectrographic analysis was given by Patterson (1942). They have concluded that the absorption curves indicate that lignin is aromatic and that the spectra of amorphous lignins would indicate that lignin is derived from precursors of the types exemplified by hydroxy derivatives of 1-(4-hydroxy-3-methoxyphenyl)-1-propanone and 1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone.

Since lignins from forage and feces are much more contaminated with nitrogenous and possibly with carbohydrate

material than wood lignin, the possible effect of some of these materials on the absorption spectrum of isolated lignin is discussed below.

Ward (1923a) reported the absorption spectra of seven amino acids. Alanine, histidine, glutamine and cystine showed absorption in the ultraviolet descreasing from 2100 Å to 3000 Å. The ultraviolet absorption spectra of tryptophane and tyrosine showed decreasing absorption from 2100 Å to a minimum at 2450 Å, then a maximum at 2800 Å. The absorption then decreased as the visible region was approached. Phenylalanine showed an absorption maximum at about 2600 Å.

Ward (1923b) has shown that various indole derivatives have an absorption maximum at about 2800 Å with the absorption getting less as the visible region is approached. He noted that the curve of B-indole aldehyde is similar in its lower part to the curves that Henri reported for aliphatic aldehydes which rose to a maximum at 2600 Å, fell off again, and then rose once more around 2200 Å.

Marchlewski & Nowotnowna (1926) reported a study of the ultraviolet absorption of amino acids and protein decomposition products. The absorption of most of these was very low above 2500 Å except in the case of tyrosine, tryptophane and phenylalanine. Keratin, obtained by treating wool with calcium hydroxide, also gave high absorption, and its curve was similar to that of tyrosine and tryptophane.

The absorption spectra of sugars have been reported by various workers, e.g., Marchlewski & Urbanczyk (1933),

Goos et al. (1930), but the sugars they studied would be removed in the pre-extractions.

EXPERIMENTAL SECTION

Materials and Methods:

The lignin materials used in this study were prepared from the forage and feces samples secured from the 1942 and 1943 digestibility trials. These samples and their methods of preparation have been described.

The lignin solutions were prepared for spectrographic analysis by the following method. Lignin was isolated on Gooch crucibles by the 72% sulphuric acid method from 0.5 gm. of forage and feces samples previously extracted by alcohol-benzene, boiling water and 1% hydrochloric acid. The isolated lignin was then chlorinated in a desiccator without drying (it was found that if the isolated lignin was dried it was very difficult to dissolve) and extracted with a 2% solution of sodium sulphite. The chlorination and extraction with sulphite were repeated until no further colour was observed in the sodium sulphite leachate. The sample was then washed with water, dried, weighed, ashed, and weighed again. The weight of lignin that could be isolated from a 0.5 gm. sample and that did not dissolve in the sulphite solution was thus known. From lignin determinations previously made, the total weight of lignin isolable from a 0.5 gm. sample was also known. Thus the weight of lignin in the sodium

sulphite solution could be calculated with a fair degree of accuracy. For the Crampton & Maynard (1938) method the same general procedure was followed. The lignin was isolated by this method from a 1 gm. sample of ether-extracted material. Then the procedure described above was followed. Since the amount of lignin that could be isolated by this method from a 1 gm. sample is not so constant as that isolated from the 0.5 gm. sample by the sulphuric acid method, the amount of material in the sodium sulphite solution could not be estimated with as high a degree of accuracy. The sodium sulphite solution of each type of lignin was diluted with distilled water to a concentration suitable for spectrographic analysis.

In order to have some material that would serve as a standard, lignin was isolated from a sample of maple woodmeal. This meal was supplied by Professor Hibbert, then of the Division of Industrial and Cellulose Chemistry of McGill University. It had been previously extracted by alcohol-benzene 24 hours, alcohol 24 hours, and by hot water for 12 hours, in that order. This lignin was chlorinated, dissolved in sodium sulphite solution and the concentration of the resulting solution calculated in the same way as that described for forage and feces lignin.

The absorption curve of the wood lignin was determined on solutions of concentration 0.036 and 0.0068 gms. per litre. Approximately the same effective concentration as the latter was used in studies on the sulphuric acid lignin samples.

Later it was found that more reproducible results could be secured with stronger solutions at higher extinction values. Therefore, solutions approximating in effective concentration the 0.036 gm. sample were used for the Crampton & Maynard lignin studies.

The absorption curves were photographed on Eastman 33 plates over a spectral range of 2510 Å - 3450 Å. The measurements were made on a large quartz spectrograph, Littrow mounting with a 30° quartz prism. The dispersion was approximately 2.5 Å per mm. at 2500 and 25 Å per mm. at 5500 (in the range used an average value is about 3.2 Å per mm.). Illumination was obtained from a 10,000 volt condensed spark between tungsten-steel electrodes. The spectrograph was equipped with a Bellingham and Stanley rotating sector from which the extinction values could be read directly. The sector speed was 100 revolutions per minute.

After development, fixing and drying, the plates were examined visually and the points of equal blackening of the two simultaneously taken photographs were marked with ink on the glass side of the plate. The distance from the match point to a tungsten line selected to serve as a standard was measured. The ink marks were then erased and the plates were re-read and measured again. The average of four readings was taken, and the wave length at the match point was then read from a graph prepared for this purpose from a standard plate. The extinction value of the match points was read directly

from the settings of the rotating sector. The absorption curves were drawn plotting E as the ordinate against wavelength in Å as the abscissa. The value of E at 2800 Å was then read from the graph. The values of k at 2800 Å were calculated from the formula $T = e^{-cdk}$. The value of k for wood lignin was found to be 38.9 at a concentration of 0.036 gm. per litre and 39.3 for a concentration of 0.0068 gm. per litre. The average, 39.1, is in fair agreement with the value of 41.7 that Glading reported for 'native' lignin from maple wood. The 'native' lignin was probably a purer lignin and hence would have a higher specific extinction than that of the sulphuric acid lignin from wood. The k values were calculated in the same way for the forage and feces lignin isolated by the sulphuric acid and by the Crampton & Maynard methods. If it is assumed that the specific extinction of the maple wood lignin is typical of relatively pure lignin, then the purity of the lignin isolated from the forage and feces can be estimated. The percentage of pure lignin = $\frac{\text{k of the test solution x 100}}{\text{k of wood lignin solution}}$. This is true only if the impurity present has no absorption at 2800 Å and no effect on the absorption of the lignin present. Table 7 shows the k values of the dissolved lignins, the concentration of the solutions used, the solubility of the lignin preparations in sodium sulphite, and the purity of the lignin solutions.

TABLE 7 Spectrographic study of lignins from the 1942 samples.

Type of Material	Sample No.	Concentration of Solution gms./litre	k	Sulphite Solubility	Solution Purity (ave.)	Purity of Sample
Lignin Isolated by 72% Sulphuric Acid Method						
Wood	a	0.036	38.9	-	-	-
	b	0.0068	39.3	-	-	-
Forage	1	0.015	20.2	83	52	43
Forage	2	0.0215	16.3	81	42	34
Forage	3	0.015	26.5	65	67	44
Feces	1	0.0175	23.8	67	61	41
Feces	2	0.0206	18.1	77	46	35
Feces	3	0.020	23.9	68	61	41
Lignin Isolated by the Crampton & Maynard Method						
Forage	1	0.103	21.3	78	54	42
Forage	2	0.077	22.4	78	57	44
Feces	1	0.098	20.7	81	53	43
Feces	2	0.097	23.7	73	60	44

Discussion of Results:

It can be seen at once from the table that the lignin isolated from forage and feces has a very much lower specific extinction than has lignin isolated from wood. This would give strong evidence that the forage and feces lignin is a very impure product. The reaction of the lignin to chlorination and sodium sulphite solution also shows that a different material is being dealt with in the case of forage and feces lignin. Wood lignin dissolves easily and completely in sodium sulphite after one chlorination to give the wine-red solution that is considered to be typical of lignin. The forage and feces lignin, on the other hand, must be chlorinated and extracted several times before the maximum amount has been dissolved and even then solution is not complete. The solubility of these lignins ranges from 65-83%. The solution produced is a yellowish-red rather than wine-red colour. It is noteworthy that when the higher percentage (83%) has gone into solution the specific extinction is low, whereas when the amount dissolved is lower (65%) the specific extinction is higher. This would indicate that much of the material dissolved was not lignin since it showed less absorption at 2800 Å. In view of this the percentage purity was multiplied by the percentage solubility in sulphite to get an estimate of the purity of the isolated lignin. These values are remarkably constant as can be seen from the table.

A similar spectrographic study was made of the lignin isolated by the 72% sulphuric acid method from the forage and feces samples obtained from the 1943 digestibility trials. These samples were described in Part A. The experimental technique was the same as described before. The results are tabulated below -- a and b refer to determinations made on two samples of extracted lignin from the same digestibility trial samples. Numbers 2, 3, etc., refer to digestibility trial samples in 1943 trials.

TABLE 8 Spectrographic study of lignins from the 1943 samples.

Type of Material	Sample No.	k	Sulphite Solubility %	Solution Purity %	Sample Purity %	Ave. %
Forage 2	a	25.7	83	66	54	54
	b	26.3	80	67	54	
Forage 3	a	-	-	-	-	45
	b	25.0	71	64	45	
Forage 4	a	23.7	64	61	39	40
	b	23.3	68	59	40	
Forage 5	a	21.3	71	55	39	39
	b	20.7	74	53	39	
Forage 6	a	25.9	67	66	44	44
	b	26.0	64	67	43	
Feces 2	a	30.5	74	78	58	57
	b	29.6	74	76	56	
Feces 3	a	30.2	74	77	57	57
	b	29.4	75	75	56	
Feces 4	a	33.1	68	85	58	56
	b	33.9	62	87	54	
Feces 5	a	31.9	42	82	34	35
	b	35.8	38	92	35	
Feces 6	a	34.2	45	88	40	42
	b	38.5	45	96	43	

The same general observations apply to this table as were applied to the study on the 1942 samples. Where sulphite solubility is low, the specific extinction is high (note especially feces samples 5 and 6). Also, in the case of duplicate samples, if one dissolves to a lesser extent in the sulphite than the other, it usually has a higher extinction value so that when the calculation is made of the sample purity there is fairly good agreement between the duplicates. No explanation can be given for the very low sulphite solubility of feces samples 5 and 6. With these samples the solution purity is high although in the final result the sample purity is low.

In general, the purity of these lignins, where the soluble material was removed before drying, is slightly higher than in the case of the 1942 samples when the samples were dried before pre-extraction.

The absorption curves for the 72% sulphuric acid forage lignin from the 1942 samples are shown in Figure 1, those for the 72% sulphuric acid feces lignin from the 1942 samples are shown in Figure 2, and those for the Crampton & Maynard lignin from forage and feces samples 1 and 2, in Figure 3. In Figure 4 is shown the absorption curve of the forage and feces lignin from samples 2 and 5 from the 1943 digestibility trials. Since the other absorption curves for the lignin from the other 1943 digestibility trials are similar, they are not shown. These curves were drawn by

plotting $E\frac{1\%}{1cm.}$ as the ordinate against wave lengths in Å as the abscissa. The $E\frac{1\%}{1cm.}$ values were calculated from the extinction and concentration of the solutions from the relationship $E\frac{1\%}{1cm.} = \frac{E}{c}$, where c = concentration in gms. per 100 cc. For the lignin from forage and feces the concentrations were corrected for the dissolved impurities (calculated by assuming wood lignin to be pure). On each of these graphs the absorption curve for wood lignin, shown over the same range 2600 - 3300 Å, serves as a basis of comparison.

Absorption curves of sulphuric acid lignin from 1942 forage materials.

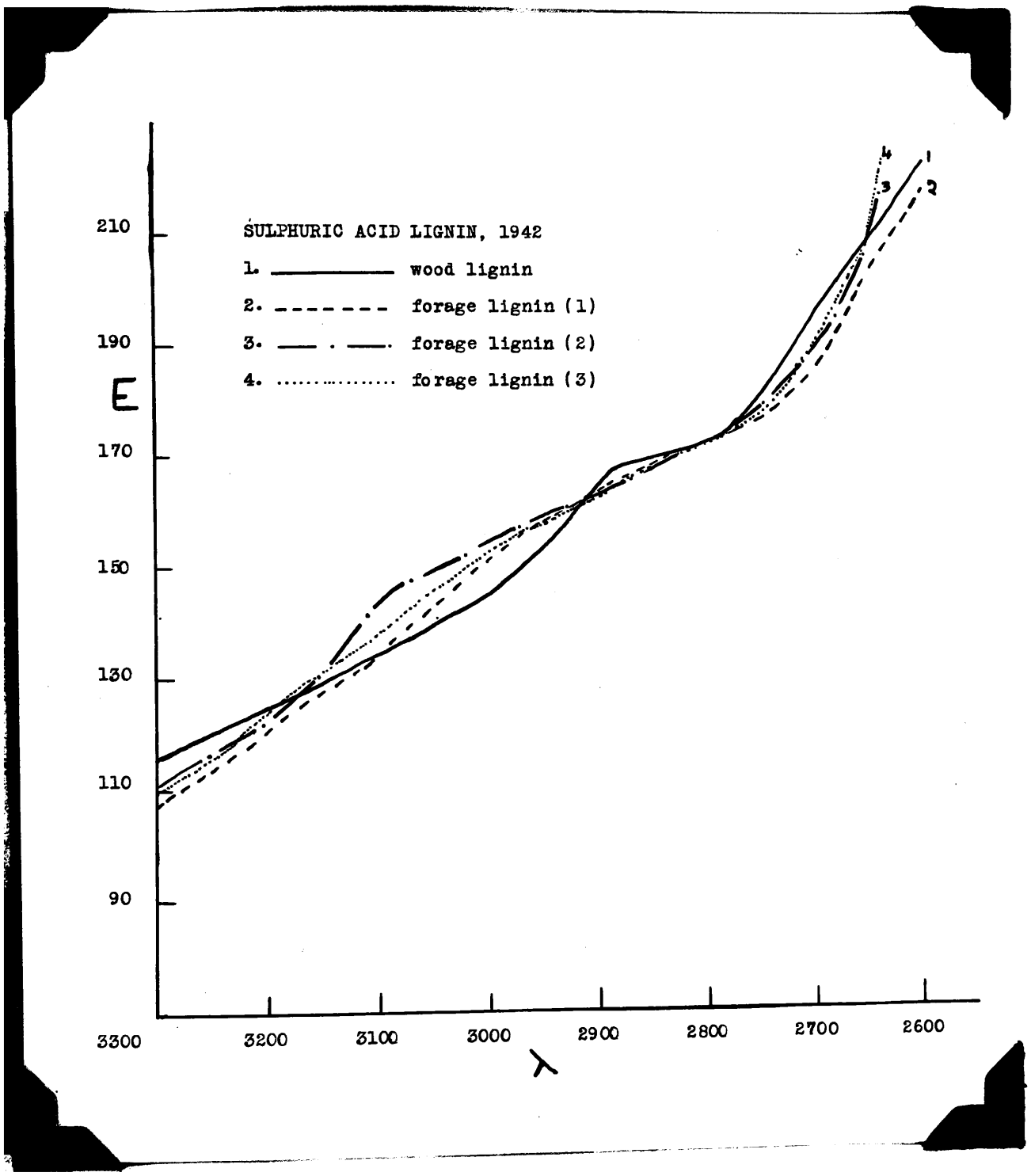


Figure 1.

Absorption curves of sulphuric acid lignins from 1942 feces materials.

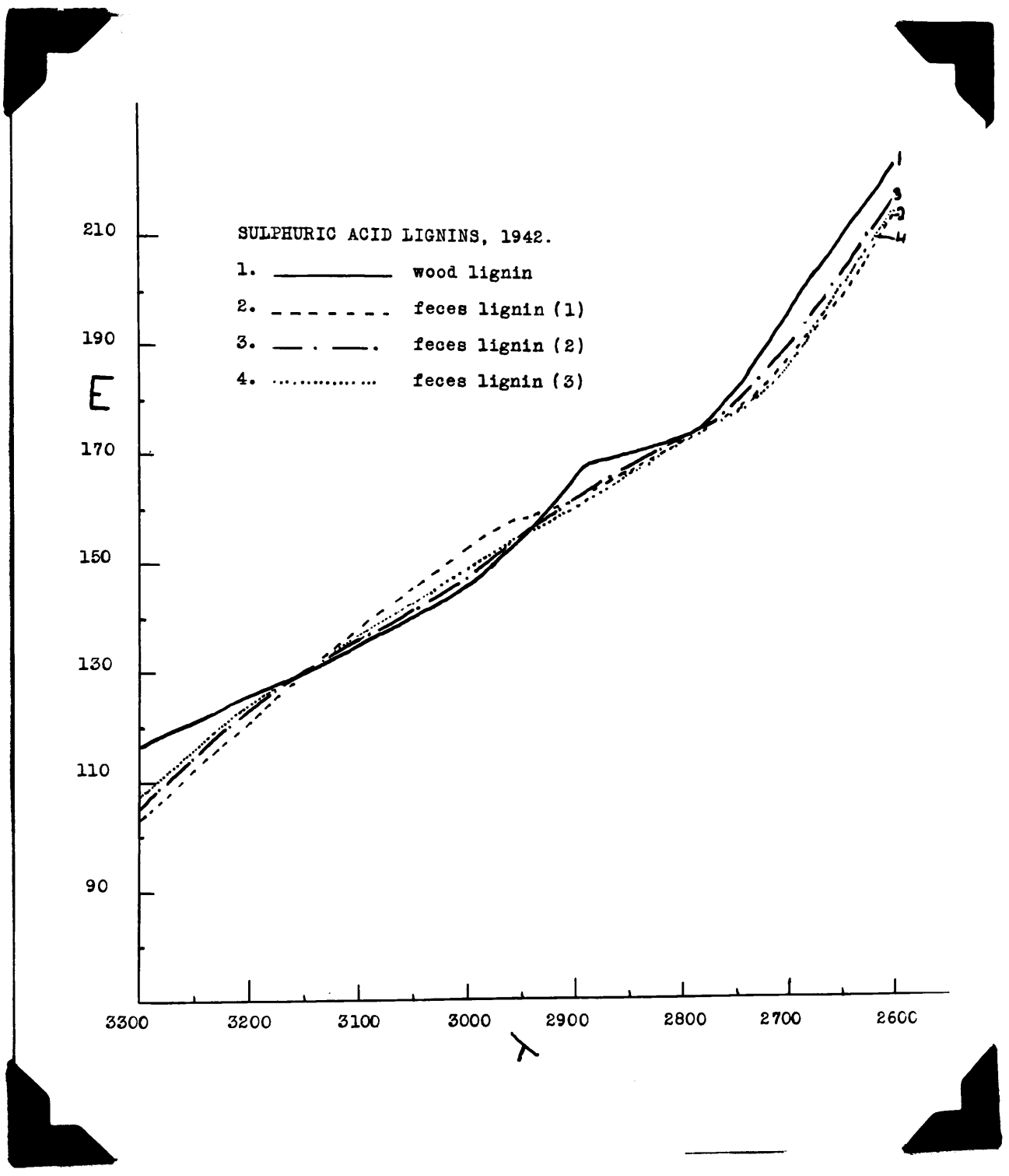


Figure 2.

Absorption curves of the Crampton & Maynard lignins from 1942 materials.

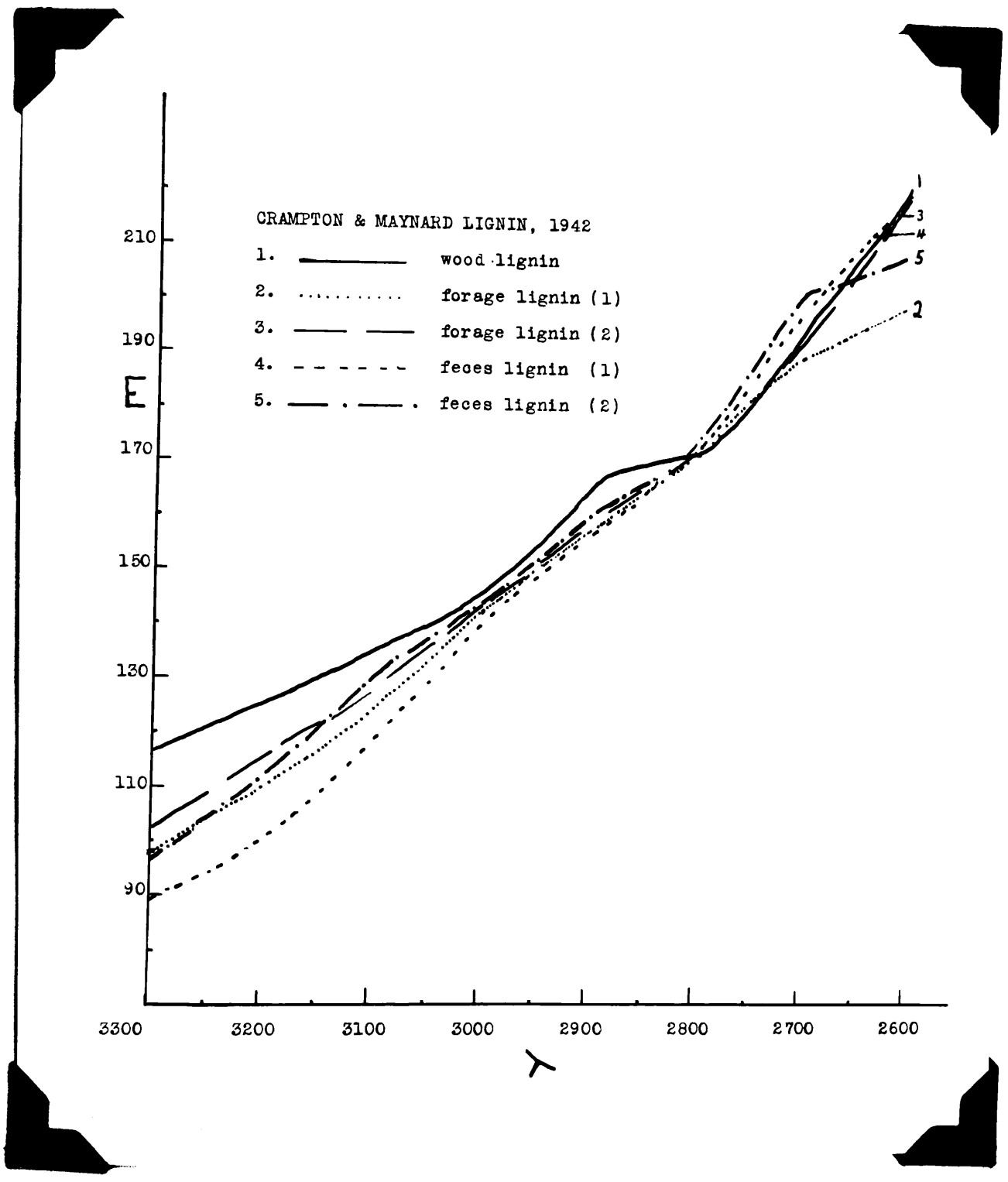


Figure 3.

Absorption curves of sulphuric acid lignins from 1943 materials.

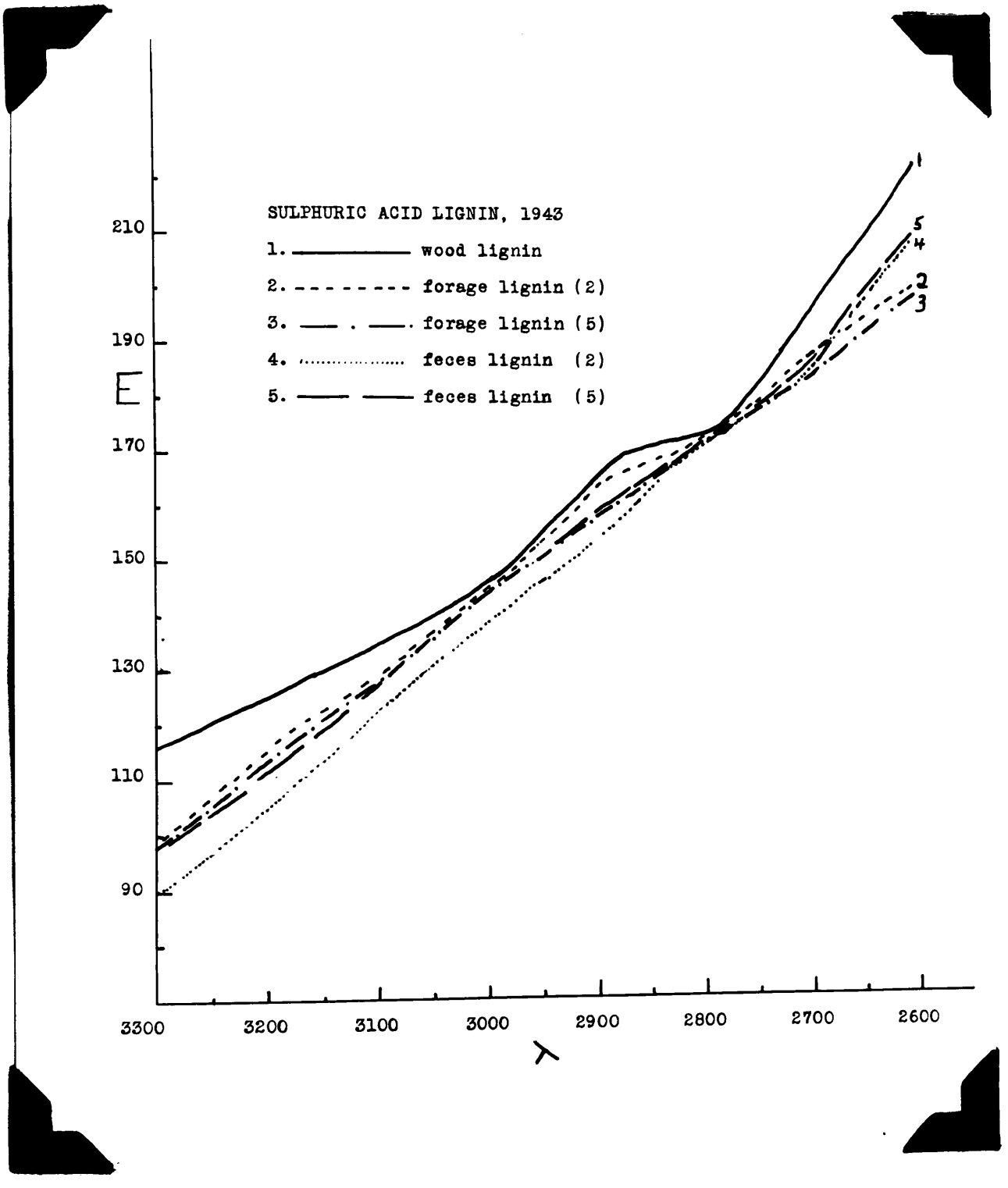


Figure 4.

Conclusions

The absorption curves for forage and feces lignin isolated by the 72% sulphuric acid method are, in general, similar to the wood lignin curve except that the break in the curve at the 2800 Å region is less pronounced and is often lacking. The absorption curve of the lignin isolated from forage and feces by the Crampton & Maynard method is also similar, but it shows no break at 2800 Å. It also shows lower absorption at the longer wave lengths.

The shape of the absorption curve for the sulphuric acid lignin isolated from the 1943 forage and feces samples would seem to indicate that, while the absorption per unit concentration at 2800 Å is as high or higher than for the 1942 lignin, the lignin is less similar to wood lignin. This might be the result of changes in pretreatment of the sample (i.e., extraction before drying the sample) but is more likely caused by the higher amount of clover in the forage samples. The percentage of nitrogen in the lignin of the 1943 samples is higher than in the 1942 samples and the nitrogenous impurity may interfere in the determination of the absorption curve.

The curve for forage lignin is, in general, similar to the curve of the lignin of the feces sample that is obtained when that forage sample is fed. The percentage purity is usually similar also, although this is more

variable in the 1943 trials than in the 1942 studies. This is true for either method of isolating the lignin and would indicate that a somewhat similar lignin fraction is isolated from forage and from feces.

The percentage of apparent lignin when estimated by the Crampton & Maynard method is much higher than when estimated by the 72% sulphuric acid method, though the spectroscopic study would indicate that the lignins are of similar purity. There are two possible explanations for this: the pre-extraction procedures employed for the 72% sulphuric acid method may remove lignin, or the Crampton & Maynard method may fail to remove non-lignin materials that absorb light at 2800 Å. This material may come from protein fragments containing tryptophane or tyrosine, or the formaldehyde might form an aldehyde grouping which would absorb light in that region.

PART C

Hydrogenation Studies

Introductory and Historical:

Much of the recent work on lignin chemistry has been concerned with the isolation, from the lignin of plant materials, of relatively simple substances whose structure could be determined. This necessarily involves comparatively mild methods of treatment of plant materials so that polymerization and condensation reactions are prevented insofar as possible. Methods used for this purpose include high pressure hydrogenation, alkaline nitrobenzene oxidation and extraction with an anhydrous solution of hydrogen chloride in ethyl alcohol.

By the use of the last named technique, Hibbert and co-workers have isolated a number of phenylpropyl units which are presumably true lignin progenitors. This work is reviewed by Hibbert (1941).

MacInnes (1941) studied the ethanolysis products of spruce, redwood, Douglas fir (gymnosperms), maple, red oak, jute (dicotyledons), bamboo, corn, and rye (monocotyledons) and reported considerable variation between species in the percentage of yield of crude ethanolysis products, based on the amount of initial Klason lignin. There was also a considerable difference in the percentage yield of

some fractions (e.g., total ethanolysis products) between angiosperms and gymnosperms. These percentages were again based on the amount of initial Klason lignin. Also, the gymnosperms yielded products containing only the guaiacyl nucleus, while the angiosperm products contained both guaiacyl and syringyl nuclei.

Creighton et al. (1944) found that gymnosperms on alkaline nitrobenzene oxidation yielded only vanillin, while the angiosperms yielded vanillin and syringaldehyde in the ratio 1:3. Creighton & Hibbert (1944) reported the isolation of p-hydroxybenzaldehyde as well as vanillin and syringaldehyde from corn stalks. They noted that this represented the first isolation of p-hydroxybenzaldehyde from lignin, and suggested that its presence may serve as a distinguishing characteristic between monocotyledons and dicotyledons.

The studies on hydrogenation have been reviewed by Hibbert (1941) and by Erdtman (1942).

Harris et al. (1938) hydrogenated methanol aspen lignin over copper-chromite catalyst and obtained 4-n-propylcyclohexanol-1, 4-n-propylcyclohexanediol-1, 2, and 3-(4-hydroxycyclohexyl)-propanol-1. Later, Harris and co-workers (1940) have shown that lignin can be hydrogenated over Raney nickel in alkaline solution at more moderate pressures to form propylcyclohexane derivatives.

Cooke, McCarthy & Hibbert (1941) hydrogenated maple ethanol lignin. They obtained the same products as Harris et al., a new unidentified substance and a high boiling resin.

Godard, McCarthy & Hibbert (1941) treated maple wood with hydrogen in the presence of copper chromite and obtained 4-n-propylcyclohexanol and 3-(4-hydroxycyclohexyl)-1-propanol.

Pepper (1943) has studied the effectiveness of various hydrogenation catalysts and concluded that Raney nickel and copper-chromium oxide are the most suitable for bringing about the degradation of maple lignin. He found that the most important factors in the extraction of lignin from maple wood by high pressure hydrogenation were (a) nature of catalyst and solvent, (b) time, (c) temperature, and (d) pH of the reaction medium.

Bower, McCarthy & Hibbert (1941) hydrogenated the holocellulose of maple wood, fractionated the products formed, and compared these with fractions isolated from the entire wood samples. They were thus able to show that certain fractions were not likely of lignin origin and that other products, e.g., 4-n-propylcyclohexanol, were probably of lignin origin. The yield of distillable oils was much greater from the entire wood than from the holocellulose.

Bower, Cooke & Hibbert (1943a) studied the products formed by the hydrogenation of maple wood and by the use

of a more efficient fractionating column were able to separate the material usually isolated as 4-n-propylcyclohexanol into two substances, 4-n-propylcyclohexanol and 3-cyclohexyl-1-propanol. In the later paper, Bower, Cooke & Hibbert (1943b) studied the formation of lignin in spruce buds using hydrogenation technique. They hydrogenated 2.5-3.0 week old spruce tips and spruce tips 3.0-4.0 months old, and fractionated the products through the column previously used. Refractive indices of the different fractions were plotted against the cumulative weight of material isolated. Failure to secure any 'flats' in this curve in the refractive index ranges of known propylcyclohexyl derivatives in the case of the young spruce tips led the authors to state that there was no lignin in young spruce buds. However, distillable substances having the same refractive index range as propylcyclohexane compounds were found. Since these compounds might not give 'flats' if they were present in very small amounts, it would seem that the argument of Bower et al. is not necessarily sound. They succeeded in isolating 4-n-propylcyclohexanol from the older material and hence concluded that lignin had been formed at this stage of growth.

It was thought that hydrogenation of pre-extracted feces and forage samples would show something of the chemical composition of the lignin fraction of these materials. Information thus secured would supplement the data obtained from the chemical and spectrographic studies of these materials.

Experimental Methods

Hydrogenation Apparatus:

The equipment used in the hydrogenation work consisted of (1) a 2500 cc. capacity high pressure hydrogenator manufactured by the American Instrument Company (#406-01a, catalogue #406, American Instrument Company); (2) a type 100 Variac manufactured by the General Radio Company, which was used to control the temperature; (3) a hydrogen pressure "booster" pump which was used to secure any desired pressure above the cylinder pressure (#406-135, American Instrument Company); (4) a slide-wire potentiometer calibrated to read temperature directly, which was used to measure voltages from a copper-constantan thermocouple, the hot junction of which was inserted in a wall in the bomb.

The arrangement of the hydrogenation apparatus is shown in the following photograph (Figure 5).

Hydrogenation Equipment

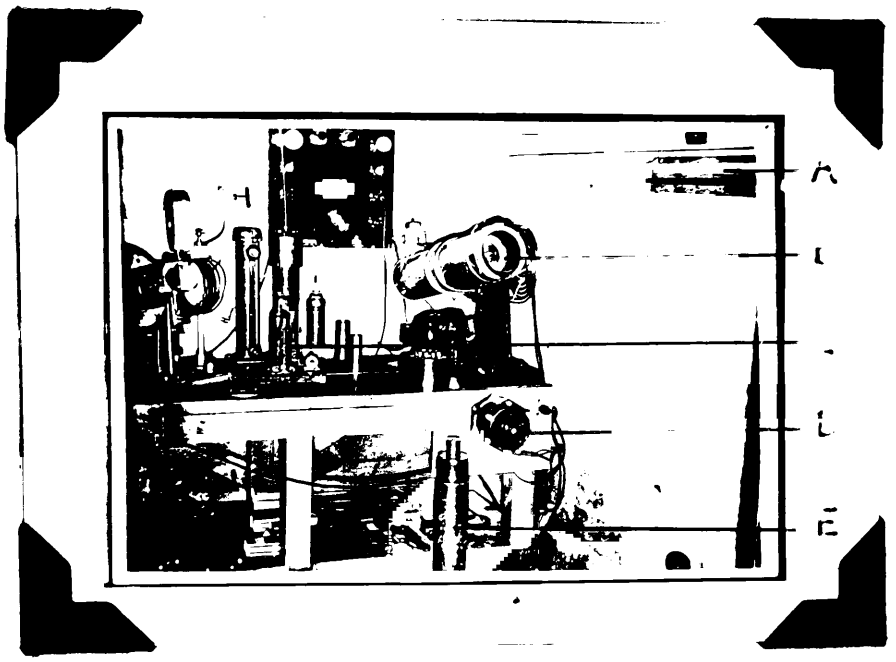


Figure 5.

A. Slide-wire potentiometer.

B. Heater for bomb.

C. "Booster" pump.

D. Variac.

E. Bomb.

Materials:

The feces material was successively extracted with boiling water, 150 cc. per gm. dry weight, 1% hydrochloric acid in the same proportion and, after drying, with alcohol-benzene.

The forage material used was young timothy cut about June 1st. The opportunity to use the hydrogenation apparatus had not presented itself when the forage digestibility trial samples were taken, and hence there was not enough of this material available for this study. Moreover, for the initial work, the use of a pure species sample had certain advantages in view of the variation in materials extracted from different species. The young timothy was comparable in age to the timothy which comprised a large percentage of the forage material used in the digestibility studies. The finely ground (40-mesh) timothy was extracted with alcohol-benzene, hot water, and 1% hydrochloric acid as for a lignin determination. A description of these materials is tabulated below.

TABLE 9

Nature of materials hydrogenated.

	Feces	Timothy*
Percentage moisture	58	-
Weight of original material (+moisture)	1370	60
Lignin soluble in sulphite	60	75
Percent purity of isolated lignin (spectrographic)	52	-
Gms. of extracted material (air-dried)	157.0	20.5
Percent lignin in extracted material	39.2	18.4
Percent nitrogen in extracted material	1.50	1.07
Percent methoxyl in lignin	6.57	7.47
Percent nitrogen in lignin	3.00	4.00

*Data supplied by Mr. D. MacDougall.

Dioxane was used as the solvent. It was dried and purified by refluxing over metallic sodium for 24 hours, then collecting the material distilling at 101.5° C. Copper-chromium oxide, prepared as described by Connor, Folkers & Adkins (1932), was used as the catalyst.

Methods and Results:

The same general procedure was followed as that described by Bower et al. (1943) since it was planned to compare the products formed by hydrogenation of timothy

and feces with each other and with the materials isolated by Bower.

The charge, consisting of 100 gms. air-dried feces material, 60 gms. of catalyst and 1 litre of dioxane, was placed in the bomb and the latter was flushed out three times with low pressure hydrogen (500-800 lbs.). The bomb was then pumped up with the "booster" pump to the required pressure of 200 atmospheres. The bomb was then started rocking, and shortly after, the heat was turned on. The temperature rose to 280° C. at which it was maintained by adjusting the Variac. Pressure and temperature readings were taken at intervals. The hydrogen concentration was calculated from the gas law, PV = nRT. The volume of hydrogen in the bomb was calculated by measuring the space not filled with the charge. The reaction was allowed to continue for 21 hours. After cooling, the charge was removed from the bomb, and the catalyst and undissolved material were centrifuged off. The liquid portion was a greenish fluorescent oil. The catalyst was found to be poisoned (i.e., had turned red) and hence the liquid was returned to the bomb with 60 gms. of fresh catalyst and hydrogenated again under the same conditions for 12 hours. On removal from the bomb the liquid was found to be a light almost colorless oil and the catalyst was black. The material centrifuged off from the first hydrogenation was dried and weighed. The catalyst and undissolved feces material weighed 88 gms.

The material from the second hydrogenation was centrifuged to remove the catalyst. The dioxane and other low boiling materials were distilled off at atmospheric pressure and up to 104° C. The lowest boiling material came over at 75-80° C. The residual material was transferred with a small amount of dioxane to a small distilling flask which had been loosely filled with glass wool to prevent bumping. The distilling flask and the glass wool had been previously weighed. The dioxane used to transfer the product was distilled off, the flask and contents were weighed, and the product was distilled under vacuum into a weighed test-tube. The first product, a colorless oil, distilled over at a vapour temperature of 65° C. and a bath temperature of 185° C. under a pressure of 300 μ. At 120° C. vapour temperature and a bath temperature of 200° C. the product was a yellow oil. The distillation was continued until crystalline material came over at a vapour temperature of 180° C. and 55 μ. pressure. The distillation was stopped at this point. Later a second fraction of very high-boiling resinous material was distilled at 220-265° C. and 20 μ. pressure. The product was too high-boiling to fractionate and, since no crystalline material could be obtained from it, it was weighed and discarded.

The first distillable fraction was dissolved in ether. On standing a short time, a white crystalline material separated out. The suspension was centrifuged and the ether solution was decanted off. The crystals

were washed four times in the centrifuge tubes (until the ether was colorless), and the ether washings were removed by centrifuging and decantation.

The crystalline material was dissolved in warm ethyl alcohol and crystallized out at 0° C. The suspension was filtered, the crystalline material recrystallized from alcohol, filtered, washed with ether to remove the alcohol, and dried in vacuo. Some additional crystalline material was recovered from the combined washings by concentration and recrystallization, and this was added to the first. The total yield was 0.36 gms. The melting point of this material was not sharp. It was then recrystallized from alcohol until the melting point was sharp (at 96.5-97° C.) and was not changed by further crystallization (Crystals I).

The ether-soluble portion of the product was transferred to a tared pear-shaped flask loosely filled with glass wool and was fractionated through a column of the type described by Bower & Cooke (1943). The rate of refluxing was 5-6 drops per minute. Fractions were removed at 45 minute intervals. The fractionation was continued until crystalline material started to distil over at a bath temperature of 234° C., a column temperature of 111° C., and 0.5 mm. pressure. The material remaining in the flask was removed by vacuum distillation into a tared test-tube. It was a brown waxy solid.

The last mentioned material was dissolved in warm alcohol. On cooling to 0° C., a white crystalline precipitate separated. The crystals were filtered off, the filtrate was concentrated, and on cooling, further crystalline material settled out. After filtration, the filtrate was diluted with alcohol, boiled with decolorizing charcoal, filtered, then concentrated to small volume, cooled at 0° C., and the crystalline material that settled out was filtered off. The filtrate was discarded. The crystal fractions were combined. Attempts were then made to purify this material by crystallization from alcohol, alcohol-water, ether, benzene and chloroform. In each case it was soluble in the warm solvent but crystallized out readily in the cold (0° C. or less). However, in spite of repeated recrystallizations a sharp melting point could not be obtained. Since the amount of material was limited, the attempt at purification was abandoned when a white crystalline material melting at 88-93° C. was secured (Crystals II).

The refractive indices at 25° C. of the fractions distilled through the Bower-Cooke column were determined using an Abbé refractometer equipped with a thermostat to control the temperature. The refractive indices were plotted against the cumulative weight of distillable material. The curve is given in Figure 6.

The young timothy was hydrogenated under the same conditions of temperature and pressure as described for the

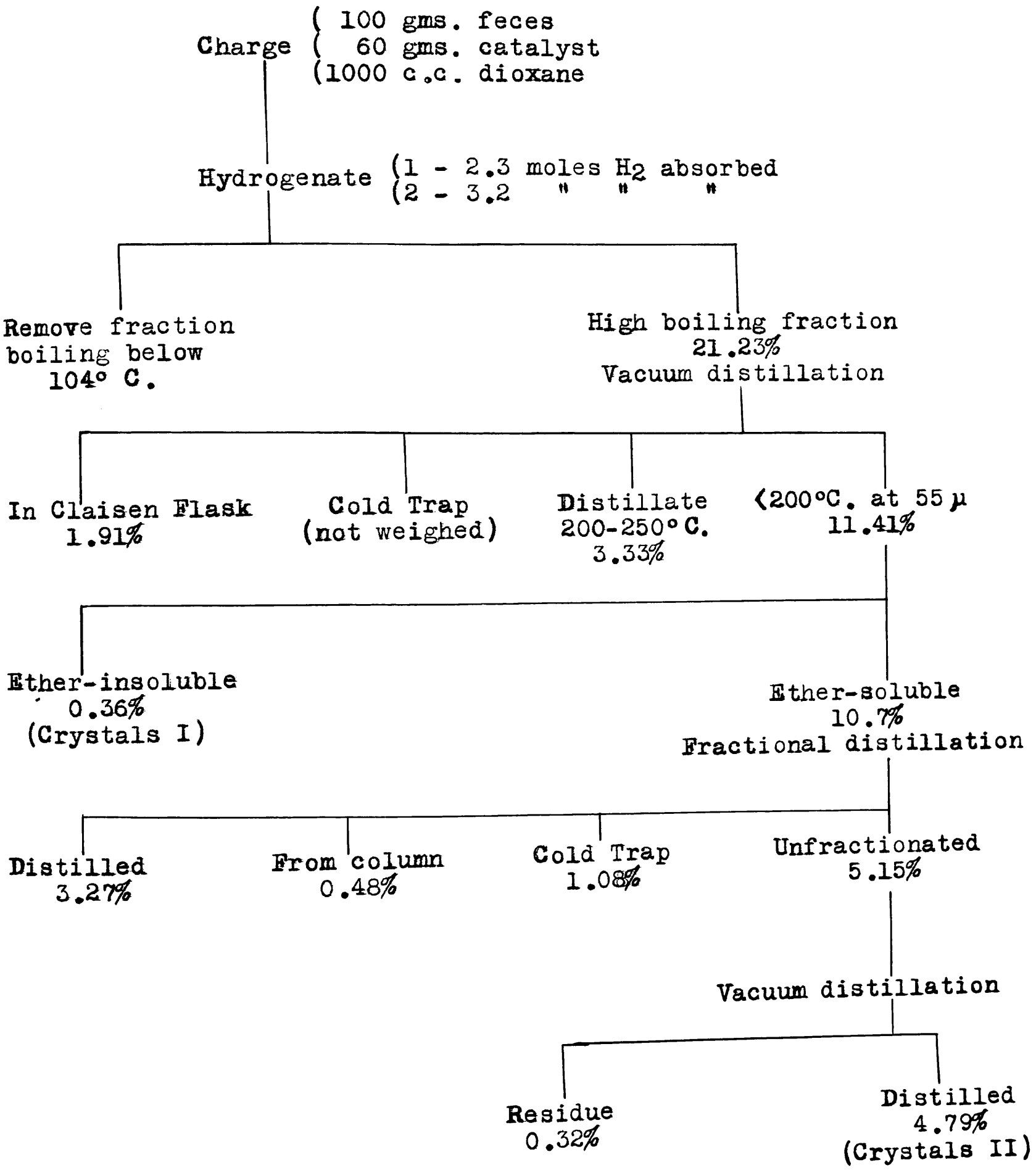
feces. The charge consisted of 100 gms. of extracted material, 60 gms. of catalyst, and 1000 cc. of dry dioxane. It was necessary to hydrogenate this material three times, since after the first two hydrogenations the catalyst was red. The poisoned catalyst was removed by centrifuging before fresh catalyst was added. After the third hydrogenation the catalyst was black and the liquid was water-white. The fraction distillable below 105° C. at atmospheric pressure was removed and the residue was transferred to a tared distillation flask loosely filled with glass wool. After boiling off the dioxane used to transfer the residue, the flask and contents were weighed. The product was then vacuum distilled into a weighed test-tube. The material started to distil at a bath temperature of 220° C., a vapour temperature of 100° C. and 30 μ. pressure. Distillation was complete at a bath temperature of 345° C., a vapour temperature of 250° C. and a pressure of 14 μ. After weighing, the product was dissolved in ether and let stand. A small amount of crystalline material settled out. This was removed by centrifuging and decantation. The crystalline material was very small in amount and relatively soluble in warm alcohol. It was later discarded. The amount of product recovered from the 100 gm. sample was too small for successful fractionation through the Bower-Cooke column. It was therefore combined with a similar hydrogenation product of another sample from the same material which had been hydrogenated by Mr. D. MacDougall in connection with another study.

The combined material was then fractionated as described for the feces study. The fractionation was stopped when crystalline material appeared in the receiver at a bath temperature of 280° C., a column temperature of 128° C., and slightly over 3 mm. pressure. On cooling, a considerable amount of material solidified in the column and in the condenser. There was a clear separation between these compounds since there was a sudden elevation of the boiling point of the material in the flask and the upper part of the column went dry just as distillation was discontinued. These fractions were removed by passing acetone through the apparatus. These two materials were readily soluble in warm acetone but crystallized out at 0° C. They were purified by recrystallization from acetone. The melting point of the material from the condenser was 54-56° C. (Crystals III) and that of the material from the column was 95-97.5° C. (Crystals IV).

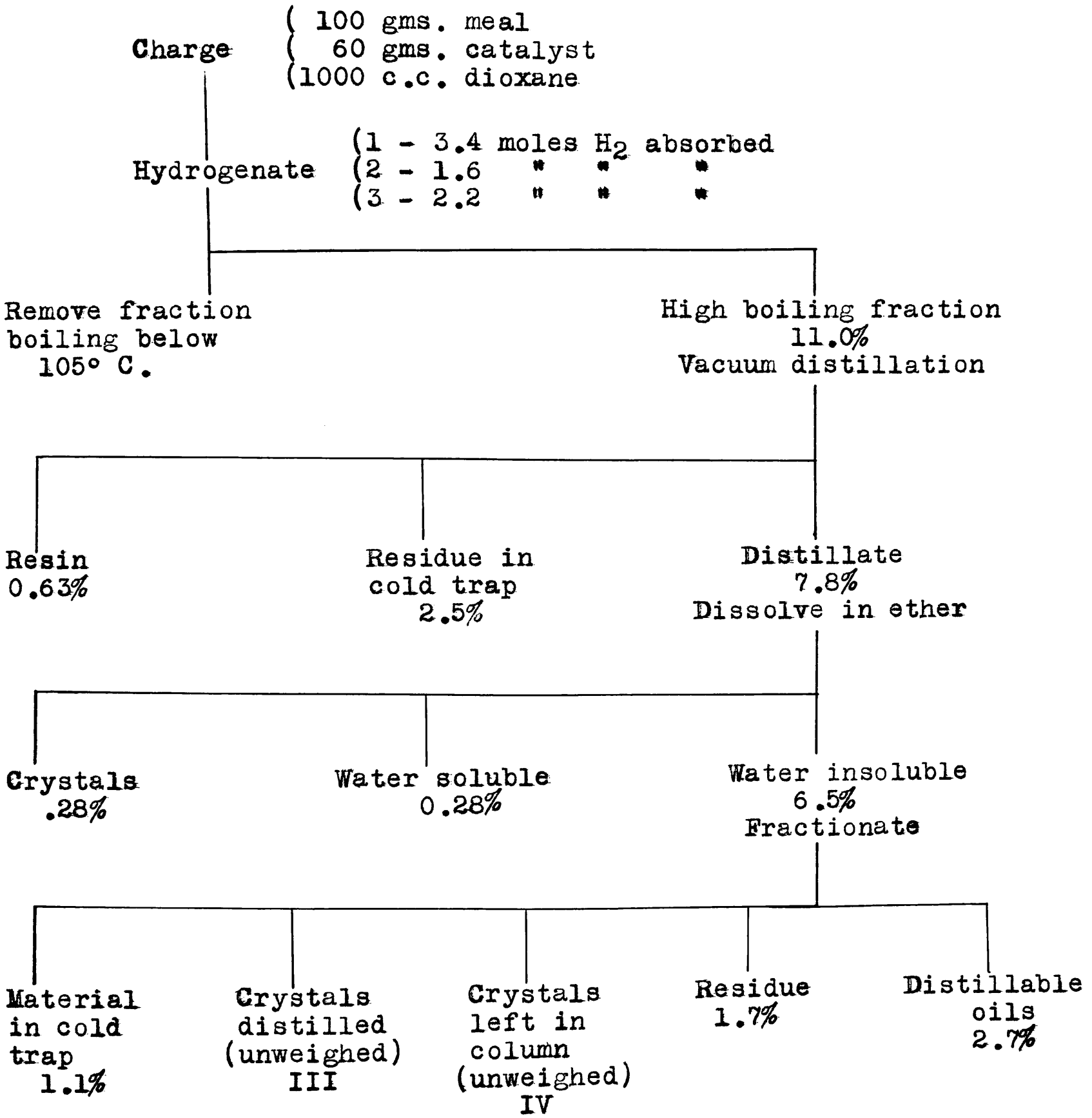
The refractive indices of the material distilled through the Bower-Cooke column were determined as for the feces sample. These values were also plotted against total weight of distillable material on Figure 6.

Flow sheets, showing the amount of material recovered in each fraction, have been prepared for the feces and timothy samples. The material from the timothy samples hydrogenated by Mr. MacDougall and by the writer was combined for fractionation, and therefore it was thought best to average the results of the treatments previous to fractionation. The average value is therefore given in the timothy flow sheet.

Flow Sheet 1 for Feces Samples



Flow Sheet 2 for Young Timothy



Fractionation curves of distillable oils from hydrogenation products of feces and timothy.

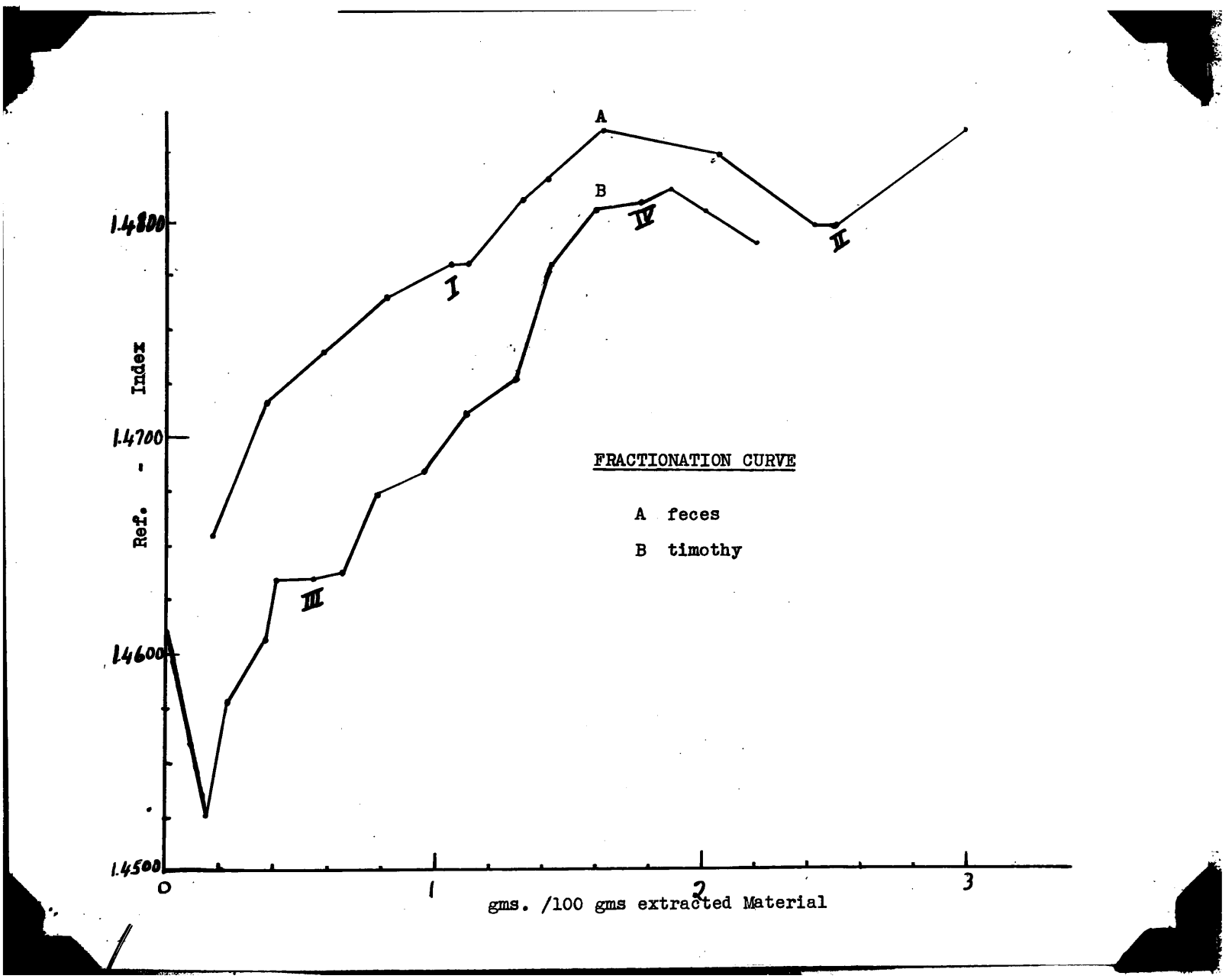


Figure 6.

The carbon and hydrogen contents of the crystalline materials isolated from the feces and from the timothy, and of four samples of the distillable oils from the fractionation products of the same materials were determined by the semi-micro method described by Niederl & Niederl (1938). The distillable oils were taken in the refractive index regions as marked on Figure 6 (I and II from feces and III and IV from timothy). The results are tabulated below.

TABLE 10

Properties of hydrogenation products.

Type of Material	Sample No.	Refractive Index at 25° C.	Melting Point °C.	Carbon %	Carbon av.dev.	Hydrogen %	Hydrogen av.dev.
Crystals feces	I	-	96.5-97	74.9	0.4(3)	10.9	0.2(3)
Crystals feces	II	-	88-93	75.6	0.2(2)	14.0	0.1(2)
Crystals timothy	III	-	54-56	80.4	0.1(2)	14.3	0.1(2)
Crystals timothy	IV	-	95-97.5	74.7	0.2(2)	13.3	0.1(2)
Oils feces	I	1.4781	-	72.0	0.2(2)	11.8	0.1(2)
Oils feces	II	1.4802	-	75.1	0.3(2)	12.5	0.1(2)
Oils timothy	III	1.4635	-	73.2	1.3(4)	12.6	0.3(4)
Oils timothy	IV	1.4810	-	78.7	0.3(2)	13.4	0.0(2)

Discussion of Results:

As can be seen from the flow sheets, the hydrogen absorption was greater for the young timothy than for the feces. This was possibly due to the fact that the feces sample did not go entirely into solution on hydrogenation (weight of undissolved material centrifuged off with catalyst = 28 gms.). This should also be borne in mind when comparing the yields of various fractions from the two materials. Based on the Klason lignin content of the hydrogenated material, the yield of most fractions was lower for the feces sample than for the timothy sample. Another distinguishing feature of the two flow sheets was the fact that the yield of distillable oils was higher in the timothy sample if based on either the Klason lignin content or on the amount of water insoluble oils that were subjected to fractionation. The percentage of unfractionated material was higher in the feces sample.

The oils that were fractionated from the feces sample were generally higher in their refractive indices than those from the young timothy. Also in the case of the feces sample there was no indication of any of the lignin products that Bower et al. (1943a) isolated from wood. There was a possibility that oil sample III (from young timothy) may be 4-n-propylcyclohexanol. The refractive index was in the proper range, and a very short 'flat' was found in the curve.

The elementary analysis shows that this material could be a propylcyclohexanol, though the carbon content is somewhat low.

The carbon and hydrogen analyses would indicate that the crystalline and oil products formed by hydrogenation were somewhat similar in nature (Carbon 72-80%; hydrogen 11-14%).

In general, the hydrogenation work showed that young timothy and feces materials yield hydrogenation products that are similar in nature. Neither showed the presence of any large amount of material that investigators have found comes from lignin. In the fractionated material from young timothy, there was one short 'flat' that might indicate material from lignin. It was not present in material from the feces sample.

GENERAL CONCLUSIONS

General Discussion

The structure of lignin is still uncertain and in view of this it is impossible to prove that the material isolated is 'pure' lignin. Since this is true, it is not possible to prove that one method of determining lignin is superior to another; if the yield of lignin is high, this may be due to the formation of artefacts and to the condensation of foreign material with the lignin; low yields of lignin may be the result of solution of the more soluble lignin components. The hydrogenation and spectrographic studies would indicate strongly, however, that the amount of true lignin in forage and feces is lower than it is estimated to be by methods of lignin determination now in use. The high nitrogen content of the isolated lignin would tend to strengthen this argument.

In view of this uncertainty regarding the chemical nature of the lignin isolated, and the possible effects of change of plant species on the amount and nature of lignin isolated, there would seem to be little likelihood of the time-consuming lignin determination being satisfactory as a measure of the amount of indigestible material in forage. Progress in the use of chemical analysis to measure the nutritive value of forage is dependent on the progress in the knowledge of plant chemistry. Knowledge of the nature

of the lignin of young plants and of the differences that may exist between lignins of young plants of different species is still indefinite. Therefore, methods of proven accuracy for the determination of lignin in young plants and in feces therefrom are still unavailable.

General Summary

A study has been made of the lignin of forage and of feces therefrom. The following general conclusions are indicated.

1. Feces samples that are subjected to drying before the pre-extraction treatments yield larger amounts of apparent lignin than those that are pre-extracted before drying. A method of pre-extracting feces samples before drying has been developed.

2. The methoxyl content of lignin from feces is usually higher than that of forage lignin. The reverse is usually true of the nitrogen percentages.

3. Results obtained by the Crampton & Maynard method are less easily reproducible than those obtained by the 72% sulphuric acid method. The former method yields a larger amount of lignin fraction that is usually higher in nitrogen and lower in methoxyl than the latter method. The two methods often give results that show widely different values for the digestibility of lignin.

4. A preliminary study of the urine of a sheep failed to identify any materials that could be proven to be degradation products of lignin.

5. Spectrographic studies of isolated lignins indicate that these may be less than 50% pure as compared to wood lignin. This is true for lignin isolated by either the Crampton & Maynard or the 72% sulphuric acid method.

6. The absorption curve of lignin isolated from forage and feces by the 72% sulphuric acid method is more like the absorption curve of wood lignin than is that of lignin isolated from these materials by the Crampton & Maynard method. There is, however, little difference in the amount of absorption per unit concentration at 2800 Å between the two materials.

7. The shape of the absorption curves and the high percentage of nitrogen in the 1943 samples would seem to show that a change in the species composition of the sward materially affects the nature of the isolated lignin. This may modify any seasonal change in the apparent digestibility of lignin.

8. High pressure hydrogenation of young timothy and feces materials failed to produce, in significant amounts, products which have been shown to be derivatives of lignin. Crystalline material and distillable oils with carbon and hydrogen contents similar to those of monohydroxy propyl-cyclohexyl derivatives were formed.

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