AN EVALUATION OF THE PHEOPHYTIN METHOD FOR
DETERMINING THE DRY MATTER DIGESTIBILITY
OF FORAGES CONSUMED BY RUMINANTS

by

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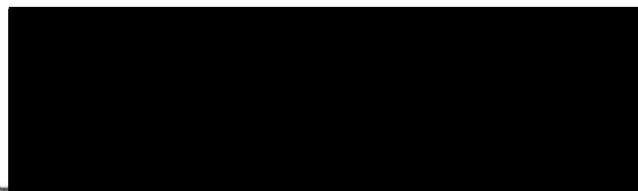A THESIS

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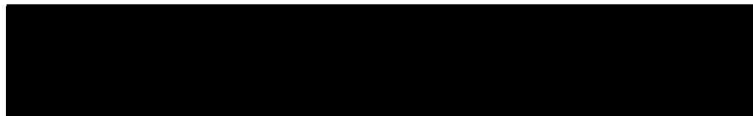degree of

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INTRODUCTION

The time and energy used in studying methods for determining the evaluation of feeds is indeed well spent. Advancements in the fields of both animal nutrition and plant breeding would proceed more rapidly than at present with a satisfactory yet rapid method for determining the nutritive value of feeds.

Because of the many variable conditions which are encountered in performing any biological study, it is necessary that similar results be obtained repeatedly by different investigators under similar conditions before a certain technique is accepted. This thesis presents the results of an attempt to evaluate an existing technique for determining dry matter digestibility.

Because of the lack of background experience on the part of the author and of reliable information in the field under study, much time was spent in varying the procedure for pheophytin determination in forages and feces. An attempt has been made to clearly and completely outline all the various techniques used so that any person may, without undue difficulty, repeat this study. Also, many of the problems which can be expected to arise have been discussed so they may be avoided.

The results obtained by the use of the pheophytin method for the determination of the dry matter digestibility of forages, as described by Kane (25, p.672) and Chesnutt (5, pp.86-89) constitute the first portion of this thesis. The pheophytin study was carried on with

samples obtained during a series of four digestion trials with dairy cattle, two with clipped pastures and two with silages. The results were compared with those obtained by the chromogen method and by the conventional digestion trial method with the same forages. Also included is a discussion of the principles of the pheophytin method, a study of some of the pigments which make up the pheophytin extract and an evaluation of the pheophytin procedure for digestibility determinations.

REVIEW OF LITERATURE

Studies relating to the digestibility of dry matter or of one or more of its components in feed stuffs are among the oldest and the most abundant to be found in the field of animal nutrition. During the period from 1867 to 1947, more than 2,500 publications have appeared in which original digestion experiments have been recorded. According to Schneider (41, pp.1-299) these publications, printed in 17 languages, contain the results of some 25,000 digestion trials.

Schneider (41) reported that the first recorded digestion trials were carried on in Germany by Henneberg and Stohman in 1864. These original trials closely resemble the conventional digestion trial used today. The method involves the collection and proximate analysis of both feed and fecal samples to determine the content of water, ash, crude protein (N x 6.25), crude fiber (Henneberg Method), ether extract, and nitrogen-free-extract (by difference). This procedure of collecting and analyzing feed and fecal samples must be carried on long enough to get an estimate of fecal constituents which will be representative of the kind and amount of feed consumed. Such a digestion trial is a long, tedious and costly project.

Since the original work of Henneberg and Stohman, digestion trials have been conducted with many and various organic constituents such as cellulose, lignin, etc., used as indicators in hopes of finding a reliable, quick method of determining the nutritive value of various animal feed stuffs, forages in particular.

Attempts have also been tried to develop an accurate method for determining the feeding and economical value of grasses and legumes in the form grazed by animals. Although pasture grass is generally considered to be the most economical of livestock feeds, research in this field has lagged far behind that conducted on other types of feeds. This is largely due to the lack of a reliable method for estimating digestibility and rate of consumption under grazing conditions. Because of the selective nature of animals, the digestion coefficient of a pasture forage consumed under hand-fed digestion trial conditions probably would be lower than that of the same feed, had it been selectively grazed (5, p.77).

The first attempts to develop a short-cut method to determine the digestibility of feeds were based on the use of an artificial indicator which could be placed in the feed and recovered in the feces.

Hendin of Sweden as reported by Hamilton (19,pp.119-120) devised an ingenous method of feeding a known amount of chromic oxide, in the feed, to animals, assuming that the chromic oxide would pass through the digestive tract without being absorbed. Hendin believed that if the chromic oxide were given in a definite amount to animals on constant feed, the concentration of this indicator in the feces would soon reach a fairly constant level. The digestibility of a particular nutrient in the feed could then be calculated from the ratio of the nutrient to chromium oxide in the feed given and in the feces excreted. For example, if the ratio of protein to chromium oxide in the feed were 100 to 1 and in the feces, 30 to 1, it would be evident that 70 parts of the

protein to each 100 consumed had disappeared from the digestive tract. Thus the digestibility of the protein would be 70 per cent. The weight of the feed consumed and of the feces excreted would not be needed in the calculations and theoretically, only one small sample of feces, taken after several days of chromium feeding, need be collected.

Reid et al. (39, p.1336) developed a formula for determining the digestibility of a nutrient in feed which can be used with the chromic oxide ratio technique or any other indicator method. The formula is as follows:

$$D = 100 - 100 \frac{a}{b} \cdot \frac{b1}{a1} \quad \text{where}$$

D = digestibility of nutrient expressed in per cent
a = concentration of indicator in feed
b = concentration of indicator in feces
a1= per cent of nutrient in feed consumed, and
b1= per cent of nutrient in feces excreted.

The chromic oxide indicator method has received study from many workers. Anderson (1, p.244) and Edin et al. (11, no.6773) reported having good success with this method. Kreula (29, p.270) however reported having difficulty in recovering all of the chromic oxide fed. On the average only 97.5 per cent of the chromic oxide consumed was excreted in the feces. In order to successfully predict the digestibility of any feed or nutrient in a feed, any indicator used must either pass through the digestive tract of the animal used in the digestion trial without being absorbed or be absorbed in a constant, known percentage. Also, if only one sample of feces is to be collected, the excretion of the indicator must be uniform throughout the trial.

Kameoka et al. (23, pp.462-467) carried out a series of four digestion trials, using goats, to examine the effect of the time of feed ingestion and kinds of feed on the diurnal variations of chromic oxide concentration in the fecal excretions. They found that there was a significant variation in the concentration of chromic oxide excreted in the feces, not only from one day to the next, but also within a day. These results are in agreement with the work of Kane et al. (27, pp.263-273) who worked with dairy cows and that of Hardison et al. (20, p.35) and Smith et al. (46, p.515). They studied the chromic oxide excretion-time pattern by both grazing and hand-fed steers. In grazing animals the peak concentration of chromic oxide in the feces appeared at night, but in hand-fed animals the maximum values appeared during the day as in Kane's experiment. If this variation occurs, and there is little doubt that it does, it does not seem likely that an accurate digestibility determination could be obtained, using the chromic oxide concentration found in one fecal sample as the representative concentration for the duration of the trial. A composite fecal sample would, therefore, seem necessary.

Bergeim, (3, p.32) one of the first to use inert chemical substances in digestibility studies, proposed a similar artificial indicator method using iron oxide, rather than chromic oxide. Iron oxide or hydroxide was considered a useful key substance for several reasons. The concentration of iron oxide can easily be determined in both feed and fecal samples, and it can readily be incorporated with the feed. Also, it is not absorbed from the intestine in appreciable

amounts and being insoluble to a great extent, it has little pharmacological action.

Hale et al. (17, p.390) however, reported that variations in the amount of iron oxide passing through the digestive tract did appear, making it unreliable for use in bovine digestibility studies. Knott et al. (28, p.555) and Moore et al. (37, p.302-303) reported similar findings.

Heller et al. (21, pp.275-282) reported that iron oxide might have the power to increase assimilation of food nutrients. In a group of five trials with rats, using both natural iron in the feed and iron oxide, it was also found that the iron oxide had a tendency to separate out of the ration, resulting in a poor recovery. Only 82.78 per cent of the total iron was recovered when both forms were used, whereas 97.43 per cent was recovered when only the natural occuring iron was used. From this information they concluded that the use of the natural occuring iron might be better than the use of iron oxide.

Forbes et al. (12, pp.373-382) and Swift et al. (46, pp.432-444) reported apparent success when they used the so called lignin ratio method for determining the digestibility of pasture forages and other feeds. According to this method the digestibility of the feed is determined from the ratio of lignin in the feed eaten to that in the feces voided. Ellis et al. (11, p.293) who also studied the lignin ratio method found that lignin was not digested to any significant amount. According to Forbes, (13, pp.9-11) lignin digestion varied from a negative to plus 71.9 per cent. The work of Crampton et al.

(7, p.337), Kane et al. (24, p.331), and Hale et al. (17, p.391) also indicate that lignin cannot be relied on to indicate digestibility trends in forage studies. The use of methoxy, a fragment of lignin isolated in lignin studies also proved unsatisfactory.

Skulmowski et al. (42, col.4715) studied the use of silica in determining the digestibility coefficients of various feeds. They reported adequate agreement between their results and those of the standard total collection procedure. The findings of Druce et al. (9, p.191) and Gallup et al. (14, p.893), however, indicate that approximately 15 per cent of the silica consumed is digested, which would mean that the amount of silica recovered in the feces is too variable to be of service in digestibility studies. Other workers including Kane et al. (26) claim that the dust in the air or the contamination of the feed with dirt interferes seriously with the results obtained when applying the silica ratio to digestibility studies.

In addition to the indicator methods already mentioned, many others have been tried and tested under different conditions, with various findings, according to Schneider (41, pp.1-299). Among these are the use of fecal nitrogen and forage protein. Others used were barium sulfate, titanic oxide, and anthraquinone violet. The use of a dry matter consumption-excretion ratio was also suggested by Garrigus et al. (15, pp.443-508) for use in pasture evaluation studies.

In 1950, Reid et al. (40, pp.60-71) reported on the first in a series of studies involving the use of chromogen(s), a material or

materials which occur naturally and in a measurable quantity in food stuffs and can be completely recovered in the feces. The proposed method of extracting and reading the chromogen(s) was as follows:

1. Samples were weighed on filter paper, then

2. Transferred to a 500 ml boro-silicate Waring blender cup which had been designed to prevent any leakage.

3. The sample was then combined with 250 to 400 ml 85% aqueous acetone, and

4. Mascerate for 3 to 7 minutes, removing the cup and cooling in an ice-water bath when necessary.

5. The sample was then transferred to a Buchner funnel and filtered by suction through Whatman No. 42 filter paper. The mascerate was washed with 85% acetone and returned to the blender, repeating the process two or more times, depending on the:

   a. degree of pigmentation of successive extractions,

   b. toughness of material being extracted, and

   c. fineness of masceration of the residue.

6. The extractions were then made up to a known volume and a portion of this, sufficiently large enough to prepare the final dilution, was filtered by gravity through Whatman No. 40 or 42 filter paper.

7. Absorption measurements were then read at 406 mμ, using a Beckman spectrophotometer.

According to well established laws and assuming a minimum of interference, solutions of similar source, absorbing the same quantity of light at a given wave length, an isosbestic point, would theoretically contain equal quantities of the same substance. Therefore, it would follow that at wave lengths where equal quantities of light were absorbed by the forage and corresponding fecal extracts, disregarding possible presence of interfering substances, some chromogenic substance(s) was present in both extracts in the same quantity. The occurrence of such isosbestic points would indicate a 100 per cent or complete recovery of the chromogen(s) in the feces. Woolfolk (50) in fact obtained an average of 101.2 per cent recovery of chromogen(s) in his study of the chromogen method. Possible explanations for this might be that the chromogen(s) is light labile and possibly more subject to destruction in the feed extracts than fecal extracts, that not all of the chromogen(s) was extracted from the feed or that a slight error was made in selecting the isosbestic point.

The wave length setting of 406 mμ which was used by Reid et al. (40, pp.60-71) and Woolfolk (50) has been accepted as the best isosbestic point for making chromogen absorption measurements. Several other isosbestic points were found, but none gave as great an absorption as that obtained at 406 mμ. This same setting gave the highest degree of absorption in studies of field, oven, and barn dried hays, silage, and pasture grasses.

The possibility of destruction of chromogenic material due to storage and light has been studied by several workers. It has

generally been considered that fecal samples are more labile to light destruction than feed samples. Reid et al. (40, pp.60-71) observed that very little chromogen destruction occurred when extracts were allowed to sit at room temperature, in the dark, for several days. He also found that chromogen analyses made at intervals of one month on the same feces stored at 1 to 5°C. were in close agreement. These findings might indicate that analysis of the samples does not have to be made on fresh material. Results obtained from more recent studies, however, seem to indicate that absorption readings should be made soon after the chromogen(s) was extracted from the samples.

Lancaster et al. (30, pp.489-496) studied the chromogen method with specific reference to the effect of light. It was found that the optical density of extracts exposed to light increased rapidly, the rate of increase depending on the intensity of light and the time of exposure. These results would indicate that light should be excluded both during and after the extraction procedures and that absorption measurements should be made within six hours of the preparation of the extracts.

Meyer et al. (36, pp.64-75) reported that chromogen extracts increased in light transmission when extracted in dim light and stored in the dark. Lofgreen et al. (32, p.268 and 33, pp.1158-1165) exposed chromogen extracts to 800 foot-candles of light for 20 minutes prior to making absorption measurements. Such standard treatment resulted in a stable solution which exhibited no change in light transmission during storage in the dark. However, when they applied the procedure

to pasture consumption and utilization studies, the chromogen results were consistently lower than those obtained by an actual measurement of feed consumption.

Woolfolk (50) found that the chromogen(s) responsible for the absorption at 406 mµ was light labile, but not as readily destroyed as the chlorophylls and carotene. He also reported that no great destruction occurred when extracts were allowed to set at room temperature in the dark for several days, nor was any consistent loss demonstrated when extracts were stored at 10-5°C. in the dark for a period of several months, Table 1.

Woolfolk (50) did find that there was a slightly greater loss of chromogenic material from extracts of silage and freshly clipped pasture grasses stored in the same manner. This might be explained by the greater concentration of the chromogen(s) in the extracts. In general, he obtained results equally good when he applied the chromogen ratio method to extracts of roughages cured in various manners and to extracts of rations which contained mixed grains. He believed that since the concentration of chromogen(s) was low in the grains, the error might be low, but another possibility of error might arise from the presence of interfering substances in the grain residue.

Table 1

Stability of Chromogen(s) Extracts Stored
in the Dark at 10-5°C (Woolfolk, 50,p.76)

| | Time (months) | Chromogen content per gram day matter | |
		Before	After
Field cured hay (mixed grasses)	11	52.8	51.9
Barn cured hay (mixed grasses)	10	55.9	55.4
Silage (mixed grasses)	11	107.9	104.0
Feces--sheep	11	189.4	189.4
Feces--cattle	11	110.3	109.6

Cook et al. (6, p.572) obtained a poor recovery of chromogen(s)

from certain plants, especially those high in ether extract. Smart

et al. (43, p.318-321) made a study of the pigments involved in the

chromogen ratio method. A chromatographic separation of the pigments

demonstrated the presence of at least seven pigments in the extracts

of hay and the feces of animals fed this hay. These pigments were

identified as: chlorophyll a, chlorophyll b, pheophytin a, pheophytin b,

luteol, violanthol, and beta carotene. The chromogen extract is essen-

tially a total chloroplast pigment extract or a mixture of the caro-

tenoids and the chlorophylls and their degradation products having a

maximum light absorption in the region of 406 mμ. The digestibility

of plant pigments has been studied by Davidson (8, p.211) with the

results shown in Table 2.

Table 2

The Apparent Digestibility of the Various Pigments
Found in Acetone Extracts of Dried Grass

Pigment	Apparent Digestibility %
Carotenoids	2-12
Xanthophyll	10-20
Chlorophyll a	81-87
Chlorophyll b	77-82
Pheophytin a	22-52
Pheophytin b	-36-17

Irvin et al. (22, pp.541-550) separated the various pigments into three classes based upon their physical and chemical properties. These classes were carotenoids, xanthophyll, and chlorophylls. The carotenoids are considered unstable, absorbed from the digestive tract to a certain degree and definitely light labile. The xanthophyll likewise are unsuitable as they are sensitive to oxygen and acid. The last class, the chlorophylls, go through a chemical change as they pass through the digestive tract. The gastric juices, especially in the fourth stomach of the ruminant, replace the magnesium with hydrogen in the porphyrin ring of the chlorophyll molecule leaving pheophytin.

Although Reid et al. (40, pp. 60-71) and Woolfolk (50) reported complete recovery of the chromogen(s) in fecal samples, thus being able to obtain results that were in close agreement with those of the conventional digestion trial, other workers did not attain this apparent success. Smart et al. (44, pp. 1331-1332) recovered only 55 per cent of the chromogenic material in trials using winter leaves of switchcane, Arundinaria sp. and 82.9 per cent chromogenic recovery

with spring leaves. These authors found that Reid's chromogen method worked well only when the principal pigments were the chlorophylls and their degradation products. Kane et al. (25, p.672) also reported unsatisfactory results obtained from the chromogen method.

Smart et al. (44, pp.1331-1332) studied the use of copper derivatives of chlorophyll in pigment ratio methods for estimating forage digestibility. They found that the introduction of copper into the porphyrin ring of the chlorophylls and pheophytins by treatment with cupric chloride stabilized these compounds against acid, alkali and light while at the same time largely destroying the carotenoids. They compared their modified method with the original method of Reid's. In the trial previously mentioned with the use of switchcane, the recovery of the chromogen(s) was increased from 55 per cent for winter leaves and 82.9 per cent for the spring leaves by the original method to 100 per cent for winter leaves and 99.3 per cent for spring leaves by the copper derivative method. These workers considered that this modified method might prove more successful than the original when the forages to be studied contained relatively small amounts of the chlorophylls and their degradation products.

Because of the varied digestibility of the pigments involved in the chromogen method, a search was started to find a single pigment which could be used as an indicator in digestibility studies. It was reported by Irvin et al. (22, pp.541-551) that no pigment exists in hay which passes unchanged completely through the digestive tract. One pigment extracted with 80 per cent ethyl alcohol from both hay and

feces, however, did prove promising enough to warrant further study. This pigment, which had a maximum light absorption at 415 mµ, behaves spectrally and chromatographically like pheophytin.

Pheophytin, whose isolation, physical and chemical properties were reported as early as 1928 by Willstatter (49, p.9), is the first breakdown product of chlorophyll. It is found to be the most significant plant pigment found in cow feces. As reported by Smart et al. (43, p.320) and shown in Table 3, apparently most chlorophyll is converted to pheophytin in passing through the digestive tract.

Table 3

Relative Amounts of Chlorophyll and Pheophytins
in 85% Aqueous Acetone Elutes from Bands of
Chromatograph Columns (Measured at 406 mµ)

	Optical density		Color %		Optical density		Color %	
	Fescue hay	Sheep feces	Fescue hay	Sheep feces	Ladino hay	Rabbit feces	Ladino hay	Rabbit feces
Chlorophyll a	1.558	0.404	41.6	11.9	0.708	0.400	23.4	11.2
Pheophytin a	0.550	1.500	14.7	44.5	0.709	1.520	26.1	42.6
Pheophytin b	0.630	0.620	16.8	18.4	0.518	0.602	17.1	16.8

After experimentation, Kane et al. (25, p.672), White et al. (48, p.216), it was found that if plant pigments extracted from freshly cut forage were treated with saturated oxalic acid, the wave length for maximum optical density reading was changed from 435 mµ (chlorophyll) to 415 mµ (pheophytin). This meant that a method was made available to duplicate the changes the chlorophyll undergoes in the digestive tract, and a single pigment would be predominant in both the feed and fecal extracts. Theoretically this would reduce any

error due to proportional changes in the amount of the various pigments found in the chromogenic extractions.

Several workers, including Kane et al. (25, p.672) and Chesnutt (5, p.96) have reported obtaining results with the pheophytin method which were in very close agreement with the results of conventional digestion trials, and in some cases in closer agreement than those obtained by the chromogen(s) method. Bateman et al. (2, pp.1-5) reported very good agreement in twelve of sixteen comparisons with the conventional and pheophytin methods, the differences ranging from 1.45 per cent to -1.00 per cent. However, in the four remaining comparisons, the pheophytin method gave results which were from 12.09 per cent to 24.45 per cent greater than the conventional method. This, he explained, might be due to light damage of the fecal samples from the four comparisons in the last two digestion trials.

The theory behind the pheophytin method does seem promising, readings being based on a single pigment with the same light transmission peak in both forage and fecal extracts. However, unless consistently superior to the original chromogen method, it is unlikely that it will become popular because of the extra steps involved in preparing the samples for absorption measurements.

EXPERIMENTAL PROCEDURE

The experimental material used in this study came from a series of four digestion trials with non-lactating dairy cows. The feeds used in the first two trials were clipped pasture forages, the first being an alfalfa-grass mixture and the second a second cutting of ladino clover-grass mixture. In both of the trials, the forages were clipped twice daily, once in the morning and once in the evening, to assure that the cows would have fresh feed, thus reducing the chance that they might go off feed because of heating of the forage. The forages used in the third and fourth trials were grass silages. The silage used in the third trial was preserved with 200 pounds of dried molasses beet pulp added per ton of forage and the silage used in the fourth trial was preserved without added preservative. As in the case of the first two trials, the animals were fed once in the morning and once in the evening.

An attempt was made to feed each animal the maximum amount of feed which she would completely consume. This amount was determined as nearly as possible during the five-day preliminary period before the collection period. However, any refusal that did occur was weighed and recorded and subtracted from the amount fed. If the refusal exceeded five pounds, it was saved for examination to determine if it had been a selective refusal, i.e. coarse stems, or just an excess which could not be consumed.

The animals used in conducting the trials consisted of one set of identical Guernsey twins, TX1 and TX2; one set of identical Holstein

twins, TQ1 and TQ2; one set of non-identical Jersey twins, TS1 and TS2; and a pair of non-related Jersey heifers, TU1 and 336. As the purpose of the trials was to be an evaluation of methods for determining the dry matter digestibility of the various forages used, the same forage being fed to all four of the cows making up a single trial, it did not seem essential that identical twins be used. Had two different forages been used in one trial, the use of identical twins would have a much greater advantage over the use of unrelated animals or even non-identical twins.

The major pieces of equipment involved in conducting the digestion trials were the four digestion stalls (5, p.84) in which the cows were confined during the collection period. These stalls were constructed so that all of the feces and urine excreted would fall onto an endless belt which was about three feet wide and five feet long. This belt moved in an uphill motion, one end being about twelve inches higher than the other. The urine would run to the lower end of the belt by gravity and drain into a tank while the feces would be carried to the upper end and dropped into another tank on the opposite side of the digestion stall. In this manner the feces was kept separate of the urine. Each digestion stall was constructed in a similar manner.

The samples of feed and feces to be used in the pheophytin method for determining the dry matter digestibility of the forages were taken from samples which were collected and sent to the Department of Agricultural Chemistry, Oregon State College, where a chromogen analysis and proximate analysis were made of the samples. The results

obtained from both the pheophytin and chromogen analysis were evaluated on the basis of a comparison with the proximate analysis or conventional digestion trial method.

The evening of the first day of the collection period, a sample of feed was taken from the forage fed each cow, prior to a weighing of the feed. The four samples were combined to form a composite sample, placed in a plastic sack and stored in a refrigerator overnight. The next morning a sample of feed was taken in the same manner, mixed with the sample taken the previous evening and called the feed of the first day. It was from this sample that the sample of the first day for the pheophytin analysis was taken. The rest of the sample was taken to the Department of Agricultural Chemistry for further analysis as soon as the fecal samples were obtained.

The feces tanks were emptied and rinsed the first day without saving any fecal samples. It was not until the morning of the second day that the feces samples were saved. This lag of one day between the feed and feces was provided to allow for the forage to pass through the digestive tract. In this way, it was assumed that the first samples of feces saved would correspond to the feed of the first day.

To ensure that a representative sample of feces was obtained, each morning at least an hour before collection time, ten pounds of water were added to each feces tank. The feces was then given a preliminary mixing and allowed to soak until collection time. As soon as the cows had been fed and the forage samples obtained, the belts

were stopped. The fecal tank from each cow was weighed and the feces thoroughly mixed. Samples taken for the pheophytin method were placed in waxed pint cartons, covered with cardboard lids and stored below 50°F until processed. If the storage date were to be a week or more, the samples were put in a pint plastic sack and tightly sealed to prevent any portion of the sample from being exposed to air. Samples were taken at the same time for the chromogen and proximate analyses. These samples were placed in one-gallon cream cans. Although the feed samples were composited, the fecal samples were kept separate.

The process of collecting both feed and fecal samples was continued for the next three mornings; whereas on the sixth or last day, only fecal samples were taken. This completed the collection period, giving a total of five composite samples of feed and five corresponding samples of feces from each cow.

The procedure for determining the pheophytin content of both forage and fecal samples was basically the same as that outlined by Chesnutt (5, pp.86-89) with a few modifications which will be explained in the appropriate section of this thesis.

1. Dry matter determination.

A 50-gram sample of forage or feces was weighed into a tared beaker on a triple beam balance. The sample and beaker were then placed in an electric oven, 95°C. for at least 48 hours. At the end of this period of drying or anytime thereafter, usually after all samples had been dried for the required period of time, the samples were removed and reweighed. The weight of the dried sample (weight of

beaker and sample after drying less the tare weight of the beaker) was multiplied by two (2) to give the percentage of dry matter in the sample.

2. Alcohol extraction.

The pigments were extracted from a 10-gram sample with a total of 750 ml of 95 per cent ethyl alcohol. A 10-gram sample was weighed into a pint screw-top fruit jar using a triple beam balance. The jars used were wrapped with black tape to protect the samples from exposure to light and to reduce the danger of breakage during the masceration process.

Approximately 150 to 200 ml of the 750 ml of alcohol was added to the sample. The jar was then screwed onto an Omni-mixer and the sample was mascerated for seven minutes by a six-edged cutting blade which rotated at 14,500 r.p.m. At the end of the seven-minute period, the sample was filtered by suction through a Buchner funner using Whatman No. 42 filter paper. The filtrate was collected in a 4-liter filter flask. After the filtration was completed, the residue and the filter paper were returned to the mixing jar, combined with a second 150-200 ml of alcohol and mascerated at the same speed for three minutes. Again the sample was filtered, combined with a third 150-200 ml of alcohol and mascerated in the same manner for one minute and refiltered. The remaining portion of the 750 ml of alcohol was used to rinse the equipment, then it was poured over the residue in the Buchner funnel. A technique of making the filtrate up to a known volume might assure more accurate results, rather than assuming that

all of the 750 ml of alcohol used in the extraction process is recovered, since a certain amount of the alcohol is absorbed by the residue.

The three maxceration periods of seven, three, and one minute, using 750 ml of alcohol, (5, p.87) appeared to be adequate for all samples, both forage and feces, with both the pasture and silage digestion trials, as long as a 10-gram sample was used. This was evident by the lack of color in the residue and the last portion of the filtrate which passed through the funnel. However, when the size of the sample was increased from 10 to 20 grams, a preliminary study was made to determine how completely the pigments were being extracted. To perform this study, two different 20-gram samples of silage from the same day were extracted with the same amount of alcohol but mascerated for a different period of time. These extracts were then each diluted in the same manner and the amount of light which was transmitted at 415 mμ was determined by means of a spectrophotometer. After the final extraction and filtration were completed, the residue of each sample usually discarded, was combined with an additional 150 ml of alcohol, mascerated for seven minutes and filtered through a Buchner funnel as before, but into a clean filter flask. The residue was rinsed as before with 100 ml of alcohol. After the filtration was complete, an aliquot of the filtrate was refiltered by gravity and read in a spectrophotometer. Any absorption of light was assumed to have been done by pigments which had not been extracted from the sample in the normal extraction time. The results of this study, Table 4,

indicate there was as much variation between two samples of the same feed extracted for the same time as there was between two samples of the same feed extracted for different periods of time. Poor agreement between duplications of the same sample is usually caused by an incomplete recovery of the pigments during the filtering process which takes place after the pigments have been oxalated. This problem is discussed in the section on the oxalation of the pigments. Since a loss of pigments would result in an increase in the amount of light transmitted by a solution, the lower transmission reading would be assumed more representative of the sample from which it had been extracted.

Table 4

The Effect of Masceration Time on the Pigment Extraction
from 20-Gram Silage Samples Using 95% Ethyl Alcohol

Day of collection (third trial)	Masceration time (minutes)	Transmission % (duplicates)	% Transmission (additional extraction)
2	7,3,1	75.6	----
2	7,3,1	56.3 59.7	89.2
3	7,5,3,1	59.8 62.0	89.9
3	7,7,7,7	72.4 60.8	92.2
5	7,5,3,1	56.8 56.0	93.0
5	7,7,7,7	52.6 51.8	87.2

3. Ether extraction.

After the pigments were extracted in alcohol, a 100 ml portion

was combined with 100 ml of NaCl, 4% w/v. Chesnutt (5, p.87) proposed that a 50 ml portion of fecal extract be combined with 150 ml of the 4% NaCl because of the greater concentration of pigments in the feces. On a dry basis there is definitely a greater concentration of pigments in the feces as a result of the absorption of the digestible nutrients as the forage passes through the digestive tract of the cow. However, since the percentage of dry matter in the feces is about one-half of that in the forage in the four digestion trials used in this study, the amount of pigment extracted from the same size sample, wet basis, was about the same for both fecal and forage samples.

Fifty ml of the combined alcohol extract-salt solution was transferred to a 150 ml separatory funnel where it was extracted with 50 ml of petroleum ether in five 10 ml portions. The first 10 ml of petroleum ether was combined with the 50 ml extract and shaken thoroughly then allowed to stand until a clear line of separation was formed. The lower layer was then drained out and combined with a second 10 ml of petroleum ether. The top layer which contained a major portion of the pigments was then poured into a 38 X 200 mm rimless culture tube to which 10 ml of 95 per cent ethyl alcohol had been added. Each successive portion of petroleum ether was added and the extracted pigment saved in the same manner. After the last extraction, only a faint yellow color remained in the alcohol-salt solution extract. A second 50 ml portion of the alcohol extract-salt was combined with another 50 ml of petroleum ether and extracted in the same manner as a check or duplication.

The purpose of the ether extraction is to remove certain non-pigmented materials such as proteins which have been alcohol extracted from the samples along with the pigments. The addition of the neutral salt, NaCl, changes the solubility of these compounds (48, p.154), and they settle into the heavier layer. The majority of the pigments, being more soluble in the petroleum ether, can thus be extracted in a relatively pure state. According to Gortner (16, p.848) chlorophyll b is insoluble in cold petroleum ether. This might suggest that chlorophyll b would not be extracted with the rest of the pigments in the petroleum ether. However, even when extreme caution was taken to separate all of the salt-alcohol solution from the petroleum ether, it was impossible to recover the complete 50 ml of salt and alcohol added. In most cases, only about 49 ml were recovered. If the one ml which was lost, probably alcohol, were absorbed into the petroleum ether, which is highly possible, the extracting solvent would be approximately 2 per cent ethyl alcohol, not pure petroleum ether. Since chlorophyll b is soluble in a 1 per cent solution of n-propanol in petroleum ether, it is possible that it would be soluble in the 2 per cent ethanol solution. Since a study of the pigments involved in the pheophytin method indicates that chlorophyll b is present, it must be extracted at least in part with the rest of the pigments.

4. Concentration of pigments.

The culture tube, containing the 50 ml of petroleum ether, 10 ml of alcohol and the extracted pigments, was next placed in a water bath at 65°C. where the petroleum ether was evaporated, thus concentrating

the pigments into the 10 ml of alcohol. As a precaution against bumping, two small glass beads were added to each culture tube and the tube shaken until a steady boiling was attained. The complete evaporation of the petroleum ether took about 15 to 20 minutes.

5. Oxalation of pigments.

As soon as the evaporation of the petroleum ether was completed, the culture tube was removed from the water bath. Two ml of saturated oxalic acid were added to the concentrated pigments, and the tube was shaken and allowed to stand until the oxalation reaction was completed. In the case of a green forage, a definite color change from green to yellow appeared. In the case of silage and fecal extracts, no color change could be noticed. The next step after the oxalation was to remove the magnesium oxalate. This was accomplished by filtering the oxalated pigments by gravity through Whatman No. 42 filter paper into a 25 ml volumetric flask. The additional alcohol required to make the pigments up to a volume of 25 ml was used to rinse the filter paper. This procedure of rinsing was very critical. Quite often a colored precipitate would form on the filter paper. The exact nature of this precipitate and the reason for its presence in some cases and not in others are not known. However, since the precipitate is not formed until after oxalation, it could possibly be a precipitation of pigment on crystals of magnesium oxalate. If this precipitate is not thoroughly washed of all color, a marked loss of pigment occurs as indicated by the final reading.

6. Reading of pigment concentration.

After the oxalated extract was made up to a volume of 25 ml, it was diluted and read on a spectrophotometer at 415 mμ. The amount of dilution would vary with the type of samples being studied. The samples should be diluted so that the final readings would fall within a range of 50 to 80 per cent transmission. If the readings are too much above or below this range, the accuracy of the results is decreased. In this study, a 10 per cent dilution, i.e. 1 ml extract and 9 ml alcohol, was used with the two pasture trials and a 20 per cent dilution, i.e. 2 ml extract and 8 ml of alcohol, was used with the two silage trials. These dilutions can be made quite easily and accurately with the use of a 1 or 2 ml volumetric pipette and a 10 ml volumetric flask.

7. Calculation of readings.

The results of the pheophytin method were evaluated by two methods. The first was the calculation of the percentage recovery of the pigments in the feces. This was accomplished by the following formula:

$$\% \text{ Rec.} = 100\left(\frac{a \text{ X D.M. excreted/5 days}}{b \text{ X D.M. consumed/5 days}}\right) \text{ where}$$

a   = units of pheophytin per gram oven dry feces,

b   = units of pheophytin per gram oven dry feed and

D.M.   = pounds of dry matter.

The other method was the comparison of the dry matter digestibility as calculated by the pheophytin method with that calculated by the

chromogen and conventional methods. The formula used for the calculation of the dry matter digestibility is:

$$\% \text{ Dig. of D.M.} = 100 - \left(\frac{\text{b consumed}}{\text{a excreted}} \text{ X } 100\right)$$

Both of these formulas include the units of pheophytin per gram of dry matter in both the feed and feces. The concentration of pheophytin is determined spectrophotometrically by determining the amount of light not absorbed by the pigments in the extract at 415 mμ. The diluted extract was transferred to a selected, round, glass cuvette and read in either a Coleman Junior or Coleman Model H spectrophotometer which had been adjusted to 100 per cent transmission using 95 per cent ethyl alcohol as reference. All readings were made at a wave length setting of 415 mμ. Since selected, rather than matched cuvettes were used, the best results were obtained when the same cuvettes were used for the reference blank and for the unknown each time and care was taken to see that the cuvettes were placed in the carrier in exactly the same position each time. The amount of light transmitted by the extracts were read on the galvanometer scale either as per cent transmittancy (T) or as absorbancy. The latter is often called optical density (O.D.). These readings are converted to units by means of a calibration or transmittance-concentration curve.

If light absorption follows the Beer-Lambert Law, the transmission readings fall in a straight line when plotted O.D. against concentration of solute. From this curve the concentration of an unknown can be determined (18, pp.259-261).

Reid et al. (38, pp.255-269) developed a curve using Na_2CrO_4 for use with the chromogen method. From a concentrated chromogen extract, a portion to be used in developing a transmittance-concentration curve was diluted sufficiently to obtain a low galvanometer deflection, i.e. 10 per cent transmission of light. A chromogen concentration value was assigned to this extract and to each successive sub dilution, based upon the concentration of Na_2CrO_4 which transmitted the same amount of light. The amount of light absorbed by a reference solution containing 5.05 mg. per cent of Na_2CrO_4 was termed equivalent to that absorbed by an extract containing 10 units of chromogen per 100 ml extract. Na_2CrO_4 was used as a reference standard because the changes in color which occured as the concentration was changed corresponded to the color changes of chromogen extracts at corresponding changes in concentration.

8. Unit of pheophytin.

Since the color of the pheophytin extracts differ from that of the chromogen extracts, the same reference curve cannot be used with the pheophytin method. Such a curve for use with this method has not been reported.

For use in this study, a transmittance-concentration curve, Figure 1, was constructed by plotting different known concentrations of extract against their respective absorbency or O.D. values. The amount of pheophytin in a solute with an optical density of .94 was arbitrarily defined as being equivalent to 8.06 units of pheophytin. From this curve, the O.D. reading of an unknown extract of pheophytin

could be plotted and the units of pheophytin read from the abscissa of the curve. A table of optical density values and their corresponding unit values was developed, Table 5. This table is not complete, but it includes the range of values encountered in this study. Once the unit value is determined, it is converted to units of pheophytin per gram oven dry sample by determining the grams of dry matter in the sample used in the extraction of the pigments.

Figure 1

Figure I. Log Transmittance — Concentration Curve
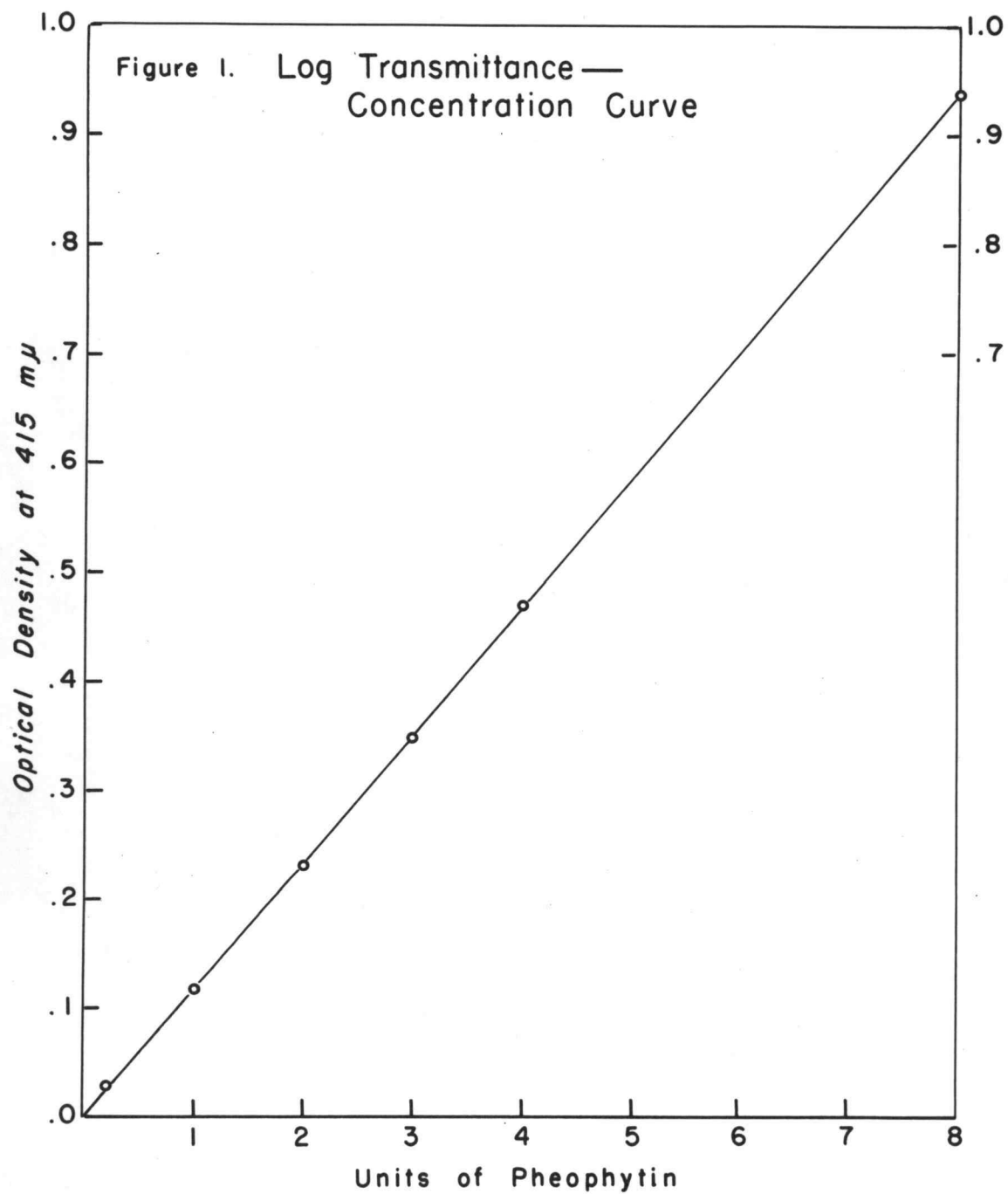
TABLE 5

Table for Converting Optical Density to Units of Pheophytin

O.D.	0	1	2	3	4	5	6	7	8	9
.05	.429	.437	.446	.454	.463	.471	.480	.489	.497	.506
.06	.514	.523	.531	.540	.549	.557	.566	.574	.583	.591
.07	.600	.609	.617	.626	.634	.643	.654	.660	.669	.677
.08	.685	.694	.703	.711	.720	.729	.737	.746	.754	.763
.09	.771	.780	.789	.797	.806	.814	.823	.831	.840	.849
.10	.857	.868	.874	.883	.891	.900	.909	.917	.926	.934
.11	.943	.951	.960	.969	.977	.986	.994	1.003	1.011	1.020
.12	1.028	1.037	1.046	1.054	1.063	1.071	1.080	1.089	1.097	1.106
.13	1.114	1.123	1.131	1.140	1.149	1.157	1.166	1.174	1.183	1.191
.14	1.200	1.209	1.217	1.226	1.234	1.243	1.251	1.260	1.269	1.277
.15	1.286	1.294	1.303	1.311	1.320	1.329	1.337	1.346	1.354	1.363
.16	1.371	1.380	1.389	1.397	1.406	1.414	1.423	1.431	1.440	1.449
.17	1.457	1.466	1.474	1.483	1.491	1.500	1.509	1.517	1.526	1.534
.18	1.543	1.551	1.560	1.569	1.577	1.586	1.594	1.603	1.611	1.620
.19	1.629	1.637	1.646	1.654	1.663	1.671	1.680	1.689	1.697	1.706
.20	1.714	1.723	1.731	1.740	1.749	1.757	1.766	1.774	1.783	1.791
.21	1.800	1.809	1.817	1.826	1.834	1.843	1.854	1.860	1.869	1.877
.22	1.886	1.894	1.903	1.911	1.920	1.926	1.937	1.946	1.954	1.963
.23	1.971	1.971	1.980	1.989	1.997	2.006	2.014	2.023	2.040	2.049
.24	2.057	2.066	2.074	2.083	2.091	2.100	2.109	2.117	2.126	2.134
.25	2.143	2.151	2.160	2.169	2.177	2.186	2.194	2.203	2.211	2.220
.26	2.229	2.237	2.246	2.254	2.263	2.271	2.280	2.289	2.297	2.306
.27	2.314	2.323	2.331	2.340	2.349	2.357	2.366	2.374	2.383	2.391
.28	2.400	2.409	2.417	2.426	2.434	2.443	2.451	2.460	2.469	2.477
.29	2.486	2.494	2.503	2.511	2.520	2.529	2.537	2.546	2.554	2.563

RESULTS AND DISCUSSION

Summaries of the results of four digestion trials, obtained with the pheophytin, chromogen, and conventional methods, are shown in Tables 6 and 7. The chromogen and conventional method analytical data was obtained by chemists in the Department of Agricultural Chemistry and is used here for method comparison only. As a means of checking the reproducibility of the pheophytin method, the samples of pasture trial 1 and the forage samples and fecal samples of cow TU1, silage trial 3, were processed twice using slightly different procedures.

The day each sample of trial 1 was collected, a dry matter determination and alcohol extraction was made. A 200 ml portion of the extract was poured into an amber-colored bottle and stored at $0^\circ C$. for about one month, at which time the processing was completed and absorption readings made. The portion of each sample not used in the above steps was placed in a plastic sack and frozen at $-10^\circ C$. for about five months. They were then thawed and completely processed in one continuous operation. The results show a definite increase in the percentage recovery of the pigments by the complete processing in one day, even after the long period of being frozen; however, this cannot be explained by an increased recovery of units of pheophytin per gram of dry matter in either feed or fecal samples. In fact, there was a definite loss of pigments in the forage extracts, especially in the samples of the last two days.

To determine whether the pigments in the alcohol extracts of feces were changed during storage, absorption readings were made on a

TABLE 6

Recovery of Pigments in Feces by Pheophytin Method and a Comparison with Results of Chromogen Method

Cow	Day	Dry Matter Consumed		Dry Matter Excreted		Per cent Recovery	
		units/gram	total units	units/gram	total units	Pheophytin	Chromogen**
			Pasture Digestion Trial No. 1* (Alfalfa-grass mixture)				
	1	518.02	8,599.13	1,140.53	6,991.45	81.30	
	2	654.92	12,895.37	1,119.66	8,319.07	64.51	
TX1	3	516.87	11,014.50	1,055.77	7,728.24	70.16	
	4	656.44	11,638.68	972.25	7,262.71	62.40	
	5	619.81	11,937.54	907.25	6,260.97	52.45	
			56,085.22		36,562.44	65.19	100.00
	1		9,205.21	1,146.25	7,427.70	80.69	
	2	Same	12,895.37	887.79	6,285.55	48.74	
TX2	3	as	11,045.00	1,043.96	7,975.85	72.21	
	4	Cow	13,089.41	906.52	6,826.10	52.15	
	5	TX1	12,352.81	925.97	7,028.11	56.90	
			58,587.80		35,543.31	60.67	102.00
	1		11,023.46	1,188.44	7,546.59	68.46	
	2	Same	14,329.65	1,075.47	8,152.06	56.89	
TQ1	3	as	12,239.48	868.03	7,308.81	59.72	
	4	Cow	14,546.71	829.95	7,203.97	49.72	
	5	TX1	13,722.59	827.16	7,477.53	54.49	
			65,861.89		37,688.96	57.22	103.00
	1		11,023.46	1,081.77	9,303.22	84.39	
	2	Same	14,329.65	993.89	7,036.74	49.11	
TQ2	3	as	12,239.48	935.28	7,351.30	60.06	
	4	Cow	14,546.71	948.80	8,472.78	58.25	
	5	TX1	13,722.59	921.49	7,676.01	55.94	
			65,861.89		39,840.05	60.49	100.00

* Samples were extracted with alcohol same day collected then stored in refrigerator ca. one month.

**Results obtained by Oregon State College Dept. of Agricultural Chemistry, Weswig,(47).

TABLE 6 cont.

Pasture Digestion Trial No. 1* (Alfalfa-grass mixture)

| Cow | Day | Dry Matter Consumed | | Dry Matter Excreted | | Per cent Recovery | |
---	---	units/gram	total units	units/gram	total units	Pheophytin	Chromogen
	1	540.14	8,966.32	862.42	5,286.63	58.96	
	2	578.42	11,389.09	1,062.85	7,896.97	69.34	
TX1	3	561.39	11,963.22	1,014.56	7,426.58	62.08	
	4	389.49	6,905.66	1,030.67	7,699.10	111.49	
	5	401.02	7,723.64	862.50	5,959.87	77.16	
			46,947.93		34,269.15	72.99	100.00
	1		9,598.29	1,168.83	7,574.02	78.91	
	2	Same	11,389.09	1,051.19	7,442.24	65.34	
TX2	3	as	11,963.22	980.62	7,491.94	62.62	
	4	Cow	7,766.43	898.79	6,767.89	87.14	
	5	TX1	7,992.33	898.78	6,821.74	85.35	
			48,709.36		36,097.83	74.11	102.00
	1		11,494.18	1,115.83	7,085.52	61.64	
	2	Same	12,655.83	1,087.64	8,244.31	65.14	
TQ1	3	as	13,293.71	959.79	8,081.43	60.79	
	4	Cow	8,631.10	912.74	7,922.58	91.79	
	5	TX1	8,878.58	941.01	8,506.73	95.81	
			54,953.40		39,840.57	72.50	103.00
	1		11,494.18	1,048.96	9,021.06	78.48	
	2	Same	12,655.83	1,002.42	7,097.13	56.08	
TQ2	3	as	13,293.71	897.18	7,051.83	53.05	
	4	Cow	8,631.10	977.44	8,728.54	101.13	
	5	TX1	8,785.83	911.56	7,593.29	86.43	
			54,860.65		39,491.85	71.98	100.00

* Samples were placed in plastic sacks and frozen for ca. 5 months then thawed and completely processed in one day.

TABLE 6 cont.

Pasture Digestion Trial No. 2 (Ladino clover-grass mixture)

Cow	Day	Dry Matter Consumed		Dry Matter Excreted		Per cent Recovery	
		units/gram	total units	units/gram	total units	Pheophytin	Chromogen
	1	592.95	11,716.69	1,205.00	8,362.70	71.37	
	2	518.38	9,745.54	---------	---------	-----	
TX1	3	521.07	10,353.66	1,001.17	6,667.79	64.40	
	4	584.14	12,734.25	952.21	6,122.71	48.08	
	5	546.03	10,259.90	1,010.48	7,083.46	69.04	
			45,064.50*		28,236.66*	62.66	95.50
	1		11,592.17	1,387.28	9,877.43	85.21	
	2	Same	8,879.85	1,263.02	10,243.09	115.35	
TX2	3	as	11,036.26	1,432.89	9,543.05	86.47	
	4	Cow	12,734.25	1,127.52	7,272.50	57.11	
	5	TX1	8,709.18	1,152.72	8,230.42	94.50	
			52,951.71		45,166.49	85.30	100.00
	1		14,847.47	---------	---------	-----	
	2	Same	11,502.84	1,045.29	9,313.53	80.97	
TQ1	3	as	11,885.61	1,456.64	11,725.95	98.66	
	4	Cow	14,007.68	910.07	7,580.88	54.12	
	5	TX1	10,740.41	1,423.58	10,819.20	100.73	
			48,136.55*		39,439.56*	81.93	104.00
	1		15,458.21	1,656.85	14,331.75	92.71	
	2	Same	12,327.08	1,319.62	12,087.72	98.06	
TQ2	3	as	11,885.61	1,301.40	10,645.45	89.56	
	4	Cow	14,305.59	971.39	9,004.78	62.95	
	5	TX1	12,116.41	1,034.84	9,065.20	74.82	
			66,092.90		55,134.90	83.42	109.00

* Calculations based on four days.

TABLE 6 cont.

Grass Silage Digestion Trial No. 3 (Dried Molasses Beetpulp)

Cow	Day	Dry Matter Consumed units/gram	Dry Matter Consumed total units	Dry Matter Excreted units/gram	Dry Matter Excreted total units	Per cent Recovery Pheophytin	Per cent Recovery Chromogen
TS1	1	167.94	1,995.13	309.02	1,334.97	66.91	
	2	167.12	2,055.58	183.05	1,087.32	52.90	
	3	189.20	2,213.64	171.56	796.04	35.90	
	4	210.31	2,832.88	200.69	934.24	33.30	
	5	202.56	2,570.49	265.50	1,229.26	47.82	
			11,667.72		5,390.83	46.20	62.20
TS2	1		2,105.97	290.82	1,596.60	75.81	
	2	Same	2,376.45	222.37	1,020.68	42.95	
	3	as	2,342.30	173.41	705.78	30.13	
	4	Cow	2,746.65	190.41	868.27	31.61	
	5	TS1	2,570.49	255.10	948.97	36.92	
			12,141.86		5,140.30	42.33	58.80
TU1	1		2,105.97	229.58	1,724.15	81.87	
	2	Same	2,239.41	137.34	784.21	35.02	
	3	as	2,342.30	194.30	969.56	41.39	
	4	Cow	2,891.76	192.25	843.98	29.18	
	5	TS1	2,734.56	264.32	1,279.31	46.78	
			12,314.00		5,601.20	45.49	63.60
336	1		1,817.11	246.41	1,197.55	65.90	
	2	Same	2,055.58	223.50	1,461.69	71.11	
	3	as	2,200.40	176.64	870.83	39.57	
	4	Cow	2,630.98	176.87	880.81	33.48	
	5	TS1	2,570.49	264.70	1,135.56	44.18	
			11,274.56		5,546.44	49.19	65.70
TU1*	1	98.46	1,244.04	226.83	1,503.20	120.83	
	2	68.70	952.66	178.19	892.09	93.64	
	3	121.86	1,612.21	162.76	803.24	49.82	
	4	144.72	2,112.91	189.45	863.76	40.88	
	5	163.53	2,297.60	205.59	962.67	41.90	
			8,219.42		5,024.96	61.13	63.60

* Rerun after samples had been frozen, all light excluded during processing.

TABLE 6 cont.

Grass Silage Digestion Trial No. 4 (No Preservative)

| Cow | Day | Dry Matter Consumed | | Dry Matter Excreted | | Per cent Recovery | |
		units/gram	total units	units/gram	total units	Pheophytin	Chromogen
TS1	1	276.21	3,245.47	337.94	1,906.61	58.75	
	2	277.16	3,048.76	340.19	1,387.97	45.52	
	3	293.43	3,154.37	340.64	1,665.73	52.81	
	4	297.57	3,192.93	343.47	1,421.97	44.53	
	5	282.69	3,149.17	337.39	1,396.79	44.35	
			15,790.70		7,779.07	49.26	63.4
TS2	1		3,245.47	314.68	1,833.33	56.49	
	2	Same	3,262.17	319.73	1,429.19	43.81	
	3	as	3,438.00	316.41	1,360.56	39.57	
	4	Cow	3,374.44	352.16	1,440.33	42.68	
	5	TS1	3,197.22	334.07	1,349.64	42.21	
			16,517.30		7,413.05	44.88	62.0
TU1	1		3,245.47	310.07	1,364.31	42.04	
	2	Same	3,262.17	308.46	1,443.59	44.25	
	3	as	3,439.00	326.70	1,470.15	42.75	
	4	Cow	3,576.79	351.56	1,638.27	45.80	
	5	TS1	3,457.30	330.21	1,410.00	40.78	
			16,980.73		7,326.32	43.14	58.7
336	1		3,237.18	297.92	1,304.89	40.31	
	2	Same	3,262.17	321.40	2,452.29	75.17	
	3	as	3,439.00	288.39	1,309.29	38.07	
	4	Cow	3,582.74	352.58	1,706.49	47.63	
	5	TS1	3,457.30	332.32	1,688.19	48.83	
			16,978.39		8,461.15	49.83	58.8

TABLE 7

Dry Matter Digestion Coefficients Calculated by
Pheophytin Method and Compared with Chromogen
and Conventional Methods

Cow	Pheophytin Method %	%	Chromogen Method %	Conventional Method %
Pasture Digestion Trial No. 1 _(Alfalfa-grass Mixture)_				
TX1	42.26	48.67*	63.80	63.50
TX2	38.44	50.71*	64.40	63.60
TQ1	36.39	50.93*	66.20	65.00
TQ2	38.90	48.88*	65.20	65.60
Pasture Digestion Trial No. 2 _(Ladino Clover-grass Mixture)_				
TX1	45.90		63.30	65.00
TX2	56.14		63.70	63.60
TQ1	53.02		66.10	64.60
TQ2	54.41		66.70	63.20
Silage Digestion Trial No. 3 _(Dried Molasses Beetpulp)_				
TS1	15.64		41.00	63.30
TS2	17.54		43.90	67.00
TU1	10.57	37.45**	40.90	62.40
336	16.11		40.40	60.80
Silage Digestion Trial No. 4 _(No Preservative)_				
TS1	16.05		42.50	63.60
TS2	10.62		44.70	65.80
TU1	12.20		41.20	65.50
336	10.32		42.60	66.20

* Rerun of first trial, after samples were frozen.
** Rerun of forage and TU1 feces, all exposure to light
  eliminated.

series of five fecal samples taken from pasture trial 2, then two portions of the alcohol extracts of each sample were stored in the dark, one at room temperature and the other at $0^{o}C$. After about one month, the processing was completed and absorption readings were made, as reported in Table 8. A statistical analysis of the results, using the 5 per cent significant level, showed that there was no significant difference between the means of the samples processed before storage and those processed after a month's storage at $0^{o}C$. There was a small but significant difference between the means of the samples processed before storage and those stored at room temperature. These results would indicate that any change which occured must have appeared in the alcohol extracts of the forage.

Table 8

Storage Effects on 95% Ethyl Alcohol Extracts of Feces
as Measured by Changes in Transmission
(Samples processed in duplicate)

Sample	Before storage %	Storage in the dark (one month)	
		Room temp. %	$0^{o}C$. %
1	80.1	80.9	79.6
	79.2	80.2	80.8
2	80.7	81.3	79.8
	80.8	81.8	80.9
3	78.7	79.8	79.4
	78.6	80.2	79.8
4	79.3	80.0	80.9
	79.1	80.7	79.6
5	78.3	79.2	77.2
	79.2	79.7	78.1
Average	79.40	80.38	79.61

Since there was considerably more pigment extracted from the forage samples which were allowed to stand in alcohol, it might appear that forage samples should be allowed to stand for a period of time in alcohol before the extraction process is completed. According to Mayer et al. (34, pp.295-296) chlorophyll does undergo certain changes when allowed to stand in an alcoholic solution, i.e. chlorophyll in ethyl alcohol forms an ester of alcohol, ethyl chlorophylic. Possibly this change aids in the extraction of the pigments from the forage samples.

The samples of the second, third, and fourth digestion trials were completely processed and absorption readings made the same day the samples were collected. An average of 78.3 per cent recovery was obtained with the second pasture trial as compared with an average of 72.9 per cent with the rerun of the first pasture trial. An average of 101.7 per cent recovery was obtained from the two trials with the chromogen method. These latter results are in close agreement with those of Woolfolk (50) who obtained an average of 101.2 per cent recovery in his study of the chromogen ratio method.

The results of the dry matter digestibility determinations with silages did not agree with the results of the standard digestion trial when either the pheophytin or chromogen method was used. Using the same procedure as in the pasture trials, an average of 46.3 per cent recovery was obtained with the pheophytin method and an average of 61.7 per cent recovery with the chromogen method. A rerun of the silage and TU1 fecal samples of the third digestion trial, with all

sources of light excluded during the complete processing of the samples, showed a definite increase in per cent recovery with the pheophytin method over extraction in ordinary room light. The recovery was increased from 45.5 per cent to 61.1 per cent. This increase in per cent recovery again, however, does not necessarily refer to an increased recovery in terms of units of pheophytin. The average units of pheophytin per gram of dry matter after the first processing was 187 for the silage and 203 for the feces as compared to 119 for the silage and 192 for the feces when all light was eliminated during the processing.

The decrease in units of pheophytin extracted per gram of dry matter in both feed and fecal samples when light was excluded during the processing led to the initiation of a study of the effect of light on the pigments. Extracts of various samples were extracted in the absence of light. This was accomplished by covering the vessels which contained the extracts with towels or in the case of the seporatory funnels, masking them with black tape. The only time the extracts were exposed to light was during the few seconds required to pipette them from one vessel to the next, a total of not more than two to three minutes during the entire processing. Drawing the shades and using a minimum of light in the room in which the extracting was being done also helped decrease the exposure to light. After the extracts were made up to their final volume, they were placed in cuvettes and sealed with a parafilm covering to prevent evaporation. The extracts were read in a spectrophotometer immediately then stored either in the

dark or in room light. Since the amount of light would vary from day to day and hour to hour, the degree of destruction would vary accordingly. The results of this study are shown in Figure 2. In all cases of storage in the dark, the solutions remained relatively stable for the six-day duration of the study. The samples of oxalated feces, non-oxalated silage, and sample of grass which was extracted with alcohol only, showed no significant change. The sample of oxalated silage increased from 62.7 per cent to 66.9 per cent transmission in a period of four and one half days. All of the samples stored in light showed a definite increase in transmission of light, especially during the first few hours. These results are in agreement with those of Meyer et al. (36, pp.162-169) and Lofgreen et al. (32, p.268 and 33, pp.1158-1165). They do not agree with those of Lancaster et al. (30, pp.489-496) who reported an increase in the optical density of acetone extracts when exposed to light. Since the results of this study show that the pigments are destroyed by light, it would be logical to assume that the amount of pheophytin recovered with light excluded from the processing would be greater than where light was permitted to destroy some of the pigments. Therefore, another explanation, such as an incomplete extraction of pigments from the samples, must be given to the decreased recovery of pigments in the rerun of the silage and TU1 fecal samples.

Lofgreen (32 and 33) suggested a procedure of stabilizing the extracts by exposing them to a standard amount of light. If this would destroy only the carotenes which are excreted in a variable pattern

Figure 2. Storage Effects on Pigments
Extracted with 95 % Ethanol
Read at 415 mμ in a Coleman Model H Spectrophotometer

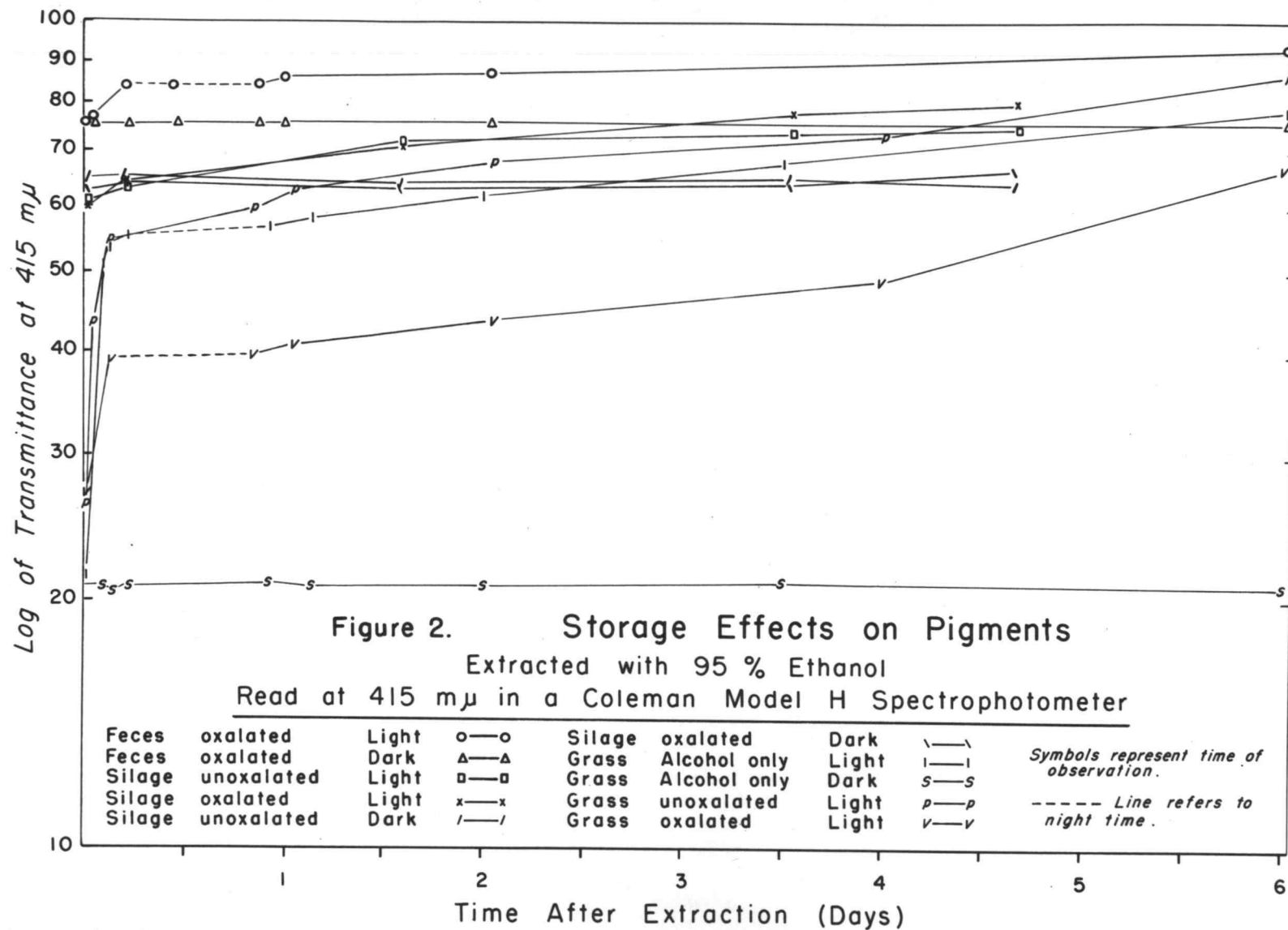|								
Feces	oxalated	Light	o——o	Silage	oxalated	Dark	\——\	Symbols represent time of observation.
Feces	oxalated	Dark	△——△	Grass	Alcohol only	Light	l——l	
Silage	unoxalated	Light	□——□	Grass	Alcohol only	Dark	s——s	----- Line refers to night time.
Silage	oxalated	Light	x——x	Grass	unoxalated	Light	p——p	
Silage	unoxalated	Dark	/——/	Grass	oxalated	Light	v——v	

(4, pp.162-169 and 35, pp.223-228), the procedure would seem justified. However, if it would also destroy other pigments such as chlorophyll, it is questionable as to whether this procedure would be reliable. It was found that when an alcohol extract of non-oxalated grass was exposed to sunlight, it changed from a dark green to a yellow in two hours and increased in transmission from 20.7 per cent to 50.7 per cent. It would appear that more than carotene was destroyed. As previously indicated, very good repeatability of results was obtained, Table 8, when extracts were processed in room light then reprocessed one month later from alcohol extracts which had been stored in the dark. Even though these results were repeatable, they were of no use in predicting digestibility trends in samples of forage.

The purpose of any short-cut method for determining the dry matter digestibility or nutritive value of a forage is to reduce the time and expense of analyzing the samples. A goal might be to develop a method which requires the collection of only one sample of feed or feces or both. In order for this to be possible, the units of pheophytin or chromogen per gram of dry matter or what ever units are to be used, must be in close agreement from one day to the next, providing the forage does not change. Also, if the units per gram in the feed increase, there should be a corresponding increase in the feces, if the digestion coefficient is to remain approximately the same. A statistical comparison of the daily variation of the units of pheophytin per gram of dry matter in both feed and fecal samples was made with the corresponding values obtained by the chromogen method. It

was found that the variation was much greater with the pheophytin

method, the average coefficient of variation being 18.5 per cent for

the pheophytin method and 10.1 per cent for the chromogen method.

A STUDY OF THE PIGMENTS
INVOLVED IN THE PHEOPHYTIN METHOD

The suggested advantage of the pheophytin method over the chromogen method is that the pheophytin extract is composed predominately of a single pigment which is common to both feed and fecal samples (25, p.672 and 2, leaf.2). Also, the absorption readings are measured at 415 mμ, the maximum absorption peak of pheophytin and a relatively level part of the absorption spectra as compared to a wave length of 406 mμ, the isosbestic point of the pigments in the chromogen extract and a relatively steep portion of the absorption spectra.

A study of the absorption spectra of various chromogen extracts showed the absorption peaks ranging from 410 mμ to 432 mμ, Table 9, (47).

Table 9

Maximum Absorption Point of Various Chromogen Extracts
(Extracted with Acetone and Read on a
Beckman DU Spectrophotometer)

Sample	Maximum Absorption Point (mμ)
Fescue, fresh	432
Fescue, dried and ground	410-412
Silage forage	410
Silage feces	412
Pasture feces	412-415

The variation in maximum absorption point as shown in Table 9, would be due to a variation in the composition of the extract, i.e. the predominance of chlorophyll in fresh forage samples would result

in a maximum absorption peak near that of chlorophyll, 435 mμ, whereas
a predominance of pheophytin in a fecal sample would shift the maximum
absorption peak towards 415 mμ. This shift is adjusted with the
chromogen method by the use of an isosbestic point which is reported
the same for all of the pigments found in the chromogen extracts. The
use of a maximum absorption point with the pheophytin method is justi-
fied by an attempt to eliminate the variation in the composition of the
extract, i.e. changing chlorophyll to pheophytin.

A study of the pigments involved in the pheophytin extract of
pasture, silage, and fecal samples was conducted to determine the
similarity of these extracts. The pigments of the various extracts
were isolated by means of paper and column chromatography.

1. Column separations.

Glass columns, approximately nine inches long and one-half inch in
diameter which had been drawn out at one end to form a smaller tube,
about two inches long and one-eighth inch in diameter were used in
the column isolations. In preparing the columns, a quarter inch of
glass wool was packed on top of the constricted portion of the column.
A similar layer of white sand was packed on top of the glass wool.
These two layers formed a firm base against which the absorption
material was packed. A mixture of 50 per cent diatomaceous earth and
50 per cent magnesium oxide, measured on weight basis, was used as the
absorbent. This material was added to the glass column in small
portions, packing each layer firmly, until a column of about five to
six inches was obtained. This left a space of about two inches on top

of the column in which the extracts were poured. It is important that the columns be firmly and evenly packed in order to obtain a narrow, well-defined band of pigment. The addition of a small layer of cotton, packed on top of the column, aided in eliminating any disturbance which might occur when the pigment extracts were poured into the column. Just prior to the addition of the pigment extracts, the absorbent material was wet with petroleum ether.

In preparing the pigments for separation, the processing was the same as for making the absorption measurements up until the concentration of the pigments. The pigments were concentrated in petroleum ether, rather than ethyl alcohol, i.e. the 10 ml of ethyl alcohol was not added to the culture tubes as before. This allowed the pigments to be concentrated into a much smaller volume without the use of a higher temperature or longer heating period. When about three ml of the petroleum ether remained, the culture tubes were removed and the pigments oxalated. Since the petroleum ether would not mix with the solvent of oxalic acid in water, the latter was removed by means of a pipette after the oxalated pigments had been filtered. The pigments were then poured into the glass columns and drawn through the absorbent material with suction. Additional petroleum ether was added as needed until either the pigment bands had stopped moving or had reached the lower end of the absorbent column.

A single separation on a diatomaceous earth-magnesium oxide column, Table 10, did not separate the pigments sufficiently to allow for their identification, but did provide an indication as to variation

among oxalated and non-oxalated pasture and silage extracts and their resemblance to non-oxalated and oxalated fecal extracts.

Since the bands were not completely separated in most cases, an attempt to identify the pigments by their absorption spectra proved unsuccessful. As a means of further identifying certain predominant bands, sections of the columns were removed from the glass tubes and the bands were separated as completely as possible by mechanical means. These relatively pure bands were then placed in centrifuge tubes to which a few ml of diethyl ether were added, to elutriate the pigments from the absorbent. The tubes were shaken and centrifuged. If all of the pigment had not been elutriated, the process was repeated. These pigments were either returned to a new absorbent column and reabsorbed to further separate any mixture of pigments which might exist or studied by means of paper chromotography. This process was carried out with only a few of the pigment bands.

Table 10

Single Column Separation of Plant Pigments
Extracted from Various Plant and Fecal Samples
(Disolved in Petroleum Ether and Absorbed on
Diatomaceous Earth-Magnesium Oxide Columns)

Band	Unoxalated forage	Oxalated forage	Unoxalated silage	Unoxalated feces	Oxalated feces
1	dk. green	lt. yellow	dk. green	yellow-green	yellow-green
2	yellow-brown	white	brown-green	white	white
3	orange	reddish-brown	dk. green	brown-green	brown-green
4	white	green	yellow	yellow	yellow
5		yellow	white	orange	orange
6			orange		

2. Paper separations and identifications.

The techniques used in this section were adapted from those
reported by Lind et al. (31, pp.325-328). The pigments to be used,
either an unresolved mixture or a single pigment obtained from a
column separation, were transferred drop by drop to a piece of
Whatman No. 1 filter paper. The number of drops required to make a
pigment spot sufficiently dark enough depended upon the concentration
of the pigments in the solvent. If not enough pigment was placed on
the paper, i.e. the spot was too light, it was difficult to follow the
movement of the pigment. Also, if too much pigment was added, or added
too rapidly, an incomplete movement or trailing of pigment prevailed.
Extreme care must be taken when building up the spot to be sure that
the preceeding drop has completely dried before adding an additional
drop. The correct procedure for building up a spot can be developed
only by practice.

The position of the pigment spot on the absorbent paper is
another point which should be considered. If a one-dimentional separa-
tion is to be made, one pigment spot may be placed about one and one-
half inches from the bottom edge of a strip of filter paper, say three
inches wide and six inches long. If a series of one-dimentional
separations are to be made, a series of pigment spots may be placed
about one and one-half inches up from the bottom of the paper and about
two inches apart. In this case, the width of the paper would depend
upon the number of spots to be used. When a mixture of several pig-
ments is to be studied, it is often advantageous to use a

two-dimentional separation technique. This allows, in most cases, a more complete separation of the pigments. With this technique, a square piece of filter paper is usually used. The pigment spot is placed about one and one-half inches up from the bottom and about two inches in from one side of the paper.

Once the pigment spots are placed on the paper, the paper is rolled and stapled into a cylinder with the pigment spots around the bottom edge of the cylinder. Care should be taken to avoid handling the paper except by the edges as any dirt or oil left by a finger print may disrupt the free movement of the pigment. To move all of the pigments in the spots to a common line, the bottom edge of the cylinder was placed in about one-half inch of acetone. As soon as the acetone had reached the top of the pigment spots, the cylinder was removed and allowed to dry. It was now ready for further separation and identification of the pigments. If a two-dimentional separation were to be made following the completion of the first dimentional separation, the cylinder was unstapled and restapled to form a new cylinder perpendicular to the original. In this way the separated pigments would be around the end of the newly formed cylinder for the start of the final separation.

The identification of a pigment by paper chromatographic means is based upon the color of the pigment spot and the distance it travels with a given solvent. The movement of a pigment depends upon its affinity for a solvent, i.e. a pigment which contains one or more polar groups is soluble in a polar solvent such as an acid or certain alcohols,

whereas a pigment which is primarily a hydrocarbon with no polar groups is not soluble. If a mixture of polar and non-polar pigments is subjected to a solvent such as ethanol, the polar pigment will follow the solvent front for a certain distance; whereas, the non-polar pigment will be left behind and this becomes separated from the polar pigment. The distance which a pigment moves in a given solvent is measured as a ratio of the distance the solvent moves to the distance the pigment moves. This measurement is designated as an Rf value and a specific pigment would have a constant Rf value in a given solvent, no matter how far the solvent front moved. In this way, an unknown pigment can be identified by comparing its Rf value with those of known pigments.

A portion of the dark green band which was characteristic of the first bands of the column separations of non-oxalated forage and silage extracts were studied. After reabsorbing the elute of one of these bands on a new absorbent column to further purify it, the elute of the new band was placed on a piece of Whatman No. 1 filter paper and prepared for identification as previously mentioned. The bottom edge of the paper cylinder was placed in petroleum ether and the cylinder covered with a large beaker. After the solvent front had moved up the paper about three inches, the paper was removed and allowed to dry. The pigment spot had remained almost stationary. The cylinder was next placed in a 1 percent n-propanol in petroleum ether solvent, and the pigment spot began to follow closely behind the solvent front. The calculated Rf value was .88. The spot, which appeared blue-green,

was concluded to be chlorophyll a. Being a polar compound, chlorophyll a would be absorbed by the magnesium oxide of the column, thus not moving down the column with the petroleum ether. For the same reason, it would not move on the filter paper with petroleum ether, a non-polar solvent. It would move with a 1 per cent n-propanol, a polar solvent. The spot was designated chlorophyll a rather than b because of its color (16, p.858). In only one case in this particular part of the pigment study was the presence of chlorophyll b indicated. This was when a band near the top of the column, which contained a mixture of yellow and green pigments, was studied. In a 1 per cent n-propanol solvent, a yellow-green spot was left behind, thus separated from the blue-green spot designated as chlorophyll a. The yellow-green spot which had an Rf value of .37 could possibly have been chlorophyll b (31, pp.489-496). Smart et al. (43, pp.318-321) indicated in their work that chlorophyll b did not appear as a separate, distinct band but rather in conjunction with chlorophyll a.

A portion of the elute designated as chlorophyll a was treated with oxalic acid and it turned olive-green. When put on filter paper and placed in petroleum ether, the pigment spot diffused over an area of about one-half inch square, rather than following the solvent front. When the paper was transferred to a 1 per cent n-propanol solvent, the pigment followed the front, resulting in an Rf value of 1, which would be of little value in identifying the pigment. On the basis that the pigment was the result of an oxalation of the designated chlorophyll a pigment and olive-green in color, it was called pheophytin a.

Now that a pheophytin a or suspected pheophytin a pigment had been isolated, steps were taken to find this same pigment on the column separations and on paper chromatographic separations of the unresolved extracts. The top yellow band taken from a column which had been washed with ethyl alcohol rather than petroleum ether, thus carrying the chlorophylls on down the column, was studied first. Since pheophytin is almost insoluble in petroleum ether and soluble with great difficulty in cold alcohol, this band was suspected as being a pheophytin. A purified elute of this band remained stationary on filter paper in a solvent of petroleum ether. It did appear olive-green on the paper, but the same diffusion pattern was not observed. When put in the 1 per cent n-propanol solvent, the spot was too faint to follow; therefore it would not be possible to say this spot definitely represented pheophytin a, but there were several reasons to believe it was. If this pigment were pheophytin a, it appeared in the oxalated forage column rather clearly. However, in the other columns it must have appeared in a mixture of several pigment bands.

Two other rather clearly separated pigment bands were studied. The first of these was an elute characteristic of the orange bands found in the non-oxalated forage and the fecal columns. This pigment followed the petroleum ether solvent front on filter paper. This plus the fact that a similar spot disappeared upon being exposed to light for several days indicated that the pigment might be a carotenoid, possibly beta carotene. The other pigment was yellow, similar to the carotenes, but it did not move with petroleum ether. It did follow

the 1 per cent n-propanol front. This pigment could very well be a xanthrophyll (31, pp.489-496).

Two-dimensional paper chromatographic separations of unresolved extracts of oxalated and non-oxalated forage and fecal samples using a 25 per cent chloroform in petroleum ether as solvent for the second dimension revealed the presence of the carotene and xanthrophylls in all cases, if the previous assumptions as to the identification of the various pigments are correct. The chlorophylls appeared predominate in the non-oxalated forage sample extract whereas the pheophytin appeared to replace the chlorophylls in the other extracts. A comparison of the unresolved extracts following the use of only petroleum ether is shown in Figure 3. This figure, a water-color reproduction, is by no means included for the purpose of identifying the pigments in the various extracts but for a general comparison of the extracts. The overall comparison shows much similarity among the oxalated and non-oxalated fecal extracts and the non-oxalated silage extracts. This observation would be in agreement with Kane et al. (37, p.672), who reported that the addition of oxalic acid was not necessary when silage extracts were used as the chlorophyll in the alfalfa silage studied had been changed naturally to pheophytin or possibly lower degradation products of chlorophyll by the acid and heat developed by the silage fermentation. The oxalated pasture extract more closely resembled the fecal extracts than did the non-oxalated pasture extract; however, both pasture extracts appeared to have a slightly different diffusion pattern.

The absorption spectra of the various pheophytin extracts did show some variation, although not as great as with the chromogen extracts. The maximum absorption points of oxalated and unoxalated fecal extracts varied from 412 to 415 mμ. The maximum absorption points of oxalated extracts of silage and forage samples were both 412 mμ.

Chromatographic Separation of Plant Pigments

(Extracted with 95 % Ethanol)

Unoxalated Forage   Oxalated Forage   Unoxalated Silage   Unoxalated Feces   Oxalated Feces

Figure 3

SUMMARY AND CONCLUSION

An evaluation of the pheophytin method for determining dry matter digestibility using a series of digestion trials with dairy cattle, two with clipped pastures and two with grass silages, indicated a poor recovery of pigments in fecal extracts. An average recovery of 75.6 per cent was obtained from the two pasture trials and an average recovery of 46.3 per cent with the two silage trials. These results are compared with those obtained by use of the chromogen method (47) with the same digestion trials, 101.7 per cent recovery with the pasture trials and 61.7 per cent recovery with the silage trials.

Furthermore it was found that the calculated units of pheophytin per gram of dry matter were more variable from day to day with the pheophytin method than with the chromogen method, the average coefficient of variation being 18.5 per cent for the pheophytin method and 10.1 per cent for the chromogen method. Since the variation in the fecal samples did not appear to be correlated with those in the feed samples, it would appear that many samples would have to be taken in order to obtain a true estimation of the nutritive value of a feed. Since these variations did not reflect normal variations which would be expected to occur in the forages analyzed, a study of the pheophytin extract was conducted in order to determine possible explanations

for the results obtained.

A chromatographic separation of the pigments in forage, silage and fecal extracts revealed the presence of at least two pigments besides pheophytin. These appeared to be beta carotene and xanthophyll. A variation in the relative amounts of these pigments present might explain the fluctuation of the maximum absorption point of the various extracts from 400 to 415 mu. Even though the maximum absorption point did vary, it would not account for the results obtained as the absorption spectras were relatively level at these wave lengths and would be of great concern, only if a spectrophotometer with a very narrow refractive index were used. Use of the Coleman Junior spectrophotometer revealed very little difference between a wave length setting of 400 and 415 mu.

Since a variation in the composition of the extracts would not account for the results, it was felt that the error must lie in either an incomplete extraction of pigments from the samples or exposure to light during the processing of the samples or a combination of the two sources of error. A study of the effect which light had on the pheophytin extracts revealed that they were rapidly diminished by light, especially during the first few hours of exposure. The amount of destruction was related to the intensity of light and the time of exposure. It was also

found that no significant change occured when extracts were stored in the dark for a period of six days. These two factors, an incomplete extraction and light damage possibly account for the results with the pasture trials.

It was observed that the units of both pheophytin and chromogen per gram of dry matter in both feed and fecal samples were much lower with the silage trials than with the pasture trials. This again could not be explained by a difference in pigment extracts as the pigments of the silage extracts appeared even more similar to the fecal extracts than did the pasture extracts.

Since with silages difficulties were encountered with both the pheophytin and chromogen methods, some consideration other than the processing technique was considered to be at fault. The low recovery in the feed samples might be the results of a partial breakdown of the forage pigments during the silage fermentation. If this partial breakdown would increase the absorption of the pigments from the digestive tract or if some interfering substance would be found in the silage, the low recovery in the fecal samples could also be explained.

With these points in mind, the following suggestions are made for use in further studies of the pheophytin method:

1. Use larger samples, especially in preparing dry

matter determinations.

2. Exclude all sources of light during the processing of samples.

3. Increase the extraction time by allowing the samples to stand in the alcohol, possibly over night, then mascerating for an additional period of time.

4. Make the alcohol extracts up to a known volume rather than assuming that all of the alcohol is recovered.

Even if the adoption of these suggestions would result in a 100 per cent recovery of pigment, one other important problem is yet to be solved. That is the problem of the time involved in preparing the pheophytin extracts. Only if the pheophytin method could be adapted to digestibility studies with silages would it be more advantageous than the chromogen method.

The results of this study indicate that the pheophytin method appears to be of little value in dry matter digestibility studies. The only advantage which this method appears to have over the chromogen method is that the pheophytin extract obeys the Beer-Lambert law.

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