THE CAROTENE CONTENT OF OKLAHOMA FEEDS

THE CAROTENE CONTENT OF OKLAHOMA FEEDS

By

ROBERT WALL

Bachelor of Science

Iowa State College of Agriculture and Mechanic Arts

Ames, Iowa

1937

Submitted to the Department of Chemistry

From the Laboratory of the

Department of Agricultural Chemistry Research

Oklahoma Agricultural and Mechanical College

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

1940

LIBRARY
A & M COLLEGE
STILLWATER OKLA

OKLAHOMA
AGRICULTURAL & MECHANICAL COLLEGE
LIBRARY
AUG 6 1940

APPROVED:

Director of Research

Head of the Department of Chemistry

Dean of the Graduate School

126831

TABLE OF CONTENTS

	Page
Introduction	1
Method	10
Experimental	16
Results	19
Summary	29
Bibliography	31

ACKNOWLEDGMENT

The author wishes to express his appreciation for the assistance and constructive criticism given by Dr. V. G. Heller throughout this investigation.

INTRODUCTION

The mass of literature, scientific studies, popular articles, and books which have been written dealing with the subject of vitamins during the past twenty-five years is undisputed evidence of the popularity that this subject has found in the minds of both scientific workers and the lay public as a whole. The foundation of research, comprising an almost incredible amount of work extending over many years and into every branch of chemistry, and carried out by some of the most able men of the times, has demonstrated that this popular interest in vitamins has a firm scientific backing, and that vitamins are essential to the proper growth and the maintenance in health of the animal organism. The emphasis on vitamins in food advertising, and the sale of vitamin-packed foods and vitamin concentrates, illustrates the acceptance of the value of vitamins by the general public.

One of the earliest articles dealt with vitamin A, and down through the years no single one of these indispensable dietary needs has been proven to be of greater importance than that which was first referred to as fat-soluble A, later as vitamin A, and which is now defined by an exact chemical formula. Early studies soon demonstrated that the primary source of this nutritionally significant material was invariably found in plant tissue, and later studies correlated its occurrence with the pigmented parts of the plants. As investigators progressed further they noted that the vitamin A activity of plants was centered in the yellow fat-soluble pigments, and finally narrowed the search down to a few members of the group of carotenoid plant pigments. It was demonstrated that these yellow pigments were the parent substance from which vitamin A is derived, and that vitamin A is present in plants almost exclusively

as carotenes. Thus it is this carotene in plant material which is the primary source of the vitamin A which is utilized and stored by animals. Recent investigations, therefore, tend to measure the vitamin A potency of plant foods as carotene content.

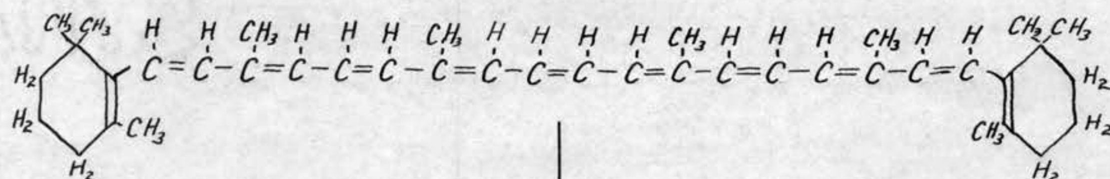
From a purely chemical standpoint carotene was known as a plant pigment long before the existence of vitamins was postulated and the biological significance of carotene as the precursor of vitamin A in animal nutrition was established. The intense yellow color which now serves so conveniently for its chromatic determination aroused the interest of many investigators and led Wackenroder to its isolation from carrots in 1831. Since then the association of carotene with chlorophyll, and later its suspected, and finally proven, significance as a growth factor (16) stimulated investigators to a study of its properties, structure, and transformation into vitamin A. The work of Karrer and his associates is outstanding in the development of the structure of β-carotene, first accomplished in 1930, in separating and determining the properties and structure of the associated carotenoids (19), and in the determination of the structure of vitamin A (20).

The biological discovery of "fat-soluble A" was first announced by McCollum and Davis in 1913 (21), almost simultaneously with a similar announcement by Osborne and Mendel (28). It was five years later, in 1919, before a connection between carotene and vitamin A was suggested by Steenbock (35), and it was ten years more, in 1929, before the conversion of carotene into vitamin A in animals was definitely proven by von Euler (39) and T. Moore (23). They demonstrated that carotene is transformed into vitamin A in the liver and that the liver is the chief storehouse of both vitamin A and untransformed carotene.

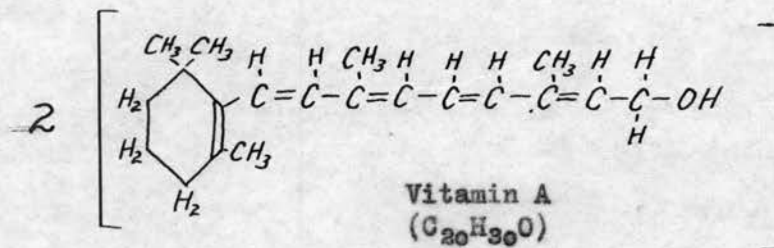
The transformation is apparently an enzymatic hydrolysis by the enzyme carotenase, in which the symmetrical molecule of β-carotene is split at the center to yield two molecules of vitamin A (27).

Shown below:

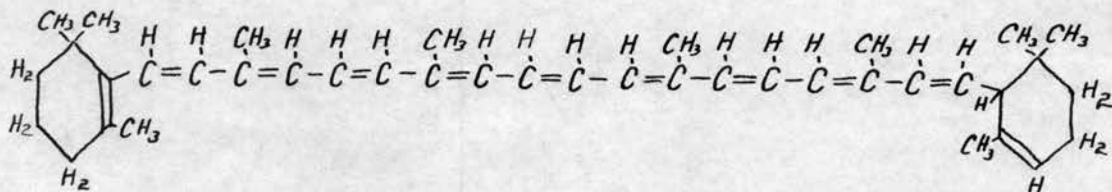
β -Carotene ($C_{40}H_{56}$)



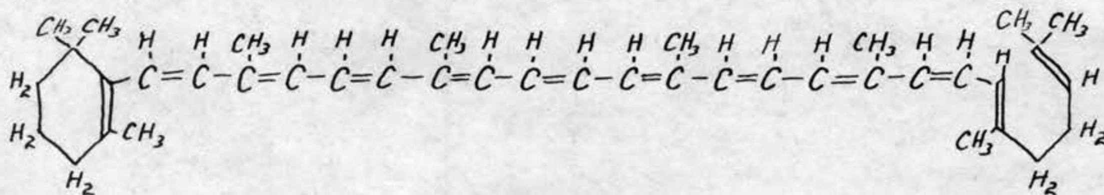
+ $2H_2O$ (Carotenase)



In α-Carotene ($C_{40}H_{56}$)



and γ-Carotene ($C_{40}H_{56}$)



only one of the two cyclic groups present in each molecule is of the same β-ionone configuration that occurs in the formula of vitamin A. Thus each α and γ carotene molecule can yield but one molecule of vitamin A upon hydrolysis in the liver as compared with two for every molecule of β-carotene, and therefore has but half the vitamin A potency of β-carotene.

α, β, and γ carotenes are the principal naturally occurring carotenoid pigments exhibiting vitamin A activity. The proportions of each vary with different plant materials; however, leaf carotene is chiefly β-carotene, so that the use of pure β-carotene as a primary standard for estimating the carotene of forage does not introduce a serious error.

Once in the liver the transformation of carotene into vitamin A is nearly complete; the absorption of carotene from the intestine, however, is far from complete, and seems less than that of vitamin A. Widely varying coefficients of utilization are given by different investigators (2,3,5,7,15,24,44), indicating that the problem is not a simple one, and that the absorption of carotene from the intestine is influenced by a large number of variable factors, including the breed, physical state, and individual peculiarities of an animal, and also the feed in which the carotene is contained. There is strong evidence, for instance, that the bile salts play an important part in the actual process of absorption, and that the quantity and kind of fat present in the diet have a marked influence upon the absorption process.

The physiological activity of carotene is accepted as being dependent upon the transformation into vitamin A; at least it has never been demonstrated that there is any physiological activity of carotene other

than that attendant to the transformation into vitamin A. While carotene has been credited with a certain healing effect when applied directly to open wounds, and is of known benefit when applied directly to xeropthalmic eyes, it has never been shown that this beneficial effect is not due to vitamin A formed from the carotene.

Vitamin A is briefly described as being essential to growth, vision, and the maintenance in normal condition of the epithelial tissues of the body, and as having a considerable effect upon the bodily resistance to infection and the general state of health. While vitamin A was the first of the vitamins to be discovered, and has been the object of a large amount of intensive study, many of its functions in the animal organism are not yet known or clearly defined, and of those functions which have been credited to the vitamin only the role it plays in the perception of light by the visual purple of the retina of the eye is considered as being very thoroughly understood. Thus an adequate supply of vitamin A or carotene will prevent night blindness, one of the first symptoms of vitamin A deficiency.

In the system vitamin A is known to have an antagonistic action to thyroxine, and seems to play some part in the oxidation-reduction system of the tissues (1,40,43). The primary effect of a deficiency of vitamin A is a general keratinization of the epithelial tissues throughout the body. This keratinization, and a subsequent bacterial infection, of the tissues of the eyes produces the xeropthalmia erroneously termed a specific deficiency disease. Apparently there is no specific disease which is characteristic of vitamin A deficiency.

Massive doses of vitamin A and of carotene have proven non-toxic, even when continued for considerable periods of time, and result in an

increased resistance to infection, including, in the case of humans, a slight decrease in the number of duration of colds. There is doubt whether this protective action is due directly to the vitamin itself, or is the result of a better state of general health resulting from an abundance of vitamin A.

Recently a permanent blindness in young calves caused by a lack of vitamin A beginning very early in life has been reported. This blindness was produced by the constriction of the optic nerve in the bony passage through the skull rather than by advanced stages of xeropthalmia (22).

A supply of vitamin A is stored within the system, chiefly in the liver, and when the diet contains large amounts of carotene and/or vitamin A it will be stored in quantities that would be sufficient to meet the minimum requirements of the body for a considerable period of deficiency if it were utilized at the minimal rate. However, in the case of a deficiency the supply is rapidly depleted and the pathological symptoms of avitaminosis A will eventually result.

Carotene occurs in plant material in association with chlorophyll, and its formation in the growing plant tissue parallels the formation of chlorophyll. Both are the result of photochemical reactions, and carotene apparently plays an important part in plant growth, although just what its function is in plant metabolism is known no more definitely than its function in animal tissue. The vitamin A of plants is almost entirely in the form of carotene. Many roots carry stores of carotene, of which the carrot is the classical example, but for the purposes of the feeder the leafy sources are of primary importance. Therefore the determination of the carotene content of a feed

is an accurate measure of the vitamin A potency of that feed, and as the chemical determination of carotene is far more rapid and economical than the biological assay of vitamin A, vitamin A potency of plants is almost always measured as carotene content by colorimetric analysis. This colorimetric method, however, was necessarily preceded by careful work to determine absolutely that the colorimetric value is an accurate index of the biological value of the material analyzed, since the value desired is not a color factor, but a biological factor.

That carotene is a very reactive substance is evident to the chemist from inspection of its highly unsaturated structure, as is the fact that this reactivity will be modified by a greater stability due to the conjugation of the double bonds and by an inertness characteristic of large molecules. It is the experience of feeders that the vitamin A potency of a feed decreases rapidly upon the curing and storage of that feed. While carotene very probably is inactivated in many ways, there are two factors producing its decomposition that are of such importance that the others may be considered as negligible in the preparation and preservation of feeds of high carotene content. The one, a chemical factor, is the oxygen of the air, which is present in a large and constant supply and is quite reactive chemically. The other is the sunlight originally responsible for the formation of the carotene in the growing plant by a photochemical reaction. Working together, as when hay is left in a field longer than is necessary for curing, they can reduce the vitamin A potency to a very small value in a surprisingly short time.

Since carotene content is known to be correlated with color of plant tissue, naturally feeders would consider the inclusion of colored

materials in animal rations desirable. For that reason certain green hays have been examined for their relative carotene content. It was discovered that the potency varied considerably with different species, and within the same species during the season. It was also proven that for comparable growing conditions there was a direct correlation between the vitamin A content and the amount of sunshine that the plant had received, which is concordant with the statement that carotene is formed by a photochemical reaction. It was found that the plants which had grown in the favorable conditions of early spring were more potent than those grown in mid-summer. Reports from many investigators demonstrated that the carotene content of hay was less than that of an equivalent weight of the green plant, and further, that the carotene content of the hay was quite variable, depending upon the method of drying. In other words, long exposure to bright sunshine proved to be quite destructive of the carotene content, while hay dried in semi-darkness was more potent, and the same hay dried artificially in tunnel driers was still more potent than that dried in the dark. Not only was there a loss in the carotene content in harvesting and in drying, but it was also discovered that the carotene disappeared from the hay after being placed in the mow, and, oftentimes, in six or eight months after the curing process the hay was of little value so far as its vitamin A potency was concerned.

A lesser destruction of the labile carotene was noted in plant material which had been preserved in the silo, indicating that that method of preservation somehow inhibited the decomposition of the carotene. It seems probable that the packing and the juices formed in ensiling exclude the atmosphere, and that aerobic bacteria soon

utilize the oxygen present, thus preventing any great action of oxygen upon the carotene.

The results of investigators in other areas are not directly applicable to the forage crops of this state, because of Oklahoma's extremely variable type of climate, with long growing seasons, relatively long hours of sunshine, high temperatures, and dry atmosphere. For these reasons it has seemed appropriate to make a study of the carotene content of those naturally occurring hays, grasses, and forage crops upon which our feeders are dependent. In this study, attention was given to the effect of the season of the year, the method of cutting and drying, and the manner of preservation, in an effort to find what conditions are productive of high initial content and good maintenance of carotene in the feeds of the state.

The problem is complicated by the nature of Oklahoma's long growing season, with its rainy spring and fall and hot dry summer, in that forage crops can be grown and can reach maturity at almost any climatic condition, varying from the most favorable to the most unfavorable. A forage may be started under ideal conditions in the spring and mature under those same ideal conditions or under unfavorable drought conditions in late spring or early summer; in the fall the same variety of growing conditions may be encountered. This extreme seasonal variation coupled with the variation in the carotene content of a plant with its state of maturity makes the prediction of the carotene content of a forage very difficult.

An adequate supply of carotene in the feed of livestock is of primary importance to the feeder. It assures the efficient utilization of a feed and rapid and normal growth of healthier livestock,

thereby giving both a greater gain in weight per pound of the relatively costly rations used in preparing livestock for market, and a better grade animal commanding a higher price on the market. It also provides the indirect saving attendant upon the greater resistance to disease and infection and the prevention of conditions characteristic of avitaminosis A.

The problem of what feeds are high in carotene and how the carotene is best maintained is thus of considerable practical importance. This problem has received much attention in New Jersey (33), Texas (10), Kansas (30), California (14), and at other agricultural experiment stations. Workers have determined the carotene content of the various feeds characteristic of their state, and the reaction of this carotene content to various methods of curing and storage has been investigated.

In order to provide comparable and characteristic values for the carotene content of the feeds of Oklahoma this investigation of the carotene content of the forage crops of the state was undertaken.

METHOD

The method adopted in this investigation was the Peterson, Hughes, and Freeman revision of the Guilbert method for carotene in agricultural materials (29), and was used as presented below:

2 grams of each sample are refluxed 30 minutes with 25 ml of strong alcoholic KOH (ethyl alcohol free from aldehydes is used). Care must be taken to keep portions of the samples from collecting on the sides of the flasks. If any material does collect it is washed down with alcohol. After refluxing the contents of the flasks are cooled, 25 ml of petroleum ether added, and after shaking for a minute and allowing the sediment to settle, the petroleum ether-alcohol mixture is decanted into

a 1/2 liter separatory funnel. This extraction of the residue and decantation is repeated twice more with 25 ml portions of petroleum ether; then the residue is broken up by shaking with 5 ml of 95% ethyl alcohol and 15 ml of petroleum ether. After 2 or 3 additional extractions with 20 ml portions of petroleum ether, it usually comes off colorless and the residues are discarded. About 125 ml of petroleum ether are required for the extraction.

100 ml of distilled water are next poured gently through the alcohol petroleum ether solution in this separatory funnel. The alkaline alcohol-water solution containing most of the chlorophyllines and flavines separates, is drawn from the bottom of the funnel, and is re-extracted by shaking gently twice with petroleum ether in another funnel. If an emulsion is encountered it may be destroyed by adding 1 ml of ethyl alcohol. The petroleum ether extracts are combined and washed with distilled water until free from chlorophylline and alkali. Washing three or four times by pouring the water through the solution and down the sides of the flask removes most of the alkali. The remainder is removed by gently shaking the petroleum ether solution with 25 ml portions of water until the wash water no longer gives a color with phenolphthalein.

The xanthophyll is extracted from the petroleum ether solution, first with 85%, and finally with 90% methyl alcohol. 20 ml portions of alcohol are used. In the first extraction with 85% methanol the lower layer may be cloudy. If it is, draw it off and extract with 20 ml of petroleum ether. Add the petroleum ether extract to the other petroleum ether fraction and continue washing with 85% methanol. About 5 or 6 extractions with 85% methanol, followed by 2 or

3 extractions with the 90% methanol, are usually sufficient. The petroleum ether solution must be washed until the wash alcohol comes through colorless.

The petroleum ether layer is finally washed with water, dried over anhydrous Na_2SO_4, and made up to 200 ml for the reading of the color.

Ethyl alcohol free from aldehydes is necessary in the saponification in order to prevent any interference by aldehydes. This alcohol is prepared from U. S. P. alcohol according to the following standard procedure. Approximately 25 grams of KOH are added to each liter of the U. S. P. alcohol, and the mixture allowed to stand 24 hours in a warm place. It is then distilled, and to the distillate is added 2 to 3 grams per liter of m-phenylene diamine dihydrochloride. This is allowed to stand on the steam hot plate for 48 hours with occasional shaking. It is then distilled, discarding the first 100 ml and leaving a residue of about 100 ml per liter distilled in the flask. The alcohol is best stored in well filled, glass stoppered bottles.

Technical grade KOH was used. The methyl alcohol used was reagent grade, free from aldehydes. The petroleum ether used was Skellysolve, B. P. 40 - 60° C.

A Bausch and Lomb universal spectrophotometer was used in reading the optical density of the carotene solutions obtained in the analysis. This instrument consists of a standard Bausch and Lomb spectrometer, a Martens type polarizing photometer, and an incandescent light source mounted on a solid base.

The method given was selected from the literature as being the simplest and most rapid of the methods described. It avoids a solvent evaporation requisite in the Guilbert and other methods, with the at-

tendant chance of loss of carotene both by errors in manipulation and by decomposition. Munsey has carried out an investigation of the relative accuracy of the U. S. D. I. method, the Guilbert method, and the Peterson-Hughes-Freeman method, and reports the accuracy of the Peterson-Hughes-Freeman method to be equal to that of the Guilbert method, and superior to that of the U. S. D. I. method (26).

The yellow color of the carotene is very difficult to read in an ordinary colorimeter, in that the yellow is an impure color formed by the absorption of blue. The eye is relatively insensitive to blue, so a change in the blue produces relatively little sensible change in the yellow. Various apparatuses have been devised to match the color with tinted glasses, but these still have some of the difficulty of matching of the ordinary colorimeter. The use of the spectrophotometer has a marked advantage, both in accuracy and in ease of reading, and is the most satisfactory method of making colorimetric readings of carotene solutions. A pure color is read, the color selected being in the spectral range of maximum absorption, where the ratio between concentration of dissolved carotene and color absorption is greatest. With carotene, this is in the deep blue. Three readings, approximately at the peak of absorption bands in the blue, are taken. The reading of the optical density and the calculation of the carotene concentration at three different points on the absorption curve provides a check upon the purity of the pigment in solution.

The use of a spectrophotometer in reading carotene has the additional advantage of avoiding the use of the standard necessitated by a colorimeter. Owing to the instability of a carotene solution, and the difficulty and cost of preparing such a standard for each set of

readings, a solution of one or more stable materials having a yellow color approximating that of carotene, and so prepared as to have a certain carotene equivalence, is used as a working standard in the color comparison. With the spectrophotometer, the optical density of the solution of carotene is read, and the concentration calculated from the absorption coefficient previously determined very accurately upon a highly purified sample of β-carotene. The calculation is developed from Beer's law. This states that $I = I_o e^{-kcl}$, in which "I" represents the intensity of the transmitted light, "I_o" the intensity of the incident light, "e" the base of natural logarithms, "K" the absorption coefficient characteristic of a substance at a definite wave length and in a particular solvent, "c" the concentration of the dissolved substance in grams per liter, and "l" the length of the path through the solution which the transmitted light travels. From the expression $I = I_o e^{-kcl}$ the ratio $\frac{I_o}{I} = e^{kcl}$ is easily developed. By definition, "D" or optical density, equals $\ln \frac{I_o}{I}$. Therefore, $D = kcl$ and $c = \frac{D}{kl}$.

In the instrument the light from the source is separated into two parallel beams of equal intensity, one of which passes through a solution of carotene of unknown concentration, and the other through the same length of solvent, into a Martens type polarizing photometer. Here the two beams are plane polarized at right angles, made adjacent to each other by an optical system, and passed through an analyzing Nicol prism into the ocular slit of a spectrometer fitted with a shutter eyepiece. The observer here sees a rectangular field in two divisions which are matched by rotating the analyzing Nicol prism of the photometer until the halves merge. The photometer is calibrated

to indicate directly the optical density of the unknown solution, and from this and the absorption coefficient the concentration is calculated.

It is evident that, aside from experimental errors in the analysis, the accuracy of the carotene determinations depends upon the accuracy with which the absorption coefficient has been determined, which, in turn, depends primarily upon the purity of the carotene standard. The carotene used in the determination of the absorption coefficient in this investigation was purchased from the American Chlorophyll, Inc., of Alexandria, Virginia, in 0.1 g. vacuum sealed vials, and purified according to the following procedure.

0.1 g. of crystalline β-carotene is dissolved in 5 ml of chloroform in a small test tube. A wad of cotton is twisted on the tip of a 10 ml pipette and the dissolved carotene drawn through the cotton into the pipette. The cotton is then discarded and the carotene solution pipetted into a 50 ml centrifuge tube. 25 ml of absolute methyl alcohol are then added to precipitate the carotene, the mixture shaken for a few minutes, and centrifuged. The supernatant liquid is decanted, and the precipitate washed by suspending in 15 ml of methyl alcohol and centrifuging and decanting as before. The precipitate is dissolved in 5 ml of chloroform, filtered through cotton, centrifuged, and decanted as described. The precipitate is then suspended in 25 ml of methyl alcohol and poured upon a no. 50 Whatman filter paper in a Buchner funnel and sucked just to dryness. The crystals are immediately transferred to a watch glass, and dried over phosphorus pentoxide in a vacuum desiccator for an hour.

The crystals of purified β-carotene were weighed and dissolved

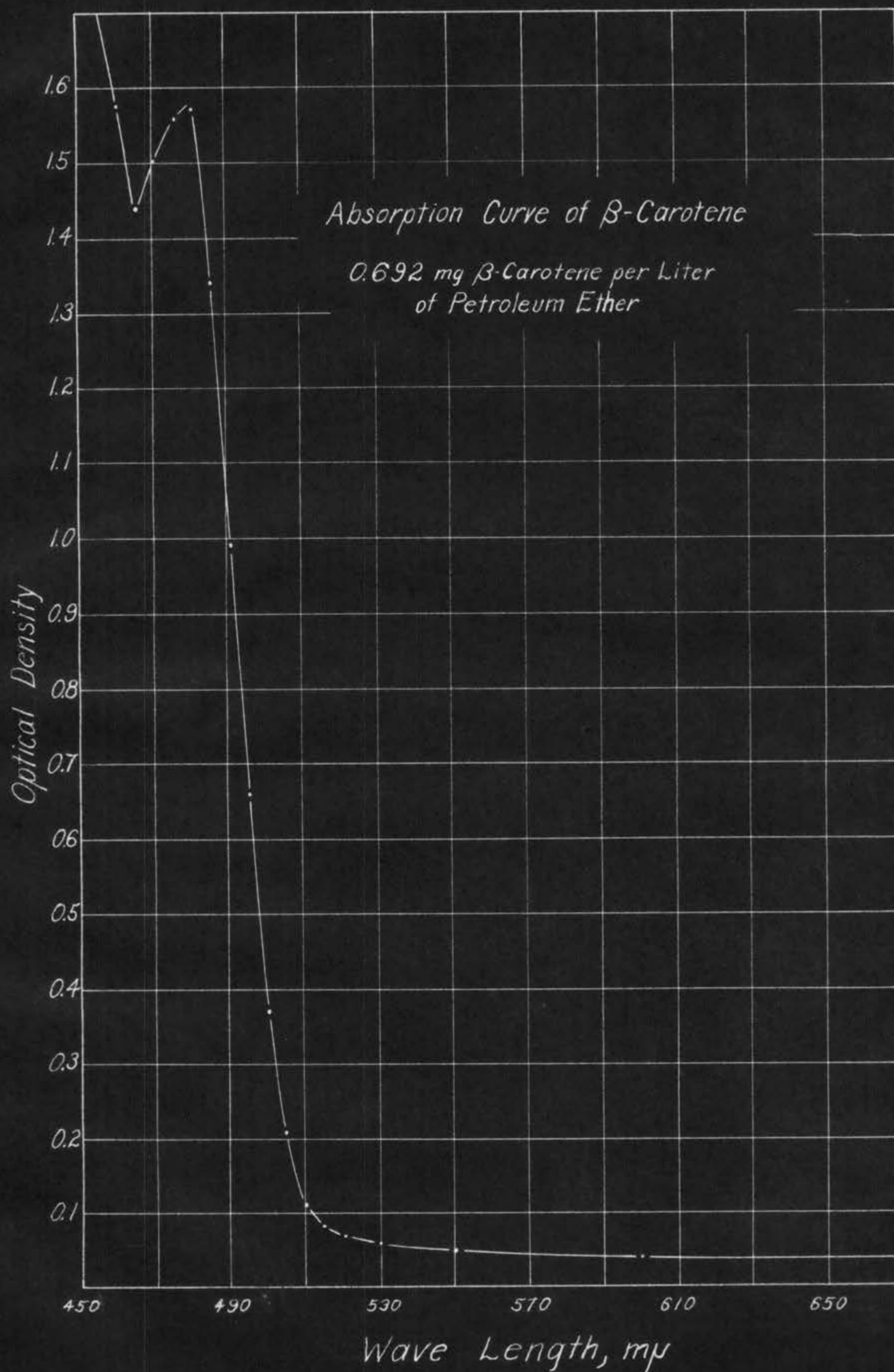
in 3 ml of chloroform, which was then made up to 1 liter with petroleum ether. From this stock solution the dilutions used in the spectrophotometer in determining the absorption coefficients were prepared. Two separate purifications and determinations were made. The figures obtained appear in table I. The absorption curve for β-carotene was determined upon one of the purified samples, and is presented in figure 1. The wave lengths indicated in the table are those occurring at the peaks of the absorption curve, and were used in reading the optical density of the unknowns. The respective absorption coefficients K average were used in the calculations.

TABLE I. Absorption Coefficient "K" of β-Carotene in Petroleum Ether at 25° C.

Sample	No. 1		No. 2	
Carotene Conc., Mg/Liter	.692	.346	.455	
Wave Length, A°	K_1	K_2	K_3	K ave.
4550	250	252	247	250
4700	220	221	217	219
4800	231	230	227	229

EXPERIMENTAL

The samples used in this investigation were all hand-harvested from pastures and cultivated areas in and near Stillwater, Oklahoma. In every case the sample was cut on a clear day, when dry, placed in



Absorption Curve of β-Carotene
0.692 mg β-Carotene per Liter
of Petroleum Ether
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Optical Density
450
490
530
570
610
650
Wave Length, mμ

a paper bag, and brought immediately to the laboratory. A weighed sample was started drying to determine the moisture content, and an analysis run at once upon the green material. The moisture content was established so that all analyses of the sample could be referred to the same basis, and the carotene values would thus be comparable. In several cases a weighed sample of green material was placed in aldehyde-free alcohol and stored at 0° C. for later carotene determinations. The purpose of this was to determine whether or not the alcohol-preservation commonly used as a method of storing plant tissue until it can be conveniently analyzed is suitable for use in carotene determinations. The samples which were run green and those that were stored in alcohol were ground to a pulp in alcohol with sand prior to analysis, to insure complete extraction of the carotene. One portion of the sample was then dried in the sun to prepare a hay comparable to field cured hay, and another portion dried in the laboratory out of direct sunlight to produce a hay that, when compared with the sun dried material, would give an indication of the effect of sunlight upon the carotene of forage. These dried samples were ground and placed in sealed glass jars, and stored upon shelves in the laboratory. Portions of several samples were also stored in a dark refrigerated room where the temperature was never in excess of 5° F. in order to determine the effect of storage temperature upon carotene decomposition. Samples from the same source were taken from time to time throughout the growing season, in order that the relative potencies could be established for the season of the year as well as for the manner of curing. Samples were taken at the growth stages at which feeders would make cuttings for forage purposes. Samples of silage were taken at the shredder as it was elevated into the silo.

The stored samples were analyzed at intervals in order to ascertain the effect of time of storage upon the destruction of the vitamin.

In summary, carotene determinations were made upon samples as follows:

Green samples.

Green samples stored in alcohol at 0° C.

Samples immediately after drying in the sun.

Samples immediately after drying out of direct sunlight.

Samples dried in the sun and stored at room temperature.

Samples dried in the sun and stored at 5° F.

Samples dried out of direct sunlight and stored at room temperature.

Samples dried out of direct sunlight and stored at 5° F.

Silages.

RESULTS

Table II presents most of the data that have been obtained in a collective form. Whenever it has been thought advisable, a smaller table has been prepared in order to make the results and discussion clearer. Table II, however, demonstrates the higher carotene content characteristic of plants dried out of direct sunlight in comparison with those dried in direct sunlight. The difference is appreciable, and the advantage is maintained throughout the storage of the material, but the figures reported here for the dark-dried forage are still well below those of the New Jersey Agricultural Experiment Station (33) and those of E. E. Gordon and W. M. Hurst of the U. S. D. A. (11) for machine dried forage. They state that the machine dried hay is very nearly equal in carotene content to that of the green forage from which it is prepared. This indicates that the speed of drying of the

forage is a very important factor in securing a hay of high carotene content, and that the destruction of carotene in the drying process is relatively rapid.

Table III presents the data obtained for the alcohol preserved samples in comparison with the values of the original green forage. While it would, in many cases, be convenient to be able to postpone the analysis of a sample until a later time, it is obvious that the method of preservation in alcohol at 0° C. is unsatisfactory as far as carotene is concerned.

TABLE III. Carotene Content of Samples Stored in Alcohol at 0° C.

Sample	Date run	Carotene of green sample (p.p.m.)	Period of storage (days)	Carotene of alcohol-stored sample (p.p.m.)
Alfalfa	6-23-37	207.3	164	60.6
Atlas sorghum	6- 9-37	79.5	180	49.7
Little blue stem	7-21-37	96.3	165	68.7
Soy beans (pod stage)	8- 4-37	106.9	160	39.2
Mung beans (bud stage)	8- 4-37	192.6	160	32.5

A surprisingly good preservation of carotene is noted in the case of the dried material stored at 5° F., according to figures presented in table IV. While the material stored at room temperature for a comparable length of time had its carotene content reduced to a very small and practically negligible value, the carotene content of the material stored at 5° F. retained its value with but little reduction below that of the original dried forage. While low temperature storage is obviously impractical for forage, it may have considerable application in maintaining a high carotene content in the case of certain more valuable concentrates.

TABLE II. The Carotene Content of Oklahoma Feeds*

Kind of Feed	Date run	Carotene of green feed (p.p.m.)	Method of drying	Carotene of dried feed (p.p.m.)	CAROTENE OF STORED DRIED FEED, IN P. P. M. ON THE DRY BASIS AT APPROXIMATE PERIODS			
					1 mo.	3 mo.	6 mo.	1 yr.
Alfalfa	6-23-37	207.3	Sun	80.9	54.3	38.7	25.5	3.7
Alfalfa			Dark	106.5	72.1	50.3	36.5	5.9
Alfalfa	4- 6-38	337.3	Sun	141.3	103.5	78.5	48.8	
Alfalfa	5-16-38	352.7	Sun	107.8	89.3	57.0	28.2	
Alfalfa	7-26-38	209.8	Sun	78.5	54.7	38.8	27.6	
Alfalfa	9-26-38	391.4	Sun	143.2	88.9	67.3	36.5	
Bermuda	6- 1-37	225.5	Sun	101.9		45.3		4.8
Bermuda			Dark	127.5		54.2		6.2
Bermuda	7-28-37	223.8	Sun	93.2		58.7	30.3	4.2
Bermuda			Dark	123.4		73.5	43.4	5.8
Bermuda	4- 6-38	394.3	Sun	175.3	128.7	81.2	40.7	
Bermuda	5-16-38	373.0	Sun	184.5	147.5	112.5	46.7	
Bermuda	7-26-38	258.5	Sun	121.2	87.3	67.3	24.1	
Bermuda	9-26-38	366.3	Sun	158.4	91.3	70.7	41.2	
White	6- 1-37	185.6	Sun	61.2	46.4		24.1	3.4
Clover			Dark	88.3	65.4		35.6	4.9
Sweet	6-30-37	190.3	Sun	98.7		66.5		5.3
Clover			Dark	139.8		89.7		7.2
Atlas	6- 9-37	99.5	Sun	55.6			20.3	
Sorghum			Dark	71.2			38.4	
Sorghum	4- 6-38	164.8	Sun	57.3	42.0	28.7	17.5	
Sorghum (mature)	9-26-38	76.6	Sun	42.5	38.5	26.3	16.1	
Rye Grass	4- 6-38	318.3	Sun	135.2	81.9	62.3	31.7	
Rye Grass	5-16-38	213.8	Sun	104.5	77.8	58.4	24.8	
Johnson	6-30-37	74.8	Sun	43.2			4.2	
Grass (seed stage)			Dark	57.5			8.9	

TABLE II CONT'D. The Carotene Content of Oklahoma Feeds*

Kind of Feed	Date run	Carotene of green feed (p.p.m.)	Method of drying	Carotene of dried feed (p.p.m.)	CAROTENE OF STORED DRIED FEED, IN P. P. M. ON THE DRY BASIS AT APPROXIMATE PERIODS			
					1 mo.	3 mo.	6 mo.	1 yr.
Johnson Grass (seed stage)	5-16-38	117.0	Sun	62.5	43.7	32.7	20.1	
Johnson Grass (seed stage)	9-26-38	69.0	Sun	38.7	27.3	18.7	11.2	
Big Blue Stem	7-21-37	116.6	Sun	48.3			9.6	
			Dark	69.4			17.6	
Little Blue Stem	7-21-37	96.3	Sun	45.2			12.7	
			Dark	63.1			20.8	
Switch Grass	7-21-37	77.3	Sun	39.3			11.6	
			Dark	47.8			13.7	
Soy Beans (pod stage)	8-14-37	106.9	Sun	48.6			23.4	6.2
			Dark	61.2			40.7	9.3
Soy Beans (young)	9-26-38	251.3	Sun	139.3	122.7	84.7	61.6	
Mung Beans (bud stage)	8- 4-37	192.6	Sun	102.4			47.9	5.3
			Dark	121.1			63.4	7.5
Mung Beans (bud stage)	9-28-38	249.3	Sun	154.3	126.5	82.1	49.7	
Mung Beans (pod stage)	9-28-38	98.7	Sun	64.6	51.3	36.5	24.2	
Wheat	4- 6-38	298.3	Sun	126.1	86.9	77.8	43.2	
Wheat	5-16-38	181.3	Sun	96.7	79.6	61.3	28.9	

*All carotene values presented in the tables in this thesis are stated in parts per million (p.p.m.) on the dry basis. To convert to U. S. P. XI Units and/or International Units of vitamin A per gram of dried material divide the p.p.m. value by 0.6. To convert to U. S. P. XI Units and/or International Units of vitamin A per pound of dry material multiply the p.p.m. value by 756.

TABLE IV. Stability of the Carotene of Dried Meals Stored at 5° F.

Kind of Feed	Date run	Carotene of green sample (p.p.m.)	Method of drying	Carotene of dried sample (p.p.m.)	STORAGE AT ROOM TEMP. Storage (days)	STORAGE AT ROOM TEMP. Carotene (p.p.m.)	STORAGE AT 5° F. Storage (days)	STORAGE AT 5° F. Carotene (p.p.m.)
Alfalfa	6-23-37	207.3	Sun	80.9	347	3.7	342	63.4
			Dark	106.5	347	5.9	342	87.6
Bermuda Grass	6- 1-37	225.5	Sun	101.9	369	4.8	364	80.2
			Dark	127.5	369	6.2	364	98.7
Bermuda Grass	7-28-37	223.8	Sun	93.2	311	4.2	307	65.6
			Dark	123.4	311	5.8	307	95.2
White Clover	6- 1-37	185.6	Sun	61.2	369	3.4	364	42.7
			Dark	88.3	369	4.9	364	61.3
Sweet Clover	6-30-37	190.3	Sun	98.7	338	5.3	333	65.2
			Dark	139.8	338	7.2	333	106.5
Soy Beans (pod stage)	8- 4-37	106.9	Sun	48.6	327	6.2	298	36.3
			Dark	61.2	327	9.3	298	47.5
Mung Beans (bud stage)	8- 4-37	192.6	Sun	102.4	358	5.3	298	72.3
			Dark	121.1	358	7.5	298	91.5

It has been observed in this laboratory that the carotene content of a forage varies from high in spring to low in mid-summer and back to high in the fall in accordance with the seasonal variation of climatic conditions from warm and moist in the spring to hot and dry in the summer and back to warm and moist in the fall. This observation has been supported in the literature (4,31). The seasonal variation of carotene content is shown in table V, A and B.

TABLE VA. Variation of the Carotene of Alfalfa With the Season

Date	Stage of growth	Carotene (p.p.m.)	Seasonal condition
4- 6-38	4" high	373.3	Spring
5-16-38	First cutting	252.7	Later spring-warmer and drier
7-26-38	Summer cutting	209.6	Summer
9-26-38	Fall growth	391.4	Fall-fall rains begun

TABLE VB. Variation of the Carotene of Bermuda Grass With the Season

Date	Carotene (p.p.m.)	Seasonal condition
4- 6-38	394.3	Spring
5-16-38	373.0	Later spring-warmer and drier
7-26-38	258.5	Summer
9-26-38	386.3	Fall-fall rains begun

As illustrated by table VI, there is considerable variation of carotene content with the stage of growth of a plant. This observation is supported in the literature by Virtanen, Hausen, and Saastamoinen (38) who state that the carotene content of a plant increases rapidly to the time of blooming, and then diminishes continuously during the remaining life of the plant. This parallelism of carotene content of a plant and rapidity of growth may be explained by the fact that carotene is formed by a photochemical reaction concurrently and in close association with chlorophyll (41), and is thus formed most rapidly when growth of the plant is most rapid. This parallelism of chlorophyll and carotene allows the green chlorophyll color of a plant to be used as a qualitative index of the relative quantity of carotene in the plant; i.e., the greener a plant is the higher its carotene content will be, and conversely, plants which have faded or yellowed will be low in carotene content. The bleaching of the chlorophyll in a hay also appears to approximate rather roughly the decomposition of the carotene in the plant, both in the curing of the hay and in the storage of the dried hay.

TABLE VI. Variation of Carotene With Stage of Growth

Kind of Feed	Stage of growth	Date run	Carotene content (p.p.m., dry basis)
Sorghum	Young	4- 6-38	164.8
Sorghum	Mature	9-26-38	76.6
Johnson Grass	Young	5-16-38	117.0
Johnson Grass	Seed Stage	9-26-38	69.0
Johnson Grass	Seed Stage	6-30-37	74.8
Soy Beans	Young	9-26-38	251.3
Soy Beans	Pod Stage	8- 4-37	106.9
Mung Beans	Young	9-28-38	249.3
Mung Beans	Bud Stage	8- 4-37	192.6
Mung Beans	Pod Stage	9-28-38	98.7
Wheat	Young	4- 6-38	298.3
Wheat	Started to Head	5-16-38	181.3

The values given for the silages in table VII indicate that the carotene of silage is relatively stable over long periods of time. This conclusion has been supported in the literature (36), with additional evidence also that the carotene content of silage preserved by the A. I. V. method, in which the silage is brought to a pH of 3 to 4 with sulfuric or hydrochloric acid, is maintained practically unchanged (32,37). There is evidence, however, that in silage the yellow color in petroleum ether used as an index of carotene content in the analysis is due to some extent to substances other than carotene, presumably produced by bacterial action. This illustrates the inherent weakness of the analytical method used, that it is a measure of yellow color, which may or may not be due to carotene entirely, and not a measure of biological activity, which is what the analysis proposes to determine. That the yellow color is due to carotene, and that the colorimetric method is an accurate estimation of biological activity, has been proven for green and dried feeds by direct comparison with biological assay (8,25,33), but apparently this does not hold as strictly in the case

of silage. However, the carotene content of silage is well preserved upon storage, and ensiling a feed is an excellent method of preservation from the vitamin A standpoint (30).

TABLE VII. Carotene Content of Silages
(p.p.m., Dry Basis)

Kind of silage	Date ensiled	Carotene when ensiled	Time of storage (months)	Carotene of silage
Corn-poor grade, burnt, H_2O added	July 1937	20	22	12.9
Kafir-shocked and headed	Dec. 1938	20	6	8.6
Mung Bean-pod stage	Oct. 1938	98.7	8	83.8
Cane-early maturity	Aug. 1938	108.1	7	46.4

Table VIII lists the carotene content of several chicken feeds which were analyzed in the course of routine work in the laboratory. In all these mashes it may be noted that the carotene content of the parent mash was zero, and that the addition of carotene-containing supplements raised the carotene content.

TABLE VIII. Carotene Content of Chicken Feeds

Description of Feed	Carotene content
Wheat Base Mix	0
Barley and Wheat Mix	0
Bleaching Mix	0
Base Mix + 20% Yellow Corn Meal	0.2
Base Mix + 50% Yellow Corn Meal	0.4
Base Mix + 2 1/2% Alfalfa Meal	1.8
Base Mix + 5% Alfalfa Meal	2.7
Base Mix + 10% Alfalfa Meal	6.8

The range of carotene values presented in these tables does not differ appreciably from values found in the literature (4,6,9,10,12, 17,18,33,34), and the literature also supports the conclusion that the chief factor in securing a high carotene value in a hay is the

speed with which the hay is cured and removed from exposure to sunlight and weathering (11,13,14,18,31,33). The range of carotene values which may be expected are summarized in table IX. The high and low values represent the range that has been found. The high values were found with young green fast growing plants, grown in the spring or fall when moisture was plentiful, and run at a sufficient state of maturity to be suitable for feeding purposes. The low values were for more mature plants, and plants growing during the summer when rainfall was slight and the temperature high. These low values were for plants that were still green, and had not been seriously wilted by exposure to sunlight or drought.

TABLE IX. Range in Carotene Values of Oklahoma Feeds (in p.p.m., Dry Basis)

Kind of Feed	CAROTENE GREEN High	CAROTENE GREEN Low	CAROTENE DRIED High	CAROTENE DRIED Low	CAROTENE 6 MO. STORAGE High	CAROTENE 6 MO. STORAGE Low
Bermuda	375	225	185	100	85	30
Alfalfa	390	200	200	80	90	20
Sorghum	165	75	90	35	40	10
Johnson Grass	90	60	50	20	20	7
Soy Beans	250	100	140	45	120	25
Mung Beans	200	100	100	45	70	25
Wheat	300	180	140	70	80	25
White Clover	185	90	65	45	25	15
Sweet Clover	100	80	55	35	30	15
Big Blue Stem	120	70	60	30	20	7
Little Blue Stem	100	65	55	30	20	7
Switch Grass	80	50	45	25	15	5
Rye Grass	320	200	160	105	70	35

The carotene content of a dried feed is subject to so many factors contributing to its variability that it is very difficult to predict what its value will be (14). Besides the influence of the stage of growth, climatic conditions and soil fertility have their effects upon the concentration of carotene in a green forage. The curing of the

forage is the most important factor in determining what the carotene content of a hay will be, with the protection given the hay in storage and the time of storage also entering in. For reasonably good hay making, however, the carotene content of the hay will be approximately half that of the green forage, and the carotene content of the hay upon storage in a good barn for six months will drop to about one-third the figure for the freshly cured forage.

All carotene values that have been given have been stated in parts per million (p.p.m.) on the dry basis. To convert into U. S. P. XI Units and/or International Units per gram of dried material divide the p.p.m. value by 0.6. To convert to U. S. P. XI Units and/or International Units per pound of dry material multiply the p.p.m. value by 756.

SUMMARY

1. The carotene content of several Oklahoma feeds has been found to have about the same range as values found in other sections of the country, with a somewhat greater variation due to the more varied type of climate characteristic of Oklahoma.

2. The carotene content of a plant varies with the state of maturity in accordance with the rapidity with which the plant is growing, i.e., the carotene content increases to the time of blooming, and then decreases.

3. The carotene content of a plant is influenced by the season in accordance with the favorableness of that season for growth; i.e., in the spring and fall, when the moisture and temperature are right for rapid growth, the carotene content of plants will be relatively high, while in the summer, when growth is retarded by the hot dry weather carotene values will be low.

4. The exposure of forage to the sun and weather in field curing should be as brief as possible, as these are the chief factors in the destruction of carotene in a feed.

5. For a hay correctly made the carotene value will be about half that of the green forage, and will drop to about one-third of that value upon six months storage under good conditions.

6. The carotene of silage appears to be quite stable over long periods of time, and may be expected to be not less than half the original value at six months storage.

7. The green chlorophyll color of a feed is a useful qualitative index of the carotene content of that feed.

8. The methods and principles proposed for the preparation of a

feed of high carotene value are identical with those uniformly recommended by agricultural experts for the curing of roughages with the minimum loss of nutritive value and palatability. Thus the feeder who follows the precautions recommended for the preparation of feeds of high carotene content will reap two-fold benefits; in addition to having a feed of higher carotene value he will also have a feed which is of higher food value.

9. The carotene content of a feed is subject to so many variables that it is very difficult to predict its value by inspection alone with any degree of accuracy.

BIBLIOGRAPHY

1. Abelin, I., "The Relations Between Carotene (Vitamin A) and Thyroxine." Z. physiol. Chem. 217, 109-14 (1933). C. A. 27, 3974 (1933).

2. Ahmad, B., and Drummond, J. C., "The Absorption of Carotene by Rats." J. Soc. Chem. Ind. 50, 184-6T (1931).

3. Ahmad, B., and Malik, K. S., "The Metabolism of Carotene in Different Animals." Indian J. Med. Research 20, 1033-8 (1933). C. A. 27, 4561 (1933).

4. Atkeson, F. W., Peterson, W. J., and Aldous, A. E., "The Carotene Content of Some Typical Pasture Plants." J. Dairy Sci. 20, 557-62 (1937).

5. Basu, N. K., "Vitamin A and Fat Metabolism." Z. Vitaminforsch. 6, 106-10 (1937). C. A. 31, 5412 (1937).

6. Buxton, L. O., and Dombrow, B. A., "The Carotenoids in Forage." Ind. Eng. Chem., An. Ed. 10, 262-3 (1938).

7. De, N. K., "The Assimilation of Vitamin A and Carotene by Rats from Some Common Foods." Indian J. Med. Research 24, 751-66 (1937). C. A. 31, 5026 (1937).

8. Drummond, J. C., and Morton, R. A., "Observations on the Assay of Vitamin A." Biochem. J. 23, 785-802 (1929).

9. Fraps, G. S., and Kemmerer, A. R., "Losses of Vitamin A and Carotene from Feeds During Storage." Texas Agr. Expt. Sta. Bul. No. 557, 1-28 (Oct. 1937).

10. Fraps, G. S., Treichler, R., and Kemmerer, A. R., "Relation of the Carotene Content of Certain Feed Materials to Their Vitamin A Potency." J. Ag. Research 53, 713-16 (1936).

11. Gordon, E. D., and Hurst, W. M., "Artificial Drying of Forage Crops." U. S. Dept. Agri., Circ. 443, 1-23 (1937).

12. Guilbert, H. R., "The Determination of Carotene as a Means of Estimating the Vitamin A Value of Forage." Ind. Eng. Chem., An. Ed. 6, 452-4 (1934).

13. Guilbert, H. R., "Problems in Range Nutrition in California." Proc. Am. Soc. Animal Production 1930, 92-7 (1931). C. A. 25, 4947 (1931).

14. Guilbert, H. R., "Factors Affecting the Carotene Content of Alfalfa Hay and Meal." J. Nutrition 10, 45-62 (1935).

15. Holmes, H. N., Corbet, R., Cassidy, H., Meyer, C. R., and Jacobs, S. I., "Biological Activity of Some Carotene Preparations." J. Nutrition 7, 321-9 (1934).

16. Ijdo, J. B. H., "The Influence of Fertilizers on the Carotene and Vitamin C Content of Plants." Biochem. J. 30, 2307-12 (1936).

17. Johnson, H. W., "Effect of Leaf-hopper Yellowing Upon the Carotene Content of Alfalfa." Phytopathology 26, 1061-3 (1936).

18. Kane, E. A., Wiseman, H. G., and Cary, C. A., "Loss of Carotene in Hays and Alfalfa Meal During Storage." J. Ag. Res. 55, 837-47 (1937).

19. Karrar, P., Helfenstein, A., Wehrli, H., and Wettstein, A., "The Constitution of Lycopin and Carotene." Helv. Chim. Acta. 13, 1084-99 (1930).
C. A. 25, 519 (1931).

20. Karrar, P., Morf, R., and Schopp, K., "Vitamin A Fish Oils." Helv. Chim. Acta. 14, 1431-6 (1931).
C. A. 26, 4359 (1932).

21. McCollum, E. V., and Davis, M., "The Necessity of Certain Lipins in the Diet During Growth." J. Biol. Chem. 15, 167-75 (1913).

22. Moore, L. A., "Relationship Between Carotene, Blindness Due to Constriction of the Optic Nerve, Papillary Edema and Nyctalopia in Calves." J. Nutrition 17, 443-59 (1939).

23. Moore, T., "Vitamin A and Carotene." Biochem. J. 23, 803-11 (1929).

24. Moore, T., "Vitamin A and Carotene; The Relative Minimum Doses of Vitamin A and Carotene." Biochem. J. 27, 898-902 (1933).

25. Morton, R. A., Heilbron, I. M., and Spring, F. S., "Absorption Spectra in Relation to Vitamin A." Biochem. J. 24, 136-40 (1930).

26. Munsey, V. E., "Report on the Determination of Carotene." J. Assoc. Official Agr. Chem. 20, 459-68 (1937).

27. Olcott, H. S., and McCann, D. C., "Carotenase. The Transformation of Carotene to Vitamin A in Vitro." J. Biol. Chem. 94, 185-93 (1931).

28. Osborne, T. B., and Mendel, L. B., "The Relation of Growth to the Chemical Constituents of the Diet." J. Biol. Chem. 15, 311-26 (1913).

29. Peterson, W. J., Hughes, J. S., and Freeman, H. F., "Determination of Carotene in Forage." Ind. Eng. Chem., An. Ed. 9, 71-2 (1937).

30. Peterson, W. H., Bird, H. R., and Beeson, W. M., "Chemical Changes in the Making of A. I. V. Alfalfa Silage and Nutritive Qualities of Milk Produced Therefrom." J. Dairy Sci. 20, 611-23 (1937).

OKLAHOMA
AGRICULTURAL & MECHANICAL COLLEGE
LIBRARY
AUG 6 1940

31. Pyke, M., "A Rapid Method for the Determination of Carotene, Xanthophyll and Chlorophyll in Artificially Dried Grass Meals." J. Soc. Chem. Ind. 55, 139-40T (1936).

32. Richardson, H. E., "The Handling, Processing and Storing of Legume Crops for Feed." Agr. Eng. 16, 469-71 (1935).

33. Russell, W. C., Taylor, M. W., and Chichester, O. F., "Effect of the Curing Process Upon Carotene and Vitamin A Contents of Alfalfa." N. J. Agr. Expt. Sta. Bull. 560, 1-8 (1934).

34. Shinn, L. A., Kane, E. A., Wiseman, H. G., and Cary, C. A., "Variations in the Carotene Content of Farm Feeds." Am. Soc. Animal Production, Rec. Proc. 27th Ann. Meeting 1934, 190-2.
C. A. 29, 7520 (1935).

35. Steenbock, H., "White Corn vs. Yellow Corn and a Probable Relation Between the Fat-Soluble Vitamin and the Yellow Plant Pigments." Science 50, 352-3 (1919).

36. Taylor, M. W., "Stability of Provitamin A in Alfalfa and Silage." New Jersey Agr. 16, No. 6, 3 (1934).
C. A. 30, 530 (1936).

37. Virtanen, A. I., "Conservation of Vitamins in Cattle Feed." Biochem. Z. 258, 251-6 (1933).
C. A. 27, 2224 (1933).

38. Virtanen, A. I., v. Hausen, S., and Saastamoinen, S., "Studies on Vitamin Production in Plants." Biochem. Z. 267, 179-91 (1933).
C. A. 28, 1740 (1934).

39. von Euler, B., von Euler, H., and Karrar, P., "The Biochemistry of the Carotenoids." Helv. Chim. Acta. 12, 278-85 (1929).
C. A. 23, 3254 (1929).

40. von Euler, H., and Enderlein, R., "Oxygen Consumption of Animal Tissue After Thyroxine Administration." Biochem. Z. 261, 226-34 (1933).
C. A. 27, 4306 (1933).

41. von Euler, H., and Hellström, H., "The Formation of Xanthophyll, Carotene, and Chlorophyll in Illuminated and Unilluminated Barley Seedlings." Z. physiol. Chem. 183, 177-83 (1929).
C. A. 23, 5487 (1929).

42. von Euler, H., and Klussmann, E., "Biochemistry of the Carotenoids and of Vitamin C." Z. physiol. Chem. 219, 215-23 (1933).
C. A. 27, 5784 (1933).

LIBRARY
A & M COLLEGE
STILLWATER OKLA

43. von Euler, H., and Klussmann, E., "Carotene (Vitamin A) and Thyroxine." Z. physiol. Chem. 213, 21-34 (1932).
C. A. 27, 754 (1933).

44. Wilson, H. E. C., Gupta, S. M. D., and Ahmad, B., "The Absorption of Carotene and Vitamin A in the Human Subject." Indian J. Med. Research 24, 807-11 (1937).
C. A. 31, 5026 (1937).

Typed By: Nona Eckenwiler