THE XANTHOPHYLL AND CAROTIN CONTENT OF CERTAIN POULTRY FEEDS AND EGG YOLKS

by

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INTRODUCTION

The present status of our knowledge regarding the relation of carotinoids of the feed to those of eggs is far from satisfactory. Due to this fact it was thought desirable to make a quantitative determination of the two best known carotinoids, xanthophyll and carotin, in poultry feeds and in eggs produced from hens receiving these feeds of known carotinoid content.

Some of the work reported in the literature has not been done with methods sufficiently accurate to warrant a statement regarding the amount of xanthophyll present in certain plant and animal substances.

Xanthophyll is present in all green plants along with carotin, but in larger quantity.

It has been definitely established by Palmer and Kempster (1919c) that xanthophyll or its isomer is present in hen eggs and in the body fat of chickens.

Carotinoids have been held to be associated with vitamin A activity for many years but only recently was it demonstrated by Moore (1929) that carotin behaves in vivo as the precursor of the vitamin.

More attention is now being given egg yolk color than ever before, not because of the higher food value associated

with color, but because of certain prejudices and preferences of the buying public.

According to Palmer and Eckles (1914) the yellow color in natural uncolored butter is due to carotin. Since carotin has been demonstrated to be the precursor of vitamin A this fact has special significance. A rich yellow color of eggs is not such a reliable index to the carotin content but rather of the xanthophyll content. As yet xanthophyll has not been demonstrated to have any function in the body. Palmer and Kempster (1919b) think of xanthophyll in the egg yolk as an excretion. During the time the hen is not laying it is excreted through the skin. This fact has led to our modern method of judging birds for past production by means of pigmentation.

It is the purpose of this study to seek more information on the exact relationship between the amount of xanthophyll and carotin in the feed and the amount in the egg yolk. It is desirable to know the xanthophyll and carotin content of poultry feeds to have some knowledge of the amounts to feed in order to produce an egg with a satisfactory yolk color as well as to know the amount of the precursor of vitamin A (carotin). This problem was undertaken in cooperation with a research fellowship established by the Kansas Carlot Poultry and Egg Shippers Association.

Very little work has been done in this country on the pigments of egg yolks due to the difficulty with which they are isolated. It was necessary to work out a method of extraction of egg yolk before the problem could be carried out. Most of the early work with the xanthophyll and carotin content of eggs and poultry feeds has been questioned due to the methods employed.

Since the discovery by Moore (1929) that carotin is the precursor of vitamin A it has become of vital interest to know if there is any relationship between the color of the egg yolk and the potential vitamin A content.

REVIEW OF LITERATURE

According to Needham (1931) the first attempt to discover the nature and properties of the pigments of the hen's egg began in 1867 when Stradeler obtained a colored solution by extracting it in various ways and concluded it was bilirubin but Thudichum (1869) noticed that the pigment was exclusively soluble in fat solvents but unsaponfiable and called it luteine. Schunck (1903) isolated the pigment and showed by spectroscopic experiments that it was identical with xanthophyll.

Willstatter and Stoll (1918) assigned the formula $C_{40}H_{56}O_2$ to crystalline xanthophyll obtained from egg yolk. To carotin they gave the empirical formula $C_{40}H_{56}$.

Palmer and Kempster (1919a) raised chicks from hatching to maturity on rations containing the merest traces, if not devoid of carotinoids. They concluded that natural yellow pigment of fowls, which is derived from xanthophyll of the feed, bears no important relation to growth or to the fecundity and reproduction. The same authors (1919a) demonstrated that cockerels fed a carotinoid-free ration showed only a faint trace of carotin, whereas, those fed on a xanthophyll rich diet for a short period of three days showed ample evidences of xanthophyll in body fat.

Palmer (1915) fed birds on a ration rich in carotin, one rich in xanthophyll, and one nearly devoid of pigments. The color of the yolks was compared at first by hard boiling the eggs and spreading the yolks before a Lovibond tintometer. In eight weeks the color of the yolks of the non-pigmented group had become so pale that the hard boiled yolks showed scarcely enough color to be measured. The eggs were desiccated with plaster of paris and extracted with alcohol and saponified. The pigment was recovered from the soap in the usual way and separated by means of 85 per cent alcohol and petroleum ether. The ether layer was concentrated and the color noted at a volume of 12.5 c.c. in each inch layer with a Lovibond tintometer.

Not all of the pigment of the egg yolk may be considered as xanthophyll and carotin. Barbeiri (1916) des-

cribed a pigment which he names ovachromin. It is soluble in equal weight of water, likewise soluble in fat and insoluble in alcohol, ether, or chloroform. At 270°C. it carbonizes without melting. It is feebly acid, does not give the biuret reaction and has neither albumin or peptone properties. Palmer and Kempster (1919b) found an unknown pigment in their nearly colorless egg yolks. An acetone extract would not reduce ferric chloride crystals to green ferrous salts as will either xanthophyll or carotin. It gave a characteristic bluish green ring with fuming nitric acid and was precipitated from solution by mercuric chloride.

The exact function of pigments in the egg yolk is unknown. It was the common belief that when a hen started to lay, xanthophyll was removed from her shanks and beak to be placed into the egg yolk but Palmer and Kempster (1919c) suggested that it is not a subtraction process but a means of diversion or excretion. Xanthophyll is excreted through the skin of the bird and is oxidized, while fecundity replaces the excretory process.

It has been frequently asserted that eggs with highly colored yolks are richer in vitamins than those having pale yolks. Palmer (1922) stated that feeds rich in xanthophyll are also rich in vitamin A, and one can rest assured that eggs with highly pigmented yolks possess an abundance of

vitamin A. Under normal conditions the degree of yellow color in the yolk is a fair index to the vitamin A content of the egg, and feeding tests have demonstrated that pale yolked eggs when fed to chicks will not produce as satisfactory growth as will eggs with highly colored yolks.

This may not always be the case, however, as not all the vitamin in the egg occurs as carotin. Euler and Klussman (1932) made the study of several eggs for vitamin A content by means of photographing a spectrometric analysis. Besides a general absorption in the ultra violet region two absorption bands at 345 millimicrons and 375 millimicrons were recognized.

Kline, Schultze, and Hart (1932) reported that xanthophyll will not serve as a source of vitamin A for growing chicks whereas carotin will. This statement has been checked by other workers.

On the other hand Virgin and Klussman (1932) report that xanthophyll is converted into either a pro-vitamin A or carotin but considering the fact that arachidis fat and cooked codfish were used the absence of vitamin A from the diet is questioned.

While the exact nature of the xanthophylls of the egg yolk is unsettled, Kuhn and Smakula (1931) stated that the yellow pigments consist of lutein and zeaxanthin in the ratio of 2:1. The calculation was made on the basis of op-

tical rotation and later confirmed by quantitative photo-electric determinations of the absorption spectra. The curves were plotted separately for the two pigments, then for the natural mixed and for the artificial mixture of 30 per cent zeaxanthin with 70 per cent lutein. The last two curves were superimposed and were found to be identical.

The function of the carotinoid pigments in plants is also unknown. Several theories have been advanced but none proven. The pigments are found for most part in the leaves, however, some occur in the seed of plants, this being especially true in yellow corn. Several workers have shown that there are different kinds of xanthophyll. Zeaxanthin, the xanthophyll of corn, differs from the others in that it takes up a molecule of methyl alcohol of crystalization. Willstatter and Stoll (1918) found egg yolk xanthophyll to have a melting point of 195° - 196°C., whereas, plant xanthophyll has a melting point of 173.5° - 174.5°C. (corrected)

The small amount of carotin in foods is demonstrated by the fact that Schertz (1925a) was able to extract only 1.13 grams of pure carotin from 50 pounds of fresh carrots. The amounts of carotin and xanthophyll are measured most accurately by means of the spectrophotometer in which readings are 2.8 per cent accurate whereas with the colorimeter readings are accurate to 17 per cent.

The stability of the two pigments as given by Schertz

(1925c) is as follows: xanthophyll is unstable in ether solutions, is very stable in absolute ethyl alcohol, and is slightly unstable in petroleum ether. Carotin is unstable in ether solution but apparently is perfectly stable in alcohol and petroleum ether.

In the dry state xanthophyll oxidizes more readily than carotin. In solution xanthophyll oxidizes more readily than carotin when kept in the ice box, but when the solutions are kept at room temperature carotin oxidizes more rapidly.

The amount of pigment that a plant contains is not constant according to the finding of Schertz (1929a) and others. Young leaves contain a larger percentage of pigments than do older plants. The amount of carotinoids is parallel to the chlorophyll. Factors affecting the amount of carotinoids in plants are soil, light, rainfall, humidity, and temperature. The nutrients that the plant receives also has its effect on the pigments. Schertz (1929c) found a positive correlation between nitrogen fertilizer and color of leaves; this is not true with potassium. The chloroplast pigments are highest during the period from June 25 to September 29.

The proportion of pigments in green leaves appears to be constant. Willstatter and Stoll (1918) state that in 1000 parts of fresh green leaves there are two parts of alpha chlorophyll, three-fourths of a part of beta chloro-

phyll, one-third of a part of xanthophyll, and one-sixth of a part of carotin.

MATERIAL AND METHODS

Samples were taken from the feeds fed individual hens in batteries on the college poultry farm. The following feeds were analyzed for pigments; (1) a cross-strain variety of yellow corn was analyzed for xanthophyll only, (2) alfalfa hay composed of 55 per cent leaves and 45 per cent stems which according to Salmon, Swanson, and McCampbell (1925) is near the average percentages. The alfalfa was about nine inches high when cut October 9. It was carried inside immediately, quickly dried and ground. The second lot of alfalfa was the same as above only it was allowed to bleach 30 days out-of-doors before being ground into meal. The third lot of alfalfa was the same cutting as above but it was allowed to bleach out-of-doors on a screen for about three months until practically yellow.

It was desirable to know the xanthophyll and carotin content of certain basal rations used in the experiment carried out in the hen batteries. It was hoped that a ration could be compounded that was nearly colorless. In this ration, feeds to be tested biologically for pigments were substituted for part of the white corn. The basal ration (IV) consisted of:

Ingredient	Per cent
White corn	70
Dried buttermilk	20
Wood pulp	4
Dried brewers' yeast	3
Cod liver oil	2
Salt	1
Total	100

The crude analysis of this ration was fiber 6.5 per cent and protein 13.5 per cent. The basal was mixed every two weeks so the cod liver oil would not lose too much of its vitamin A potency.

Another basal was made to test the effect of alfalfa hay on yolk color. It consisted of:

Ingredient	Per cent
White corn	62
Dried buttermilk	18
Wood pulp	4
Dried brewers' yeast	3
Cod liver oil	2
Salt	1
Total	90

To this ration was added 10 per cent of alfalfa hay. Analysis was run on the ration before the alfalfa hay was added.

The white corn used in the ration was Pride of Saline, a variety of Kansas origin. The meat and bone scraps were of a commercial brand that is used on the poultry farm. The dried buttermilk was made by the Fairmont Creamery Company. The Spruce Wood Pulp fiber was obtained from the University

of Wisconsin and was used to add bulk to the ration. The dried brewers' yeast was of a yellowish brown color but most of the color was water soluble and was neither carotin nor xanthophyll. The cod liver oil was of a commercial brand and of a yellow color. The alfalfa used was that previously described.

The green feeds analyzed consisted of young wheat plants not over six inches high and the entire top of young alfalfa plants. The height was not in excess of eight inches.

As many of the methods previously used were found to give untrustworthy results it was necessary to develop a method whereby xanthophyll could be extracted and determined quantitatively from certain poultry feeds.

Two analyses were the minimum number of extractions made on each sample. In case the results did not check and the sample could not be replaced the higher result is probably the more accurate as the pigments are very unstable and oxidize readily.

The method of sampling was to pour the sack of feed on the clean floor and mix by turning it over with a shovel five times. The feed was then reduced by quartering and mixing, then quartering until the balance was about a pint. The feed was then ground on a carborundum grinder until it would pass through a one-half millimeter sieve then it was

rolled in a roller mill to expose more surface for extraction. After this process it was stored in air tight containers until extracted. Before the sample was taken the jar was alternately inverted and rolled 25 times in order to get a uniform sample.

In the case of green feed or alfalfa hay an improvised pebble mill was made by using a two liter glass bottle fitted with a glass stopper. The feed was chopped finely and ground with 100 c.c. of acetone until the sample was thoroughly disintergrated. The samples were taken early in the morning. Plants comprising the sample were taken at random from scattered parts of the field.

Extractions were made and readings taken on the spectrophotometer as quickly as possible in order to lessen the loss of pigments due to oxidation.

The method of extraction is primarily that used by Schertz (1928) together with modifications. A 10-gram sample of finely ground feed was weighed, wrapped in a filter paper, placed in a thimble and put in a 100 c.c. Soxhlet extractor, 150 c.c. of acetone was placed in a 500 c.c. round-bottom flask. The flask was heated in a water bath over an electric heater. The feed was extracted until the acetone in the extractor was colorless. It usually was necessary for the acetone to trip over about three times. Care was taken to keep the temperature as low as possible.

The feed was removed and further extracted with 150 c.c. of diethyl ether. The ether solution was filtered and added to the acetone. From this point on the procedure is the same for both feeds and green plant material.

After the combined extracts were obtained it was divided, poured into two 500 c.c. separatory funnels, and the acetone was washed out by means of distilled water. Great care had to be taken to avoid emulsions. The sample was first washed by pouring the water through the ethereal acetone solution by means of a long stemmed funnel. A small amount of water was added at first in the above manner, until a colorless or only slightly tinted aqueous layer separated, and then the aqueous layer was discarded. The yellow color in the aqueous layer consists of flavones which are water soluble. They were tested by adding alkali which causes the color to deepen. This was repeated several times and then washed by very gently rotating the liquids. When emulsions formed a wash water containing NaCl was used to break them.

In order to remove the remainder of the flavones, and any anthocyanins that may be present the sample was washed with a 1 per cent solution of sodium carbonate.

The separatory funnel should not at any time be shaken because emulsions will form, the chlorophyll will go into the water in the colloidal state, and the separation will be

more difficult. The washing should remove none of the pigments if properly done. The ether solution of the pigments was then put in a 250 c.c. flask. To this was added 15 c.c. of methyl alcohol which had been saturated with KOH. This solution should have a clear color without a trace of yellow. It was shaken thoroughly and then set in the ice box until the next day when the chlorophyll would all be saponified and separated as a layer in the bottom of the flask; the upper ethereal layer will contain the yellow pigments. This step is followed for feeds that contain no chlorophyll because the pigments occur in plants in a combined form and it is necessary to saponify the lipins.

The alkaline solution of the pigments was poured into a separatory funnel. The bottle was washed several times with distilled water and the washings added to the separatory funnel. Then the bottle was washed with 50 c.c. of ether to remove any of the yellow pigments. To this 100 c.c. of ether was added and the funnel shaken vigorously and allowed to stand 30 minutes and a yellowish ethereal layer of carotin and xanthophyll separated above, while the potassium salts of chlorophyll separated as a greenish layer below. The greenish layer was run off and extracted once or twice with 100 c.c. of ether to remove any traces of yellow pigments. The ether solutions were combined and washed with a dilute solution of KOH (10-25 c.c.) and twice with distilled

water (10-25 c.c.) to remove traces of the alkali and of the chlorophyll salts.

The combined ether extracts were evaporated in a small distilling flask at 50°C. or lower, to a few cubic centimeters, and then evaporated rapidly, using suction to remove all traces of ether.

The residue was transferred by means of about 110 c.c. of petroleum ether and about 50 c.c. of 85 per cent methyl alcohol (prepared by making 85 c.c. of absolute CH_3OH to a volume of 100 c.c. with water) to a separatory funnel. The funnel was shaken and allowed to stand about five minutes until the layers fully separated, and the methyl alcohol layer run off. From this petroleum ether the xanthophyll was further extracted by means of two extractions with 25 c.c. of 85 per cent methyl alcohol, one extraction with 25 c.c. of 90 per cent methyl alcohol, and two extractions with 92 per cent alcohol using 25 c.c. for each extraction. If the last extraction was not colorless further extractions with 92 per cent methyl alcohol were made. In this way by means of the methyl alcohol, the orange yellow xanthophyll was separated from the orange colored carotin, which remains in the petroleum ether layer.

To the combined methyl alcohol extractions of xanthophyll were added 200 c.c. of ether. The mixture was poured into a large separating funnel; 175 c.c. of distilled water

added, the solution shaken vigorously and allowed to stand for about 15 minutes. The xanthophyll collected in the upper ether layer, with the colorless aqueous methyl alcohol layer below. It was often necessary to treat the mixture with a saturated solution of sodium chloride to break emulsions, which if unbroken would have made the separation of xanthophyll incomplete and unsatisfactory. The aqueous methyl alcohol solution was run off and extracted a second time with ether. The combined ether extract was washed once or twice with distilled water to remove traces of the salt and methyl alcohol. The ether solution of xanthophyll was run into a flask and evaporated at low temperature (using suction) to about 50 c.c. The solution was poured into a separatory funnel and ether was added, then a little water, to separate any methyl alcohol from the ether solution. The lower layer was run off and the ether washed with water and allowed to stand for a moment until most of the free water separated. It was then made up to 100 c.c. (or a convenient volume) in a volumetric flask and the pigment estimated spectrophotometrically.

If the ether solution was not clear it may be cleared by allowing it to stand for several hours by which time the water will settle out; or by drying it by filtering through a layer of anhydrous sodium sulfate.

The petroleum ether solution of carotin was washed two or three times with distilled water to remove traces of

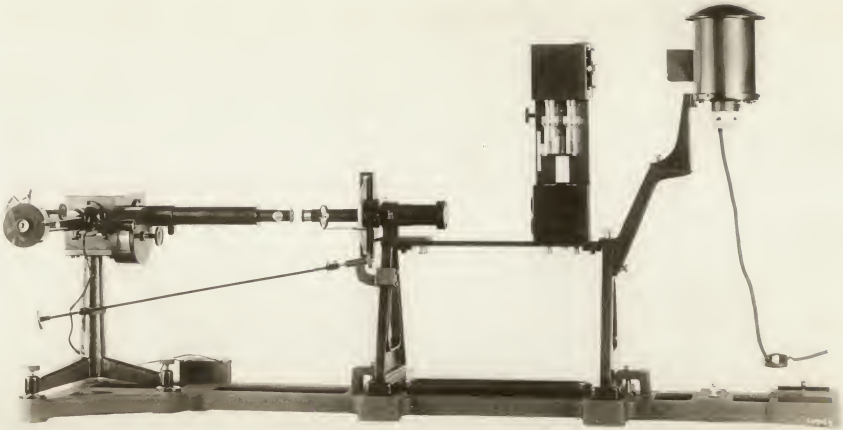
methyl alcohol. The solution of carotin was then allowed to stand until the free water separated or was dried by filtering through anhydrous sodium sulfate and then made to a convenient volume and the pigment estimated by means of a Bausch and Lomb spectrophotometer. The spectrophotometer used in determinations is shown in Plate I.

The spectrophotometer was used because it is more accurate in that transmittancies as measured depend upon the physical properties of the substance involved, and not upon the variability of light, physiological factors, or tint of solution, all of which greatly affect the readings in any colorimeter. The results are also independent of abnormalities of the observer's color vision.

The method of determination is briefly reviewed in the following paragraph.

The samples were accurately weighed on analytical balances, extracted and the pigment determined by means of the spectrophotometer. The wave length used for determining both xanthophyll and carotin was 435.8. Five readings were taken of the large angle then the field was reversed and five readings taken of the small angle. A mean of these angles was taken and used in calculating the value of the transmittancy. The cotangent of the large angle times the tangent of the small angle gives the value for transmittancy. The log of the value was taken giving the value of

PLATE I



-log T which was used in determining the amount of pigment present. The thickness of the cell in centimeters was given the symbol b. The specific transmissive index or extinction coefficient (K) of xanthophyll at mercury line of 435.8 in ether was found by Schertz (1925b) to be 2.089; for carotin in petroleum ether the value was given by Schertz (1923) to be 1.915.

The volume to which the extract of the pigment was diluted depended upon the concentration as determined by microscopic examination. The actual concentration (C) was found by means of a formula $C = \frac{1}{Kb}$ (-log T). This gave the concentration of the solution in centigrams. This value was then transposed into milligrams x 10^{-3} per gram of sample.

The eggs used for analysis were produced by hens receiving the rations given in Table 1.

Table 1. Rations Fed Hens from which Eggs were Examined (expressed in percentage)

Ingredient	Lots					
	I	II	IV	IX	X	XI
White corn	--	35	70	62	62	62
Yellow corn	70	35	--	--	--	--
Dried buttermilk	20	20	20	17	19	18
Wood pulp	4	4	4	5	3	4
Alfalfa leaves	--	--	--	10	--	--
Alfalfa stems	--	--	--	--	10	--
Alfalfa hay	--	--	--	--	--	10

To each of the rations were added the following:

3 per cent of dried brewers' yeast
2 per cent of cod liver oil
1 per cent of sodium chloride

Due to the difficulty with which xanthophyll is recovered from egg yolk very little work has been done along that line. With the exception of Palmer's work several years ago all the work has been done abroad. After much research a method whereby egg yolks could be extracted for pigments was finally adopted. All extractions given in this paper were made by the following method.

The egg yolk was weighed and placed into 100 c.c. of acetone in a 250 c.c. beaker. The yolk mixture was beaten thoroughly and the acetone filtered off. The coagulated yolk was wrapped in a filter paper and put in the Soxhlet extractor and extracted until colorless. The acetone was

then evaporated off. The filtrate was evaporated and the residue saponified by adding 25 c.c. of 20 per cent methyl alcoholic potash and allowed to stand over night. The alcoholic potash should be free from color.

The resulting soap was dissolved in 75 c.c. of distilled water. This solution was shaken with an equal volume of pure ether in a separatory funnel. The extraction was repeated using 50 c.c. more of ether until there was no more color in the ether after extraction. The combined ether extracts were washed many times with distilled water, care being exercised at all times to avoid emulsion. Egg yolk is widely known for its emulsifying properties and it is very difficult to avoid emulsion. Some emulsions may be broken by washing with water containing NaCl while other emulsions are only aggravated by its use. The ether was washed until it no longer reacted alkaline to phenopthalein. The pigments were then separated by the method used in plants, and were estimated spectrophotometrically.

DISCUSSION OF RESULTS

In Table 2 is shown the xanthophyll content of feeds. The yellow corn used in these analyses was found to have approximately 13.7×10^{-3} milligrams of xanthophyll per gram. This value was the average of three analyses, the highest of which was 18.9×10^{-3}.

Table 2. Xanthophyll Content of Feeds

Sample	Weight in grams	Volume	(b) c.m. depth of cell	-log T transmittancy	$\frac{1}{Kb}$	Mgm. pigment per gram x10-3
Yellow corn	10	50	2.0	0.87786	0.239	10.5
Yellow corn	10	50	0.5	0.39496	0.957	18.9
Yellow corn	10	100	1.0	0.24467	0.479	11.7
Alfalfa basal	10	100	2.0	0.38404	0.239	9.2
Alfalfa basal	10	50	1.0	0.41378	0.479	9.9
Basal IV	10	25	4.0	0.06737	0.120	0.3
Basal IV	10	25	4.0	0.05240	0.120	0.2

The basal ration to be used for the alfalfa showed considerable color. The average xanthophyll content was 9.55×10^{-3} milligrams per gram sample. The cod liver oil contained a yellow color, the nature of which was not determined. It may have been due to bile pigments. The eggs from hens fed cod liver oil as well as the feeds that contained cod liver oil gave a deep orange color upon the addition of methyl alcoholic potash. This color one might expect if flavones or anthocyans were present. A similar color may be produced by the use of a methyl alcohol that contains aldehydes and forms alhedyde resins upon the addition of an alkali. There were no aldehyde resins present as only absolute methyl alcohol was used. The potassium hydroxide used was boiled in order to eliminate an error from this source. The coloring material that developed during the process of saponification was not thought to be of any serious consequences as the soap was extracted with ether until the ether removed no further color.

The dried brewers' yeast contained some coloring material most of which when tested proved to be water soluble. The drying process may cause some of the material upon which the yeast is cultured to become brown. The yeast plant, itself, contains a yellowing coloring matter. A fat soluble pigment was isolated from the dried brewers' yeast used in this ration. No effort was made to establish

the identity of it, however.

The wood pulp, as far as could be determined, was colorless. The presence of the pulp in the ration made grinding difficult because its light fluffy nature would not pass through the mill readily.

Some of the color probably came from the dried buttermilk. During the process of making butter, annatto is added to give the butter a golden yellow color. As a result some of the phospholipins may have absorbed the pigment and carried it into the buttermilk. Annatta coloring is a reddish orange color and is used in very small quantities.

In order to test whether or not the annatto pigment would color the yolk two hens which had been on a xanthophyll free ration were fed "Dandelion" butter coloring at first by means of capsules, later by means of a pipette. The hen that received 1 cubic centimeter a day, at the end of seven days, had a golden yolk. The hen that received 2 cubic centimeters a day had an orange colored yolk that was less intense than the hen fed 1 cubic centimeter a day.

It was not determined whether this pigment would separate into the carotin solvent (petroleum ether) or the xanthophyll solvent (methyl alcohol).

Analyses of the basal ration (IV) showed a minute quantity of pigment. It must be remembered that readings

on the spectrophotometer were made for the wave length 435.8. This is in the violet region and readings are difficult to make. The amount of xanthophyll was only a trace. The range was found to be between 0.2×10^{-3} and 0.5×10^{-3} milligrams of pigment per gram sample.

The quantity of carotin in the two basal rations was very small. The hens were fed two per cent cod liver oil and it was not necessary to rely upon the carotin as the sole source of vitamin A. The carotin content in basal ration (IV) was found to be approximately equal to the xanthophyll content as shown by Table 3. The average analyses of both carotin and xanthophyll was 0.25×10^{-3} milligrams per gram. About a third more xanthophyll was found in the alfalfa basal than carotin. Although yellow corn contained considerable carotin the amount was not determined because at the time the analyses were made the author was not aware of the amount of carotin contained in the egg yolk.

Table 3. Carotin Content of Feeds

Sample	Weight in grams	Volume	(b) c.m. depth of cell	-log T transmittancy	$\frac{1}{Kb}$	Mgm. pigment per gram x 10^{-3}
Alfalfa basal	10	50	1.0	0.18729	0.522	4.9
Alfalfa basal	10	50	1.0	0.28549	0.522	7.5
Basal IV	10	25	4.0	0.09849	0.131	0.3
Basal IV	10	25	4.0	0.05765	0.131	0.2

The average of the analyses, given in Table 4, showed that the green alfalfa plant contains more of the xanthophyll pigment than does the green wheat plant. This statement is borne out in an experiment carried out on the Kansas State College poultry farm in which green alfalfa gave darker colored egg yolks than did green wheat. The results obtained showed that green wheat contained on an average 1.13 milligrams of pigment per gram as compared with 1.25 milligrams of pigment found in alfalfa.

The effect of drying of alfalfa hay was clearly shown by results given in Table 4. The green alfalfa used in the analyses was not the same as that used for making hay. The alfalfa sample was collected about March 15 from a small plot that was being used as pasturage for a few hens. The stand was seedling and had never been cut. According to Schertz (1929a) the chloroplast pigments in leaves are highest during the period of June 25 to September 29. It is possible that the alfalfa cut for hay originally contained more pigment than did the young plants analyzed. If the contents of the two samples were comparable about three-tenths of a milligram per liter was lost by curing this amount to about a third of the pigment during the drying process. Approximately the same amount was lost by exposure for 30 days as compared with that cured inside the house.

Table 4. Xanthophyll Content of Green Feed

Sample	Weight in grams	Volume	(b) c.m. depth of cell	-log T transmittancy	$\frac{1}{Kb}$	Mgm. pigment per gram x 10^{-3}
Green wheat	10	500	0.5	0.26793	0.957	1.22
Green wheat	10	500	0.5	0.23589	0.957	1.13
Green alfalfa	10	100	0.1	0.28470	4.787	1.36
Green alfalfa	10	100	0.1	0.23779	4.787	1.14
Alfalfa hay I	10	100	0.2	0.38412	2.393	0.92
Alfalfa hay I	10	100	0.2	0.37650	2.393	0.90
Alfalfa hay II	10	100	0.5	0.60896	0.957	0.58
Alfalfa hay II	10	100	0.5	0.58949	0.957	0.56
Alfalfa hay III	10	100	0.5	0.54602	0.957	0.52
Alfalfa hay III	10	100	0.5	0.54976	0.957	0.53

Very little xanthophyll pigment was lost after thirty days of bleaching. The amount lost was less than 1 per cent. Schertz (1925a) states that carotin is more easily oxidized in plant tissue than is xanthophyll. This fact was borne out in results shown here. With xanthophyll there was a marked reduction during the first 30 days of exposure. With carotin the amount decreased from 0.978×10^{-3} milligrams to 0.347×10^{-3} during the first 30 days of bleaching. In 90 days of bleaching it decreased to 0.199×10^{-3} milligrams per gram.

Table 5 gives the results of the carotin determinations for green feed used. It is regrettable that the carotin content of green alfalfa was not determined. According to Russell and Massengale (1929) the amount of carotin in hay cured under favorable conditions is less than one-seventh of the original content. The color of alfalfa hay used in these determinations was found to be a poor index to the amount of xanthophyll that the hay may contain.

From the results given here, considering the amount of xanthophyll that remained after curing alfalfa hay in the shade as 100 per cent, 62.6 per cent of the xanthophyll remained after 30 days exposure to the sun; and 58.2 per cent remained after 90 days exposure to the sun. In a similar manner if the carotin content of the alfalfa hay which was

Table 5. Carotin Content of Green Feed

Sample	Weight in grams	Volume	(b) c.m. depth of cell	- log T transmittancy	$\frac{1}{Kb}$	Mgm. pigment per gram x 10^{-3}
Green wheat	10	100	0.5	0.79376	1.044	0.820
Green wheat	10	100	0.5	0.75894	1.044	0.792
Alfalfa hay I	10	100	0.1	0.18734	5.221	0.978
Alfalfa hay I	10	100	0.1	0.13624	5.221	0.711
Alfalfa hay II	10	100	0.5	0.33211	1.044	0.347
Alfalfa hay II	10	100	0.5	0.33238	1.044	0.347
Alfalfa hay III	10	100	0.5	0.19097	1.044	0.199
Alfalfa hay III	10	100	0.5	0.14169	1.044	0.148

dried in the shade as 100 per cent, the percentages of the original amount were as follows: 41.1 per cent remained after 30 days of bleaching and 20 per cent remained after 90 days of bleaching. It must be remembered, however, that much of the pigment is lost even when the hay is dried in the shade.

The pigment content of yolks was difficult to determine because of the ease with which emulsions were formed. Several analyses were run before a satisfactory method of extraction could be determined. Results obtained by use of the few methods given in the literature, were far from satisfactory. It was necessary to formulate a method whereby the yolks could be extracted for pigments. Since most of the time was spent on the development of this method very little time was left in which to extract many eggs. As a result the analyses obtained are by no means conclusive.

The early work of Palmer (1922) who found that the principal natural yellow pigment of the egg yolk was xanthophyll was confirmed. Carotin or a carotin-like compound was also found to be present in the yolks. Tests made upon the pigment revealed that it had absorption bands 435.8, 546.1, and 577-579. These bands are identical with those of carotin.

The xanthophyll content of the eggs analyzed is shown in Table 6. There is a range of xanthophyll content from

13.2 x 10^{-3} milligrams per gram to 0.7 x 10^{-3} milligrams. The egg with highest xanthophyll content was laid by a hen receiving 70 per cent yellow corn. The lightest yolk was laid by a hen receiving the basal ration (IV). The hen receiving 35 per cent yellow corn in the ration put about two-thirds the amount of xanthophyll in the egg yolk as did those receiving 70 per cent. The hen that received xanthophyll in excess apparently must dispose of it in another manner than through the yolk.

Of the two hens fed basal ration (IV), No. 2603 laid at rapid rate and hen No. 2589 laid at a slow rate. The results of analyses of these hens' eggs showed that No. 2603 had 0.7 x 10^{-3} milligrams of pigment per gram of yolk as compared with 1.4 x 10^{-3} milligrams of pigment for hen No. 2589. The results indicate that the color of yolk of eggs produced by hens receiving feeds of the same xanthophyll content depends upon the rate of laying. The hens laying fewer eggs will have higher pigment content.

Due to the fact that the method of analysis was not consistant throughout, the results obtained from hens receiving alfalfa hay (lot IX), alfalfa stem (lot X), and alfalfa leaves (lot XI), cannot be compared. Results would indicate that hens fed the leaves, laid eggs containing considerable more pigment than did either of the other lots.

Table 6. Xanthophyll Content of Eggs

Lot	Weight in grams	Volume	(b) c.m. depth of cell	- log T transmittancy	$\frac{1}{Kb}$	Mgm. pigment per gram x 10^{-3}	Mgm. pigment per egg x 10^{-3}
I	14.2907	200	2.0	0.39495	0.239	13.2	188.8
II	5.6236	100	2.0	0.19619	0.239	8.3	46.9
II	5.6236	100	2.0	0.21597	0.239	9.2	51.6
IV Hen 2603	14.9688	50	4.0	0.16485	0.120	0.7	9.9
IV Hen 2603	14.8972	50	4.0	0.16329	0.120	0.7	9.8
IV Hen 2589	18.**	50	4.0	0.43661	0.120	1.5	26.2
IV Hen 2589	18.5901	50	4.0	0.40186	0.120	1.3	24.1
IX	15.1584	50	2.0	0.39934	0.239	3.1	47.7
X Hen 2604*	15.2632	50	2.0	0.44590	0.239	3.5	532.9
X Hen 2601*	17.1845	50	2.0	0.61452	0.239	4.3	734.4
XI Hen 2596*	15.5760	100	1.0	0.35498	0.479	10.9	170.0
XI Hen 2587	17.4964	50	0.5	0.22237	0.957	6.1	106.7
XI Hen 2596	24.5883	100	0.5	0.24742	0.957	9.6	236.8

* Pigments not separated

** Approximately

The results obtained from measurement of the actual pigment are likely to differ slightly from results which determine intensity of color since both of the pigments are very intense colors.

The results obtained from the analyses of egg yolk for carotin are given in Table 7. The range is from 0.6 to 0.8 x 10^{-3} milligrams of carotin per gram of egg yolk. The amount was nearly constant. The amount of carotin in the egg does not appear to vary with the feed nor with the rate of laying. There is probably very little relationship between the color of yolk and carotin content. The results obtained here, however, are not of sufficient extent to warrant a positive statement to that effect.

Table 7. Carotin Content of Eggs

Lot	Weight in grams	Volume	(b) c.m. depth of cell	- log T transmittancy	$\frac{1}{Kb}$	Mgm. pigment per gram x 10^{-3}	Mgm. pigment per egg x 10^{-3}
II*	5.8236	25	3	0.10027	0.174	0.8	0.44
IV Hen 2603	14.9688	25	4	0.30446	0.131	0.7	9.97
IV Hen 2603	14.8972	25	4	0.30473	0.131	0.7	9.98
IV Hen 2589	18.**	25	4	0.31601	0.131	0.6	10.35
IV Hen 2589	18.5901	25	4	0.32899	0.131	0.6	10.78
IX	15.1584	25	4	0.27478	0.131	0.6	8.00
IX	14.5883	25	3	0.19740	0.174	0.6	8.60

* One-half egg yolk

** Approximately

SUMMARY

1. The basal ration (IV) designed to give near colorless egg yolks was found to have traces of both xanthophyll and carotin in approximately equal amounts.
2. The green alfalfa which was analyzed contained a trifle more xanthophyll for a given amount than did the green wheat.
3. The alfalfa hay used in this experiment lost considerable more xanthophyll in the first 30 days than it did in the following 60 days.
4. Alfalfa hay loses its carotin very rapidly when allowed to bleach. The xanthophyll content of alfalfa hay can not be judged by color.
5. The difficulty with which pigments are extracted from egg yolks prevented making enough analyses for conclusive evidence but results obtained tend to show that a highly colored egg is not necessarily high in carotin.
6. The color of yolk of eggs produced by hens receiving feeds of the same xanthophyll content depends upon the rate of laying. The hens laying fewer eggs will have higher pigment content.
7. In all probability the amount of carotin or carotin-like compound in the egg yolk is about constant.

8. A method is described whereby pigments may be extracted practically quantitatively from egg yolk.
9. Results confirm early work that but little of the color of egg yolk is due to carotin, most of the color being xanthophyll.

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