THE XANTHOPHYLLS OF OLIVE COLORED EGG YOLKS

by

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INTRODUCTION

It has frequently been observed by experienced poultrymen that hens when first turned out in the early spring on grass ranges sometimes produce eggs which are off-color. Also, hens receiving oat grass silage have been found to produce olive colored yolks. Some of these eggs possess such a disagreeable taste that they are not edible, while others are. Although the taste in the latter case is not in the least objectionable, the consumer invariably finds them unsatisfactory for psychological reasons, for when an olive colored egg is opened, it has such a contrasting appearance to the normal egg that to the buying public it appears spoiled. Such eggs have a low commercial value. It should, therefore, be advantageous to the poultry industry if production of these off-colored eggs could be prevented. The feeds responsible for such discolorations are, in some cases, known, and to a certain extent the factors can be eliminated. A solution to the entire problem, however, would appear to lie in a determination of the exact chemical nature of the substance or substances which cause the discolorations.

Since previous studies in the chemistry laboratories of Kansas State College of Agriculture and Applied Science had indicated that the olive pigmentation of eggs produced by feeding an oat grass silage to hens was related to the alcohol soluble carotenoid pigments, it was decided to make a comprehensive study of the distribution of such pigments in the olive egg yolks.

REVIEW OF LITERATURE

Off-colored Egg Yolks

Payne (11) reported the finding of olive colored egg yolks (commonly called by the trade "grass eggs," "alfalfa eggs," or "green rots") in the early spring when the eggs were candled at collecting points. Buyers have had the opinion that these were produced when the hens ate abundantly of grass, alfalfa, or green wheat. Payne, however, attributed the cause to either of two weeds belonging to the mustard family, Shepherd's purse (Capsella Bursa-pastoris), and Penny cress (Thlaspi arvense), either of which is readily consumed by hens. The olive color makes its appearance in the yolk within a week after the hens are placed on grass plots containing these two weeds.

The eggs produced are, also, reported to be so strong in flavor that they cannot be relished.

Berry (1) pointed out that when a flock, during the winter months, had an alfalfa range so large that the birds did not consume all exposed parts of the plants, practically all the eggs graded low in quality. The yolks of eggs produced under these conditions were very unattractive in appearance. They were not only of a very dark color, approaching an olive shade, but were mottled, being nearly black in some spots. Such eggs continued to be produced until the season had advanced sufficiently for the plant to become full grown, after which the eggs lost their unattractiveness and became mild in flavor.

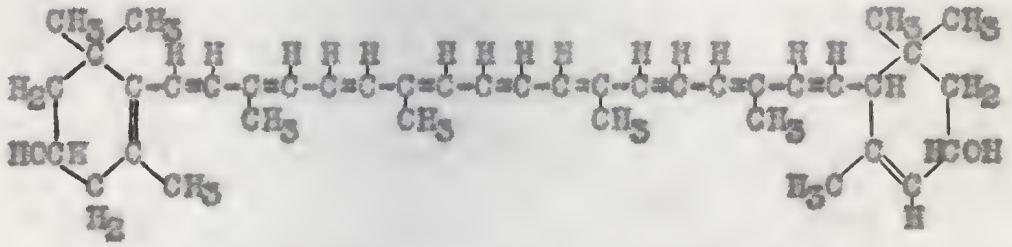
It has been observed by Schaible, Moore and Moore (14) that egg yolks from hens fed cottonseed meal, when placed in an atmosphere of ammonia, changed in a short time to an olive, brown or chocolate color depending on the level of this ingredient in the ration. They discovered that this discoloration was caused by gossypol since it was present either free or bound (as in cottonseed) in those rations producing the discolored egg yolks, and absent in those not producing discolorations.

The Xanthophylls of Egg Yolks

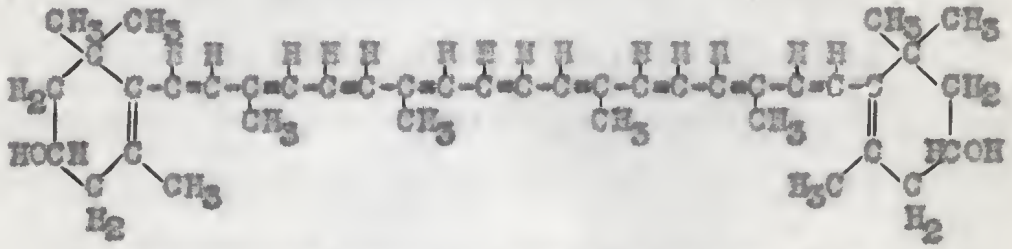
The carotenoid pigments according to Bogert (2) may be defined as a nitrogen free polyene pigment, consisting wholly or chiefly of a long acyclic chain of carbon atoms united in an uninterrupted sequence of conjugated double bonds; this system of conjugations functions as the chromophore.

The carotenoids may be divided into two classes according to their composition: one, the hydrocarbons (carotene, lycopene, etc., $C_{40}H_{56}$) which are readily soluble in ether and petroleum ether, but are quite insoluble in aqueous alcohol; and two, the oxygen containing pigments, the xanthophylls, $C_{40}H_{56}O_n$, which are soluble in aqueous alcohol and ether. Another classification is based on solubility: the petroleum soluble pigments, which, besides the hydrocarbons, includes the monohydroxy β-carotene, cryptoxanthin; and the alcohol soluble pigments which include the remainder of the oxygen derivatives. The most common of the alcohol soluble pigments are the dihydroxy α- and β-carotenes which are lutein and zeaxanthin, respectively. Lutein is the chief pigment of green leaves, while zeaxanthin comprises the bulk of the corn pigments.

The formulae of these two pigments may be represented as follows:



Lutein (Karrer, Zubrys and Morf, 7)



Zeaxanthin (Karrer, Zubrys and Morf, 7)

Willstatter and Escher (19) were successful in obtaining a crystalline pigment from egg yolks which in most of its properties resembled leaf xanthophyll. In a more recent study, however, Kuhn, Winterstein and Lederer (9) reported that the pigment of egg yolk was not homogeneous but consisted of two pigments which were separated by means of a Twsett adsorption column. The principal constituent was found to be identical with the leaf xanthophyll, lutein, while the other was identical with zeaxanthin, the pigment which had previously been isolated from corn (6).

Determination of Xanthophylls

The separation of xanthophylls from a pigment extract is based on their partition between two immiscible solvents. The carotenes are removed from an alcoholic potash extract of the pigments by washing with petroleum ether. This, also, removes some xanthophylls. By washing the petroleum ether extract with 90 per cent methanol, the xanthophylls, being preferentially soluble in this solvent are removed. The remaining xanthophylls are removed from the alcoholic potash residue with diethyl ether, while the chlorophyll and carotenoid acids remain in the residue.

For general purposes the quantitative estimation of the xanthophylls may be accomplished by first removing the carotenes by the Peterson, Hughes and Freeman (12) method, followed by the extraction of the xanthophylls with ether. The method is reviewed briefly:

The desired quantity of the finely ground material is saponified by refluxing one-half an hour with 10 per cent alcoholic potassium hydroxide, filtered and the residue washed alternately with Skellysolve B and ethanol until each comes through colorless. The alcoholic potash

extract is washed with Skellysolve until all the carotene is removed. The Skellysolve fraction is washed several times with water to remove alkali. The xanthophylls are extracted from the Skellysolve fraction by repeated washings with 90 per cent methanol until the wash solution becomes clear. By washing the xanthophyll-free Skellysolve fraction with water, the methanol is removed. The Skellysolve phase is then dried by pouring through a filter containing a small amount of anhydrous sodium sulfate. The filtrate is made to a convenient volume and the concentration of carotene determined either spectrophotometrically (Peterson, Hughes and Freeman, 12) or colorimetrically (Frape and Kemmerer, 4).

Continuing from the previous method, the determination of the total xanthophylls may be carried in the following manner:

To the 90 per cent methanol wash solution an equal volume of diethyl ether is added and the mixture diluted with water until a separation of the ether phase is brought about. Usually the water drives all the pigment into the ether phase the first time. The alcoholic potash residue is treated with ether in the same manner. Frequently, however, at least three ether extractions are necessary to remove the last traces of pigment. The ether

extract from the alcoholic potash residue is combined with that obtained from the 90 per cent methanol fraction. The combined ether fractions should be washed at least three times with an equal volume of water to remove the alcohols. The ether is then dried by filtering through anhydrous sodium sulfate. Some difficulty often arises in that the ether filtrate comes through cloudy. Washing with alcohol and water and filtering again over anhydrous sodium sulfate usually removes the cloudiness. This cloudiness or colloidal suspension is probably due to sterols which escape saponification. After a clear ether solution is obtained the concentration of the xanthophylls is measured by means of the spectrophotometer.

Separation of the Xanthophylls

The development of the field of chromatography, or the separation of pigments by their adsorption on a Tswett column has been extremely helpful in the study of the closely related carotenoids. Tswett (17), who was responsible for the original discovery, found that if a carotenoid mixture in a solution of carbon disulfide, benzene, or petroleum ether was poured through an evenly packed column of finely divided calcium carbonate, inulin,

or sucrose, the pigment separated into well-defined zones by virtue of selective adsorption on the adsorptive material used for the different pigments. The most satisfactory column is one in which the pigments will separate far enough, when pure solvent is poured through, to be mechanically separated.

Strain (15) found that a special brand of magnesium oxide (Micron Brand Magnesium Oxide, No. 2641, manufactured by the California Chemical Corporation, Newark, California) possesses the greatest number of desirable properties of the adsorbents studied. This oxide is highly active and permits a ready separation of the carotenes with minimum decomposition. It has the additional advantage in that adsorbed pigments can easily be eluted with ethanol.

By mixing this magnesium oxide with varying amounts of heat treated siliceous earth (Hyflo Super Cel F. A. 501, manufactured by Johns-Manville), Strain (16) found that the rate of filtration may be increased to the desired speed, and that it, also, permits even filtration and does not adsorb the pigments.

The factors affecting the relative positions of the pigments on a column are due to their chemical nature, namely, the degree of oxygenation, the saturation and

the position of the double bonds. The most highly oxygenated pigments are the most strongly adsorbed. Of the hydrocarbons which are the least strongly held, the adsorption decreases with a decreasing saturation.

A study of these relationships of chemical composition and structure to the behavior of the pigment on a column was made by Winterstein and Stein (21). Winterstein (20) prepared a table showing the order of adsorption of the most important carotenoids. A diagramatic representation of the adsorbability of some of these carotenoids appear below:

Most strongly adsorbed	Fucoxanthin	$C_{40}H_{56}O_6$	Alcohols, $CaCO_3$ (adsorbent)
	Violaxanthin	$C_{40}H_{56}O_4$	
	Taraxanthin	$C_{40}H_{56}O_4$	
	Flavoxanthin	$C_{40}H_{56}O_3$	
	Zeaxanthin	$C_{40}H_{56}O_2$	
	Lutein	$C_{40}H_{56}O_2$	
	Cryptoxanthin	$C_{40}H_{56}O$	
	Lycopene	$C_{40}H_{56}$	Hydrocarbons, Al_2O_3 (adsorbent)
	γ-carotene	$C_{40}H_{56}$	
Most weakly adsorbed	β-carotene	$C_{40}H_{56}$	
	α-carotene	$C_{40}H_{56}$	

For the preparation of a xanthophyll chromatograph, the solution of pigments to be separated is evaporated under vacuum in an inert atmosphere, such as, nitrogen, to prevent the formation of oxidation products.

Strain (16) reported that one of the best solvents for the separation of xanthophylls upon columns was 1, 2,

dichloroethane. Dichloroethane is a commercial solvent of remarkable uniformity. It may be purified readily and keeps well.

A successful separation of xanthophylls by the chromatograph is dependent upon the careful preparation of the adsorption columns to insure the formation of even and well defined bands of the adsorbed pigments. The adsorption columns may be prepared of pyrex glass tubing (15 cms. x 2 cms.), one end of which is constricted and sealed to a smaller piece of tubing. A small wad of cotton is placed in the constriction and pressed into place. Small portions of the adsorbent (equal parts of magnesium oxide and siliceous earth) are then added and pressed into place by a metal disk which is slightly smaller than the adsorption tube.

The separation of the xanthophylls is accomplished by inserting the completed column in a suction flask attached to an aspirator. A concentrated solution of the xanthophylls in 1, 2, dichloroethane is quickly poured over the surface of the column. When the band of adsorbed pigments has reached one-sixth of the length of the column, pure solvent is poured over the surface of the adsorbent. This causes the adsorbed pigments to move slowly over the adsorbent forming bands of the different pigments in the

order of the previously appearing adsorption series. Usually one-half to one hour is sufficient time to complete the adsorption.

The various zones are isolated mechanically by carefully removing each zone (if the separation warrants) with a long spatula. Each zone is eluted with ethyl alcohol, the suspension filtered and the concentrations of clear alcohol solutions are read spectrophotometrically. Frequently, however, the pigments are transferred to ether by the partition method before determining their concentrations. Thus by employing modifications in the manner just described, it is possible to obtain a relative distribution of the several xanthophyllic pigments present in a given sample.

Absorption Spectra

Probably no other property of the carotenoids, with the possible exception of their adsorption behavior, is more readily determined with small amounts of pigment than the adsorption spectra. Each pigment in a given solvent exhibits a characteristic maximal or minimal effect in its absorption band which furnishes a means of its identification and quantitative determination with

the spectrophotometer.

The absorption of light of the pigments is ordinarily represented by a curve obtained by plotting the logarithm of the specific absorption coefficient against the wave length. However, the method usually used is that by Miller (10) in which the specific absorption coefficient is plotted against the wave length.

From Beer's law:

$$I_x = I_o \, 10^{-\alpha c x}$$

$$\alpha = \frac{\log_{10} I_o / I_x}{cx}$$

$\alpha = D/cx$, where D (optical Density) $= \log_{10} I_o / I_x$

I_o = intensity of light transmitted by solvent cell
I_x = intensity of light transmitted by solution cell
x = thickness of solution layer in centimeters
c = concentration of carotenoids in grams per liter
α = extinction or absorption coefficient

Some specific absorption coefficients for the xanthophylls which have been interpreted from the absorption curves of the literature are shown in Table 1.

EXPERIMENTAL PROCEDURE

Work upon olive pigmentation in the past has been limited due to the fact that olive yolks were available at only certain seasons of the year and, also, that the

Table 1. Specific absorption coefficients (α) of the xanthophylls (13). (Wave length in Angstrom Units)

Pigment	Solvent	1st maximum	Minimum	2nd maximum
Flavoxanthin	Ethanol	4220	4385	4510
	α	(229)	(116)	(234)
Violaxanthin	Ethanol	4440	4600	4720
	α	(252)	(138)	(224)
Zeaxanthin	Ethanol	4530	4690	4810
	α	(244)	(200)	(218)
	CS_2	4820	5000	5120
	α	(180)	(150)	(165)
Lutein	Ethanol	4465	4625	4750
	α	(257)	(182)	(234)
	CS_2	4740	4920	5050
	α	(196)	(148)	(180)
	Ether	4450	4600	4740
	α	(260)	(162)	(234)
Isolutein	Ethanol	4440	4600	4730
	α	(240)	(166)	(222)
Cryptoxar-thin	Ethanol	4520	4700	4820
	α	(254)	(209)	(226)
	CS_2	4840	5020	5120
	α	(202)	(172)	(180)
β-carotene (12)	Petroleum ether	4500	4700	4800
	α	(238)	(200)	(212)

substance responsible for the olive color was usually unknown. Recently, however, it was found that olive yolks could be produced by an oat grass silage when fed at the rate of four pounds per hundred birds. In a lot of one hundred birds on this ration, 50 per cent of all eggs laid by six hens during a five month period were olive in color, and 87 per cent of the eggs laid by two hens were olive. A total of 31 per cent of all eggs laid by hens giving green eggs or 13.3 per cent of all eggs laid by all the hens in the lot were olive in varying degrees.

In a preliminary feeding experiment it was found that a hen receiving an ether extract of the silage in its ration produced eggs whose yolks were darker than they were previous to the feeding of the ether extract. This fact suggested a possible relationship between the olive pigmentation and the carotenoid content of the egg yolk. When the olive eggs from the above mentioned hens became available, the gross carotene and xanthophyll concentration of the yolks were compared with those of normal colored eggs obtained from hens receiving the same ration. These studies (Table 2) revealed that the olive egg yolk contained an average of 61.2 per cent more pigment than the normal egg yolk. This fact immediately suggested a chromatographic comparison of the xanthophylls of olive

Table 2. A comparison of the total carotenoids of olive colored and normal egg yolks.

Kind of yolks	No. of eggs in sample analyzed	Petroleum soluble pigments mg/100g	Alcohol soluble pigments mg/100g	Total pigments mg/100g
Olive colored egg yolks	12	0.20	5.01	5.21
	1	0.21	4.91	5.12
	1	0.18	5.83	6.01
	2	0.34	4.78	5.12
	6	0.26	6.85	7.11
Average pigments		0.24	5.48	5.72
Normal egg yolks	12	0.08	2.94	3.02
	1	0.16	2.75	2.91
	1	0.14	4.07	4.21
	17	0.18	3.88	4.06
Average pigments		0.14	3.41	3.55

and normal yolks. Previous work upon the carotenes revealed no promising results.

The methods of isolation and separation, typical of the ones used in this investigation, follow closely those previously described. For the comparative study a dozen yolks each, of the olive, weighing 188 grams, and normal, weighing 186 grams, were extracted with ethyl alcohol in the cold. The yellow color was removed from the egg yolk by squeezing out the alcoholic extract through a cheese cloth. Portions of pure alcohol were added to the residue and squeezed out until the yolks were white. The combined alcoholic extracts were saponified overnight with alcoholic potassium hydroxide. The petroleum soluble carotenes were removed by the Peterson, Hughes and Freeman (12) method and washed with 90 per cent methanol. The 90 per cent methanol soluble pigments were extracted with ether and combined with the ether soluble pigments extracted from the alcoholic potash residue. The ether extract was washed with water, filtered through anhydrous sodium sulfate and then evaporated to dryness over a steam bath and taken up in 10 cc. of dichloroethane. This was then columnated using a Strain column of magnesium oxide and siliceous earth (1:1). The method of packing the adsorption column used by Strain was found to be improved by digging

out a part of each tightly packed layer of adsorbent before the next portion of adsorbent was added. This technique gave the column a more uniform appearance by partially removing the packing lines.

After pure solvent was drawn through the column for one-half hour, the pigments had arranged themselves into four main zones. A chromatogram typical of the xanthophylls of the olive colored yolks is shown in Plate I. They were removed mechanically by means of a spatula, eluted with alcohol, transferred to ether and their concentrations determined spectrophotometrically using an absorption coefficient of 250 at 4500 Å. The calculated percentages were based on the amounts of pigment recovered from the column. The results of these several studies are shown in Table 3.

A study was also made on the xanthophylls of the silage which was responsible for the olive pigmentation. One hundred gram portions of the silage were saponified overnight at room temperature with alcoholic potash. The alcoholic potash extract was treated in the same manner described for the alcoholic potash extract of the egg yolks. When the concentrated xanthophyllic pigments in dichloroethane were columnated, they adsorbed in such a manner that five zones were roughly separated mechanically.

EXPLANATION OF PLATE I

Fig. 1. A chromatogram of olive colored egg yolks.

Plate I

Fig. 1

Table 3. Distribution of the xanthophylls (alcohol soluble) in olive and normal colored egg yolks and oat grass silage.

Pigment	Olive egg yolks		Normal egg yolks		Silage	
	Distribution on columns in per cent		Distribution on columns in per cent		Distribution on columns in per cent	
	Range	Average	Range	Average	Range	Average
Strongly adsorbed pigments	10.43-15.65	13.63	1.83-4.12	2.98	18.38-23.28	20.83*
Zeaxanthin	25.35-38.76	34.26	30.06-33.92	31.99	13.72-30.92	22.32
Isolutein	7.24-18.03	12.63	16.78	16.78	10.58-24.65	17.62
Lutein**	32.78-51.75	39.47	45.18-51.32	48.25	38.35-40.12	39.23

*This percentage was composed of two fractions, a very strongly adsorbed zone and a less strongly adsorbed fraction whose percentages were respectively, 2.23 and 18.60.

**Contains small amounts of cryptoxanthin.

The percentage distribution of these zones is also shown in Table 3.

DISCUSSION OF RESULTS

An examination of Plate I reveals the four typical zones from which the percentage distribution of the olive yolk pigments was calculated. In the upper, most strongly adsorbed zone designated as strongly adsorbed pigments in Table 3, there may be distinguished two bands. The narrow band at the top was pale green in color, while the band just below it was pinkish yellow. Although these two bands were separated and their concentrations determined individually, they were both included in the "strongly adsorbed pigments" of the table since they were not identified. Both pigments had an absorption maxima at 4500 Å (Fig. 2). The correspondingly adsorbed pigments of the normal yolks consisted only of a yellow-brown band shading off to a pale yellow zone. The percentage of the most strongly adsorbed pigments of the olive yolk was 13.62 as compared with 2.98 for those of the normal yolk.

In the middle of the column is a dark zone, pinkish-orange in color, corresponding to zeaxanthin. This fraction comprises 34.25 per cent on the average as compared

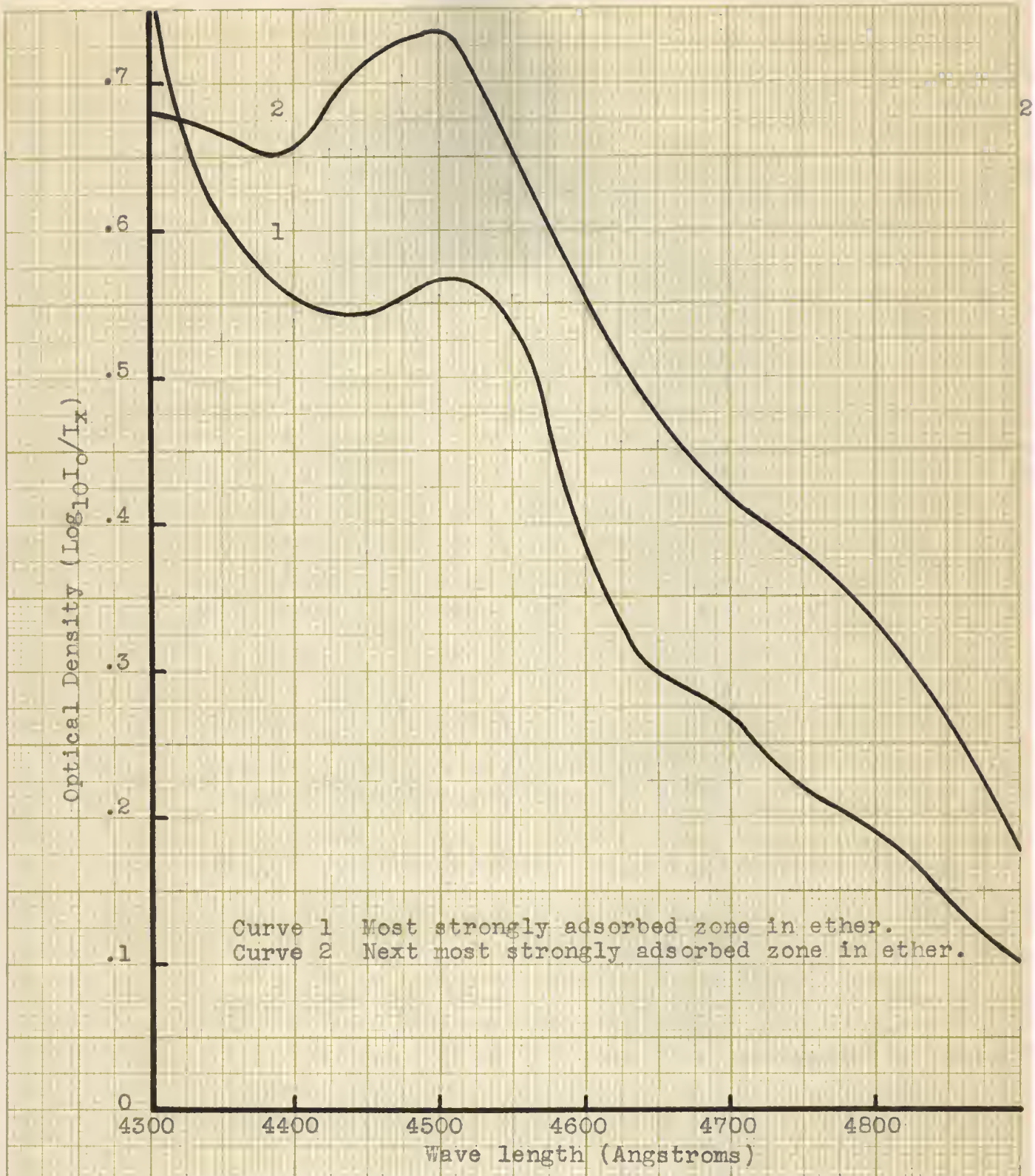


Fig. 2. Absorption curves of the most strongly adsorbed xanthophylls of olive colored egg yolks.

with 31.99 per cent for the normal yolks.

The concentrated region which was brownish-yellow in color at the bottom of the column was recognized as lutein. Just below it may be seen a narrow line of cryptoxanthin. No attempt was made to separate the cryptoxanthin from the lutein so that the pigment fraction called lutein in Table 3 consists, also, of small amounts of cryptoxanthin. The lutein fraction of the olive yolks as determined in this manner composes 39.46 per cent while that of the normal yolk was 48.25 per cent.

Between the zeaxanthin and lutein zones there was a light yellow zone. This does not show well in the plate. According to Strain (16) this pigment is isolutein. The zone was so poorly defined that it was difficult to obtain at best only a crude separation between the isolutein and lutein zones. However, the percentages determined from the various attempts are roughly 12.63 per cent and 16.78 per cent for the olive and normal yolks, respectively.

It was extremely interesting to learn that the distribution of the xanthophyllic pigments in the oat grass silage consisted of 2.23 per cent of a greenish-brown pigment having an absorption maxima at less than 4300 Å, and 18.60 per cent of an orange-red pigment having an absorption maxima at 4500 Å, thus making a total of 20.83 per

cent for the strongly adsorbed pigments. The absorption curves of these two fractions are shown in Fig. 3, in which the $\log_{10} I_o/I_x$ or optical density is plotted against the wave length. The absorption curves of Fig. 2, for the two strongly adsorbed fractions of the olive pigments are quite similar in most respects. It is quite conceivable that the fraction represented by Curve 1, Fig. 2, for the most strongly adsorbed pigment of the olive yolk could be contaminated with small amounts of the fraction represented by Curve 2, Fig. 2, which would easily account for the absorption maxima at 4500 Å.

CONCLUSIONS

It cannot be stated with certainty from this study that the difference in distribution of the carotenoids are necessarily involved in the formation of the olive colored yolks. Perhaps these differences should be explained on the basis of individual physiological differences in silage consumption. However, the greater concentration of pigments and the predominance of highly oxygenated pigments in the olive colored egg yolk is certainly suggestive. As seen from a comparison of the graphs and Table 3, the oat grass silage offers a

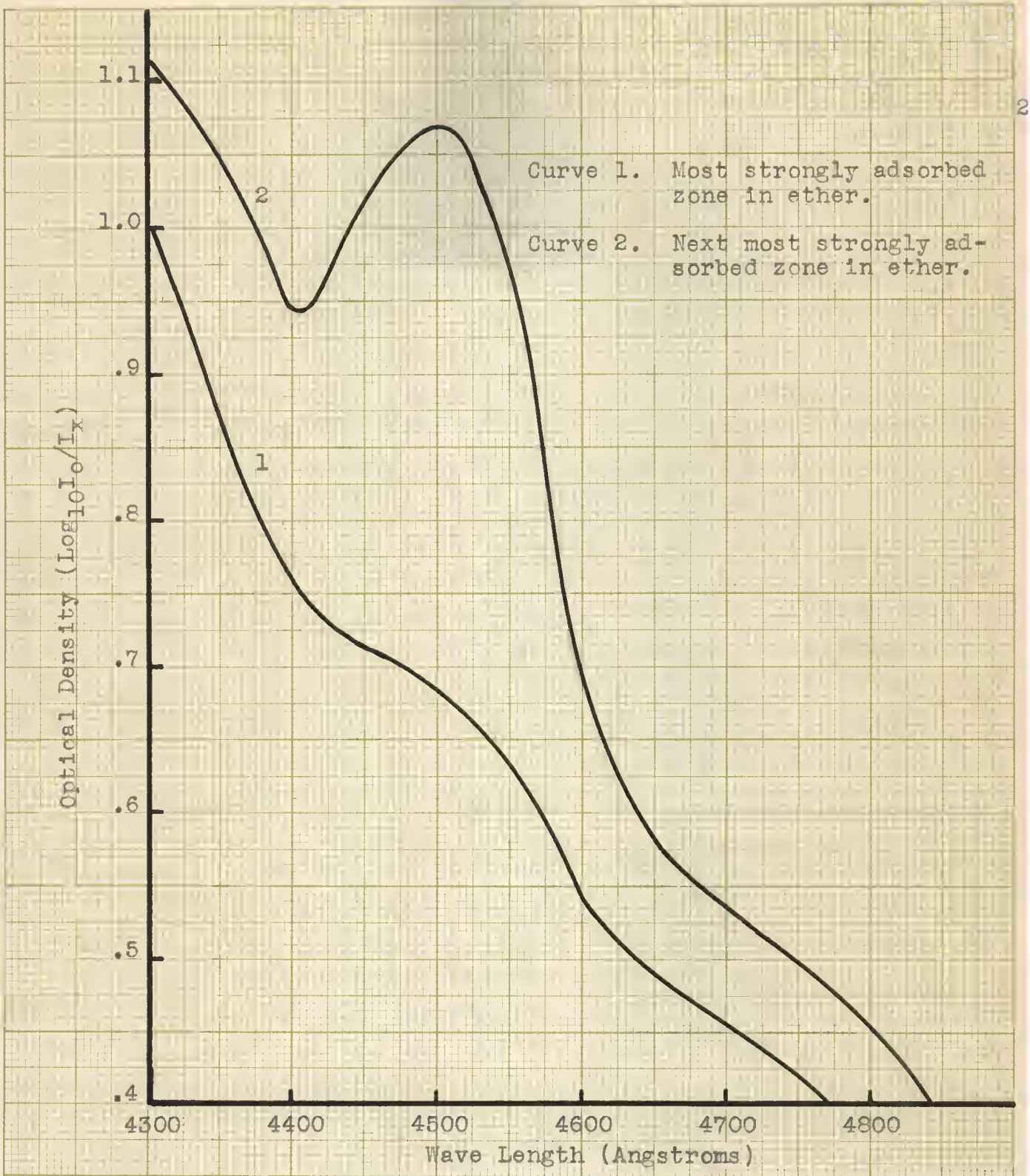
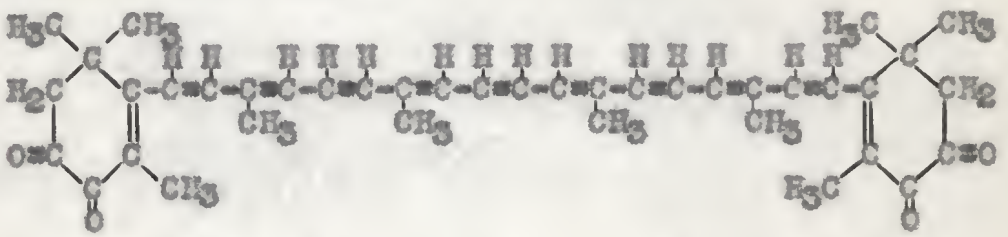


Fig. 3. Absorption curves of the most strongly adsorbed xanthophylls of oat grass silage.

convenient source of highly oxygenated pigments.

In reviewing the literature it was found that a variety of green pigments not related to chlorophyll occurs in nature. Green to blue-black chromoproteins of crustacea have been described by Verne (18), and Chatton, Lwoff and Parat (3). In contrast to the carotenoids of plants, the pigments in these cases appear to be polyenes coupled with protein which results in a water-soluble deeply colored complex. Verne (18) reported that the native blue-green pigment of the lobster is protein coupled to a carotenoid having the formula, $C_{40}H_{56}$. Vitellorubin, tetronerythrin, crustaceorubin, zoonerythrin, astroviridin, etc. are the names reported for the chief compounds of this class.

In studying the pigment of the lobster (Astacus gammarus L.) Kuhn and Lederer (8) gave the name "astacin" to the polyene portion. The formula of astacin, $C_{40}H_{56}O_4$, is represented as follows:



Astacin (Karrer, Loewe and Hubner, 5)

When the blue-green chromoprotein is decomposed by hot water, dilute hydrochloric acid, acetone, or alcohol there develops a red color which is due to the freed pigment, astacin.

It seemed quite significant that in a similar manner attempts to isolate the green pigment of olive egg yolks with alcohol and other organic solvents resulted in the disappearance of the green color and appearance of the yellow color of normal egg yolks. It can only be suggested at the present time that the highly oxygenated xanthophylls may combine with the protein of the egg yolk to produce the olive color. When a ration composed of 10 per cent lobster shell was fed to hens, orange-red egg yolks containing astacin was produced. No olive colored yolks were obtained with this ration.

Another possibility lies in the fact that carotenoid pigments react with certain organic and mineral acids to give blue compounds. Strain (16) pointed out that the ether solutions of most of the xanthophylls containing more than two oxygen atoms when treated with concentrated hydrochloric acid forms a blue color in the acid layer. When a variety of organic and mineral acid solutions of dehydrated oats and several xanthophylls were fed to the hen for a period of ten days only normal eggs were

produced. This does not eliminate, however, the possibility that the olive pigmentation is associated with the blue-green colors obtained with acids on carotenoids. If such a relation does exist, it is possible that a more unique control of conditions would be necessary.

The writer feels that the first possibility is the more likely because egg yolks contain an abundance of protein which might couple with the highly oxygenated pigments occurring in olive colored yolks in more than four times the amounts present in normal yolks.

SUMMARY

Preliminary experiments having indicated that olive colored egg yolks contained 61.2 per cent more carotenoids than normal yolks, it was concluded that olive color might be associated with the xanthophyllic carotenoids. Chromatographic adsorption studies were made on the xanthophylls of olive and normal egg yolks and of an oat grass silage which had been shown to produce olive colored yolks. It was found that the olive egg yolks contained 13.63 per cent of strongly adsorbed highly oxygenated pigments as compared with 2.98 per cent in the normal egg yolks. It is suggested that some of these highly oxygenated pigments might be combined with the protein of the egg yolk to form the olive pigmentation in a manner typical of the formation of the blue-green color of crustacea.

ACKNOWLEDGMENT

The author wishes to take this opportunity to express his indebtedness to Dr. J. S. Hughes for the selection of the problem, especially to Dr. W. J. Peterson, his major instructor, for his untiring interest and useful suggestions throughout the course of the research, and to Professor L. F. Payne for the use of the experimental fowls.

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