Discolorations in Stored Shell Eggs Produced by Hens Fed Cottonseed Meal

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The chromogen that appears in yolks of eggs produced by hens fed rations containing cottonseed meal and is responsible for the brown coloration that develops in such eggs (stored under refrigeration in the shell) is a pH indicator. A brown color develops in an alkaline medium, and reverts to yellow when the medium is acidified. The relative chromogen concentration may be estimated photographically, using color film, where portions of the yolk are adjusted to pH 4.6 and 10.4. Differences in the transmittance spectra of the positive transparencies were used as a measure of relative chromogen concentration in the yolk. The color data correlate well with the intensity of coloration estimated visually. Correlations between intensity of coloration in the yolks from cottonseed meal–fed hens and total gossypol, "free" gossypol, chemically uncombined gossypol, and gossypol-like pigments were all poor. Evidence indicates that the chromogen in cottonseed meals responsible for the brown coloration in stored shell eggs is heat-stable.

A LARGE PROPORTION of stored shell eggs produced by hens fed rations containing cottonseed meal are of poor quality. The most obvious adverse quality factors include a brown or salmon discoloration of the yolk, a pink discoloration in the white, an abnormal enlargement of the yolk, a waxy or semisolid yolk condition, or a combination of some or all of these characteristics.

The brown discoloration of the yolks has been attributed to the presence of free gossypol in the cottonseed meal (*5*, *8*, *9*), while a salmon discoloration in the yolk and a pink discoloration of the white have been attributed to the material in cottonseed oil that gives a positive Halphen reaction (*7*). It is also presumed (*7*) that this Halphen-positive constituent remaining in the meal after oil extraction is responsible for an abnormal enlargement of the yolks. Apparently the development of a waxy or semisolid yolk has not been associated with any particular cottonseed meal constituent.

The relationship between the amount of free gossypol in the meals fed and the brown discoloration which develops in the yolks of stored shell eggs has not been critically studied. Actually, a lack of correlation is implied in the work of Grau *et al.* (*4*), who suggested a test—the AGU (active gossypol units) measurement—for grading cottonseed meals for use in rations for laying hens.

Experimental

Cottonseed Meals. Properties of the 21 meals used in this study are listed in Table I. All the meals were of commercial origin, with the exception of CM–68, –69, –70, and –71.

CM–68 was obtained from prime cottonseed by extraction of the raw flakes with commercial hexane. CM–69 was prepared by extracting raw flakes obtained from prime cottonseed with acetone. CM-70 was prepared by treating raw cottonseed flakes obtained from prime seed with an equal weight of water which contained sodium hydroxide equivalent to 0.8% the weight of the flakes. Isopropyl alcohol was then added, and the mixture was permitted to stand over-

Table I. Chemical Characteristics of Cottonseed Meals Used in Rations Fed to Laying Hens

Meal		Total Gossypol, %	Free Gossypol, %			Free ε-Aminolysine, G./16 G. Nitrogen	Oil, %
No.	Source		Total	Uncombined	Gossypol-like pigment		
CM–52	Screw press	1.25	0.03	0.011	0.019	2.59	4.33
53	Screw press	0.66	0.02	0.006	0.014	3.07	2.82
54	Prepress solvent	1.11	0.04	0.014	0.026	3.42	0.74
55	Prepress solvent	1.40	0.04	0.017	0.023	3.53	0.46
56	Screw press	0.97	0.05	0.028	0.022	3.38	5.66
57	Screw press	1.07	0.03	0.009	0.021	3.40	4.10
58	Screw press	1.00	0.02	0.008	0.012	3.64	2.20
59	Screw press	0.83	0.02	0.004	0.016	2.70	4.93
60	Prepress solvent	0.97	0.02	0.003	0.017	3.12	1.75
61	Screw press	1.24	0.03	0.015	0.015	2.52	3.66
63	Prepress solvent	0.82	0.04	0.017	0.023	3.70	0.60
64	Prepress solvent	0.75	0.02	0.008	0.012	3.60	0.96
65	Prepress solvent	0.77	0.04	0.010	0.030	3.33	0.76
66	Prepress solvent	0.75	0.03	0.016	0.014	3.48	1.76
67	Prepress solvent	0.82	0.03	0.010	0.014	3.40	1.43
68	Laboratory-prepared meal, hexane-extracted	1.28	0.80	0.79	...	4.21	0.39
69	Laboratory-prepared meal, acetone-extracted	0.27	0.08	0.044	0.036	4.32	0.28
70	Laboratory-prepared meal, isopropyl alcohol–extracted	1.27	0.00	0.00	0.00	3.46	1.09
71	Laboratory-prepared meal, hexane-extracted glandless seed	0.00	0.00	0.00	0.00	4.53	0.45

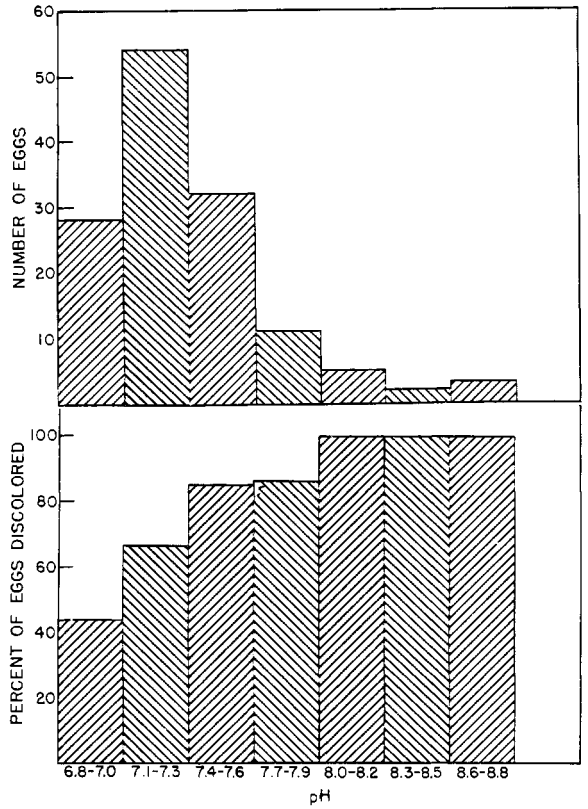

Figure 1. Influence of pH

Top. On distribution of yolks of stored shell eggs
Bottom. On percentage of eggs having discolored yolks

night. The liquid phase was then removed by decantation, and the oil was removed from the solid residue with hot isopropyl alcohol. CM-71 was prepared by extracting the raw flakes obtained from glandless cottonseed (U. S. Cotton Field Station, Shafter, Calif.) with commercial hexane.

The free and total gossypol and oil contents of the meals were determined by the methods of the American Oil Chemists' Society (*1*). The free gossypol of the meals, as determined by the method for free gossypol, is composed of chemically uncombined gossypol and other constituents which are referred to here as gossypol-like pigments. The chemically uncombined gossypol, which is merely stipulated here as gossypol, and the gossypol-like pigments in the cottonseed meals were determined by the method of King, Frampton, and Altschul (*6*). The free ϵ-aminolysine of the proteins in the meals was determined by the method of Conkerton and Frampton (*2*).

Feeding Experiments. The cottonseed meals were fed to laying hens at a level of 20% of the total ration and replaced 80% of the soybean meal in the following control ration formula (per cent):

Ground yellow corn	62.4
Soybean meal	25.0
Alfalfa meal	5.0
Ground limestone	2.5
Steamed bone meal	4.0
Manganized salt	0.5
Butyl fermentation solubles (500 units of riboflavin per gram)	0.3
Vitamin A and D feeding oil 2500 I.U. A, 400 I.U. D)	0.3

The meals were fed in four experiments, each limited to five or seven meals, with five hens for each meal. The hens were caged individually and were kept on the control ration for 30 days before being put on experimental rations containing cottonseed meals. Eggs were collected daily for 60 days, starting 10 days after feeding of the experimental meals was initiated, and were stored under commercial cold storage conditions. This same procedure was followed in collecting control eggs. Some of the eggs from each hen were opened at Glendale, Ariz., for visual estimation of color intensity after 3 and 6 months of storage. Color intensity of yolks of eggs from each hen was estimated at New Orleans, La., by the photographic method after 3, 6, and 8 months of storage.

Visual Estimation of Yolk Color. Visual estimation of color intensities was made independent of the photographic method on the yolks of 10 to 15 eggs from each of five hens on each meal. The intensity of coloration in the yolks was graded from 0 to 5. The value of 0 was assigned to normal appearing yolks, of 1 to yolks with very light brown coloration, of 2 to light brown yolks, of 3 to brown yolks, of 4 to dark brown yolks, and of 5 to those that were virtually black. The average yolk color value for eggs from feeding tests for a given meal was taken as a measure of the chromogen concentration in the meal.

Photographic Estimation of Yolk Color. The chromogen in the yolks of eggs produced by cottonseed meal-fed hens, and which is responsible for the brown coloration, was found to act as a pH indicator. Although the colors of the chromogen in acidic and alkaline media have not been determined, the yolks become brown when they are exposed to ammonia; the color reverts to the normal yellow in the presence of acetic acid vapors. It is presumed, because of the invariably high alkalinity of the white of the stored eggs, that the pH values of yolk areas adjacent to the white are also high. At any rate, the brown coloration of the yolks of stored eggs from cottonseed meal-fed hens is almost invariably restricted to the yolk surface—the interior of the yolk is usually yellow.

A histogram (Figure 1) for the pH data obtained for yolks of 153 shell eggs laid by hens fed cottonseed meal and stored for 6 months at 50° to 55° F. indicates that the mode for the distribution occurred at a pH value of about 7.2, while the pH values ranged from about 6.8 to about 8.8. pH values for yolks from the control eggs ranged from 6.6 to 7.8.

No effort was made to determine either the radial pH gradient in the yolks or the pH values at the periphery of the yolks.

The ratio of discolored to normal yolks increases as the pH values of the yolks increase (Figure 1). Apparently one of the chief contributors to the variation in the intensity of coloration of the yolks of stored shell eggs is the variation in the pH of the yolk.

Extended, but unsuccessful, efforts were made to separate the chromogen from the yolks. In addition, there was no success in repeated efforts to prepare optically void solutions of the yolks to obtain an objective estimation of the relative chromogen concentration. Therefore, color data for regression analysis were obtained by color photography, where Kodachrome film Type F:36 was used. Each yolk was divided into two portions. Ten milliliters of one portion were brought to a pH value of 10.4 with carbonate buffer, while 10 ml. of the other portion were brought to a pH value of 4.6 with acetate buffer. Each preparation was poured into a Petri dish and photographed. The photographic procedure was standardized as much as possible by using a single lot of film for each experiment, by process-

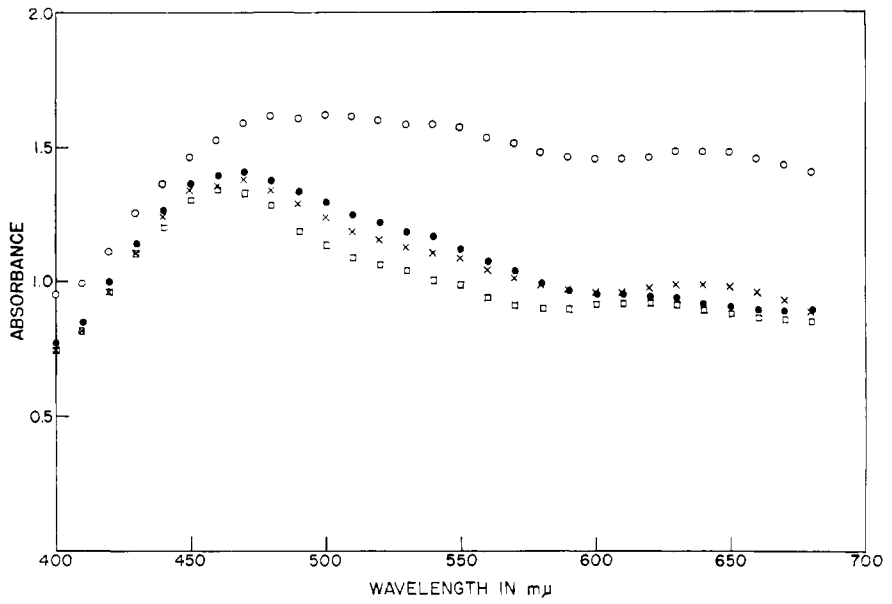

Figure 2. Transmittance spectra of positive transparencies of color photographs of yolk material

Control yolk □ pH 4.6 ● pH 10.4
Yolk from cottonseed meal–fed hen X pH 4.6 ○ pH 10.4

ing all of the film for a given experiment at the same time, by using identical lighting conditions, and by maintaining identical camera settings for all exposures.

The transmittance spectra in the visible region of the positive photographic transparencies obtained at the two pH levels for the yolks from hens fed the control and a cottonseed meal–containing ration are given in Figure 2. The transmittance spectra of the positive color transparencies for the acidified yolks of the control and experimental eggs were almost identical.

The difference in the areas under the curves obtained at the two pH levels was taken, for purposes of computation, as a measure of the relative chromogen concentration in the yolk. However, there is some pH-sensitive chromogen in the control yolks (Figure 2). Therefore, the ratio of the average differences in area obtained with the experimental eggs to the average differences in area obtained with the control eggs was taken as a measure of the quantity of chromogen contributed to the experimental yolks by an experimental meal. These ratios are referred to as the relative chromogen concentrations in the yolks.

Results

Comparison of Methods of Color Estimation. The average visual color values and the average relative chromogen concentration values for the yolks of stored shell eggs from hens fed the control and experimental rations are recorded in Table II.

Three portions of CM–63 and two of CM–66 were given different code numbers for the fourth experiment, D, and then submitted to test, in order to determine the reproducibility of the color values obtained by each of the two color methods.

The average variance for the visual estimation of color intensity in the yolks of the stored shell eggs from cottonseed meal–fed hens is 0.54, where an average of 57 eggs were examined for each meal. The average variance for the photographic estimation of the relative chromogen concentration in the yolks is 0.46, where an average of 17 eggs were examined for each meal. The coefficients of correlation between the two methods for color estimation are 0.89, 0.86, and 0.83 for experiments A, B, and C, respectively. The odds for significance are better than 50 to 1, and these correlations may be taken as an indication of the reliability of the photographic method in estimating the relative chromogen concentration in the yolks. A higher degree of correlation might have been calculated, had the variance due to pH been eliminated in the visual method.

Regression analyses of color on cottonseed meal constituents were carried out with the photographic data, rather than with the visual data, chiefly because the variance due to pH was reduced to insignificance.

Correlation between Relative Chromogen Concentration and Cottonseed Meal Constituents. Data for the relative chromogen concentration in the yolks are plotted in Figure 3 against the total gossypol in the meal used to produce the yolks. The correlation between the total gossypol (total detectable gossypol derivatives in the meals) and the relative chromogen concentration, as calculated from these data, is essentially zero.

Table II. Visual Color Values and Relative Chromogen Concentration of Egg Yolks

(Eggs produced by cottonseed meal–fed hens and stored in the shell at 50–55° F.)

Cottonseed Meal Used in Rations	*Visual Color Value*	*Relative Chromogen Concentration*
Experiment A. 6 months' storage		
CM–52	5.0	2.44
53	4.8	1.87
54	4.5	1.93
55	4.8	2.13
56	5.0	2.06
57	5.0	1.94
58	1.7	1.89
Control	0.0	1.00
Experiment B. 3 months' storage		
CM–59	2.0	1.86
60	2.0	2.41
61	2.5	2.54
63	2.0	2.40
64	1.8	1.55
Control	0.0	1.00
Experiment C. 3 months' storage		
CM–65	0.79	2.06
66	0.96	2.45
67	0.46	2.28
69	0.68	2.60
70	0.10	1.03
Control	0.00	1.00
Experiment D. 3 months' storage		
CM–633 (CM–63)	2.2	2.27
8 (CM–63)	1.3	2.42
811 (CM–63)	1.8	2.61
666 (CM–66)	1.1	2.06
866 (CM–66)	1.9	2.35
71	0.0	1.36
Control	0.0	1.00

Data relating free gossypol (chemically uncombined and soluble gossypol-like pigments) and the relative chromogen concentration are also plotted in Figure 3. The coefficient of correlation calculated from these data is 0.68, with 17 degrees of freedom, and may be regarded as having some significance. The coefficient calculated between the gossypol (chemically uncombined gossypol) content of the meals and the relative chromogen concentration is 0.60, which may also be regarded as having some significance. However, the coefficient calculated between the gossypol-like pigments and the relative chromogen concentration is 0.70, which, for 17 degrees of freedom, may be regarded as having a higher order of significance than the other two coefficients. Data relating the gossypol-like pigments in the meals and the relative chromogen concentration are plotted in Figure 4. The variation in the chromogen concentration in the yolks is not satisfactorily accounted for by the variation of the gossypol-like pigments, gossypol, total gossypol, or free gossypol in the meals.

The relative importance of gossypol

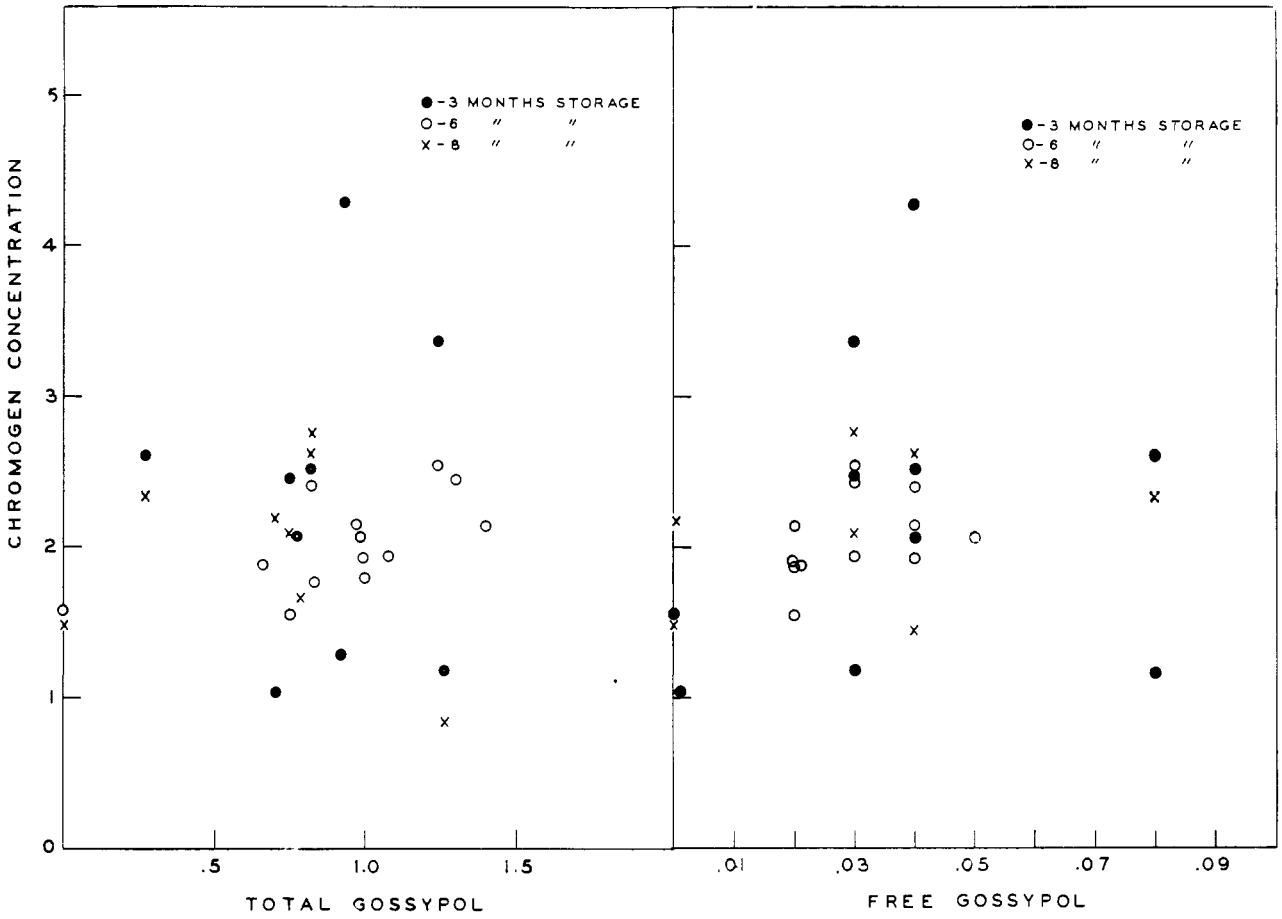

Figure 3. Relation of average relative chromogen concentration in yolks to gossypol of meals

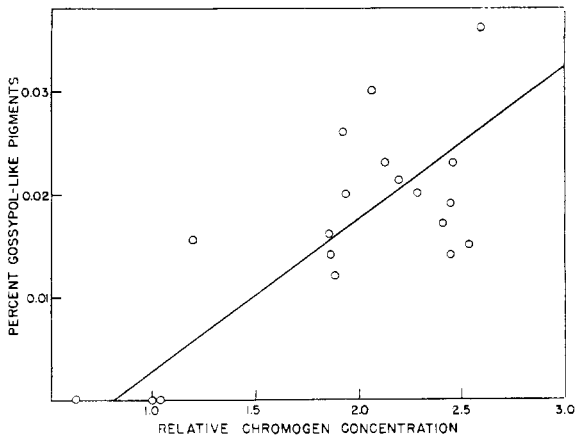

Figure 4. Gossypol-like pigments of meals plotted against average relative chromogen concentration in yolks

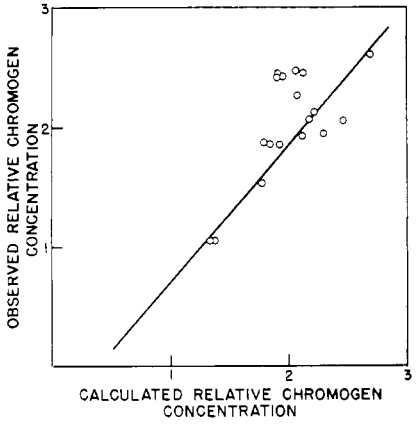

Figure 5. Relation between average observed and relative chromogen concentration in yolks calculated for multiple regression analyses

and the gossypol-like components in inducing coloration in yolks may be obtained from a multiple regression analysis of these two components on color. The equation obtained from such an analysis is:

$$C = 1.34 + 35.4S + 3.5g$$

where C is the relative chromogen concentration in the yolks, S is the gossypol-like pigment, and g is the gossypol contents of the meals. The regression coefficient for the gossypol-like pigments is tenfold greater than the coefficient for gossypol. The multiple coefficient correlation for the gossypol and gossypol-like pigments in the meals on the relative chromogen concentration in the yolks was calculated as being 0.70. The average observed and calculated relative chromogen concentrations in the yolks are plotted in Figure 5.

Other Adverse Quality Factors. No effort was made to measure the intensity of the pink discoloration in the white. However, the percentages of eggs produced by the several meals, and which showed pink discoloration in the white, are recorded in Table III.

Regression analyses of percentage occurrence of pink egg whites on the free ε-aminolysine content of the meals indicated that there is no simple rela-

Table III. Percentage of Shell Eggs with Pink Egg Whites

(Eggs stored for 6 months at 50–55° F.)

Cottonseed Meal Used in Rations	Eggs with Pink Egg Whites, %
CM–52	71
53	67
54	45
55	41
56	94
57	93
58	38
65	67
66	62
67	46
70	80
71	0.0

tionship between the two factors. Apparently the severity of processing of cottonseed for oil does not influence the extent to which pink discoloration will appear in white of eggs from hens fed cottonseed meals.

Also, on visual examination of the eggs, the appearance of abnormally enlarged yolks is not related in a simple way to the presence of pink coloration in the white. In many cases where the white was a deep red the yolks were of normal size.

The appearance of pink coloration in the white seems to be independent of the appearance of brown coloration in the yolk. Many eggs that had deep brown yolks had normal appearing whites. Conversely, many eggs with deep red egg whites had no brown coloration in their yolks.

The appearance of waxy or semisolid yolks does not seem to be related in a simple way to the appearance of either pink coloration in the white or brown coloration in the yolks.

Discussion

The two most important factors which determine the intensity of coloration in yolks of stored shell eggs from cottonseed meal–fed hens are: the chromogen concentration in the yolk, and the pH at the yolk periphery. The pH level in the white, and therefore at the periphery of the yolk, increases with time of storage. The coloration in the yolks stored for 6 months (Table II, experiment A) is much greater than the coloration in yolks stored for shorter periods of time (Table II, experiments B, C, and D).

The difference in color intensity indicated by the data for experiments B and C (Table II) may be accounted for by differences in storage. The experiments which produced these data were carried out at different times and the eggs were stored in different locations. For this reason all of the data were not pooled in a comparison of the two methods for estimation of the relative chromogen concentration; comparisons were limited to individual experiments.

The variance due to pH was eliminated by use of the photographic method in obtaining an objective measurement of the relative chromogen concentration. The degree of correlation between the two methods would have been much higher if this source of variation had also been eliminated from the visual estimation of relative chromogen concentration. The order of agreement found, as indicated by the coefficients of correlation calculated from the data, supports the feeling that both methods measure the same thing.

The relationship between the adverse quality factors in stored shell eggs produced by laying hens receiving cottonseed meal appears to have been oversimplified by earlier workers who ascribed all of the effects to two cottonseed meal constituents—free gossypol and Halphen acid. While the inclusion of gossypol in the diets of laying hens does result in a discoloration of the yolks of the stored shell eggs produced, the experiments reported here indicate that total gossypol in cottonseed meals fed to laying hens is not the predominant factor in inducing the brown discoloration. The regression coefficient for gossypol is only a tenth that for the gossypol-like constituents in the meal. These gossypol-like constituents, whose chemical identities are unknown, are much more potent than gossypol. These constituents are removed from the meals with 70% aqueous acetone, used as the solvent in the determination of free gossypol, but it is not known what proportions of the total amounts of gossypol-like constituents of the meal present were removed from the meals in the extractions for the determination of free gossypol by the method used.

The analytical procedures used for free and total gossypol and for gossypol-like pigments do not measure the concentration of the gossypol derivatives in the meal that cause the yolk discoloration.

Conditions that reduce the free ϵ-aminolysine—i.e., heat—especially under relatively dry conditions (*3*), apparently have no effect on the chromogen concentration in the meal that is responsible for the brown yolks, as the correlation between the ϵ-free aminolysine of the meals and the intensity of yolk coloration produced in the eggs is virtually zero. This fact would indicate that the chromophore in the meal is heat-stable in the range of temperatures used in commercial cottonseed processing.

One conclusion reached on the basis of the data reported in this paper is that the chromogen concentration in the yolk is not a function of the length of time the eggs are stored. The reason for this conclusion may be readily seen from the data plotted in Figure 3, where the distribution of the color data is not influenced by time of storage. The color develops on storage of the eggs because of the increase with time in the pH of the yolk.

Acknowledgment

Many of the commercial cottonseed meals used in these experiments were kindly supplied by Anderson, Clayton & Co. Cottonseed meals CM-68, CM-69, CM-70, and CM-71 were prepared with the assistance of W. H. King. The free ϵ-aminolysine determinations were carried out under the guidance of Fairie Lyn Carter.

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