

Dye Binding by Soybean and Fish Meal as an Index of Quality¹

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Soybean meal must be heat processed for maximal nutritional value. However, either underheating or overheating will produce a product of inferior nutritional value. Apparently underheating does not completely destroy or inactivate toxic substances (Alumot and Nitsan, '61; Liener, '53) or the proteins are not denatured to a point where they are completely digested (Fisher and Johnson, '58). Overheating, on the other hand, apparently results in reducing availability of lysine so that a deficiency of this amino acid is produced (McGinnis and Evans, '47). The degree of lysine inactivation depends upon time and temperature under appropriate moisture conditions. The mechanism responsible for the inactivation of lysine is thought to be primarily the browning or Maillard reaction in which the epsilon amino acid group reacts with free reducing sugar, such as glucose, under appropriate conditions or temperature and moisture to form a complex which is unavailable to the animal or from which the lysine is released too late to be utilized with the other amino acids that are released more rapidly (Lea, '50; Block et al., '46).

An analysis that would indicate the degree of lysine availability would in effect indicate the degree of heating of the soybean meals. Frölich ('54) first used dye binding as a method of indicating soybean protein quality after processing. This method was based on the increased capacity of heated protein to bind Cresol Red under acidic conditions. Olomucki and Bornstein ('60) later modified Frölich's method with respect to classification of quality with dye binding.

Udy ('56) used Orange G (1-phenylazo-2-naphthol-6, 8-disulfonic acid sodium

salt) in the determination of protein content of wheat. Orange G, under acidic conditions, binds specifically to either free amino groups, the imidazole group of histidine, or the guanidyl group of arginine provided they are in a free or dissociated state (Fraenkel-Conrat and Cooper, '44). If the lysine or soybean meal is inactivated by complexing at the epsilon amino group during processing, then differences should be expected in its capacity to bind Orange G.

It was the purpose of the studies reported in this paper to examine the Orange G binding properties of both soybean and fish meals subjected to different processing treatments and to determine whether a relationship could be demonstrated between dye binding capacity, protein quality, and heat treatment.

EXPERIMENTAL

Heat treatment and assay of the soybean meal. Whole ground soybean meal was spread approximately one inch thick (2.5 cm) on stainless steel pans and heated at 120° for various allotted times (table 1) in a Scanlan Morris steam-operated laboratory autoclave. Samples from each treatment were then reground in a small laboratory hammermill to a consistency of 24 mesh; 900- to 1,000-mg portions were weighed out to the nearest milligram and transferred to 250-ml Erlenmeyer flasks. The dye solution (one mg of Orange G/ml in distilled water buffered² to pH 2.2) was delivered to the flask with a calibrated 50-ml automatic buret at the rate of 1 ml/10

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² Buffered to pH 2.2 with the following per liter: citric acid monohydrate, 20.7 gm and disodium phosphate dodecahydrate, 1.44 gm.

TABLE 1
Effect of autoclaving soybean meal on dye binding

Auto-claving	Dye bound ¹	Ratio ²	Protein ³	Protein ⁴
min at 120°	mg/gm meal		%	Δ%
0	70.8(a) ⁵	1.00	43.8	—
15	68.5(b)	0.97	41.6	-2.2
30	68.4(b)	0.97	41.5	-2.3
45	68.5(b)	0.97	41.6	-2.2
60	63.8(c)	0.90	37.2	-6.6
90	63.2(c)	0.89	36.6	-7.2
120	61.2(d)	0.86	34.7	-9.1

¹ An average of three determinations on a moisture-free basis.

² Ratio of milligrams of dye bound per gram of the respective treatments over that of the original raw meal.

³ As determined by the dye binding technique.

⁴ The change in protein content of the respective treatment in comparison with the original raw meal as determined by the dye binding technique.

⁵ Letters in parentheses refer to significance at the 1% level; treatments with a common letter are not statistically different from each other, whereas those treatments without a common letter are.

mg of the soybean meal (on a dry-weight basis) to the nearest tenth of a milliliter. The mixture was agitated by hand approximately every 5 minutes for one hour. The mixture was then filtered through Whatman no. 1 filter paper and a 1-ml aliquot of the filtrate was diluted 1:100 in a volumetric flask.

Optical density of the diluted filtrate was obtained at a wave length of 470 mμ in a Beckman DU spectrophotometer. The amount of unbound dye in solution was then calculated from a predetermined regression [$Y = 0.019 + 2.216X$, where Y is the concentration of Orange G in mg/ml and X is the optical density at 470 mμ ($r = 0.994$)]. This regression equation was obtained by appropriately diluting the original dye solution with distilled water over a range of 0.1 to 1.0 mg/ml at 0.1-mg intervals and determining the optical density of each. A second regression equation [$Y = 0.945X - 23.1$ ($r = 0.961$), where Y is the percentage of protein ($N \times 6.25$) and X is the milligrams of Orange G bound per gram of meal] was obtained after plotting the amount of dye bound by different amounts of protein from raw soybean meal after reacting with a constant amount of the dye solution. The quantity of raw meal was varied to represent meals ranging from 35 to 45% of protein ($N \times 6.25$). By reference to this equation, the amount of protein as determined by the dye bind-

TABLE 2
Basal diet¹

	%
Whole ground soybean meal ²	35.0
Ground corn	57.7
Dehydrated alfalfa (17% protein)	3.0
Limestone	2.0
Dicalcium phosphates	1.0
Methionine hydroxy analogue	0.5
NaCl	0.3
Choline chloride (25% mix)	0.2
Supplement ³	to 100.0

¹ Contains 18.9% of protein ($N \times 6.25$).

² All samples regardless of heat treatment assayed 43.5% of protein ($N \times 6.25$) on a moisture-free basis.

³ Supplemented with the following per pound (454 gm): vitamin A, 1,500 IU; vitamin D₃, 200 ICU; riboflavin, 1 mg; calcium pantothenate, 2 mg; niacin, 5 mg; vitamin E, 2 IU; ethoxyquin, 200 mg.

ing method for an unknown was calculated. The difference in protein content as determined by this procedure and the Kjeldahl method ($N \times 6.25$) reflects the destruction or inactivation of three basic amino acids (including lysine).

Diets and design. To study dye binding as an index of protein quality, chick growth trials were conducted on the same heat treated soybean meals, both with and without supplementary lysine. The basal diet (table 2) was formulated to contain a suboptimal amount of protein (18.9% of protein, $N \times 6.25$). Thus, any amino acid deficiencies, as created by heat treatment, would be readily reflected in the growth trials.

Five male and five female chicks (New Hampshire \times Delaware) were randomly allotted to pens of electrically heated and temperature controlled multitiered battery brooders with raised wire floors. Three pens of birds were fed each diet for a period of 4 weeks. Individual body weights and group feed consumption were recorded at weekly intervals. Feed and water were supplied ad libitum.

The 4-week chick results and dye binding data were analyzed statistically in compliance with the completely randomized design (Federer, '55), followed with Duncan's multiple range test (Duncan, '55) to find lot differences.

Assay of fish meal. Various samples of fish meals³ which had been biologically

³ The samples of fish meal that had been growth-rated and analyzed for available methionine and lysine were supplied by Dr. L. E. Ousterhout, Bureau of Commercial Fisheries, Department of the Interior, College Park, Maryland.

tested as the sole source of protein for chicks and analyzed for available methionine and lysine were obtained and analyzed for dye binding capacity. The samples were extracted with diethyl ether in a soxhlet apparatus before proceeding as with the soybean meals. Because of the wide range of protein content ($N \times 6.25$), samples for dye binding were weighed out to contain 70 mg of nitrogen. To this was added a constant volume of dye solution (100 ml). Thus, a ratio of protein to dye was maintained so that dye binding capacity would be the only variable.

RESULTS

Dye binding results of soybean meal. The capacity of the soybean meal to bind Orange G was decreased by heat treatment (table 1). The shortest heat treatment significantly reduced the amount of dye bound ($P < 0.01$). However, there was no further significant reduction until the meal had been heated for 60 minutes. The meals autoclaved for 60 and 90 minutes gave similar results, but were significantly different from the meal heated for 120 minutes ($P < 0.01$).

By using a ratio of the dye binding capacity of a processed meal to that of the raw meal, or a change in the percentage of protein as determined by the regression equation, an index of quality was established. The quality, as measured by these indices, did not change significantly until the soybean meal was autoclaved for more than 45 minutes. A further significant de-

crease in quality was observed in the meal autoclaved for more than 90 minutes.

Protein quality, as estimated by chick growth (table 3) correlated well with dye binding and the suggested indices with the exception of the raw meal. Although the raw meal gave optimal dye binding values, it supported poor growth which is attributed primarily to factors other than lysine availability. The meals autoclaved for 15, 30, 45 and 60 minutes were not statistically different among themselves, but significantly different from the 90-minute autoclaved meal ($P < 0.01$). The meal treated for 120 minutes gave a significantly lower gain than all other meals. Both the chick growth and dye binding indices demonstrate no significant differences between the effects of heat treatment for 15, 30, and 45 minutes. This suggests a rather extensive period of time in which little damage to the protein occurs, under conditions used in this test.

Lysine supplementation of all meals increased chick growth except for the meal heated 45 minutes. This then indicates that lysine was the most limiting amino acid in all treatments. When the growth data of the lysine supplemented diets were statistically analyzed, only the raw meal and that heat-treated for 45 minutes were significantly different from the others ($P < 0.01$). The difference obtained with the raw meal was expected, but not with the 45-minute heat treatment. The latter is not understood, but it may have been caused by factors other than the dietary variable studied.

TABLE 3
Effect of autoclaving soybean meal on chick growth and feed utilization

Autoclaving treatment	No supplementation		0.5% Lysine supplementation ²	
	4-Week wt ¹	Gain/feed consumed	4-Week wt	Gain/feed consumed
<i>min at 120°</i>	<i>gm</i>		<i>gm</i>	
0	229 ± 7 ³ (a) ⁴	0.43	240 ± 9(a)	0.45
15	312 ± 8 (b)	0.54	328 ± 8(c)	0.55
30	315 ± 8 (b)	0.51	347 ± 8(c)	0.55
45	309 ± 6 (b)	0.52	276 ± 8(b)	0.48
60	293 ± 7 (b)	0.50	339 ± 6(c)	0.54
90	265 ± 7 (c)	0.47	313 ± 7(c)	0.52
120	185 ± 7 (d)	0.36	315 ± 8(c)	0.52

¹ Each average represents three pens of 10 chicks per pen.

² Feed grade lysine, containing 109 gm L-lysine per pound (454 gm), was replacing an equivalent amount of corn in the basal ration.

³ Mean ± SE of the mean.

⁴ See footnote 5 of table 2.

TABLE 4
Fishmeal quality and dye binding¹

Meal	Species	Type of drier	Growth rating	Protein ²	Available ³ sulfur amino acid	Available ⁴ lysine	Dye binding
				%			mg bound/ 70 mg N
GH1-1D70	Herring	vacuum	superior	57.8	high	7.96	77.3 ± 1.5 ⁵
GM1-1A70	Menhaden	steam	superior	62.9	high	—	70.0 ± 1.0
GR1-1C79	Redfish	flame	superior	54.8	high	7.8	66.0 ± 0.0
GG1-1B79	Menhaden	steam	excellent	62.9	high	7.7	72.0 ± 1.4
GM1-4G60	Menhaden	flame	excellent	56.9	high	—	69.5 ± 0.7
GM2-2B60	Menhaden	flame	good	60.8	almost high	7.43	72.7 ± 0.6
GG1-6C79	Menhaden	steam	fair	59.6	low	7.0	66.0 ± 0.0
GM1-7B80	Menhaden	steam	fair	57.4	fair	—	66.0 ± 0.0
GG1-6F79	Menhaden	flame	fair	59.4	fair	6.6	60.7 ± 1.5
GM1-4B120	Menhaden	steam	poor	60.4	low	—	64.3 ± 0.6
GG1-365	Menhaden	flame	poor	62.0	low	5.9	41.3 ± 1.5
GE-8	Tuna	flame	poor	48.6	low	5.0	54.0 ± 3.0

¹ All data in this table, other than the dye binding results, were supplied by Dr. L. E. Ousterhout, Bureau of Commercial Fisheries, Department of the Interior, College Park, Maryland.

² Protein (N × 6.25) on an "as is" basis.

³ As determined by the method of Ousterhout et al. ('59) and Ousterhout, L. E., and D. G. Snyder 1960 The nutritional evaluation of fishmeals. Poultry Sci., 39: 1281 (abstract).

⁴ On an "as is" basis by the method of Carpenter ('60) as percentage of protein.

⁵ Standard deviation of three determinations.

Dye binding results of fish meal. A very definite tendency toward a decreasing ability to bind dye with decreasing quality was observed (table 4). However, there were samples for which dye binding values would indicate good quality in contrast to a lower quality rating based on chick feeding trials. These meals which would have a high nutritional rating based on dye binding were low in sulfur amino acid content, and in some instances, they were prepared from different species of fish or through different processing methods. Dye binding values do not give an indication of sulfur amino acid content.

DISCUSSION

Soybean studies. The use of Orange G in the determination of soybean meal quality was selected because of its specificity, under acidic conditions, to bind either free amino groups, the imidazole group of histidine, or the guanidyl group of arginine provided they are in a free and dissociated state (Fraenkel-Conrat and Cooper, '44). The decreased Orange G binding by soybean meal, observed with increased autoclaving, is probably caused by the binding of the epsilon amino group of lysine with reducing sugars. Del Cueto et al. ('60) found that autoclaving the chick pea at 121° for 60 minutes destroyed about 10% of the lysine whereas the arginine

and histidine content were unaffected. The fact that supplementing overheated meals with lysine completely overcame the chick growth depression suggests that lysine was the principal, if not the only, amino acid involved in the changes in dye binding of soybean meal by autoclaving.

In contrast with the results of Frölich ('54) and Olomucki and Bornstein ('60) where binding of Cresol Red increased with heat treatment, Orange G binding decreased. The increased capacity to bind Cresol Red dye (nonspecific) is probably due to an increased availability of amphoteric groups resulting from increased protein denaturation.

With the use of either method, dye binding and the quality of the protein are well correlated with bioassay results. However, the Orange G method is considered to be more specifically related to nutritional value as it reacts with amino acids of critical importance in nutrition.

Fish meal studies. Because the quality of fish meal varies considerably after processing, it appeared possible to apply dye binding to this product also. However, it was apparent that this one simple test could not be used to indicate quality alone. Samples of fish meal that had low nutritional value because of low sulfur amino acid content still showed high dye binding properties. Dye binding can be

used to estimate the lysine content, but some other method would have to be used to estimate the available sulfur amino acids. Even then, dye binding may not be a reliable index to quality of fish meal as it appears to be for soybean meal either because of the fluctuations in histidine and arginine content among species, or parts of fish used in fish meal production, or both. These fluctuations may indirectly be observed in the discrepancies between Carpenter's method for the detection of available lysine and the dye binding values (table 4).

Some factors that must be considered when using the Orange G binding method are hydrogen ion concentration, particle size of sample, reaction time and fat content. The pH of 2.2 was used because Fraenkel-Conrat and Cooper ('44) indicated that complete dissociation of basic protein groups was approached at this point in the presence of dyes. Assuming that 2.2 is the optimal pH for groups associated with Orange G binding, the deviations from this could adversely affect the accuracy of this method.

Particle size of the meal is of great importance because the finer the meal the faster dye binding occurs. This is critical in this analysis because of the relatively short reaction time. Thus, the meals should be ground as finely and uniformly as possible, both within and between series of analyses.

Because dye binding is a process of equilibration, time is an important factor. Fraenkel-Conrat and Cooper ('44) showed that equilibrium could be attained in 20 hours with most proteins. Udy ('56) used a three-minute equilibration time with wheat; however, he used flour and the mixture was pulverized in a special mill.⁴ It is felt that the present reaction time of one hour used in this analysis is sufficient to obtain the relative dye binding capacity accurately.

Since the fat content of samples may interfere with the ability of the water-soluble dye to bind to the protein, the amount present should be constant or it should be extracted. The fat content of the soybean meal was constant in all treatments and, therefore, was not removed. The fishmeals, in contrast, varied consider-

ably in this respect and extraction was necessary prior to dye binding tests.

SUMMARY

A chemical method for the determination of soybean meal quality after processing is proposed. The method is based on the reaction of the dye, Orange G, with the basic amino acids in the meal. The amount bound depends upon the availability of either free amino, imidazole, or guanidyl groups. Differences in the binding capacity of soybean meal are caused primarily by differences in the availability of the epsilon amino group of lysine.

The dye binding capacity of soybean meals heated for varying periods of time was closely correlated with growth of chicks fed the meals.

This method was also applied to fish meal, but because of variations in methionine (which was not detected by this method), and differences in arginine and histidine content between various meals, the correlation with chick growth was not satisfactory.

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⁴ React-R-Mill, Udy Analyzer Company, Boulder, Colorado.

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