Effect of Time and Temperature of Storage on the Free and Total Gossypol Content of Cottonseed Meals and of Mixed Diets

R. P. KUPPERMAN and M. L. KARON, Southern Regional Research Laboratory,[1] New Orleans, Louisiana

THE nutritive value of cottonseed meals and the effect of gossypol and the related compounds on the nutritive value have been studied extensively. Recent results have indicated that the concentration of gossypol added to diets appears to decrease with time, as measured by chemical analyses; and that these results are in good agreement with data from poultry feeding experiments on the inactivation or loss of gossypol in mixed diets by Heywang, Bird, and Kupperman (1). These authors showed that the effect of gossypol on the nutritive value of cottonseed meals or mixed feeds appears to depend on the concentration of free gossypol at the time the diet is fed. The effect of gossypol bound to the cottonseed meal or to the constituents of the mixed diet on the nutritive value of the diet is not known at present.

This investigation was conducted in order to determine the extent of the changes in gossypol content occurring in cottonseed meals and in diets containing gossypol. The experimental work was divided into three phases: 1) the determination of the effect of temperature and duration of storage on the free and total gossypol content of different cottonseed meals and of cottonseed meals to which gossypol had been added; 2) the determination of the effect of time and temperature of storage on the free and total gossypol content of commercially blended and laboratory-blended stock diets to which gossypol had been added; and 3) the actual fate of gossypol which was lost during storage.

In each of the three phases of this investigation, analyses for free and total gossypol content of both cottonseed and diet materials were conducted according to the methods of Pons *et al.* (2, 3).[2] Free gossypol is regarded as the gossypol and gossypol-like pigments which are extracted with 70% aqueous acetone at room temperature over a one-hour period. Total gossypol is regarded as the gossypol and closely related gossypol-like pigments which are extracted with a solution of oxalic acid in water-butanone azeotrope at 75°C. over a minimum of 6 to a maximum of 16 hrs. Bound gossypol content is obtained, then, by difference between the analytically measured free and total gossypol contents.

Experimental

1. *Effect of Temperature and Duration of Storage of Cottonseed Meals.* Preparation of the samples is described in Table I, along with the changes in gossy-

[1] One of the laboratories of the Southern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

[2] A procedure specifically designed for the determination of free gossypol content in mixed feeds has been published recently (5) but was not available at the time of this study.

TABLE I

Effect of Time and Temperature of Storage on the Free and Total Gossypol Content of Cottonseed Meats and Meals

Sample Number[a]	% Moisture	Temperature of Storage °C.[b]	% Free Gossypol after Storage Period (Days) of:					% Total Gossypol after Storage Period (Days) of:				
			0	15	30	60	90	0	15	30	60	90
1	9.5	28	0.83	0.79	.....	0.89	0.85	0.97	1.06	.....	1.05	0.97
		37.5		.....	0.81	0.74	0.72		.....	.....	1.07	0.92
		60		0.50	.....	0.19	0.11		0.78	.....	0.48	0.31
2	6.4	28	0.55	0.48	.....	0.41	0.37	1.02	0.99	.....	0.90	0.92
		37.5		.....	0.33	0.31	0.27		.....	0.92	0.86	0.77
		60		0.12	.....	0.043	0.032		0.82	.....	0.62	0.50
3	8.7	28	0.016	0.016	0.015	.....	0.015	0.24	0.23	0.21	.....	0.21
		37.5		.....	0.012	0.014	0.012		.....	0.22	0.22	0.21
		60		0.013	0.012	.....	0.009		0.21	0.18	.....	0.15
4	....	28	0.067	0.067	0.062	.....	0.065	0.33	0.31	0.29	.....	0.29
		60		0.056	0.048	.....	0.047		0.29	0.26	.....	0.24
5	9.1	28	0.23	0.17	0.15	.....	0.098	0.79	0.77	0.70	.....	0.38
		60		0.016	0.019	.....	0.007		0.73	0.42	.....	0.30
6	6.4	28	0.21	.....	0.21	0.21	0.20	0.90	.....	0.90	0.92	0.87
		37.5		.....	0.20	0.21	0.19		.....	0.93	0.90	0.85
		60		.....	0.14	0.12	0.081		.....	0.78	0.70	0.56
7	7.3	28	0.076	0.076	0.078	.....	0.074	1.17	1.16	1.09	.....	1.14
		37.5		.....	0.060	0.063	0.055		.....	1.11	1.09	1.01
		60		0.061	0.047	.....	0.026		1.08	1.02	.....	0.85
8	....	28	0.13	0.11	0.11	.....	0.099	1.26	1.27	1.20	.....	1.20
		60		0.067	0.050	.....	0.031		1.17	1.02	.....	0.94

[a] Descriptions of the samples are as follows:
1. Cottonseed flakes prepared by rolling cottonseed meats through smooth rolls.
2. Cottonseed flakes prepared by rolling cottonseed meats through smooth rolls, followed by extraction with hexane.
3. Flaked cottonseed meats, extracted first with commercial hexane, then with butanone containing 3% water.
4. Flaked cottonseed meats, extracted first with commercial hexane, then with butanone containing 3% water (same material as Sample No. 3) to which 0.08 grams gossypol was added to each 100 grams c/s flakes.
5. Cottonseed flakes prepared by cracking and rolling cottonseed meats, followed by autoclaving at 15 pounds pressure for one-half hour.
6. Cottonseed meal prepared by differential settling (4).
7. Commercially prepared screw-pressed cottonseed meal.
8. Commercially prepared screw-pressed cottonseed meal (same material as Sample No. 7) to which 0.075 grams gossypol was added to each 100 grams c/s meal.

[b] Samples stored at 3 to 5°C. did not indicate any appreciable change with time of storage.

TABLE II

Effect of Time and Temperature of Storage on the Free Gossypol Content of Diet Mixtures Containing 0.1% Added Gossypol

Time of Storage (Days)	Free Gossypol Content (%)							
	Diet Mixture No. 1 Stored at		Diet Mixture No. 2 Stored at		Diet Mixture No. 3 Stored at		Diet Mixture No. 4 Stored at	
	3-5°C.	25°C.	3-5°C.	25°C.	3-5°C.	25°C.	3-5°C.	25°C.
0		0.040		0.052		0.054		0.060
1	0.042	0.040	0.067	0.053	0.068	0.054	0.076	0.058
2		0.028		0.051		0.054		0.059
3	0.031	0.030	0.051	0.052	0.046	0.054	0.049	0.054
4		0.028		0.056		0.056		0.064
8	0.044	0.027	0.068	0.055	0.071	0.057	0.076	0.059
9		0.021		0.039		0.041		0.045
10	0.038	0.024	0.054	0.048	0.060	0.047	0.065	0.049
11		0.023		0.046		0.055		0.059
14	0.013	0.010	0.026	0.020	0.030	0.021	0.034	0.025
37		0.018		0.038		0.041		0.048
57		0.010		0.018		0.022		0.030

pol contents at the various times and temperatures of storage. All samples were in tightly sealed Mason jars and were stored in dark storage chambers; therefore no analyses for moisture contents or relative humidities were made during storage. The gossypol values are reported on an "as is" basis. Storage of the original samples of meals at the temperature of 3 to 5°C., for periods up to 90 days, did not result in any perceptible loss in either free or total gossypol content. Storage of the original samples of meals at high temperatures for long periods of time appears to cause, without exception, a considerable decrease in free gossypol content. The rate of decrease in free gossypol content varies with the different type of cottonseed materials and is greater with increasing temperatures of storage. At the high temperatures of storage the decrease in total gossypol, in many cases, appears to accompany the decrease in free gossypol content. It appears, then, that the original gossypol present is converted, possibly, to something other than a bound gossypol compound.

In those cases in which gossypol was added to screw-pressed and solvent-extracted cottonseed meals, only 86% and 69%, respectively, of the added gossypol could be accounted for as free gossypol by analyses made immediately after preparation of the mixtures. While the entire amount of the added gossypol could be accounted for as total gossypol at the time of preparation, a disappearance of total gossypol did occur after storage. The rate of disappearance of gossypols differed, depending on the type of cottonseed material and upon the temperature of storage.

From the experimental results obtained, several general conclusions can be drawn. These are as follows: a) the entire amount of pure gossypol added to cottonseed meals cannot be accounted for by chemical analysis for free gossypol content, even at the time of preparation of the sample; b) with samples stored at high temperatures, that portion of the gossypol which cannot be accounted for by analysis for free gossypol rarely can be accounted for as total gossypol; and c) the fate of the free gossypol content is affected by the conditions of storage and the type of material to which gossypol is added.

2. *Effect of Temperature and Duration of Storage of Mixed Diets.* A series of investigations were made to determine the changes in gossypol content in four stock diets to which gossypol had been added in dry form. These were as follows: Diet No. 1, a Purina Laboratory Chow which was a commercially blended diet; Diet No. 2, a Western Regional Research Laboratory stock diet consisting of 73% cornmeal, 10% linseed oil cake meal, 10% casein, 2% alfalfa, 2% cod liver oil, 1.5% bone ash, and 0.5% sodium chloride; Diet No. 3, the same as Diet No. 2 to which an additional quantity of casein had been added (at the expense of some fractional proportion of the other constituents) to make the final concentration of casein 19%; Diet No. 4, the same as Diet No. 2 to which an additional quantity of casein had been added (also at the expense of some fractional proportion of the other constituents) to make the final concentration of casein 28%. Pure gossypol was added in dry form to each of these diets at the 0.1% gossypol level. After thorough mixing the samples were analyzed immediately and then were stored at temperatures of 3 to 5°C. and 25°C. for a period of 57 days. In the analyses for gossypol in these mixed diets the official procedures which are used for determinations of gossypol in cottonseed materials were modified slightly.[2] The results of the analyses are compiled in Table II.

At the time of preparation of the mixtures only 40% of the gossypol added to Diet No. 1, and only 50 to 60% of the gossypol added to Diets No. 2, 3, and 4 could be accounted for by analysis for free gossypol. Following 14 days of storage at the two different temperatures, only 10% of the gossypol added to Diet No. 1, and 20 to 30% of the gossypol added to Diets No. 2, 3, and 4 could be found present by chemical analysis for free gossypol.

In general, the results of the experiments indicate that: a) the entire quantity of gossypol added to the diets cannot be accounted for as free gossypol at the time immediately after preparation; b) after a storage period of 10 to 14 days at 3 to 5°C. and 25°C. there appears to be a further appreciable loss of free gossypol; c) the Purina Laboratory Chow, Diet No. 1, appears to undergo a greater percentage loss of gossypol than that of the Western Regional Research Laboratory Diets No. 2, 3, and 4; and d) samples stored at 3 to 5°C., while exhibiting a trend similar to samples stored at 25°C., lose gossypol at a slightly slower rate.

In order to determine whether these losses of free gossypol in the four diet mixtures were due to a binding effect of gossypol or to an actual disappearance of gossypol, freshly prepared gossypol-diet mixtures were made for determinations of total gossypol content. To subsamples of each of the original four stock diets, aliquots of known concentrations of gossypol dissolved in solvent solution were added immediately prior to total gossypol analysis. Gossypol in wet rather than dry form was added in this specific instance to insure that an extremely accurate measure

of gossypol would be added to and be present in the diet mixtures at the onset of and throughout the 16-hr. digestion period of the analysis.

The results of the total gossypol analyses revealed that the gossypol theoretically present could not be accounted for as total or bound gossypol by use of present methods. Recovery values of percentage total gossypol content ranged from a low of approximately 43% to a high of 80% on the four different diets.

In evaluating the data obtained on analyses of the four different diet materials for the free and total gossypol content, it was evident that while each of the diet mixtures was handled in the same manner, at the same time and with the same concentration of gossypol: a) the entire quantity of added gossypol could not be found by analysis for either free or total gossypol, and b) the gossypol values experimentally determined on Diet No. 1, the Purina Laboratory Chow, were persistently lower than those obtained on Diets No. 2, 3, and 4, the Western Regional Research Laboratory diets which were basically the same in their composition.

3. *Fate of Gossypol in Mixed Diets.* It appeared that the destruction and/or inactivation of the added gossypol might occur where gossypol is added to and is in contact with the diet material throughout the 6- to 16-hr. digestion period in the analysis for total gossypol; or the 1-hr. solvent-extraction period in the analysis for free gossypol. At this stage in the analysis it is possible that some constituent or constituents in the diet material could either prevent or interfere with the solvent-extraction of the added gossypol; and therefore the gossypol would not be present in the solvent filtrate solutions of the samples. The destruction and/or inactivation of the added gossypol might occur also where the aliquots of the solvent filtrate solutions of the samples undergo the p-anisidine treatment in preparation for colorimetric determination. It is possible that soluble components of the diet are extracted along with the gossypol and would be present in the solvent filtrates. These extracted, soluble constituents of the diet may either prevent the p-anisidine-gossypol reaction or may mask the developed color reaction.

In order to determine the cause and/or fate of the gossypol loss and disappearance, duplicate subsamples of the four original diet materials were weighed, and known concentrations of gossypol, in solution, were added to each of the subsamples of each diet at two different stages of the gossypol analyses. To one of each of the diet subsamples the gossypol was added at the beginning of the analysis and was in contact with the diet material throughout the entire 1-hr. extraction or the 16-hr. digestion period, respectively, for free or total gossypol determinations. To the duplicate subsamples of each of the diet materials, gossypol, in solution, was added immediately after the extraction or digestion period on the plain diets, *i.e.*, immediately before filtering the sample material from the solvent. In these cases the gossypol was in contact with the diet approximately 5 min. in the free gossypol determination and 20 min. in the total gossypol determination. Results of these analyses are compiled in Table III.

These results indicate that a) in all the samples the entire amount of gossypol added could not be accounted for or recovered either as free or total gossypol; and b) that these samples in which the gossypol was in contact with the diet material for the longer periods of time, *i.e.*, throughout the extraction and digestion periods, had lower gossypol recoveries; and c) that the recovery of total gossypol in all the samples was much lower than that of free gossypol.

TABLE III

Effect of Time of Contact of Added Gossypol in Diets on the Free and Total Gossypol Contents of the Diet Mixtures

Gossypol-Diet Mixture	% of original gossypol recovered as free gossypol when diet material is in contact with gossypol		% of original gossypol recovered as total gossypol when diet material is in contact with gossypol	
	5 minutes	1 hour	20 minutes	16 hours
Diet No. 1	78.6	74.4	50.6	43.7
Diet No. 2	89.1	81.9	73.4	66.4
Diet No. 3	87.0	76.9	78.6	69.1
Diet No. 4	82.8	83.2	84.2	74.9

In performing the analyses in the work described immediately above, it was noticed that when duplicate aliquots of each sample were withdrawn and reacted with p-anisidine for the preparation of the colorimetric reading solutions, there were significant differences between results on the duplicate aliquots of the same samples when the reacted colorimetric solutions were read at two different periods of time after reaction and preparation. This was an unusual phenomenon because the methods of analyses by Pons *et al.*, designed for analyses of gossypol primarily in cottonseed materials, indicate that the final colorimetric reading solutions are stable and can yield reliable values even up to 4 hrs. after the p-anisidine reaction and preparation of the reading solution. It was observed that the colorimetric solutions which were read immediately after the p-anisidine treatment yielded lower gossypol content values than their duplicates which were read 1 hr. after p-anisidine treatment and preparation. Results are given in Table IV.

TABLE IV

Percentage Recovery of Total Gossypol Added to Diets

Gossypol-Diet Mixture	Length of Time that Gossypol was in Contact with Diet Material	% of Original Gossypol Recovered as Total Gossypol when Colorimetric Determinations were made:	
		Immediately after p-anisidine treatment	1 hour after p-anisidine treatment
Diet No. 1	20 minutes	48.2	52.9
	16 hours	41.2	46.2
Diet No. 2	20 minutes	71.8	75.0
	16 hours	64.6	68.2
Diet No. 3	20 minutes	75.4	81.9
	16 hours	66.8	71.4
Diet No. 4	20 minutes	82.7	85.7
	16 hours	72.8	77.1

This observation implied that the factor responsible for the loss in gossypol content was some soluble constituent of the diet material which was extracted and present in the solvent filtrate. Duplicate aliquots of the filtrates obtained on original diet materials for total gossypol determination were investigated further. One aliquot was treated with p-anisidine, and the color developed was determined according to the methods specified in the official procedures. To the second aliquot, standardized gossypol solution was added immediately before the addition of p-anisidine. In all cases the developed color was determined at the same time after the addition of p-anisidine. The results of the percentage of the original added gossypol that was recovered (Table V) correlated well with

the values obtained in Table IV, described under "Gossypol in Contact with the Diet for 20 Minutes"; and also these values fall into the same range of percentage recovery obtained in the previous experimental work reported.

TABLE V

% of Original Gossypol Recovered as Total Gossypol When Gossypol is Added Directly to Colorimetric Reading Samples and Treated with p-anisidine

Description of Sample	Mg. Gossypol Found by Analysis	% of Original, Added Gossypol Recovered by Analysis
Solvent Blank	0.013	.....
Solvent Blank plus Gossypol	0.102	102.0
Aliquot of Filtrate of Diet No. 1	0.007	.....
Aliquot of Filtrate of Diet No. 1 plus Gossypol	0.067	52.1
Aliquot of Filtrate of Diet No. 3	0.004	.....
Aliquot of Filtrate of Diet No. 3 plus Gossypol	0.089	80.7

Thus it appears that while there is a percentage loss or inactivation occurring when the gossypol is mixed and is in contact with the diet materials, the greatest percentage of the loss of gossypol is accountable to some unidentified, soluble constituent of the diet which is extracted or digested along with the gossypol and therefore is also present in the filtrates of the samples.

Discussion of Results

Storage of commercially processed cottonseed meals at low and moderate temperatures for extended periods of time—similar conditions as customarily employed in commercial mills and warehouses—has little or no effect on the total or free gossypol contents of the meals. However storage of commercially processed cottonseed meals and cottonseed materials at elevated temperatures of 37.5°C. and above appears to cause a considerable decrease in free gossypol content, which in many cases is accompanied by a decrease in total gossypol content.

When gossypol was added to commercially processed screw-pressed and to solvent-extracted cottonseed meals, only 86% and 69%, respectively, of the added gossypol could be accounted for as free gossypol by analysis immediately after preparation of the mixture. The entire amount of gossypol added could be accounted for as total gossypol at the time of preparation. Upon storage over a 90-day period, the total gossypol, as well as the free gossypol content, consistently decreased with length of time to a slight extent at 28°C., and more appreciably at 60°C. The rate of disappearance of gossypols differed, depending on the type of cottonseed material and upon the temperature of storage.

When gossypol in pure form is added to mixed diet materials, the entire quantity of added gossypol cannot be accounted for, by the methods used, as either free or total gossypol at the time of preparation of the mixtures. Further there is an additional decrease in free gossypol content over relatively short periods of time at low and at moderate temperatures of storage. In addition to the influence of temperature and duration of storage, the rate of disappearance or inactivation of gossypol in mixed diets differs, due to differences in the various components of the diets.

As previously cited in the text, the methods of analyses employed for determining the gossypol content in these mixed feeds were primarily designed for cottonseed materials; and therefore the scope of their direct application to other materials may be limited. For adaptation of the official method to mixed diet materials, one slight modification was made, the substitution of aqueous ethanol for aqueous acetone as the solvent for extraction of free gossypol. In earlier studies where these methods of analyses were employed, chemical data obtained on the concentration and on the inactivation or loss of gossypol added to animal feeds exhibited the same pattern and also correlated with nutritional data of poultry feeding experiments (1).

Summary

With different types of cottonseed products and cottonseed products to which gossypol is added, there is a loss or disappearance of gossypol upon storage, as determined analytically. The rate of disappearance of gossypol varied for the different type cottonseed and was increased at higher temperatures and over the longer periods of storage.

When gossypol is incorporated into different types of animal diet materials, there is a loss or inactivation of some portion of the added gossypol as determined analytically immediately after preparation of the gossypol-diet mixtures, and a further additional loss or inactivation upon storage. The factors which may contribute to this loss or inactivation of gossypol are: a) the components of the diet materials; b) the temperature and length of time of storage; c) the concentration of gossypol added and the final percentage of gossypol in the mixture.

REFERENCES

1. Heywang, B. W., Bird, H. R., and Kupperman, R. P., Poultry Science, *31*, 35 (1952).
2. Pons, W. A. Jr., Hoffpauir, C. L., and O'Connor, R. T., J. Am. Oil Chemists' Soc., *27*, 390 (1950).
3. Pons, W. A. Jr., Guthrie, J. D., J. Am. Oil Chemists' Soc., *26*, 671 (1949).
4. Spadaro, J. J., Persell, R. M., Reuther, C. G. Jr., Vix, H. L. E., Laborde, E. J., Latham, J. W., Jaeger, R. L., Pollard, E. F., Gastrock, E. A., J. Am. Oil Chemists' Soc., *27*, 336-343 (1950).
5. Storherr, R. W., and Holley, K. T., J. of Agric. & Food Chem., *2*, 745-747 (1954).

[Received March 23, 1954]