THE JOURNAL OF THE American Oil Chemists' Society

Volume 31 APRIL, 1954 No. 4

Detoxification of Cottonseed by Salts and Alkalies

EDWARD EAGLE, H. F. BIALEK, D. L. DAVIES, and J. W. BREMER, Research Laboratories, Swift and Company, Chicago, Illinois

THE earliest recorded statement on the harmful effect of cottonseed is attributed to Voelker in England in 1859 (1). Böhm, in an address before the Scientific Society at Marburg in 1881, ascribed the adverse effect to choline, which he had found to be present in cattle feed prepared from cottonseed (2). Other investigators have blamed a host of materials including lint, oil content, high protein content, a toxalbumin or toxic alkaloid, betaine, resin, and decomposition products (3). Crawford (4) believed a salt of pyrophosphoric acid to be the poisonous principle in cottonseed meals, and Withers and Brewster (5) placed the responsibility for the toxicity of cottonseed meal on a constituent group of the protein molecule containing loosely-bound sulfur.

Although a crude pigment was isolated from cottonseed oil "foots" by Longmore in 1886 (6), and a purified, yellow, polyphenolic pigment, which he named gossypol, was extracted from the same material by Marchlewski in 1899 (7), both of these investigators were concerned with the properties as a dye and made no mention of physiological activity. It was not until 1915 that Withers and Carruth published their preliminary note (8), in which they reported separation of a toxic substance from cottonseed kernels which appeared to be identical with the material described by Marchlewski 16 years before. Three subsequent papers by the same group (9, 10, 11) also bore titles referring to gossypol as the toxic substance in cottonseed. In one of these (11) gossypol was isolated from crude ether extracts of cottonseed by reacting them with aniline, acetic acid, or dilute aqueous sodium hydroxide.

Marchlewski had found in 1899 (7) that gossypol is quickly oxidized in a solution of sodium hydroxide, but it was not until 1912 that Withers and Ray (12) demonstrated by rabbit feeding experiments that the toxicity could be destroyed by boiling cottonseed meal with alcoholic sodium hydroxide. Other investigators subsequently reported varying degrees of successful detoxification of cottonseed with alkali (9, 11, 13), with iron salts (5, 9, 14) and by cooking, steaming, or autoclaving (9, 10, 14, 15, 16). It may be noted that all the research cited above was published no later than 1918, at approximately the time when Osborne and Mendel (15) stated, "the treatment of the cottonseed so as at least to render it harmless now seems to lie within the range of ready possibilities." Despite all this McGowan and Crichton in 1924 (17), Curtis *et al.* in 1926 (18), and Halverson and Sherwood in 1930 (19) were still of the opinion that cottonseed injury was not due to a toxic material.

Our studies on the detoxification of cottonseed pigment glands with ferrous sulfate (20), and on the effect of fractionation and treatment on the acute oral toxicity of cottonseed pigment glands (21) led us to the use of this technic for screening various chemical agents for their ability (as 2% aqueous solutions) to decrease the previously determined acute oral toxicity of the same pigment glands when suspended in distilled water. This LD_{50} procedure applied to a total of 28 compounds led to the selection of a group, which was then tested in detoxification studies on cottonseed meals specially prepared for maximal toxicity.

Experimental

In the preliminary test 200 g. of hexane-extracted cottonseed meal of established toxicity was mixed thoroughly with 100 ml. of distilled water (to make the moist heat-treated sample) or with 100 ml. of a 2% solution of the chemical agent being tested. Thus the final treated meal would contain 1% of the chemical used, unless otherwise noted for special cases. The moistened meal was placed in a vacuum oven at 100° C. and 125 mm. Hg. pressure for 6 hours. Moisture removed from the meal during processing was condensed in conventional traps containing dry ice plus acetone. The resulting meal samples were ground in a Wiley Mill through a 20-mesh screen (openings 0.033 in.).

In the second test hexane-extracted, air desolventized (unheated) cottonseed flakes were used as the starting material. The samples were treated in the same manner as described above, except that a new pump and oven provided better evacuation, *i.e.*, 23 mm. Hg. pressure instead of the previous 125 mm. Hg.

The third and fourth tests were performed on samples of solvent-extracted cottonseed meal subjected to minimal heat. This untreated meal contained 7.55% moisture, 2.6% fat, 40.9% protein, 19.2% soluble protein in 3% NaCl, and 0.65% free gossypol and was identified as sample No. 87, series 6, from the Southern Regional Research Laboratory. Either 273 g. of water or 262 g. of water plus 11 g. of the treating chemical were added to 1,135 g. of meal, mixed thoroughly, and placed in a steam-jacketed cooker (steam pressure 20 lbs./sq. in.) for a period of 90 minutes. The moistened meal was kept agitated during the cooking and drying process. Continuous temperature determinations made by means of a thermocouple attached to a recording potentiometer showed that it required 20 minutes for the inside temperature to reach 215°F., after which a range of between 215° and 225°F. was maintained during the remaining 70-minute toasting period. On completion of the 90-min-

TABLE I

Effect of High Level Feeding of Chemically Treated Cottonseed Meals on the Body Weight of Rats

Diet	Variable in diet	No. rats per group	Average starting weight	Avg. weight after 1 wk.	Avg. weight after 2 wks.
			gms.	*gms.*	*gms.*
1a	Untreated cottonseed meal A	4	48	....[a]	....
1b	A–dry heated	4	48	....[b]	....
1c	A–treated with aqueous alcoholic NaOH[c]	4	48	51	61
1d	A–moist-heat-treated	4	48	61	75
1e	A–treated with 1% sodium hypochlorite	4	48	64	77
1f	A–treated with 1% disodium phosphate	4	48	62	91
1g	A–treated with 1% trisodium phosphate	4	48	69	93
1h	A–treated with 1% sodium chloride	4	48	68	94
1i	A–treated with 1% sodium alkaline pyrophosphate	4	48	65	97
1j	Stock diet control	7	46	82	127

[a] 4/4 dead within 7 days.
[b] 3/4 dead within 7 days; 4/4 dead within 11 days.
[c] In this case 200 g. of untreated meal A was moistened with a mixture of 50 ml. 0.5 N NaOH plus 50 ml. 95% ethyl alcohol before being placed in the vacuum oven; the final meal contained 0.5% NaOH.

ute cycle the charge was removed and ground in a Mikro-Samplmill through a $\frac{1}{16}$-in. round perforation screen.

The dry heated cottonseed meals (used in diets 1b, 2b, and 3b) were made by subjecting the untreated cottonseed meal to the particular heat treatment given to the other treated meals in the same experiment. All cottonseed meals in this report were incorporated into rat diets at a level of 67%, the remaining 33% of the diet consisting of the following: dextrose (Cerelose) 14%, lard 10%, Jones and Foster salt mixture 4%, Wilson 1:20 liver concentrate 3%, vitamin A & D oil (2,250 U.S.P. A; 300 U.S.P. D_3 per g.) 1%, and yeast (AB 300) 1%. The soybean oil meal which replaced the usual 67% level of cottonseed meal (control diet 3g) was from a special lot of control meal set aside for groups engaged in collaborative studies. The stock diet used as the laboratory control diet for all tests consisted of whole ground wheat 21%, meat and bone scraps 19.6%, skim milk powder 15%, soybean oil meal 15%, ground yellow corn 13%, lard 10%, salt and yeast mixture 2.5%, alfalfa leaf meal 2%, wheat germ oil 1%, vitamin A and D oil (2,250 U.S.P. A; 300 U.S.P. D_3 per g.) 0.5%, and Wilson 1:20 liver concentrate (N.F. IX) 0.4%.

Determinations of free gossypol were made by the method of Pons and Guthrie (22). In most cases free gossypol analyses were performed both on the cottonseed meal itself and on the finished diet which contained 67% of the particular meal.

Weanling male rats of the Sprague-Dawley strain (Holtzman) were fed the stock diet for a period of one to three days, after which they were distributed into the various groups according to body weight and placed on the experimental diets. All rats were kept in individual wire-bottom cages in an air-conditioned room maintained at 79° ± 1°F. and *ca.* 45% relative humidity. Food and water were allowed *ad libitum*. The rats were weighed daily for the first 14 days and at least twice weekly thereafter.

Results and Discussion

The results obtained in the four tests are given in Tables I, II, III, and IV. It may be seen that dry heat treatment does not detoxify toxic cottonseed meals for the rats began losing weight from the start, and survival was very poor; the over-all mortality from dry-heated meals within three weeks was 18/22 or 82%. Moist heat treatment provided only partial detoxification, less than any of the chemical treatments used. There was considerable variation however in the detoxifying performance of the chemicals tested. Sodium hydroxide gave the best results although all the alkalies were not very far apart and were in the following order of decreasing effectiveness:

$$NaOH > KOH > NH_4OH > Ca(OH)_2$$

Results from analyses for free gossypol in the various meals and in the mixed diets, which routinely contained 67% of these meals, were in good agreement. It may be seen from Tables III and IV that various diets containing high levels of free gossypol (diets 3f, 4e, 4f, and 4h) showed better growth performance than other diets containing lower levels of free gossypol (3d, 4b, 4c). It may likewise be noted from Table III that diets 3c, 3d, 3e, and 3f which contained, respectively, 0.20, 0.17, 0.19, and 0.20% free gossypol, showed the following different body weight results

TABLE II

Effect of High Level Feeding on Chemically Treated Cottonseed Meals on the Body Weight of Rats

Diet	Variable in diet	No. rats per group	Average starting wt.	Average body weight[a] after:							
				1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.
			gms.	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*
2a	Untreated cottonseed meal B	8	50	44	47^8	....[b]	....	....	....	....	....
2b	B–dry heated	8	49	61	69^7	80^4	87^4	96^3	116^2	110^2	106^2
2c	B–moist heat-treated	8	50	50	58^6	65^2	69^2	83^2	112^1	114^1	118^1
2d	B–treated with 1% disodium phosphate	8	50	56	66	77^5	94^5	....[c]	....	....	....
2e	B–treated with 1% trisodium phosphate	8	50	66	85	102	115	128	134	133	135
2f	B–treated with 1% sodium alkaline pyrophosphate	8	50	57	75	97^6	116^6	139^6	147^6	150^6	149^6
2g	B–treated with 1% sodium chloride	8	50	81	116	145	149	147	152	149	151^7
2h	B–treated with 1% sodium hypochlorite	8	50	67	93	115	127	147	158	161	159
2i	B–treated with 0.25% $FeSO_4$+1% NaCl	8	50	74	115	146	155	170	180	182	186
2j	B–treated with 1% ammonium carbonate	8	50	72	102	147	150	159	175	190	202
2k	Stock diet control	8	50	83	129	176	209	249	279	302	322

[a] The figure appearing as an exponent indicates the number of surviving rats averaged.
[b] 5 rats died within 2 weeks; 8/8 dead within 20 days.
[c] Discontinued after 4 weeks.

TABLE III

Effect of Chemical Treatment of Cottonseed Meals on Free Gossypol Content and Body Weight[a] Performance After High Level Feeding in Rats

Diet No.	Variable in diet	Free gossypol content of diet[b]	No. rats per group	Average starting weight	Average body weight after:							
					1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.
		%		*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*
3a	Untreated cottonseed meal C	0.43	10	60	....[c]	....	....	....	....	....	....	....
3b	C–dry heated	0.42	10	60	46^{7}	....[d]	....	....	....	....	....	....
3c	C–moist heat-treated	0.20	10	60	72	78	85^{8}	101^{8}	115^{8}	129^{8}	140^{8}	147^{8}
3d	C–treated with 1.5% NaCl	0.17	10	60	86	111	131	147	160	165	162	159^{9}
3e	C–treated with 1% ammonium carbonate	0.19	10	60	85	108	132	150	172	177	205	229
3f	C–treated with 1% NaOH	0.20	10	60	95	133	165	188	211	227	237	246
3g	Soybean meal control	0	10	60	103	144	182	218	253	272	291	304
3h	Stock diet control	0	10	60	97	135	173	212	250	273	301	322

[a] The figure appearing as an exponent indicates the number of surviving rats.
[b] The free gossypol content of the cottonseed meals would be approximately 50% higher than that given for the diet since cottonseed meal comprised ⅔ of the diet.
[c] 10/10 dead within 7 days.
[d] 10/10 dead within 9 days.

after 8 weeks on test: 147 g. (2/10 died within 3 weeks), 159 g. (1/10 died within 8 weeks), 229 and 246 g. Furthermore Table 4 shows six diets whose free gossypol content varied within the narrow range of 0.11 to 0.13% (diets 4b, 4c, 4d, 4e, 4f, and 4h). Despite this the respective average body weights after 8 weeks on test were 196, 225, 272, 283, 294, and 303 g.—a 107-g. difference between the first and the last groups fed two diets which contained the same amount of free gossypol (0.13%).

These results and similar ones obtained on 29 other samples of treated cottonseed meals (23) show that the residual toxicity of treated cottonseed meals cannot be explained solely on the basis of their analyzed free gossypol content. A similar conclusion had been made on the basis of toxicity studies on gossypol and cottonseed pigment glands in rats (21, 24, 25, 26).

There has been some confusion with respect to the use of the term "nutritive value of cottonseed." Many authors have used this designation to refer primarily to the biological value (and digestibility) of the protein in cottonseed. This depends not only on the presence of essential amino acids but also on the relative amounts that are absorbed by the digestive tract. Thus reference is really being made to the biological utilization of cottonseed protein, which can be determined by the effect on maintenance and growth of animals fed threshold levels of the cottonseed protein, with similar levels of standard proteins fed as controls. The use of protein levels much higher than 9-10% for such an evaluation would exceed the level imposing a stress upon the organism and obscure small differences in protein quality.

Other investigators have used the term "nutritive value of cottonseed" in the broad sense to include the net effect obtained from feeding high levels of cottonseed meal, with due consideration to protein quality and contributions by fat, carbohydrate, vitamins, minerals, etc., as well as to the growth-depressing effects from toxic factors. In these cases they are dealing with the algebraic sum of positive and negative effects, which is the over-all food value.

Summary

In a series of four tests in which three deliberately chosen toxic cottonseed meals were treated with aqueous solutions of salts and alkalies, it was found that the best detoxifying effect was obtained with sodium hydroxide, followed very closely by potassium and ammonium hydroxides.

Dry heat treatment alone did not detoxify, and mortality was high. Treatment with moisture plus heat gave partial detoxification. Of the 22 chemically-treated cottonseed meal samples tested, those treated with alkalies showed the best weight gains, the order of decreasing effectiveness being

$$NaOH > KOH > NH_4OH > Ca(OH)_2.$$

TABLE IV

Effect of Chemical Treatment of Cottonseed Meals on Their Free Gossypol Content and Their Body Weight Performance After High Level Feeding in Rats

Diet No.	Variable in diet	Free gossypol content of diet[a]	No. rats per group	Average starting weight	Average body weight after:							
					1 wk.	2 wks.	3 wks.	4 wks.	5 wks.	6 wks.	7 wks.	8 wks.
		%		*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*
4a	Untreated cottonseed meal C	0.41	5	55	42[b]	....	....	....	....	....	....	....
4b	C–moist heat-treated	0.13	5	55	86	121	152	176	202	207	195	196
4c	C–treated with 1% sodium hypochlorite	0.11	5	55	76	107	136	153	170	199	210	225
4d	C–treated with 1% calcium hydroxide	0.13	5	55	85	122	152	172	198	232	249	272
4e	C–treated with 1% trisodium phosphate	0.14	5	55	89	127	164	182	210	245	259	283
4f	C–treated with 1% ammonium hydroxide	0.12	5	55	91	128	163	185	212	252	268	294
4g	C–treated with 1% potassium hydroxide	0.09	5	55	91	129	166	187	223	257	275	296
4h	C–treated with 1% sodium hydroxide	0.13	5	55	89	131	165	191	223	261	278	303
4i	Stock diet control	0	5	55	92	130	168	203	238	271	293	315

[a] The free gossypol content of the cottonseed meals would be approximately 50% higher than that given for the diet since the cottonseed meal comprised ⅔ of the diet.
[b] Average of 2 rats; 5/5 dead within 8 days.

The residual toxicity of treated cottonseed meals cannot be explained on the basis of their free gossypol content as analyzed for meals with high values gave better growth performance than some with lower levels of free gossypol. There were also very marked differences in final body weight after 8 weeks of feeding six different treated cottonseed meal samples having practically the same free gossypol content.

REFERENCES

1. Voelker, A., cited by Crawford, A. C., J. Pharmacol. Exp. Therap., *1*, 519-48 (1910).
2. Böhm, R., cited by Macy, I. G., J. Dairy Sci., *4*, 250-65 (1921).
3. Editorial, Expt. Sta. Record, *22*, 501-7 (1910).
4. Crawford, A. C., J. Pharmacol. Exp. Therap., *1*, 519-48 (1910).
5. Withers, W. A., and Brewster, J. F., J. Biol. Chem., *15*, 161-6 (1913).
6. Longmore, J., J. Soc. Chem. Ind., *5*, 200-6 (1886).
7. Marchlewski, L., J. prakt. chem., *60*, 80-90 (1899).
8. Withers, W. A., and Carruth, F. E., Science, *41*, 324 (1915).
9. Withers, W. A., and Carruth, F. E., J. Agr. Res., *5*, 261-88 (1915).
10. Withers, W. A., and Carruth, F. E., J. Agr. Res., *12*, 83-102 (1918).
11. Carruth, F. E., J. Am. Chem. Soc., *40*, 647-63 (1918).
12. Withers, W. A., and Ray, B. J., Science, *36*, 31-2 (1912).
13. Withers, W. A., and Ray, B. J., Proc. 33rd Ann. Meeting, Soc. Prom. Agr. Sci., 19-21 (1913).
14. Withers, W. A., and Carruth, F. E., J. Biol. Chem., *32*, 245-57 (1917).
15. Osborne, T. B., and Mendel, L. B., J. Biol. Chem., *29*, 289-317 (1917).
16. Withers, W. A., and Carruth, F. E., J. Agr. Res., *14*, 425-52 (1918).
17. McGowan, J. P., and Crichton, A., Biochem. J., *18*, 273-82 (1924).
18. Curtis, R. S., Halverson, J. O., Williams, V. M., and Combs, S., J. Oil and Fat Ind., *3*, 170-2 (1926).
19. Halverson, J. O., and Sherwood, F. W., N. Carolina Agr. Expt. Sta. Tech. Bull., *39*, 5-158 (1930).
20. Eagle, E., Proc. Soc. Exper. Biol. and Med., *72*, 444-6 (1949).
21. Eagle, E., Hall, C. M., Castillon, L. E., and Miller, C. B., J. Am. Oil Chemists' Soc., *27*, 300-3 (1950).
22. Pons, W. A., and Guthrie, J. D., J. Am. Oil Chemists' Soc., *26*, 671-6 (1949).
23. Eagle, E., Bialek, H. F., Davies, D. L., and Bremer, J. W., to be published.
24. Eagle E., Castillon, L. E., Hall, C. M., and Boatner, C. H., Arch. Biochem., *18*, 271-7 (1948).
25. Eagle, E., and Bialek, H. F., Food Research, *15*, 232-6 (1950).
26. Eagle, E., and Bialek, H. F., Food Research, *17*, 543-9 (1952).

[Received January 4, 1954]

Determination of the Phosphorus Content of Lipids[1]

W. D. HARRIS, research engineer, and PRANJIVAN POPAT, graduate assistant, Texas Engineering Station, Texas A. & M. College System, College Station, Texas

NUMEROUS procedures, including gravimetric, titrimetric, and colorimetric, have been suggested for the determination of phosphorus in organic materials. Of these, colorimetric procedures are usually preferred for their rapidity and adaptability to microanalysis. A considerable amount of work has been done on colorimetric methods and in particular, on the "molybdenum blue" reaction suggested by Bell and Doisey (1) and developed by Fiske and Subbarrow (2), which appears to be the one most generally accepted. This method is extremely sensitive, and very small samples may be used without loss of accuracy.

However an examination of the literature on this subject reveals the existence of many variations and modifications of the molybdenum blue method. Experience with the most authoritative procedures (3, 4) has shown that color development is very critical and varies with the nature and the concentration of the reducing agent, acidity, time, and temperature. Because of this, the analyst has had to do considerable experimentation in order to develop a technique which would give reliable results.

In this work a number of molybdemum blue procedures were investigated, and a method was developed for the determination of phosphorus in cottonseed lipids which considerably reduces the effect of the variables. The method utilizes perchloric acid for sample digestion and p-methyl-amino-phenol sulfate (elon, metol) as a reducing agent for color development. Samples analyzed included crude and semi-refined cottonseed oil as well as phospholipid fractions separated from the oil.

Digestion of Lipid Samples

Perchloric acid has found considerable favor for the digestion of organic materials due to its high oxygen content and its speed of reaction. It has been recommended by King (5) and Frampton (6) for the digestion of lipids for phosphorus determination. A disadvantage however is its incompatibility with stannous chloride, which is an excellent reducing agent for the molybdenum blue reaction. Moreover perchloric acid is a hazardous chemical, and explosive conditions may occur unless proper care is taken.

The hazard may be reduced by a) the use of a minimum size sample which reduces the heat liberated, b) the use of sufficient acid to avoid an explosive mixture, and c) the use of a small amount of nitric acid to supplement the perchloric acid.

Since the sensitivity of the molybdenum blue method permits the use of small samples, a maximum of 0.1 gm. of oil and a minimum of 1.0 ml. of 72% perchloric acid have been found desirable. Larger samples are much more difficult to digest, and less perchloric acid will form an explosive mixture.

It was found that one drop of concentrated nitric acid would initiate the reaction at a lower temperature and, after the major action had subsided, two or three more drops helped to complete the oxidation. The digestion procedure involved four stages which were as follows: first, a foaming reaction involving the nitric acid; second, the major oxidation which liberates considerable heat; third; a clean-up with additional nitric acid; and fourth, a final clarification with strong heating.

It is most important that the final clarification be complete, otherwise erratic results will be obtained. Yet it is well to avoid excessive heating since some phosphoric acid may be vaporized.

The use of hydrogen peroxide has been suggested for clarification of the digest (7), but it was not found as satisfactory as the nitric acid. A test has shown that residual nitric acid will not interfere with the color reaction. However Greenberg (8) has found that the nitrite ion interferes when the phosphoric-molybdic complex is reduced with $SnCl_2$ and recom-

[1] The findings reported in this article were determined in research dealing with the separation and properties of the constituents of crude cottonseed oil by the use of liquid-liquid solvent extraction, which is being conducted by the Texas Engineering Experiment Station in cooperation with the Cotton Research Committee of Texas.