FEED ANALYSIS

Determination of Free Gossypol in Mixed Feeds

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As less toxic cottonseed meals are becoming commercially available methods for free gossypol assay in mixed feeds are needed for experimental use and for control. The method here proposed is based on the color developed in the reaction of phloroglucinol with gossypol in strong acid at room temperature. The extracting solvent excludes many substances that react with phloroglucinol. Interference by reactive substances brought in by the solvent is minimized by carrying out the reaction at room temperature.

IN ATTEMPTS TO FOLLOW CHANGES in free gossypol in mixed feeds, spectrophotometric methods applicable to cottonseed meal gave obviously erroneous results when applied to prepared rations containing the same meals. Alfalfa meal, yellow corn, barley, oats, and wheat contain substances which either react with the reagent or absorb at the wave length at which measurements are made in the case of the *p*-anisidine method (*5*). Moreover, extracts prepared by the latter method were cloudy when the sample contained yellow corn or oats and no satisfactory color measurement could be made. After this experience, extracting solvents and gossypol reagents which might be applied to the determination of free gossypol in mixed feeds were investigated. As a result 2-butanone-water azeotrope (*6*) containing aniline was developed as the solvent for the extraction and phloroglucinol (*1*) was adopted as the gossypol reagent.

Absorption curves of the red solution developed from the reaction of phloroglucinol with pure gossypol are shown in Figure 1. The one peak at 550 mμ is evident at three gossypol concentrations. The red color is apparent at 0.006 mg. of gossypol per 25 ml.

The term "free gossypol," apparently introduced by Clark, Nelson, and Jones (*3*), has come into use with little organized experimental background of either biological or chemical nature to support it; consequently it is not well defined even by an empirical standard. As usually understood, free gossypol refers to that portion of the gossypol or gossypol derivatives in cottonseed meal which may be toxic to animals. In the absence of exact information as to this relationship, the term can be applied only to the amount of gossypol brought into solution by a specified solvent under specified conditions.

Results of extraction studies with butanone-water azeotrope containing aniline show that a very soluble form of gossypol is extracted from a screw-pressed meal containing a low level of this fraction in about 10 minutes (Figure 2). A similar rapid extraction is shown from a hydraulic meal containing still more of the same soluble form and also from a solvent meal with a very high level of this fraction. The latter solvent meal was prepared in the laboratory in order to obtain a product with a very high free gossypol content. It seems reasonable to assume that free gossypol should be very soluble in appropriate solvents and should come into solution readily. Therefore, the very soluble gossypol extracted in the first 10 minutes under the conditions out-

Figure 1. Absorption curves of gossypol solutions

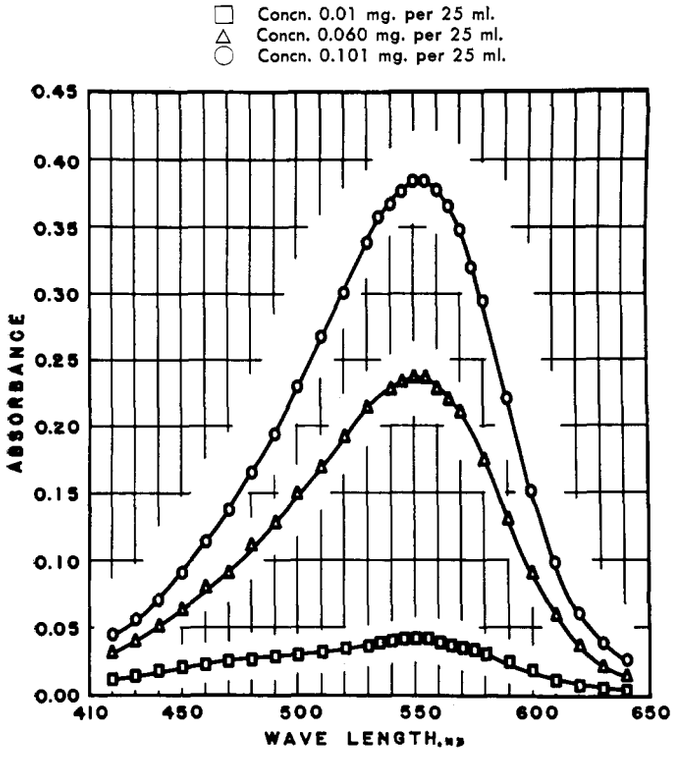
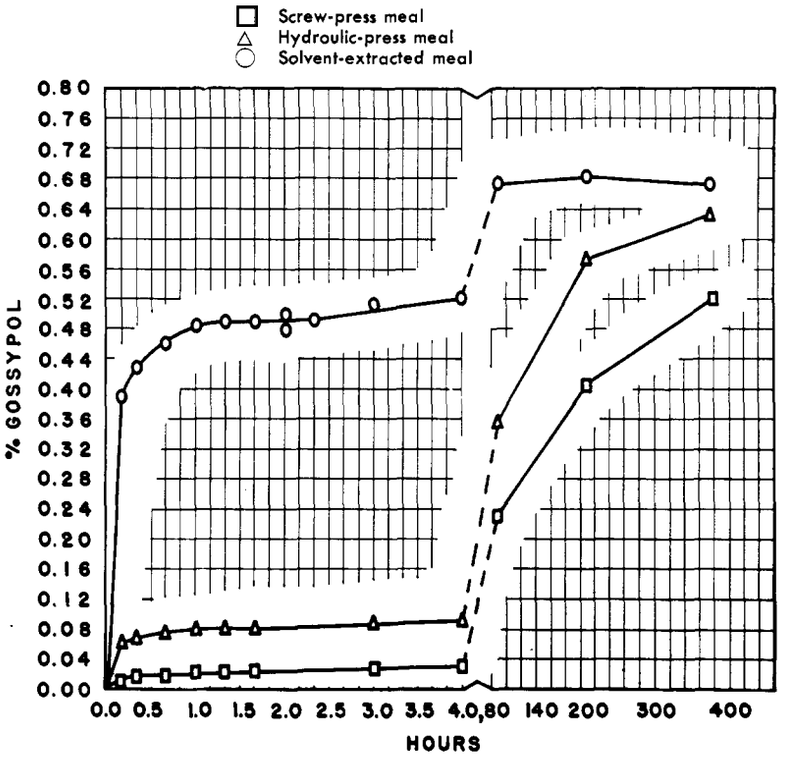


Figure 2. Rate of extraction of gossypol from cottonseed meals



lined here should include the free or toxic substances in these meals. This premise is supported by the rather sharp break in these extraction curves after about 10 minutes. The second break after the 4-hour period is due to the change in the time scale. Actually, the curves show that after the initially rapid extraction there is a gradual increase in the gossypol extracted up to 380 hours. This slowly extracted gossypol must be bound in a manner very different from that extracted so readily in the first few minutes. In fact, further investigation may show that free gossypol is defined by this 10-minute extraction limit. For the present, however, because of the uncertainties in the meaning of these values, the method here reported is based on 1-hour shaking extraction of the meals with butanone-water azeotrope containing aniline as the solvent. Preliminary results on cottonseed meals and feeds of low free gossypol content showed no difference in static or shaking extraction. As Figure 2 shows, 1-hour extraction places the free gossypol well on the plateau of the extraction curves. Yet there is little practical difference, as far as toxicity estimates are concerned, in the amount of gossypol extracted from meals of the common types, at either 10 minutes or 1 hour.

Apparatus

Coleman Model 11 Universal spectrophotometer.

Reagents

Ethyl alcohol, 95%.

Isopropyl alcohol, 98 to 99%.

Hydrochloric acid, concentrated C.P.

Phloroglucinol solution, 0.4 gram per 100 ml. of ethyl alcohol. Purify phloroglucinol by boiling a saturated water solution with Darco for 5 minutes, filtering, cooling, and then filtering off and drying the resulting crystals.

Aniline, redistilled from zinc (*4*).

Butanone-water-azeotrope–aniline solution. Distill a mixture of 2-butanone and water (10:1) and collect the azeotrope boiling at 73° C. Dissolve 5 ml. of redistilled aniline in azeotrope and dilute to 1000 ml. Use 50 ml. for extractions.

Procedure

Standard Gossypol Curve Dissolve 0.038 gram of pure gossypol in butanone-water-azeotrope–aniline solution and make to 250 ml. Take 2-, 5-, 10-, 15-, 20-, 25-, 30-, 35-, 40-, and 45-ml. aliquots and dilute to 50 ml. Pipet duplicate 2-ml. aliquots of each of these into 25-ml. volumetric flasks, and add 1 ml. of phloroglucinol reagent and 2 ml. of concentrated hydrochloric acid. Allow to stand 20 minutes at room temperature for full color development and dilute to volume with ethyl alcohol or isopropyl alcohol. Use 1 ml. of ethyl alcohol for the blank in place of the phloroglucinol reagent, dilute immediately after the addition of 2 ml. of concentrated hydrochloric acid, and make to the mark after 20 minutes. Measure the absorption at 550 mμ in a 1.0-cm. cell in a spectrophotometer, reading the sample against the blank.

The standard solutions up to and including a concentration of 0.182 mg. per 25 ml. are stable in ethyl alcohol for 48 hours in darkness. Prepare a standard absorbance curve from the values, as illustrated in Figure 3. It is advisable to work in the range 0.02 to 0.10 mg. per 25 ml. for best results.

Determination Of Gossypol in Cottonseed Meal And Feedstuffs Weigh 0.2 to 2.0 grams of cottonseed meal or 5- to 10-gram samples of mixed feed, ground to pass a 1-mm. screen, into 250-ml. Erlenmeyer flasks, add 50 ml. of butanone-water-azeotrope–aniline solution, stopper, and shake meals 1 hour and feeds 2 hours on a mechanical shaker. Filter the extract, transfer 2-ml. aliquots to 25-ml. volumetric flasks, and proceed as in the preparation of the standard curve. The filtered extracts of meals and feeds must be assayed within 1 hour for accurate results; otherwise the free gossypol increases (up to 60 hours) owing to breakdown of soluble combined forms. Calculate the percentage of gossypol in the sample from the standard curve.

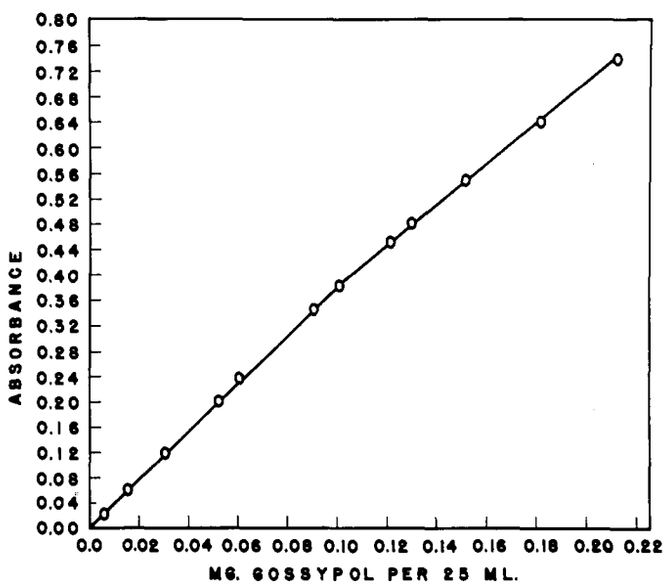


Figure 3. Absorbance - concentration curve

Table I. Free Gossypol in Cottonseed Meal

Meal	Method: *p*-Anisidine	Method: Phloroglucinol
	Free gossypol, %	
Screw-pressed	0.013	0.018
Hydraulic 2	0.097	0.093
Hydraulic 3	0.080	0.082
Solvent-extracted 1	0.390	0.440
Solvent-extracted 2	0.444	0.448

Results

Extracts from cottonseed meal prepared by this method show absorption curves similar to those for pure gossypol (Figure 4). The curve is characteristic of meals of three representative types.

The phloroglucinol and *p*-anisidine methods (*5*) are compared in Table I, where each value is the mean of two or more determinations. The evident agreement between these two methods on these meals is significant because the background of the *p*-anisidine method (*5*) in animal experiments shows that it is useful in differentiating toxic and nontoxic meals.

Experience has shown that many substances in common feed mixtures may interfere in the spectrophotometric determination of gossypol. The absorption curve of an extract prepared by the above method from a mixture of 20% hydraulic meal, 71% wheat, 8% alfalfa meal, and 1% mineral mix is shown in Figure 5. Substitution of corn, oats, or barley for the wheat had no appreciable effect. The curve is typical of the gossypol-phloroglucinol reaction, except that the ascending leg is slightly displaced. The magnitude of this displacement appears to be related to the age of the feed, but there is no detectable influence on peak absorption at 550 mμ.

In the determination of the free gossypol in mixed feeds, it was found that a 2-hour extraction on a mechanical shaker is necessary if the volume of solvent is the same as that used on meals. The size of sample necessary probably accounts for the slower extraction from mixed feeds.

Results by this method on feed mixtures prepared from meals of various types and different carbohydrate sources

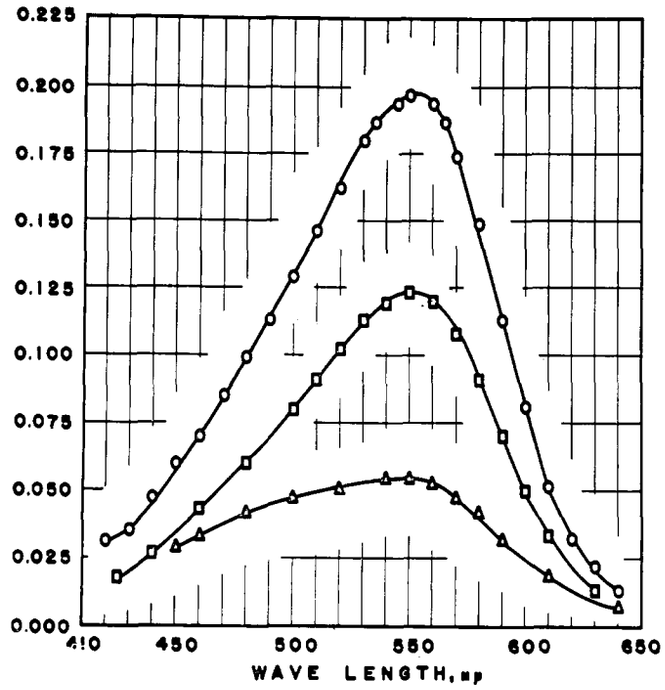


Figure 4. Absorption curves of cottonseed meal extracts

△ Screw-press meal
□ Hydraulic-press meal
○ Salvent-extracted meal

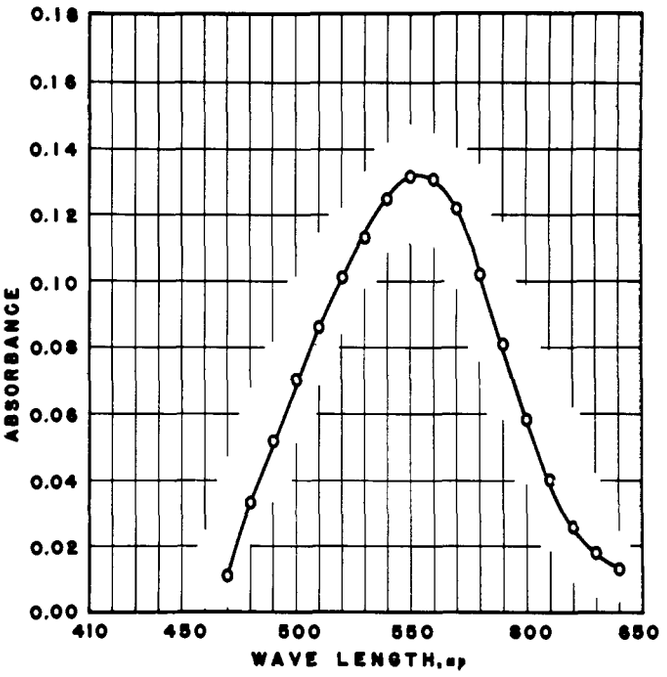


Figure 5. Absorption curve of mixed feed extract

are illustrated in Table II. Each meal constituted 20% of a mix and the carbohydrate source indicated at the top of the column 71%. Each mixture contained 8% alfalfa meal and 1% mineral mixture. Percentages of free gossypol in the mixtures have been multiplied by 5, because the free gossypol in the parent meal affords the only standard of reference. All results are the mean of two or more determinations and when more than four determinations were made on a sample, standard deviations are reported. The well-known tendency of free gossypol to decrease on storage (*2*), as shown in the case of the screw-pressed meal, made it necessary to determine free gossypol soon after mixing if valid comparisons with the meal from which it was derived are to be made.

Discussion

The formulations in Table II include the principal components of the usual concentrated mixed feeds. The amount of cottonseed meal in them is at about the upper limit of practical use. Free gossypol levels are in the range significant in nonruminant animal feeding and the results show that the method gives repeatable and accurate results on mixed feeds at these levels.

Although all results are reported here as free gossypol, other gossypol-like derivatives may be included, as at this time no distinction can be made between the various forms which react with phloroglucinol. The fact that results on meals are in substantial agreement with those obtained by the *p*-anisidine method (*5*) indicates that the phloroglucinol method can differentiate between toxic and nontoxic feeds.

Glucose and other reducing sugars under the conditions outlined do not interfere in the determination. The gossypol apparently reacts preferentially with the aniline in the extracting solvent and thus competing reactions with soluble feed substances are avoided. Aniline does not interfere with the phloroglucinol reaction which follows the extraction. The method is not applicable to cottonseed meals treated with aniline, because the dianilinogossypol would be determined as free gossypol.

Literature Cited

(1) Bailey, A. E., "Cottonseed and Cottonseed Products," pp. 481–2, New York, Interscience Publishers, 1948.
(2) Castillion, L. E., Hall, C. M., O'Connor, R. T., and Miller, C. B., *J. Am. Oil Chemists' Soc.*, **26**, 655–9 (1949).
(3) Clark, E. P., Nelson, E. M., and Jones, D. B., *Oil & Fat Inds.*, **6**, No. 7, 15–19 (1929).
(4) Fieser, L. F., "Experiments in Organic Chemistry," 2nd ed., p. 150, Boston, D. C. Heath and Co., 1941.
(5) Pons, W. A., Jr., and Guthrie, J. D., *J. Am. Oil Chemists' Soc.*, **26**, 671–6 (1949).
(6) Pons, W. A., Jr., Hoffpauir, C. L., and O'Connor, R. T., *Ibid.*, **27**, 390 (1950).

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Table II. Free Gossypol in Cottonseed Meals and in Mixed Feeds Prepared from Them

	Gossypol in Cottonseed Meal, %	Gossypol in Mixed Feeds, % × 5 — Carbohydrate Source			
		Yellow corn	Oats	Barley	Wheat
Hydraulic M1	0.0923	0.0935[a]	...	...	...
Hydraulic M3	0.0918	0.0862	0.0888	0.0864	0.0860
σ	0.0012	0.0012	0.0019	0.0010	0.0007
Hydraulic B1	0.0795	0.0796	...	0.0805	0.0785
Screw-pressed A	0.0202	0.0203	0.0202	...	...
σ		0.0014			
Screw-pressed B[b]	0.0171	0.0170	0.0198	0.0215	0.0180
σ	0.0004				
Screw-pressed C[c]	0.0146	0.0141	0.0150	0.0156	0.0159
σ	0.0004	0.0008	0.0001	0.0006	0.0006
Solvent 39	0.0132	0.0130	...	...	...
Solvent 39A	0.0343	0.0300	...	...	...

[a] 10% blackstrap molasses substituted for part of corn.
[b] Screw-pressed A after 4 months' storage.
[c] Screw-pressed A after 6 months' storage.