trucks as well as tank cars, the title and text should so indicate.

All of these minor changes will be made by the editor of Methods with the assistance and approval of the chairman of the F.A.C.

Depending upon the length of the pipe line, through which oil is being pumped, variable pressure will develop which will affect the volume of the sample taken so that, under certain conditions, more than a 50-gal. sample may result. The Uniform Methods Committee requests the Fat Analysis Committee to investigate the following matters and recommend appropriate action to be taken in revising this method, if possible, before the next Fall Meeting.

1. Specify, within reasonable limits, the total volume of sample to be drawn, *e.g.*, 25 to 45 gal.
2. Devise some method for controlling the flow of oil through the bleeder line so as to assure a volume of oil in the drum within the limits specified. The U.M.C. suggests possible use of a number of reducing nozzles, each with a fixed outlet orifice, to be screwed on the discharge end of the bleeder line. These devices will apply only to reducing the volume of oil drawn during a normal pumping.

3. *Glycerine Analysis Committee, W. D. Pohle, chairman*

a) The Glycerine Analysis Committee recommends advancement from Tentative to Official status of the following methods:

1. Total and Organic Residue at 175°C., Ea 3-56.
2. Moisture by Karl Fischer Method, Ea 8-56.

These methods have been shown to be reliable, and the Uniform Methods Committee approves their advancement from Tentative to Official status. Adopted.

b) The Glycerine Analysis Committee has recommended additions to the following methods to show the degree of precision which can be expected in their use:

Ea 3-56; Ea 6-51; Ea 7-50; Ea 8-56; Ca 14-56, and Da 23-56.

The Fat Analysis Committee likewise recommends similar additions, for the same purpose, to Method Cd 11-57. The Uniform Methods Committee approves in principle these valuable contributions to the usefulness of these methods but, in the interests of uniformity and the best possible presentation of this information, requests that before the Fall Meeting these data be reviewed by and with our Statistical Committee and that a standard format for such presentation be formulated. In the course of this study a decision should be made on whether to use such terms as "inter" for "between," and "intra" for "in the same" laboratories. In general, data upon which conclusions are based should be submitted with the recommendation, *e.g.*, number of samples, number of laboratories, number of analysts, number of analyses, and when performed.

The Uniform Methods Committee is requesting the Statistical Committee to assist our technical committees by prescribing a standard format for expressing precision to be expected. Other A.O.C.S. Methods in which this information is presently shown should be scrutinized and, if possible, brought within the standard format. The Statistical Committee furthermore is requested to prepare a new section for A.O.C.S. Methods on a uniform method for determining the precision of an analytical method and a standard format for its expression. If possible, this project should be completed during the coming year.

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Quantitative Determination of Traces of Free Gossypol in Fats, Oils, and Fatty Acids by Paper Chromatography[1]

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COTTONSEED and cottonseed products contain the pigment gossypol. Trace amounts of this pigment, when fed to hens in rations, cause olive egg yolk discoloration. This discoloration is associated with free or labile-bound gossypol. Analytical methods which determine free or loosely bound gossypol in any such cottonseed product are of major importance.

The investigations by Pons and co-workers (8, 9) have led to the adoption of a tentative method for the analysis of gossypol in oils by the American Oil Chemists' Society (12). In this method the oil is dissolved in (4:6) hexane-isopropyl alcohol solution and reacted with p-anisidine to develop a colored complex, which is measured quantitatively by spectrophotometry. The lower limit of detection by this method is about 100 p.p.m. Since other pigments and aldehydes give similar color reactions, this method does not differentiate between gossypol and these other substances. Therefore the determined values are frequently higher than the actual gossypol content.

Smaller amounts of free gossypol (50 p.p.m.) are detected when phloroglucinol in acid solution is used for the color development (10). Since phloroglucinol is not a specific reagent for gossypol, other compounds in the sample give similar color reactions. Therefore the method is not specific. In addition, these colorimetric methods cannot be used to measure small amounts of gossypol in highly colored samples because of background absorption.

Grau and co-workers (2) developed a biological method, which measures both free and labile forms of gossypol. In this method the sample is fed to hens in a ration over a period of days. The egg yolks from the hens are extracted with acetone and then with 3:1 hexane-acetone. The absorbance of the latter extract at 400 mμ is proportional to the gossypol fed. Although this method is very sensitive, compounds other than gossypol will cause egg yolk discoloration. Therefore the method is not specific In addition, the method suffers from being time-consuming.

Because these methods lack specificity and because they do not detect trace amounts of gossypol, an improved analytical method was required. In the present work a specific and sensitive method for the determination of as little as 10 p.p.m. free gossypol was developed. The method is based on the concentration of gossypol by extraction and quantitative paper chromatography of the extract.

To separate gossypol from the bulk of the sample, a preferential extraction is necessary. Dimethylformamide-water solution extracts gossypol quantitatively from fats and fatty acids and does not form emulsions

[1] Presented at the fall meeting of the American Oil Chemists' Society, Cincinnati, O., September 30 to October 2, 1957.

with the fatty layer. When chromatographed in the heptane-chloroform-acetic acid solvent, the extracted material does not migrate on the chromatogram. Extraneous materials occlude the gossypol, which therefore cannot migrate. To remove materials interfering with the paper chromatography, the aqueous dimethylformamide solution is diluted with water and back-extracted with a 9:1 heptane-chloroform solution. This extract contains the gossypol and only small amounts of extraneous material and is applicable to highly colored glyceride and fatty acid samples.

The solvent system of heptane-chloroform-acetic acid was used for most of the paper chromatograms. Gossypol has an R_f value of 0.6 in this solvent. If desired, the R_f value of gossypol can be changed by changing the concentration of the components in the solvent. Increasing the acetic acid concentration in the solvent system raises the R_f. Higher concentration of heptane in the solvent decreases the R_f.

Effect of Heptane-Chloroform-Acetic Acid Ratios on R_f Value

Ratio	Comment
80:10:5	Optimal ratio, is presently used
80:10:10	Higher R_f than 80:10:5 ratio
90:10:5	Lower R_f
90:10:10	Slightly lower R_f

Gossypol can be visualized on the developed chromatogram with antimony trichloride (1, 3) or with phloroglucinol (10). Both sprays give a reddish color with gossypol. The limit of detection with antimony trichloride is 2 micrograms and with phloroglucinol 0.5 microgram. Phloroglucinol also gives a more stable color and is therefore preferred.

A few cottonseed fatty acid samples, analyzed by the proposed method, showed a red streak on the paper chromatogram, which was very close to the point of application. To find out whether or not this was gossypol which had been retained at the starting point, the concentration of acetic acid in the solvent was increased. In the changed solvent, gossypol had a higher R_f value (0.8), and the unknown had formed a red spot with an R_f of 0.2. This established that the unknown was different from gossypol.

Method

Apparatus. The chromatographic container is a cylindrical jar, 10 x 18 in. and covered by a glass plate. A sheet of Whatman No. 1 paper (16.25 x 22.5 in.) is placed on the inner wall of the jar. The solvent is placed on the bottom of the jar and swirled around so that the entire paper on the inner wall is soaked with the solvent. To equilibrate the chromatographic container, this is done each time before a chromatogram is started. Fresh solvent is used every three days. Whatman 3MM (sheets of 16.25 x 22.5 in.), a thick paper, is used for the paper chomatographic separation.

Reagents. Chromatographically pure gossypol is used as reference material. Since it is not obtainable commercially, it is prepared according to Pons (5) or King (4). The n-heptane (Matheson, Coleman, and Bell) has a b.p. of 98–99°C., and the N,N-dimethylformamide (Matheson, Coleman, and Bell) a b.p. of 152–154°C. All other chemicals used are reagent grade. The chromatographic solvent consists of 80 parts heptane, 20 parts chloroform, and 5 parts acetic acid. The phloroglucinol indicator spray is made up of 2 g. of phloroglucinol (m.p. 217–218°C., Eastman, Catalog 40) dissolved in 10 ml. of 95% ethanol and 5 ml. of concentrated hydrochloric acid. The solution is freshly prepared before use.

Procedure. Ten grams of the sample are dissolved in about 100 ml. of heptane and extracted twice with 25-ml. portions of 2:1 dimethylformamide-water solution. The lower layers are combined and diluted with 150 ml. of distilled water. This mixture is extracted three times, each with 50 ml. 9:1 heptane-chloroform solution. Since the separation of the two layers is difficult, it is centrifuged and the upper layer is removed after each centrifugation with a siphon. The combined upper layers are dried with anhydrous sodium sulfate, filtered, and concentrated to 5-ml. volume. If more than 1% free gossypol is present in the sample, the extract is concentrated to 10 ml. Unless chromatographed within 1 to 2 hrs., the concentrated solution is kept under refrigeration.

Gossypol standards in the range of 0.5 to 10.0 micrograms and the unknowns are spotted 2 in. from the long edge on a sheet of chromatography paper. Depending on the expected amount of free gossypol in the sample, 50 to 1 lambdas of the concentrate are used for the range of 10 to 10,000 p.p.m. (1%). Thus for 10 p.p.m., 50 lambdas are used, and for 10,000 p.p.m., 1 lambda. For gossypol concentrations between these values proportional aliquots are chromatographed.

The chromatogram is formed into a cylinder, stapled together, and chromatographed in the equilibrated chromatographic container by the ascending technique. After chromatographing for 1.5 hrs., the chromatogram is dried at room temperature and sprayed thoroughly with phloroglucinol solution. The red color develops after 1 to 5 min. at room temperature and is stable for one day. Free gossypol in the unknown is measured by visual comparison with the standards. The concentration of free gossypol in the sample is calculated.

$$\text{p.p.m.} = 2{,}000\ m\ \gamma/g\ \lambda$$

m = final volume of concentrated sample
g = grams of sample
γ = micrograms of free gossypol estimated on the chromatogram
λ = microliters of concentrated sample spotted on the chromatogram

Results

To evaluate the method, pure gossypol was added to tallow and analyzed by the complete procedure. The results are shown in Table I. The recovery was consistently 50%, and therefore a calibration factor of 2.0 was required. This results from the partitioning of gossypol between the aqueous dimethylformamide and the chloroform-heptane solution during the second series of extraction. This chloroform-heptane extraction is essential to remove substances which interfere with the migration of gossypol on

TABLE I

Evaluation of Extraction Procedure

Gossypol—added	Gossypol—extracted	Calibration factor
(p.p.m.)	*(p.p.m.)*	
100	50	2.0
50	25	2.0
50	24	2.1

the paper chromatogram. The conditions specified in the method give reproducible results. Other tests demonstrated that the aqueous dimethylformamide extraction and the paper chromatography are quantitative. Since the recoveries are consistent and very reproducible, a factor of 2.0 is used to calculate the amount present.

Recovery of Gossypol. Known amounts of gossypol were added at various levels to representative samples, such as fat and fatty acid samples, containing no free gossypol and a fatty acid sample containing free gossypol. The samples were analyzed by the proposed method. To eliminate prejudice and to increase the precision, the visual judgments were done separately by three to four persons. Each value is the average of three to four determinations. Data on the recovery of the method are shown in Table II. The average recovery was 97%.

TABLE II

Recovery of Gossypol

Sample	Number analyzed	Gossypol			Recovery
		Present	Added	Found	
		(p.p.m.)	(p.p.m.)	(p.p.m.)	(%)
Fat	3	0	100	95	94
Fat	4	0	50	49	98
Fat	2	0	10	11.6	116
Fatty acid	1	0	100	80	80
Fatty acid	1	0	50	44	88
Fatty acid	1	0	10	10	100
Fatty acid	1	20	50	63	86

Reproducibility of the Method. Samples of gossypol alone, tallow with added gossypol, and an acidulated cottonseed foots, containing an unknown amount of free gossypol, were analyzed by this method. Each value represents the average of three to four determinations. The standard deviation from the mean for all samples examined was ± 3 p.p.m. Data on the reproducibility of the method are presented in Table III.

TABLE III

Reproducibility of the Method

Sample	Number analyzed	Gossypol		Standard deviation
		Added	Found	
		(p.p.m.)	(p.p.m.)	(p.p.m.)
Gossypol solution	4	40	42.5	3.1
Fat	8	100	97.5	1.6
Fat	4	50	50	0
Unknown	6	0	87	2.0

Discussion

The method, with slight modifications, was also applied to meals. One gram of cottonseed meal was extracted with 50 ml. of 7:3 acetone-water solution (6, 11) and filtered. The filtrate was diluted with 200 ml. of distilled water, acidified with 2 ml. of concentrated hydrochloric acid, and extracted twice with 50-ml. portions of chloroform. The chloroform layer was dried with anhydrous sodium sulfate, concentrated to a suitable volume, and chromatographed for free gossypol. The recovery is quantitative, and therefore no calibration factor is required.

Total gossypol (7, 8) can also be determined by this method. The sample is hydrolyzed, then analyzed by the given procedure.

Gossypol derivatives, such as dianilino- and 2,3-dimethyl-butadiene-gossypol, were chromatographed in the heptane-chloroform-acetic acid solvent. The derivatives have different R_f values, and they give different color reactions with phloroglucinol. Therefore this solvent can be used for the differentiation between gossypol and its derivatives.

Summary

A quantitative method for the determination of traces of free gossypol in oils and fatty acids was developed. The method is based on the concentration of gossypol by extraction and quantitative paper chromatography of the extract. The method is specific for free gossypol and is not subject to interferences. The new method is both accurate and reproducible. The lower limit of detection is 10 p.p.m. The method is intended primarily for p.p.m. levels but is suitable for all concentrations. With slight modifications the method is applicable to meals.

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Erratum

THROUGH AN OVERSIGHT the References were omitted for the paper entitled "Solubility of Cottonseed Proteins in Hydrochloric Acid," by Mann, Rubins, Carney, and Frampton, which was published in the May 1958 issue of the Journal of the American Oil Chemists' Society (*35*, 244–246). The bibliography follows.

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