Modified Procedures for Determination of Free and Total Gossypol Pigments

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Abstract

The inclusion of hydrochloric acid and thiourea in the gossypol blanks in present official AOCS methods for free and total gossypol produced equivalent absorbances identical to those obtained in the AOCS procedure for gossypol in oils. The treatment relieved the darkening of reagent blanks and allowed the same calibration data to be used for all three methods. Aniline was the reagent in each.

Introduction

PRESENT OFFICIAL AOCS METHODS for the determination of free gossypol pigments, total gossypol, and gossypol in oils (1) require separate calibration procedures. In these standardizations, series of duplicate aliquots of standard gossypol solutions are pipetted into volumetric flasks. In each case one of these sets (gossypol blanks) is diluted to volume with the appropriate solvent, and the absorbances are determined against the solvent as reference. The other sets are heated, e.g., with aniline, to develop the dianilinogossypol chromophore. After dilution to volume, the absorbances are determined against heated reagent blanks as reference. Subtraction of the absorbance of the gossypol blanks from those heated with aniline provides the net absorbance owing to the reaction of gossypol. This technique, when applied in a like manner to sample extracts, allows correction for the absorbance of the nongossypol pigments in extracts of cottonseed products.

It is noted, in the three standardization techniques, that the absorbance of equivalent weights of gossypol which are reacted with aniline differ only slightly, but the absorbances of the gossypol blanks are quite different for each procedure. Moreover the reagent blanks, particularly for the total gossypol procedure, are frequently high and variable. These results indicate solvent effects on the absorbance of gossypol in the gossypol blanks of each procedure and probably tautomeric effects because of the presence of acetic acid in these blanks in the total gossypol procedure.

The present work was undertaken to study the causes of the variable absorbances of the gossypol blanks and of the high reagent blanks in the total gossypol method, also to develop modified procedures which would incorporate a single calibration for all three methods.

Experimental Procedure

Reagent Blanks

Reagent blanks in the free gossypol or gossypol in oils methods are usually low whereas those in the total gossypol procedure are frequently high and variable. High blanks in the latter procedure are probably caused by the oxidation of the reaction product of aniline with methyl ethyl ketone. Hoffpauir and Pons (2) and Pons et al. (3) have suggested the incorporation of the antioxidant, thiourea, in a *p*-anisidine reagent as a means of preventing excessive reagent blanks in total gossypol assays. Accordingly 21 identical reagent blank aliquots, from the same solution, were heated with aniline. Two drops (0.10 ml) of 10% aqueous thiourea were added to three of these aliquots prior to heating. The blanks, containing thiourea, had optical densities between 0.007 and 0.009; the others varied between 0.013 and 0.031. In other cases, reductions in the absorbances of these blanks ranged from 0.039 to 0.009, 0.033 to 0.003, and 0.015 to 0.006.

The darkening of these reagent blanks was not compensated by an equivalent darkening in the sample aliquots. The addition of thiourea to these aliquots, prior to heating with aniline, had very little effect, probably because of the presence of gossypol and other antioxidants in cottonseed extracts.

TABLE I

Comparison of the Optical Densities of Gossypol Blanks in Calibrations With and Without Hydrochloric Acid and Thiourea

Treatment	Optical Densities[a] The mg gossypol per 25-ml solution				
	0.020	0.040	0.060	0.100	0.200
Standard free[b]	0.008	0.015	0.022	0.029	0.053
Free + HCl[c]	0.005	0.005	0.005	0.011	0.019
Free + HCl + TU[d]	0.000	0.001	0.005	0.006	0.013
Standard total[e]	.....	.....	0.016	0.025	0.034
Total + HCl + TU[f]	0.000	0.003	0.004	0.006	0.013
Standard oil[g]	0.003	0.004	0.005	0.007	0.012

[a] All measurements made at 442 mμ with a Beckman DU spectrophotometer.
[b] Standard free gossypol calibration.
[c] As in Footnote 2, with one drop of dilute hydrochloric acid.
[d] As in Footnote 2, with one drop of dilute hydrochloric acid and two drops of thiourea.
[e] Standard total gossypol calibration.
[f] As in Footnote 5, with one drop of dilute hydrochloric acid and two drops of thiourea.
[g] Standard calibration for gossypol in oil.

Gossypol Blanks. In calibration procedures for the three gossypol methods the conditions under which the absorbance of the diluted aliquots without aniline (gossypol blanks) are measured involve different solvent systems and, in the total procedure, the presence of acetic acid. Thus, for equivalent gossypol concentrations, different values are obtained for the gossypol blank in each procedure (Table I). When one drop (0.05 ml) of 1.2N hydrochloric acid and two drops (0.10 ml) of 10% aqueous thiourea were added to comparable gossypol blanks in the free and total gossypol procedures (Table I), the absorbances of the gossypol blanks for all three procedures were reduced to comparable levels. Acid treatment was not required for the gossypol blanks for the gossypol in oil procedure. In the absence of thiourea the acidified blanks apparently tended to darken slightly, e.g., in free gossypol calibrations, although this darkening has not been noted in assays. It should also be noted that aeration did not alter the optical density of a free gossypol extract of cottonseed meats. However aeration of a gossypol solution for the free gossypol calibration caused a significant increase in its optical density, but the addition of acid decreased the optical density of the aerated and nonaerated aliquots to the same value.

Aniline Reaction

In the normal AOCS calibration procedures the absorbances of aliquots of equivalent gossypol concentrations heated with aniline are equal for both the total gossypol and gossypol in oils methods as shown in Table II. The absorbances of comparable aliquots for the official free gossypol procedure are slightly lower but are increased to comparable values by the addition of acetic acid prior to

TABLE II

Comparison of the Optical Densities of Aliquots Heated with Aniline in Gossypol Calibrations

Treatment	Optical Densities[a] The mg gossypol per 25-ml solution				
	0.020	0.040	0.060	0.100	0.200
Free gossypol[b]	0.056	0.119	0.184	0.311	0.629
Free + acetic acid[c]	0.063	0.129	0.193	0.324	0.639
Total gossypol[d]	0.064	0.132	0.195	0.327	0.645
Gossypol in oil[e]	0.070	0.134	0.196	0.326	0.652

[a] All optical densities read at 442 mμ with reference to reagent blanks except Footnote 5 read versus solvent.
[b] Standard free gossypol calibration.
[c] As in Footnote 2, with one drop of 15% acetic acid added.
[d] Standard total gossypol calibration.
[e] Gossypol in oil calibration.

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or after reaction with aniline (Table II). Although not shown, similar results were obtained when dilute hydrochloric acid and thiourea were added to the aliquots prior to heating with aniline. When free gossypol assays were performed on cottonseed meats, the addition of acetic acid, prior to heating with aniline, did not alter the optical density. This suggests that a slight error would result in free gossypol assays if the slightly lower optical densities of the aliquots heated with aniline in the present free gossypol calibration were used.

Comparative Assays

Comparative free and total gossypol analyses on four samples of cottonseed meals were conducted by using the present official AOCS methods (1) and the modified procedure in which one drop (0.05 ml) of 1.2N HCl was added to the gossypol blanks and two drops (0.10 ml) of 10% aqueous thiourea were added to the reagent blanks prior to heating with aniline. In the calibrations for these modified methods both acid and thiourea were added to each gossypol blank.

The data, set forth in Table III, indicate that the modified method for free gossypol in these meals gives results from 0.8% to 8.2% lower than those of the official method. However the modified method for total gossypol gives results averaging 4.6% higher than those by the official method. It is of interest that Pons et al. (3) have also reported gossypol values averaging 4.5% higher than those obtained by the official AOCS gossypol method by using thiourea in their *p*-anisidine reagent.

Discussion

The addition of a drop of dilute hydrochloric acid to each gossypol blank aliquot for free and total gossypol is useful. It reduces the optical density of these aliquots in calibrations to the same values as those obtained in the calibration for gossypol in oil. Two drops of 10% aqueous thiourea were also added, along with the acid in these calibrations, to prevent darkening prior to reading their optical densities. The addition of thiourea to gossypol blanks in free and total gossypol assays has not been necessary. Because it is added to the reagent blank in these assays, it might be advisable to add it to the samples as well for the most precise work.

TABLE III

Comparison of Assays by the Official and Modified Methods of Gossypol Analysis

Standard Procedure			Modified Procedure			
			Free Gossypol			
Aniline OD	Blank OD	Gossypol %[a]	Aniline OD	Blank OD	Gossypol %[b]	Difference %[c]
0.175	0.030	0.131	0.177	0.012	0.130	99.2
0.419	0.048	0.335	0.423	0.020	0.317	94.6
0.383	0.041	0.310	0.380	0.018	0.285	91.4
0.362	0.039	0.294	0.361	0.017	0.270	91.8
			Total Gossypol			
0.281	0.025	0.810	0.282	0.012	0.849	104.8
0.326	0.024	0.956	0.328	0.011	0.997	104.3
0.441	0.034	1.29	0.445	0.015	1.35	104.7
0.282	0.021	0.826	0.286	0.011	0.865	104.7

[a] The % gossypol calculated from the best data for free gossypol calibration by the standard method.
[b] The % gossypol calculated from the results of the modified calibration by using the total gossypol procedure with hydrochloric acid and thiourea in the gossypol blanks.
[c] Amount of gossypol measured by the modified method as a percentage of that measured by the standard method.

The addition of acid to the aliquots to be heated with aniline has not been necessary either. However it might be advisable to add acid since, in some samples, it might alter the optical density because of the nongossypol pigments present. Also oxidized gossypol might give a lower optical density on heating with aniline if acid is not present, according to the present official calibration method for free gossypol. If acid is added, two drops of aqueous thiourea should also be added prior to heating to avoid possible oxidation of the aniline.

The addition of two drops of aqueous thiourea, prior to heating with aniline, eliminates undue darkening of the reagent blanks in free and total gossypol assays. The addition of thiourea to free gossypol aliquots, before heating with aniline, had little effect on the resulting optical densities. In total gossypol assays thiourea gave a reduction in these optical densities of about 0.002 to 0.005.

Acid should not be added to reagent blank aliquots because it tends to cause darkening of the aniline, which may not be completely prevented by the thiourea used.

Proposed Modification of Gossypol Procedures

Based on these results, the following modifications of the official AOCS procedures for free and total gossypol are suggested.

One drop (0.05 ml) of 1.2N HCl and two drops (0.10 ml) of 10% aqueous thiourea should be added to each gossypol blank aliquot in both calibrations and assays for free and total gossypol and the mixture diluted to volume with 80% aqueous isopropyl alcohol as soon as possible. These same reagents should be added to the sample aliquots before heating with aniline.

Two drops (0.10 ml) of 10% aqueous thiourea should be added to the appropriate reagent blank aliquots before heating with aniline in free and total gossypol assays and calibrations. No acid should be added to these reagent blanks. The calibration by the procedure for total gossypol, or for the gossypol in oil procedure, should be used for these determinations as well as for free gossypol assays.

Much of the free gossypol in meals of lower free gossypol content consists of soluble-bound gossypol (4). For these, and for the precise determination of gossypol in oil, an additional operation is required. This will be published later.

REFERENCES

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3. Pons, W. A. Jr., R. A. Pittman and C. L. Hoffpauir, JAOCS *35*, 93–97 (1958).
4. Martin, J. B., Proceedings of the Conference on the Chemical Structure and Reactions of Gossypol and Nongossypol Pigments of Cottonseed, Southern Regional Research Laboratory, New Orleans, La., March 19–20, 1959, pp. 71–90.

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• *Names in the News*

R. E. MUSTO has been appointed Manager of International Sales for Hodag International SA, Skokie, Ill., according to Sheldon Kent, president. For the past two years, Mr. Musto was International Sales Manager with Matheson Scientific, Inc., and Matheson, Coleman and Bell.

E. A. DAY (1959), Vice President of Research & Development, International Flavors & Fragrances, Inc., has announced the addition of Morton Rubel to the R&D staff. Mr. Rubel joins the Systems Development Department as Senior Systems Analyst. Previous experience includes positions with Allied Chemical Corp. and Standard Brands, Inc., in the areas of research chemical engineering and applied statistics.

G. W. CHAILLE has been appointed regional sales manager of the Austin Company's Process Division with responsibility for service to chemical electrochemical, petrochemical and food industries in Michigan, Indiana, Ohio, Kentucky, western Pennsylvania and western New York states, and parts of West Virginia, as well as Delaware and Virginia.

R. A. BAKER, senior Fellow, Mellon Institute, Pittsburgh, Pa., will receive the 1968 Max Hecht Award presented by the American Society for Testing and Materials' Committee D-19 on Water at a meeting of the Committee in West Palm Beach, Fla., on Jan. 25, 1968. He will receive the award for his "outstanding leadership in the development of chemical and instrumental methods for water analysis and for his administrative contributions to Committee D-19.

R. W. SPRAGUE has been elected secretary of Waters Associates, Inc., Framingham, Mass. The announcement was made by J. L. WATERS, president and chairman of the board of directors. Before joining Waters Associates, Mr. Sprague was corporate controller at Technical Operations, Inc., Burlington, Mass.