Determination of Total Gossypol at Parts-Per-Million Levels

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The official AOCS method Ba 8-78 for total gossypol has been modified by introduction of two clean-up steps. These steps reduce sample blanks and also substantially increase the concentration of dianilinogossypol available for spectrophotometric measurement. An improved method of correcting sample absorptions for blanks, which reduces the possibility of over or under compensation, and criteria for judging the quality of the results have been added. With these modifications, the method is more than adequate for grading glandless cottonseed under Rule 112 of the National Cottonseed Products Association. As little as 1 ppm can easily be measured in sound cottonseed samples. The method is not universally applicable to mixtures that may contain TG, but the criteria of quality should permit identification of samples to which it is not applicable and, hence, prevent reporting of inaccurate TG concentrations. Additional modifications for use with foods and other complex mixtures are suggested.

Several years ago the National Cottonseed Products Association issued Trading Rule 112 (1), which defined three classes of glandless cottonseed on the basis of total gossypol (TG) content. Class A must contain no more than 400 ppm TG, class AA no more than 100 ppm and class AAA no more than 10 ppm. Although gossypol is a well characterized sesquiterpenoid phenolic aldehyde, TG is a variable mixture of gossypol and related compounds. In effect, TG is defined as the apparent gossypol content found using AOCS official method Ba 8-78 (2). Unfortunately, this method is unreliable at TG levels much below 100 ppm. Other possible grading methods that are suggested in the rule measure gossypol, not TG. This paper describes simple modifications of method Ba 8-78 that make it applicable to seed containing less than 10 ppm TG.

MATERIALS AND METHODS

Materials. Except for the thiourea and citric acid solutions, the following reagents are those proposed by Pons et al. (3) and incorporated in AOCS Method Ba 8-78:

- Reagent grade 2-propanol, hexane, N,N-dimethylformamide (DMF), 3-aminopropanol (AP), aniline, glacial acetic acid, thiourea and citric acid.
- Complexing reagent: Two ml AP and 10 ml acetic acid diluted to 100 ml with DMF. Store in refrigerator.
- Dilution solvent: 2-Propanol:hexane, 60:40 by volume.
- Thiourea solution: One g dissolved in 100 ml distilled water.
- Aniline: Freshly redistilled over zinc dust, discarding first and last 10% of distillate. Excess can be stored in a brown bottle in a refrigerator and used for a few days, but it should be redistilled when absorbance of reagent blank at 438 nm exceeds 0.022.
- Citric acid solution: Dissolve 384 g citric acid in water and dilute to one l.

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Sample preparation. The official method is satisfactory for general use but if completely glandless samples are required (e.g. for measurement of recovery of added gossypol), dehull seeds carefully, slice into 2-mm sections with a clean razor blade and inspect the sliced kernels under a low power microscope, rejecting those that contain any glands.

Analysis. The analytical procedure involves the following 10 steps:

(i) Weigh sample (w) containing 1–50 μg of TG, but not more than 1.25 g, into a 15-×-150-mm screw-capped culture tube.
(ii) Add 2 ml complexing reagent. Break up large particles with a glass rod and rinse rod with 0.5 ml reagent. Cap tube tightly using a Teflon lined cap. Include a reagent blank with each set of samples.
(iii) Immerse tubes to ca. 1 cm above the reagent level in a boiling water or steam bath and heat for 30 min.
(iv) Cool to about 50 C, add 10 ml solvent, recap tubes and mix thoroughly. Heat to insipient boiling (ca. 80 C), remix, and cool to room temperature. Volume of diluted extract (V_1) is 12.5 ml.
(v) Centrifuge briefly to get a clear upper layer. Carefully pipette a 5-ml sample aliquot (V_2) and a 4-ml blank aliquot to separate capped culture tubes. The 5-ml aliquot of the reagent blank is treated as a sample in the succeeding steps.
(vi) Add 2.5 ml thiourea solution to each sample and 2.0 ml to each blank, shake, and centrifuge to obtain two clear layers. Carefully draw off upper hexane layers and discard them.
(vii) Add 0.5 ml aniline to each sample tube and cap all tubes tightly.
(viii) Heat samples and blanks for 30 min in a boiling water or steam bath, immersing just deeply enough to cover the liquid in the tubes.
(ix) Cool for 1 hr. Add 2.5 ml citric acid solution and 1.0 ml hexane (V_3) to each sample and 2 ml citric acid solution and 0.8 ml hexane to each blank. Shake tubes vigorously and centrifuge to obtain two clear layers.
(x) Carefully transfer hexane layers to tightly stoppered cells. With the reagent blank without aniline as the reference, measure absorbances of samples (S_{nm}), sample blanks (R_{nm}), and aniline-blank (B_{nm}) at 418, 438 and 458 nm. Use of semi-micro cells may be necessary with some spectrophotometers. With our spectrophotometers, maximum absorbance is at 438 nm. Before using the method, a 1-mg portion of glanded seed should be carried through the first nine steps of the procedure and the spectrum for the sample vs the aniline-blank determined from 400 to 500 nm. The observed position of the maximum, with corresponding adjustment of the other two wavelengths, should be used for the absorbance measurements in step (x).

Calculations. Determinations of the constants used in the calculations, which need not be repeated by the user, are described in subsequent sections. The rationale for

the method of calculating corrected absorbances (A_{nm}) is covered in the Discussion section.

Three equations (I, II and III) are used to correct observed absorbances and calculate TG:

$$A_{nm} = S_{nm} - R_{nm} - FB_{nm} \quad [1]$$

$$F = [1.06(S_{418} - R_{418}) - S_{458} + R_{458}]/(1.06B_{418} - B_{458}) \quad [2]$$

$$c \text{ (conc of TG in ppm)} = 16.3A_{438}V_1V_3/wV_2 \quad [3]$$

where:

A_{nm} is corrected absorbance at wavelength nm.
S_{nm}, R_{nm} and B_{nm} are the corresponding absorbances of the sample, sample-blank and aniline-blank, respectively.
F is a correction factor.
V_1 is the volume of the diluted extract (usually 12.5 ml).
V_2 is the volume of the sample aliquot (usually 5 ml).
V_3 is the volume of the hexane solution (usually 1 ml).
w is sample weight in grams.
16.5 is the calibration constant.

If A_{438} is less than 0.02, report zero TG. If A_{438}/A_{458} (F_1) is 1.15 ± 0.05, calculate TG (ppm) using Equation [3]. If neither of these criteria is met, check for errors in data, recalculate by subtracting B and applying the correction factor to R, and/or run a duplicate sample. If A_{438} is consistently greater than 0.02 but less than A_{458}, there is interference that precludes calculation of TG, so report "method not applicable." If F_1 is consistently greater than 1, but outside the limits for calculation of TG with complete confidence, results should be considered semiquantitative or the method should be considered not applicable to the sample.

Determination of absorbance ratios for dianilinogossypol (DAG): Solvent corresponding to the aqueous phase from Step (vi) was prepared by mixing 10 ml of extraction reagent, 24 ml of 2-propanol and 25 ml of thiourea solution. Aliquots of concentrated solutions of DAG in DMF were dissolved in 5.9 ml of this solvent, and the DAG was transferred to hexane as in Step (ix) above. Nine concentrations covering the 0.1 to 1.4 absorbance range were used. Absorbances (A_{nm}) were measured against hexane. Absorbances at the three wavelengths were highly correlated (r = 0.9999) with ca. zero intercepts. As measured by the slopes of the regression lines, absorbance ratios were A_{458}/A_{418} (F_1) = 1.06, and A_{438}/A_{458} (F_2) = 1.15. Means (standard deviations) of the ratios calculated for each concentration of DAG were F_1 = 1.06(0.01) and F_2 = 1.15(0.001).

Calibration constant. The constant required to convert corrected A_{438} to concentration of TG in μg/ml was determined using gossypol-acetic acid as the standard. Aliquots of stock solutions in acetone were transferred to extraction tubes, evaporated to dryness, and converted to DAG by the modified method. Observed absorbances were corrected using Equations [1] and [2] of the Calculation section. A total of 18 samples covering an absorbance range of 0.01 to 1.5 were run over a period of several months using different stock solutions and different lots of reagents. Results were pooled for calculation of the slope of the regression line, which is the required constant. The regression equation was μg TG/ml = $16.3A_{438}$ − 0.006 (r = 0.9999) with a standard error of 0.1 for the slope. The very small intercept, corresponding to about 0.01 ppm TG, was neglected in formulating Equation [3].

TABLE 1

Reproducibility of Method

Sample	F[a]	A_{458}/A_{418}	A_{438}	TG[b]
1a	1.5	0.87	0.0027	0.0
1b	1.5	0.16	0.0002	0.0
1c	1.1	1.11	0.0065	0.2[c]
1d	1.1	1.27	0.0084	0.3[c]
1e	1.4	−0.05	0.0003	0.0
1f	1.3	0.88	0.0075	0.0
1g	1.9	1.14[d]	−0.0046	−0.1[c]
1h	1.5	1.4	0.0079	0.3[c]
1i	1.0	1.21[d]	−0.0026	−0.1[c]
2a	2.0	1.19	0.0258	0.8
2b	1.2	1.19	0.0294	1.0
3a	0.9	1.13	0.0991	4.0
3b	0.9	1.12	0.0940	3.8
4a	0.8	1.13	0.2588	8.5
4b	0.8	1.14	0.2552	8.4

[a]Calculated by Equation 2.
[b]Total gossypol.
[c]TG was calculated whenever the unforced ratio was greater than 1. These values normally would be reported as 0.0 because A_{438} is less than 0.02.
[d]All three corrected absorbances were negative, so there is a minimum at 438 nm.

Recovery. Aliquots of stock solutions of gossypol-acetic acid in acetone, equivalent to about 1 to 20 ppm, were evaporated under nitrogen in extraction tubes and 1.25 g ground glandless cottonseed added to each tube. The spiked samples were analyzed by the proposed method. Three lots of seed and three stock solutions were used to prepare 13 spiked samples.

DISCUSSION OF METHOD

The principal causes of the poor sensitivity of AOCS Method Ba 8-78 are the high absorbance of the sample blank and the measurement of absorbances on dilute solutions. The final liquid-liquid partition (Step ix) in the revised method removes the polar substances that contribute to the high absorbance of the sample blanks and simultaneously increases sixfold the concentration of DAG. Citric acid is used to assure retention of unreacted aniline in the aqueous layer. Further reduction in overall dilution of sample was achieved by use of .4 of the total extract, instead of .2 as used in Ba 8-78, for reaction with aniline. Use of a larger sample aliquot and liquid-liquid partition were facilitated by substituting sealed tubes and centrifugation for volumetric flasks and filtration. This revised technique also eliminates an error of up to 10%, caused by the presence of the solid sample in the extraction flask, while avoiding the possibility of evaporation during filtration.

For Grade A or AA seed the method can be simplified by omission of Step vi. If this is done, two drops of 10% thiosulfate are added at Step vii. The water that is normally added at Step vi is added with the citric acid solution at Step ix, and no hexane is added at this step. V_3

becomes 1.2 ml, i.e., the volume of hexane present in the 5-ml sample aliquot. Tubes must be capped very tightly to avoid loss of hexane in Step viii.

Step vi is essential for Grade AAA seed, for which errors of a few ppm would be critical. This step removes unidentified non-polar components that have absorption maxima at 420, 444 and 470 nm. On reaction with aniline, these components cause a general increase in absorbance below 500 nm and the maxima disappear. The net result is negative interference, i.e. c = −1 or −2 ppm for seed containing no TG, which could result in misgrading seed that contains a little more than the 10 ppm TG permitted for Grade AAA. When Step vi is included, blanks must be heated in Step viii because presence of water increases both blanks. Thiourea reduces darkening of sample blanks as well as aniline blanks (4).

The rather elaborate method of calculating TG was based on the premise that corrected absorbance at 438 nm should not be attributed to DAG and used to calculate TG unless it is part of a corrected spectrum that has the right shape for DAG, i.e. a maximum at 438 nm with shoulders at 418 and 458 nm. At high TG concentrations the correction specified in Ba 8-78 met this criterion (3), so measurement of absorbance only at the maximum was considered adequate. However, we found that at low TG concentrations blank absorbances were large compared to corrected sample absorbances, and spectra obtained by using the sample blank in the reference cell and subtracting the aniline blank often had no maximum near 438 nm. Both over- and under-compensation were observed. More or less arbitrarily, we assumed that if the ratios of the corrected absorbances at 418, 438 and 458 nm were close to the ratios for DAG ($A_{418}/A_{438}/A_{458}$ = 1/1.06/1.22), valid results would be obtained for TG and that over- and under-compensation can be corrected by application of the proper correction factor(s) to the observed absorbances.

Equations with two correction factors, which forced corrected absorbances to have exactly the correct ratios, frequently gave impossible results, e.g. corrected absorbances much larger than observed absorbances and very large factors, both positive and negative. The successful approach forces one ratio to be correct by use of one correction factor and uses a second, unforced ratio to judge the acceptability of the results. This approach assumes that due to interferences or errors in the data it may be impossible to calculate accurate TG concentrations for some samples. In these cases, all one can do is detect the problem and avoid reporting inaccurate results. All nine variations of this method of correction were evaluated. The variation given in Equation [2] was selected for manual use because it almost always gave satisfactory results with cottonseed and gave very good recovery of gossypol from spiked samples. Those variations that apply the factor to S_{nm} or force A_{438}/A_{458} = 1.15 consistently gave poorer results than the other four. We routinely use a computer program that uses the four variations to calculate TG with a single input of data. No extra work is required and, on the rare occasions when Equation [2] gives poor or marginal results, the other three results may help identify the source of the problem.

DISCUSSION OF RESULTS

The results of the determinations of absorbance ratios for DAG and the calibration factor justify considering this to be a primary method. That is, one does not have to calibrate the method with standards in order to use it. The calibration factor and absorbance ratios were not affected by changing reagent or by day-to-day variations in reaction conditions. Considering differences in methods, our values are in good agreement with the ratios of 1/1.07/1.20 reported by Pons et al. (5) and with the value of 16.0 recommended for the calibration constant in a method being considered for adoption by the International Standards Organization (6). Furthermore, a 10% change in F_1 would change calculated TG concentration by only 5%.

Reproducibility was very good for cottonseed (Table 1). For samples that contained TG, the values of F and F_2 were reasonable. The results with lot 1, hand-sorted completely glandless seeds, were particularly gratifying, because samples having no TG were not considered in formulating Equation [2]. Although the absolute values of absorbances of both blanks were highly variable, the absorbance ratios were quite constant; $B_{458}/B_{438}/B_{418}$ = 1.0/2.0 ± 0.1/3.6 ± 0.3 and $R_{458}/R_{438}/R_{418}$ = 1.0/1.4 ± 0.1/1.7 ± 0.2 for TG levels below 50 ppm. Sample blank ratios increase slightly at higher TG levels. Observed ratios that are significantly different from the means probably indicate an error in one or more of the absorbances.

Three sets of spiked samples, prepared over a period of several months using a different lot of glandless cottonseed and a fresh stock solution of gossypol-acetic acid for each set, were used to establish the accuracy of the method. Except at the higher level in cottonseed 2, recoveries were very good even at 1 ppm levels (Table 2).

In view of the reproducibility and accuracy of the results, routine analyses of duplicate samples were not specified in the method. Conservatively, analyses that

TABLE 2

Recovery of Gossypol Added to Glandless Cottonseed

Added (ppm)	Found[a] (ppm)	Recovery[b] (%)
0.75[c]	0.70	93
1.33[d]	1.22[e]	92
1.33[d]	1.30[e]	98
1.50[c]	1.40	93
3.36[f]	3.20	95
3.74[c]	3.31	89
6.72[f]	6.68	99
7.48[c]	7.49	100
13.30[c]	11.85[e]	89
13.30[d]	11.57[e]	87
13.44[f]	13.66	102
14.98[c]	14.63	98
20.16[f]	20.63	102

[a]Using F from Equation 2.

[b]Mean (SD) recovery was 95(5)%. Found = 0.98; Added − 0.15 (r = 0.996).

[c]Stock solution, 1; cottonseed, 1.

[d]Stock solution, 2; cottonseed, 2.

[e]Corrected for 0.3 ppm TG found in cottonseed, 2.

[f]Stock solution, 3; cottonseed, 3.

give acceptable values for F_2 or for A_{438} should be accurate to 1 ppm or 10% of TG found, whichever is larger. As with any method, replicates will be required if the TG content is near the limit for a given grade. Duplicate samples should be run if the results do not meet the criteria given in the section on calculations.

Applicability of the modified method to substrates other than glandless cottonseed was investigated briefly when reports appeared showing that some other methods gave false TG concentrations for substances that were known to be free of TG. Stipanovic et al. (7), using a method that converts TG directly to DAG (8), found 60 ppm TG in soybeans and 120 ppm TG in glandless cottonseed, Paymaster 464, but were unable to detect DAG in either one using a TLC method that was sensitive to 20 ppm TG. Reber et al. (9) reported that the official method gave moderate TG concentrations for food ingredients such as salt, sugar, coffee and vanilla extract. Samples (1 g) of the six substances mentioned above, including a sample of Paymaster 464 supplied by Dr. Stipanovic, and rancid peanuts were analyzed by the modified method. Rancid peanuts were included because Stipanovic et al. (7) attributed their false TG values to oxidized lipids. As shown in Table 3, the method gives correct values of zero for Paymaster 464, soybeans, peanuts, sugar and salt. For instant coffee and vanilla extract, values of A_{438} outside the prescribed range with no maximum at 438 nm indicate that the method is not applicable (NA) to these samples. Simple subtraction of both blanks at 438 nm would give 52 ppm TG for coffee and 9 ppm for vanilla.

Even though this method does a much better job of eliminating or detecting interference than other methods do, caution should be exercized in attempting to apply it to mixtures that may contain low levels of TG in combination with components that may react with aniline. For example, calculations based on simulated data indicate that zero TG could be reported for a mixture of 1 part of vanilla extract with 10 parts of cottonseed containing 4 ppm TG. One part of coffee added to the same cottonseed would make the method inapplicable, but would cause a 2 ppm error if added to cottonseed containing 15 ppm TG. In general, inaccurate TG concentrations were based on marginal values of A_{438} or F_2 and high values of F, which clearly indicated absorbance by something other than DAG. Hence, for mixtures, we recommend that the method be considered inapplicable if $F > 2$, if F_2 is not between 1.14 and 1.16, or if $A_{438} > 0.01$ and $F_2 < 1$. If there is any reason to suspect that A_{438} may not be due to DAG, the final hexane solution can be analyzed by a method, such as TLC (7) or HPLC (10), that is specific for DAG. HPLC in particular may offer a viable method for mixtures of unknown composition.

For mixtures of known composition, another modification of the method should extend its applicability. With glandless cottonseed, two blanks must be used because there is no way to remove TG and react the rest of the sample with aniline to provide a single true blank; but for mixtures of known composition one has the option of omitting the cottonseed product that may supply TG, or replacing it with another oilseed product, to get a control that can be reacted with aniline to provide a single blank. Although precise controls may not be possible, except for in-house quality control, similar mixtures should provide better compensation than is provided by separate sample and aniline blanks. The control and samples should be run at the same time. When a control mixture is used to provide a single blank, sample blanks and aniline blanks are omitted, absorbances of the control are substituted for B_{nm} in Equations [1] and [2], and R_{nm} is eliminated.

TABLE 3

Interference of Food Ingredients

Sample[a]	A_{438}[b]	F_2[c]	F[d]	TG[e] (ppm)
Cottonseed (CS)[f]	−0.0024	1.12[g]	1	0
Soybeans (S)	−0.0141	1.49[g]	3	0
Peanuts (P)	0.0044	1.09	2	0[h]
Salt	−0.0024	0.97	1	0
Sugar	0.0092	0.97	2	0
Coffee (C)	0.4298	0.98	40	NA
Vanilla (V)	−0.2760	2.08[g]	24	NA

[a]1 g each. Coffee is freeze-dried instant. Vanilla is imitation vanilla extract.

[b]Corrected (Equation 1) absorbance at 438 nm.

[c]Ratio of A_{438} to A_{458}. Values less than 1.01 indicate no maximum.

[d]Correction factor (Equation 2).

[e]Total gossypol concentration in ppm (Equation 3). NA means method is not applicable to this sample.

[f]Paymaster 464.

[g]All corrected absorbances are negative, so these are minima.

[h]From Equation 3, TG = 0.2 ppm, but $A_{438} < 0.02$ so TG = 0 is reported (cf. text).

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