Quick Method for Estimating Free Gossypol in Cottonseed, Meats, Collets, and Extracted Meals[1]

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ABSTRACT: A method for estimating free gossypol (FG) has been developed that decreases sample-determination time from over 2 h to about 25 min per sample. With auto pipetters and bottle-top dispensers, six sample determinations can be completed in approximately 50 min. The method consisted of adding water and acetone separately to a fixed sample weight, mixing, filtering, diluting with 65% acetone, and reading absorbance on a spectrophotometer. Absorbance was plotted against the official American Oil Chemists' Society's FG method for samples that contained FG between 0.02 and 0.9%. Quadratic least squares regression for 31 samples had a correlation coefficient of $r^2 = 0.986$ and a standard error of estimated FG of 0.032%.
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KEY WORDS: Analysis, cottonseed, environment, gossypol, plant breeder, polyphenol, spectrophotometer.

Gossypol (G) is a highly reactive polyphenolic, binaphthyl aldehyde compound. It occurs extensively in the seed, foliage, and root of most varieties of the cotton plant. In seed, G is found as the predominant pigment contained in discrete glands, 100 to 400 μ in size. Nutritionally, whole cottonseed is an excellent source of energy and protein and, when fed to dairy cows, it results in an increase in milk fat. Because G is toxic to nonruminants, such as poultry and swine, however, the use of whole seed is restricted to ruminant feed (1). After extracting or pressing the edible oil from the seed, the resulting meal is again mainly used as ruminant feed. Because of the reactive nature of G, processing seed with heat and moisture, in preparation for oil extraction, results in significant complexing or binding of physiologically active free gossypol (FG) to carbohydrates, phospholipids, and the alpha and omega amino groups of proteins and amino acids to produce so-called biologically inactive bound gossypol (BG). Although once thought to be totally inactive, it is suspected that some BG can revert to FG in the digestive tracts of some ruminants (2). During solvent extraction of conditioned seed, a small amount of G is also extracted with the oil but is easily removed during oil-refining operations. Various commercial processing procedures significantly reduce FG levels by binding and oil removal, from approximately 1.4% in kernels to 0.02–0.5% in processed meal (1). Feed with FG levels of up to .04%, along with a small amount of iron salt, is deemed safe for broilers and swine (3). Further, cottonseed products containing up to 0.045% FG have been approved by the Food and Drug Administration for use as food additives (4). To determine the safe use of cottonseed products, it is thus important that reliable analytical methods be developed to accurately determine FG. The first quantitative method for estimating FG was developed by Withers and Carruth in 1915 (5). Their method was based on gravimetric precipitation of G as the dianilino derivative. This and other gravimetric precipitation methods were too time-consuming and gave unreliable results. They were eventually replaced with spectrophotometric methods. Various derivatives produced by the reaction of G with *p*-anisidine, antimony trichloride, and phloroglucinol were tried at our research center and elsewhere (6–8). The dianilinogossypol derivative was eventually shown to give the most reliable results, and the American Oil Chemists' Society (AOCS) adopted it in its official method. The official AOCS FG spectrophotometric method specifies that it measures G and G-like compounds that are soluble in 70% aqueous acetone (9). The G-like compounds are thought to consist of phospholipid gossypol and G amino acid or peptide complexes that exchange with aniline. Other methods, for the measurement of FG based on paper, gas, and liquid chromatography, have been reported but, because they only measure pure G, most have significantly lower FG content than those reported by the official AOCS spectrophotometric method. One exception, however, has been the use of high-performance liquid chromatography (HPLC) in the determination of FG in fresh seed samples. The results compare favorably to the official AOCS method, probably because essentially all G in fresh seed is in the pure, unreacted form (private communication). The existing methods to determine FG are not fast; the quickest method, HPLC for FG in fresh seed, takes at least 2 h to run. Presently, a majority of cottonseed-processing plants uses expanders in a seed-preparation step prior to extraction. The basic purpose of an expander is to bind FG to protein and to simultaneously make a dense but porous collet from which oil, containing a minimum of G, can be subsequently solvent-extracted. This is the primary G-binding step and, as such, essentially determines the level of

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FG in extracted meal. It is therefore apparent that a quick FG test method for collets to predict FG in extracted meal would be highly beneficial to a processor. A quick FG method would also be an asset to our pilot plant research to improve the binding or physiological inactivation of G in an expander operation. During the preparation of experimental samples for analysis of G by the official AOCS method, we noted distinct color variations in the 70%-aqueous acetone extracts. This observation led us to the development of a quick method for the estimation of FG.

EXPERIMENTAL PROCEDURES

Materials. Twelve acid-delinted cottonseed samples were supplied by Steven Calhoun, Louisiana Agricultural Center, Louisiana State University (Baton Rouge, LA). The samples consisted of three replications of four cotton strains: Deltapine 41, a conventional variety; a high-G variety; and two different Louisiana varieties with intermediate G levels. The seed samples, with seed coats intact, were dried for 1 h at 45°C in a Blue M Touchmaster forced draft oven (General Signal, Blue Island, IL) prior to being finely ground (Wiley mill; 20 mesh) to minimize pigment-gland rupture and subsequent binding of FG. Mill-run cottonseed meats and meal samples from the 1992 crop year were obtained from commercial oil mills located across the cotton-growing belt. The meats and meals were finely ground (Wiley mill; 20 mesh) prior to being analyzed.

Apparatus. Absorbance was measured at a wavelength of 430 nm in a Spectronic 20D spectrophotometer (Milton Roy Co., Rochester, NY).

Solvents and reagents. Water was obtained from a Milli-Q water system (Millipore, Marlborough, MA). Purified, crystalline potassium dichromate and spectrophotometric-grade acetone were purchased from J.T. Baker, Inc., (Phillipsburg, NJ). The final dilution solution consisted of acetone:water (65:35, vol/vol). A standard potassium dichromate solution (0.0514 g in 250 mL Milli-Q water) was used to calibrate wavelength on the spectrophotometer. At a wavelength of 430 nm, the absorbance should approximate 0.360.

Free gossypol (FG). The method is applicable to full-fat seed, meats, or expanded collets and solvent-extracted cottonseed meal that contain FG within the ranges of 0.02–0.25% and 0.9–1.8%. Appropriate sample weights were 5.0 g for 0.02–0.25% FG and 1.0 g for 0.9–1.8% FG. Knowing the approximate FG range, a finely milled (Wiley mill; 20 mesh) sample was accurately weighed into a 150-mL beaker. Fifteen mL treated water was pipetted in the beaker, and the slurry sample was swirled to rupture pigment glands. The slurry was then allowed to stand 5 min. Thirty-five mL acetone was added to the slurry, making the equivalent of a 70%-aqueous acetone solution, and the slurry was swirled and again allowed to stand 5 min. The slurry was then filtered through fluted Whatman #2 filter paper into another 150-mL beaker. After filtering for approximately 5 min, 1 mL filtrate was pipetted into a 10-mL volumetric flask, and the flask filled to mark with 65%-aqueous acetone. Sample absorbance was then read on the spectrophotometer at a wavelength of 430 nm against a 65%-aqueous acetone background. The wavelength setting on the spectrophotometer was periodically adjusted by using the fixed absorbance reading of a standard potassium dichromate solution. FG was determined by inserting the sample's absorbance reading into the appropriate linear least-squares calibration equation corresponding to sample weight.

Standardization. To determine % FG in a sample by the quick method, standardization or calibration equations must first be obtained. Indirect standardization was used after attempts to use G acetic acid as a standard proved unsatisfactory. Absorptions were measured, as described, for 31 cottonseed products of known FG contents (by the official AOCS method) ranging from 0.02 to 0.9%.

RESULTS AND DISCUSSION

Because the official AOCS method for FG measures G and G-like compounds extracted with 70%-aqueous acetone, it is important to look at the ultraviolet visible (UV) spectra of pure G dissolved in 70%-aqueous acetone, as shown in Figure 1A. Pure G shows a maximum absorbance at about 374 nm (filled circle), as does full-fat, glanded cottonseed and expander collets shown in the UV spectrum in Figure 1B. The glandless cottonseed UV spectrum, shown in Figure 1C, however, also has a fairly high absorbance at 374 nm (filled circle), due to non-G components that also are soluble in aqueous acetone. Because of this high absorbance of non-G compounds, it would be difficult to spectrophotometrically differentiate G from the non-G compounds in any type of cottonseed sample. Because of this fact, researchers in the 1940s looked at various derivatives of G with high absorbance at wavelengths of 440 nm or higher, where non-G cottonseed components, soluble in 70% acetone, would have little or no effect on a spectrophotographic analysis. There is an alternative to the use of derivatives, however. Figure 1D shows the UV spectrum for a typical, commercially extracted meal in 70%-aqueous acetone. There is a major absorbance peak at around 350 nm, as shown by a square, which unfortunately also coincides with a major absorbance peak at this same wavelength in a glandless (G-free) sample, shown as a square in Fig. 1C. The commercial meal, however, also shows a minor absorbance peak at 430 nm, where non-G pigments show only a slight absorbance, as shown for the glandless meal in Fig. 1C. In developing the quick method, it was decided that, although G absorption at 430 nm is not as pronounced as it is at other wavelengths, it is fairly significant, as shown in Fig. 1E. If FG could be accurately measured at that wavelength, it would eliminate having to use a glandless extract blank in each series of determinations, significantly simplifying the method.

To determine the sensitivity of the quick method, 31 samples, consisting of full-fat collets and various laboratory-produced and commercially manufactured meals with FG con-

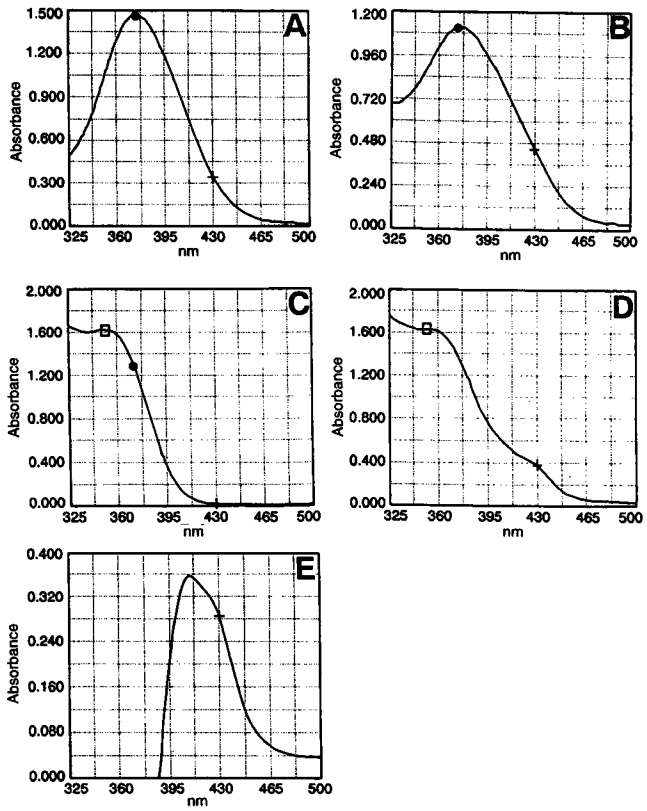

FIG. 1. Ultravioletvisible spectra for various materials in 70%-aqueous acetone: A) G; B) glanded, full-fat cottonseed kernels containing G; C) laboratory solvent-extracted glandless cottonseed meal containing no G; D) commercially solvent-extracted cottonseed meal containing G; E) resulting absorbance after subtracting part C) from part D). ●, wavelength at maximum absorption of G; □, wavelength at maximum absorption of cottonseed meal without G; +, wavelength used in test method.

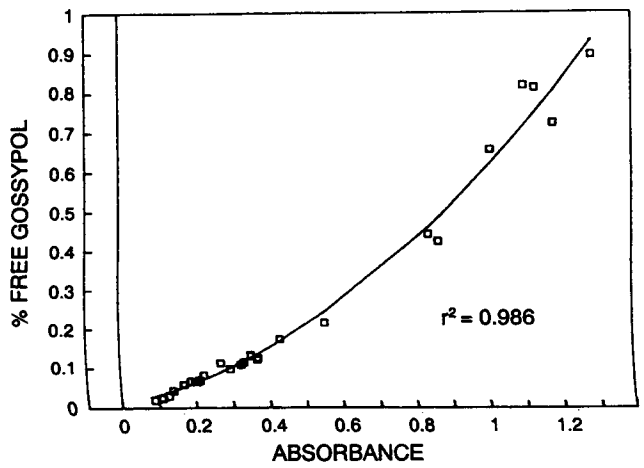

FIG. 2. Quadratic relationship of %FG determined by the official AOCS FG method and plotted against absorbance readings obtained by the quick FG method.

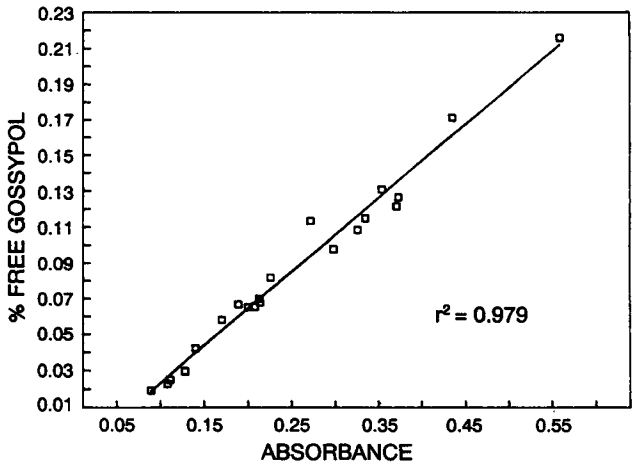

FIG. 3. Linear relationship of %FG determined by the official AOCS FG method and plotted against absorbance readings obtained by the quick FG method on full-fat collets and solvent-extracted meals.

tents ranging from 0.02 to 0.89%, were run in triplicate to produce the quadratic least-squares relationship shown in Figure 2. Although the correlation coefficient $r^2 = 0.986$ and the standard error is 0.033%, it is obvious that high absorbance readings, roughly above 0.6 absorbance units, produce a large scatter in FG readings this. Necessitates the use of a quadratic relationship. This is a concentration factor due to the use of a fixed sample weight for too broad a range of FG. By specifying a particular sample weight for a specific FG range, much as the official AOCS FG method does, the absorbance values obtained lie on the linear portion of the curve.

Generally, the major concern of processors and of feed formulators is in obtaining FG values below 0.25%, where a distinct linear relationship exists, as shown in Figure 3. The standard error for this regression was 0.008%. Application of this linear relationship to individual absorbance readings of 21 different samples showed no differences in mean values between the quick and the AOCS methods.

Cotton breeders normally evaluate large numbers of experimental cottonseed varieties, and a quick, economical FG method is desirable. Normally, seed samples containing lint are cracked, and only the meat fraction is analyzed. Because a method was being tested, however, acid-delinted seed with seedcoat was used to give a more replicable sample. Because of the high concentration of G involved, the sample size was reduced to one gram to obtain absorbance readings in a sensitive range. The 12 seed samples were run five times against the AOCS FG method, which was run in duplicate, with the results shown in Figure 4. The samples covered a range from 0.92 to 1.81% FG and gave a linear least-squares correlation factor r^2 of 0.989 and standard error of 0.032%. A % FG determination can easily be obtained in less than 25 min for a single, unground sample. By using auto pipetters and bottle-top dispensers, six samples can be ground and analyzed in less than 50 min. Because FG closely approximates total gossypol (TG) in fresh, unprocessed cottonseed, the relationship between the AOCS- and quick-method determinations of TG was also examined. The same absorbance readings

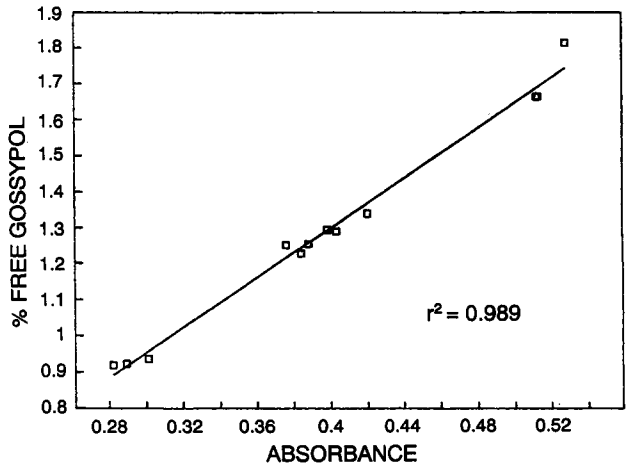

FIG. 4. Linear relationship of %FG determined by the official AOCS FG method and plotted against absorbance readings obtained by the quick FG method on acid-delinted cottonseed samples.

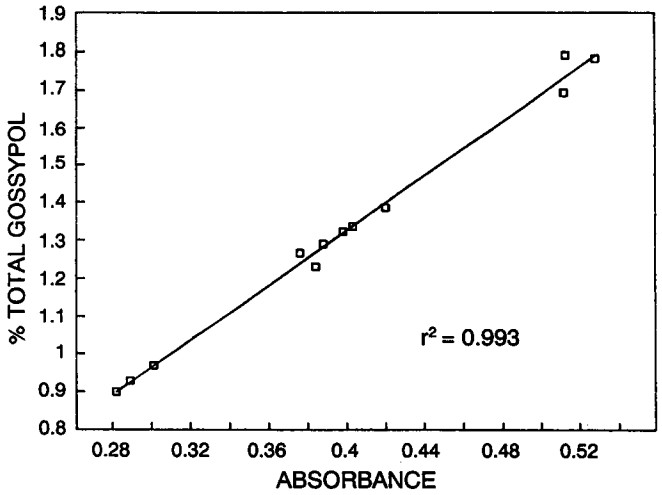

FIG. 5. Linear relationship of %TG determined by the official AOCS TG method and plotted against absorbance readings obtained by the quick FG method on acid-delinted cottonseed samples.

used in the FG correlation (Fig. 4) were plotted against AOCS TG results, done in duplicate, and the result is shown in Figure 5. The samples covered a range of 0.9–1.79% TG, and a correlation factor of r^2= 0.993 was found. With the least-squares correlation, the standard error was 0.026%, which was slightly better than the FG correlation.

The method enables plant breeders to quickly screen seed samples and allows processors to quickly estimate the extent of FG binding occurring in the flake cooker and expander. Additionally, and possibly more importantly, the method is "environmentally friendly" and should cause minimal waste-management problems, as it only requires water and acetone.

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REFERENCES

1. Berardi, L.C., and L.A. Goldblatt, in *Toxic Constituents of Plant Foodstuffs*, 2nd edn., edited by I.E. Liener, Academic Press, New York, 1980, pp. 183–237.
2. Calhoun, M.C., J.E. Huston, C.B. Calk, B.C. Baldwin, Jr., and S.W. Kuhlmann, in *Cattle Research with Gossypol Containing Feeds*, edited by L.A. Jones, D.H. Kinard, and J.S. Mills, National Cottonseed Products Association, Memphis, TN, 1991, pp. 39–51.
3. Martin, S.D., *Feedstuffs 62(33)*:14 (1990).
4. Fine, S.D., *Fed. Regist. 37*:13713 (1972).
5. Withers, W.A., and F.E. Carruth, *J. Agric. Res. 5*:261 (1915).
6. Pons, W.A., Jr., and J.D. Guthrie, *J. Am. Oil Chem. Soc. 26*:671 (1949).
7. Hall, C.M., L.E. Castillon, W.A. Guice, and C.H. Boatner, *Ibid. 25*:457 (1948).
8. Storherr, R.W., and K.T. Holley, *J. Agric. Food Chem. 2*:745 (1954).
9. *The Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., American Oil Chemists' Society, Champaign, 1989.

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