Determination of Free and Total Gossypol by High Performance Liquid Chromatography

R.J. Hron, Sr.*, M.S. Kuk and G. Abraham
Southern Regional Research Center, Agricultural Research Service, USDA, P.O. Box 19687, New Orleans, LA 70179

Existing HPLC methods determine only pure gossypol whereas the official AOCS method determines both gossypol and other physiologically active gossypol-like compounds that react with 3-amino-1-propanol and aniline. The feed industry uses the official AOCS method, which is complex and produces results that do not correlate well among laboratories. HPLC methods were developed, using 3-amino-1-propanol as a complexing agent, for the quantitative determination of free and total gossypol in cottonseed meal, oil, and ethanolic miscella. These methods are simple, sensitive, and provide reproducible results. In addition the use of toxic aniline is eliminated.

The gossypol contained in glanded cottonseed limits the markets for cottonseed products in at least three ways: (i) Because this phenolic compound is toxic to monogastric animals and young ruminants, this limits use of raw, delintered seed as a direct feed to ruminants; (ii) in processing, it affects the color of oil and can thereby seriously affect quality along with production costs and sale price; and (iii) it generally limits the use of extracted meals to ruminant feeding unless it is chemically bound during processing. However, such binding causes a loss of nutritive value.

Gossypol is a terpenoid aldehyde that composes approximately 0.4 to 1.7%, by weight, of U.S. glanded cottonseed (1). It is found in discrete pigment glands within the seed, leaves, stems, bracts, and roots of the plant. Although gossypol constitutes approximately 95% of gland pigments, Boatner (2) reported at least 15 other gossypol-like pigments or derivatives. About nine of the fifteen have been isolated and characterized (2-4) but most may be oxidation or condensation products of gossypol (5).

Gossypol exists in cottonseed products in two forms—free and bound. Free gossypol (FG), as defined by AOCS official methods, are those gossypol and gossypol derivatives that are soluble in aqueous acetone and are physiologically active. Bound gossypol (BG) forms during conventional cooking and processing of cottonseed by the reaction of gossypol with free amino groups of proteins and peptides. It is insoluble in ether, chloroform, or aqueous acetone (6). BG for the most part is physiologically inactive (3). It is not measured directly but is calculated as the difference between total and free gossypol. Total gossypol (TG) is briefly defined as the amount of gossypol, and gossypol derivatives, both free and bound, and gossypol-like pigments extracted during hydrolysis.

FG's toxic effect on monogastric animals is well-known, and BG has received increased attention because it reduces the nutritional value of oilseed meal as well as possibly being liberated in the gastrointestinal tract of nonruminants (3). It is thus apparent that cottonseed oil mills and feed formulators need reliable analytical methods for quantitatively determining both FG and TG content in their various products.

In 1977, Pons (6) suggested that high performance liquid chromatography (HPLC) might offer a good approach to accurate, precise determination of gossypol and gossypol derivatives. HPLC has since been used to determine gossypol and other individual terpenoid aldehydes in the seed, stems, roots and leaves of the cotton plant and in processed oils and meals (7-11). However, the gossypol determined by these HPLC methods does not correlate with the results obtained by the official AOCS method. The difference is because the AOCS method measures gossypol, gossypol analogs, and gossypol derivatives having an available aldehyde moiety. Total gossypol is a much better determinant of the physiological effects of a feed on animals than the amount of "pure" gossypol. Although HPLC research to date has separated and quantified some constituents of gossypol containing pigment glands, no important toxicity findings have been reported. This leaves the AOCS spectrophotometric method—which is slow, complex and unreliable (Table 2)—as the only indicator of toxic pigment-gland components other than FG. The quick, reliable HPLC methods presented here give results comparable to the official AOCS methods for FG and TG without the use of toxic aniline.

EXPERIMENTAL PROCEDURES

Apparatus. The liquid chromatograph (LC) was an IBM/Nicolet LC/9560 equipped with a Waters NOVA-PAK 3.9 mm × 15 cm, 4 μ, C_{18} reverse-phase column and a 2 cm disposable C_{18} Supelguard column. The LC was operated isocratically with a mobile-phase flow rate of 1.0 ml/min. Samples were injected into a Rheodyne 1725 injector equipped with a 50 μl sample loop. Gossypol was detected as the gossypol-aminopropanol (GA) complex by an IBM/Nicolet model LC/9563 variable wavelength UV detector at 254 nm. Peak areas were determined with an IBM 9000 computer. A three-dimensional chromatographic scan of the GA complex was obtained, using the above columns and conditions, on a HP 1090 LC equipped with a diode array detector and HP 300 work station.

Solvents and reagents. The mobile phase consisted of methanol:water (87:13, v/v) with 0.1% phosphoric acid (7,8). Complexing reagent was made by mixing 4 ml of 3-amino-1-propanol with 20 ml glacial acetic acid. The solution was cooled and diluted to 200 ml with N,N,dimethylformamide (DMF). Standard gossypol solution was made by dissolving 25 mg of standard gossypol acetic acid (12) in 25 ml of complexing reagent.

Standardization. Standard gossypol solutions of 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 ml were pipetted into 100-ml volumetric flasks. Complexing reagent was added to give a total volume of 20 ml. The samples were heated in a hot-water bath (95°C) for 30 min, cooled, and filled to

*To whom correspondence should be addressed.

100 ml with mobile phase. Approximately 10 ml of each sample was filtered through a 0.45 μm Millex-HV filter. The 50 μl injector loop was then flushed and filled with filtered sample. The sample was injected into the LC, and the total run time was 10 min. The weights of pure gossypol used (wt of gossypol acetic acid × 0.8962) and peak areas determined were then used to calculate the linear calibration equation:

$$\text{mg pure gossypol} = \text{m (peak area)} + \text{b}$$

Total gossypol. A ground sample (Wiley mill; 20 mesh) containing 0.2–5 mg of gossypol was accurately weighed into a 125-ml stoppered Erlenmeyer flask. Appropriate sample weights were 0.2 g for >0.5% expected TG; 0.5 g for 0.1–0.5%; and 5 g for <0.1%. Twenty-five ml of complexing reagent was added, and the slurry was heated in a water-bath (95–100°C) for 30 min. The slurry was cooled, and 100 ml of mobile phase was added. The slurry was filtered through Whatman No. 2 filter paper, then through a 0.45 μm Millex-HV filter. The 50 μl injector loop was flushed with sample, and the sample injected. For miscella and oil samples, an appropriate size sample (10–20 ml samples were used for gossypol contents <0.01%) was weighed into a 100-ml volumetric flask. Twenty ml of complexing agent was added, and the sample was heated in a water-bath (95–100°C) for 30 min. The solution was cooled, diluted to exactly 100 ml with mobile phase, mixed well, filtered through a 0.45 μm filter, and injected as above. Twenty min was allowed for miscella samples because an additional small peak eluted at approximately 13–15 min.

Free gossypol. A ground sample (Wiley mill; 20 mesh) was accurately weighed into a 250-ml screw-capped Erlenmeyer flask, which had a glass bead-covered bottom. Appropriate sample weights were 0.5 g for >0.5% expected FG; 1 g for 0.1–0.5%; 5 g for 0.01–0.1%; and 20 g for <0.01%. Fifty ml of 70% acetone was added, and the flask was mechanically shaken for 1 hr. The slurry was then filtered through Whatman No. 2 filter paper, and the first 5 ml of filtrate was discarded. Twenty ml of the remaining filtrate was pipetted into a 100-ml volumetric flask, and 20 ml of complexing reagent was added. The sample was heated, cooled, diluted, and injected as described in the TG section.

Calculations. The percentage of gossypol was calculated as follows: % Gossypol = K{m (peak area) + b}/sample weight (g) where K is a constant that converts mg/g to % and, corrects for differences in dilution between standardization and analysis. For TG of solids, TG of liquids, and FG of solids, K = 0.125, 0.100, and 0.25, respectively. The constants m and b are from the calibration equation.

Spectrophotometry. Gossypol was determined spectrophotometrically in-house according to official AOCS methods Ba 8-78 for TG and Ba 7-58 for FG (13).

RESULTS AND DISCUSSION

Following the official AOCS TG method, as developed by Pons *et al.* (14), FG, BG and other gossypol-like pigments were hydrolyzed and complexed with aminopropanol in dimethylformamide to form a GA complex. The filtrate, containing the complex, was then reacted with aniline, an extremely hazardous liquid, to form dianilinogossypol. Gossypol was determined before and after its reaction with aniline, by the difference in its spectrophotometric absorbance of duplicate aliquots of the filtrate. Pons *et al.* (14) showed that the absorption spectra of pure gossypol before treatment with aminopropanol had a maxima at 370 nm. After treatment with aminopropanol, a high absorption was found at 376–398 nm. Dianilinogossypol

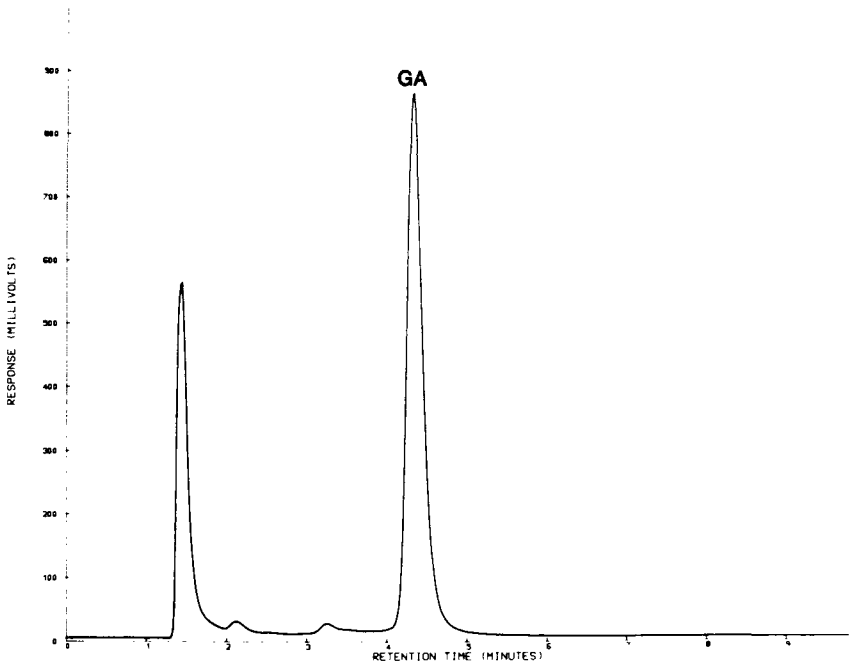

FIG. 1. HPLC chromatogram of GA complex that was produced by the reaction of gossypol acetic acid with 3-amino-1-propanol. Chromatographic conditions as described in text.

produced by the reaction of pure gossypol and GA complex with aniline gave a high absorbance at 440 nm. Pons *et al.* (14), apparently used the dianilino derivative because of the high absorbance of the sample blank at 254 nm. This resulted in a problem of differentiating it from GA which also had its maxima in this general area. In the present study, the excellent separation characteristics of the HPLC were used to determine gossypol quantitatively in the GA complex at 254 nm (Fig. 1). This eliminated the use of highly toxic aniline, and still gave results comparable to the spectrophotometric method. Although the maximum peak height, or sensitivity, was 247 nm (Fig. 2), a 254 nm wavelength was chosen because it is the standard used in the readily available single wavelength detector. Further, with a variable wavelength detector it may be advantageous to use 400 nm where there is less sensitivity but the interfering reagent peak is at a minimum. The single and three-dimensional chromatograms (Figs. 1 and 2) of the GA complex were produced by dissolving a small amount of gossypol-acetic acid standard in the complexing reagent used in the official AOCS method. The solution was treated as described under Standardization in the Experimental Procedures Section to produce the GA complex. The mobile phase was basically the same as reported by Abou-Donia *et al.* (7), and Nomeir and Abou-Donia (8) in their determinations of pure gossypol by HPLC. The early small peaks in the GA complex chromatogram (Fig. 1) may indicate a trace of gossypol-related compounds in the gossypol-acetic acid standard. Similar small peaks also were found in other cottonseed chromatograms as in Figure 5. The standardization plot (Fig. 3) was developed by applying linear regression analysis to peak area data obtained from a series of injections with varying complex concentrations. A straight-line relationship between Mg of pure gossypol and peak area, with a correlation coefficient of $r^2 = 0.999$ resulted.

Figure 4 shows the standardization plot for low levels of gossypol to a minimum of about 1 ppm in a 5 g sample ($r^2 = 0.999$).

Figure 5 shows typical chromatograms for total gossypol in raw cottonseed flakes (Fig. 5A) and in a cottonseed meal extracted with hexane (Fig. 5B). Other constituents of unextracted seed seemed to have little effect on the method.

The HPLC total gossypol method also worked well in determining gossypol in crude cottonseed oil and ethanolic miscella (Figs. 6A and 6B). The only significant difference in these chromatograms is that miscella samples showed an additional small peak, probably a reaction product of raffinose and stachyose with the reagents, that eluted at 13–15 min. The oil contained 2.10% and the miscella 340 ppm of gossypol.

The HPLC method and the official AOCS methods (performed in-house) for % TG in various full-fat cottonseed and extracted meal samples yielded quite similar results (Table 1). A least squares regression of the data had a correlation coefficient of $r^2 = 0.982$. The results fell within the standards of a proposed international TG method, which states that duplicate samples run at the same time should not vary by more than 10% (15). The variance is also small when compared to the differences obtained from four commercial laboratories who used the official AOCS method to determine TG in various seed and meal samples (Table 2).

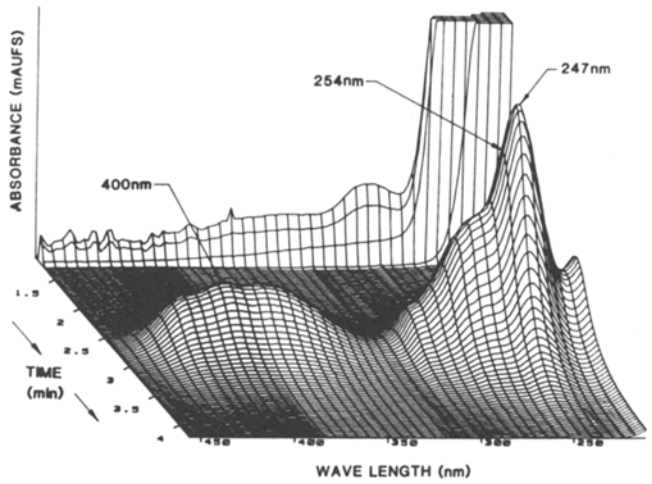

FIG. 2. Three-dimensional chromatogram of GA complex. Chromatographic conditions as described in text.

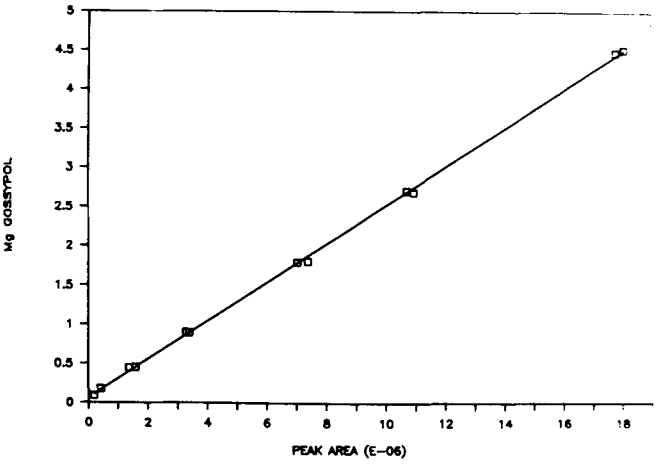

FIG. 3. Standardization plot for measuring high levels of gossypol (>100 ppm) showing the relationship between mg of gossypol and HPLC peak area.

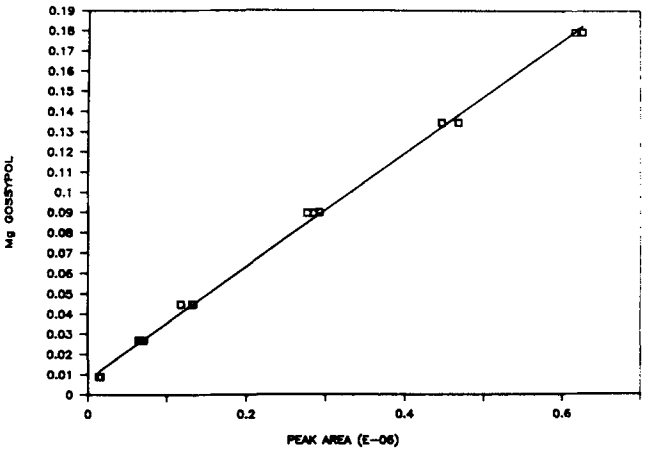

FIG. 4. Standardization plot for measuring low levels of gossypol (1–100 ppm) showing relationship between mg of gossypol and HPLC peak area.

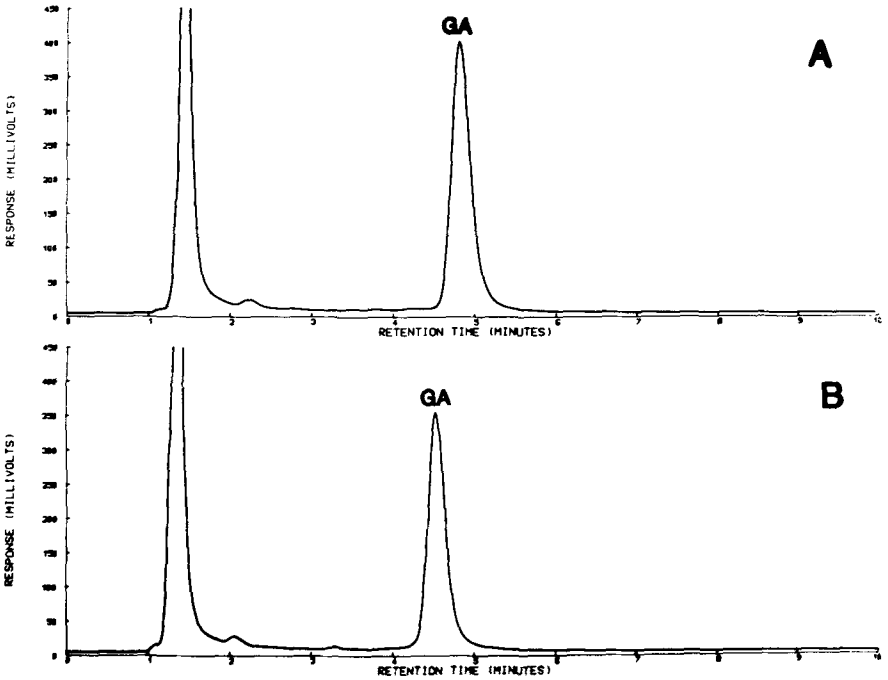

FIG. 5. HPLC chromatograms of GA complex that was produced from (A) raw cottonseed flakes, and (B) a hexane extracted meal by the HPLC TG method. Chromatographic conditions as described in text.

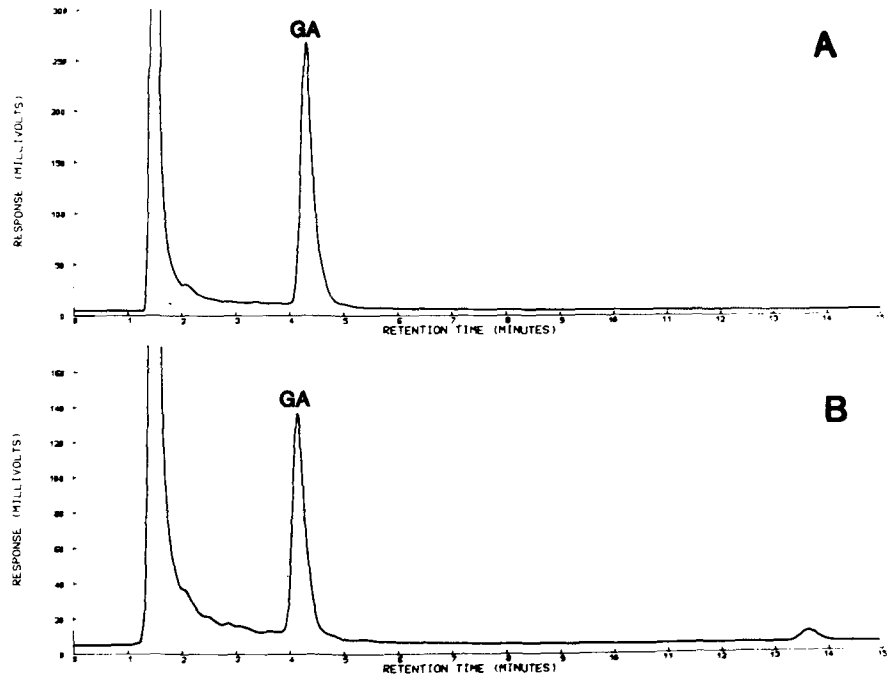

FIG. 6. HPLC chromatograms of GA complex that was produced from (A) crude cottonseed oil, and (B) ethanolic miscella by the HPLC TG method. Chromatographic conditions as described in text.

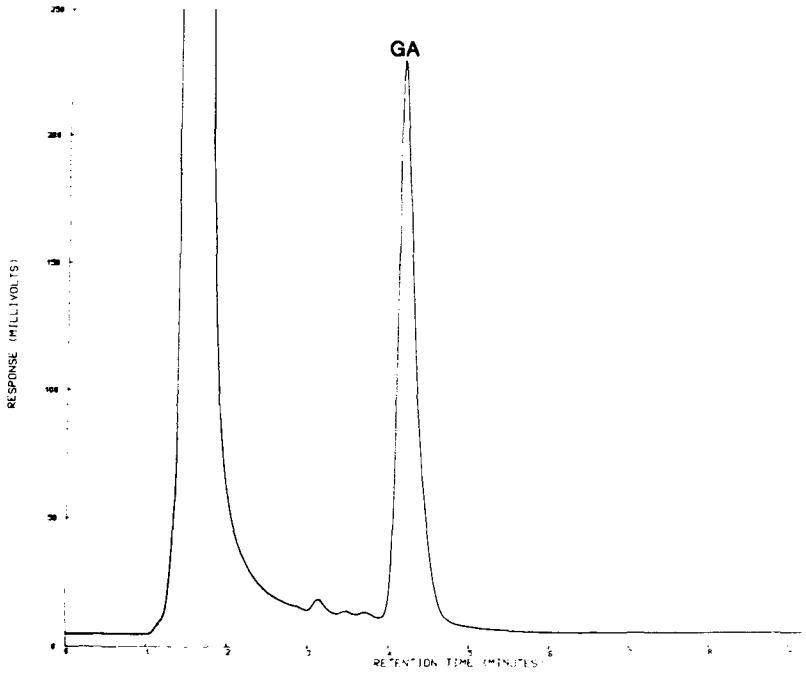

FIG. 7. HPLC chromatogram of GA complex that was produced from raw cottonseed flakes by the HPLC FG method. Chromatographic conditions as described in text.

TABLE 1

Comparison of Percent TG Determined by HPLC and the Official AOCS Method

HPLC	AOCS	HPLC/AOCS (× 100)
0.146	0.142	102.8
0.518	0.531	97.6
0.607	0.609	99.7
0.657	0.646	101.7
0.707	0.748	94.5
0.798	0.793	100.6
0.894	0.876	102.1
0.903	0.890	101.5
1.054	0.973	108.3
1.073	1.126	95.3
1.093	1.050	104.1
1.099	1.125	97.7
1.125	1.095	102.7
1.132	1.086	104.2
1.132	1.030	109.9
1.147	1.125	102.0
1.209	1.172	103.2
1.211	1.144	105.9
1.368	1.313	104.2
1.980	2.086	94.9
	AVG.	101.6

A HPLC method for FG was also developed that is identical to the TG method except that it uses 70% aqueous acetone, as in the AOCS method, to extract FG. Pons *et al.* (14) could not adapt animopropanol to the AOCS determination method for FG because acetone and aminopropanol react to form colored solutions. However, HPLC differentiated these colored solutions and provided a single peak of the GA complex for cottonseed (Fig. 7).

TABLE 2

Comparison of Percent TG Determined by Commercial Laboratories Using the Official AOCS Method and HPLC

Sample	Lab 1	Lab 2	Lab 3	Lab 4	HPLC
1	1.38	1.83	1.40	0.96	1.34
2	0.14	0.07	0.17	0.09	0.16
3	1.36	1.57	1.27	0.97	1.31
4	1.24	1.42	1.16	0.95	1.17
5	0.90	0.80	0.97	0.60	0.88

TABLE 3

Comparison of Percent FG Determined by HPLC and the Official AOCS Method

HPLC	AOCS	HPLC/AOCS (× 100)
0.0168	0.0173	97.1
0.0191	0.0182	104.9
0.0199	0.0208	95.7
0.391	0.398	98.2
0.483	0.523	92.4
0.740	0.740	100.0
0.779	0.768	101.4
0.858	0.827	103.7
0.978	0.917	106.7
	AVG.	100.0

Comparing the present HPLC technique and the official AOCS free gossypol methods among various cottonseed and cottonseed meal samples gave a standard error of analysis, $r^2 = 0.996$ (Table 3).

Future research on the HPLC methods should investigate their use in determining gossypol in mixed feeds. The methods could be refined by examining other GA complex sensitive wavelengths in conjunction with the substitution of another solvent in place of the highly absorbent DMF.

ACKNOWLEDGMENTS

G. S. Fisher provided valuable advice and free and total gossypol determinations using official AOCS methods. V. Jordan performed chromatographic analysis and A. M. Spanier provided the three-dimensional chromatogram of GA complex.

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