Determination of "Free" Gossypol in Cottonseed and Cottonseed Meals by Second-Derivative Ultraviolet Spectrophotometry

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A new spectrophotometric method for the determination of "free" gossypol in cottonseed and cottonseed meals has been developed. The method involves extraction of free gossypol with aqueous acetone, hydrolysis of the "soluble-bound" forms of gossypol with hydrochloric acid, partitioning of the pure compound into chloroform, and analysis by derivative spectrophotometry. Reaction of the analyte with a chromogenic reagent is not required, since the second-derivative transformation and measurement of the conventional analytical band around 300 nm permits direct quantitation of gossypol in sample extracts. The method was evaluated at concentration levels normally encountered in cottonseed meals. Precision and accuracy data suggested an overall precision of 4.0% and an overall accuracy of 91.2 ± 3.6%. The new method gave results comparable with those obtained by the official AOCS method, for all but one of the samples analyzed.

INTRODUCTION

The utilization of cottonseed and cottonseed meals in animal nutrition is limited by the presence of the polyphenolic binaphthyl aldehyde gossypol [1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-binaphthalene-8,8′-dicarboxaldehyde] and some structurally related compounds that occur naturally or form as a result of processing and storage of cottonseed meals. Gossypol and related compounds are toxic, particularly to poultry (Phelps, 1966; Wedegaertner, 1981) and swine (Altschul et al., 1958; Eisele, 1986). As cottonseed meal is a relatively low cost good protein supplement, the occurrence of these toxic compounds, conventionally termed "free" gossypol, is of significant economic importance. Therefore, precise and accurate analytical methods for the determination of free gossypol are valuable both for process control in the industry and for feed inspection purposes.

Numerous analytical methods for the determination of free gossypol in cottonseed and cottonseed meals have been published. Early methods are gravimetric procedures (Carruth, 1918; Halverson and Smith, 1937) based on the precipitation of gossypol as its dianilino derivative. Other methods that have not been widely used are titrimetric (Podol'skaya, 1944), polarographic (Markman and Kolesov, 1956), chemiluminescent (Vil'kova and Markman, 1958), and paper chromatographic (Schramm and Benedict, 1958) procedures. Preferred methods are spectrophotometric procedures based on the reaction of gossypol with antimony trichloride (Boatner et al., 1944; Hall et al., 1948), *p*-anisidine (Pons and Guthrie, 1949), phloroglucinol (Storherr and Holley, 1954; Crouch and Bryant, 1982), or aniline (Miller, 1955; Smith, 1968; AOCS, 1975).

Methods for analyzing individual terpenoid aldehydes have been reported. They include gas–liquid chromatographic (GLC) procedures (Raju and Cater, 1967; Chan et al., 1983), high-performance liquid chromatographic (HPLC) procedures (Abou-Donia et al., 1981; Greenblatt and Stipanovic, 1984; Mahoney and Chan, 1985; Wang, 1987), and nuclear magnetic resonance (NMR) procedures (Stipanovic et al., 1977; Waiss et al., 1978).

Most studies of gossypol toxicity have been based on the spectrophotometric procedures and, primarily, on Official Method Ba 7-50, later Ba 7-58, proposed by the American Oil Chemists' Society (AOCS, 1975). As evidence has been presented that this method does in fact measure physiologically active gossypol-related compounds (Pons, 1977), it is currently considered to be the most appropriate procedure for routine quality control of cottonseed products. It has been shown, however, that procedures based on reaction of gossypol with a chromogenic reagent cannot differentiate between gossypol and other compounds reacting with the reagent (Storherr and Holley, 1954). Heating the extracts in the presence of an aromatic amine may produce colored products of extraneous compounds, leading to erroneous readings (Stipanovic et al., 1984, 1988). On the other hand, application of the highly specific GLC, HPLC, and NMR methods seems to be of limited practical importance in free gossypol analysis unless all other classes of physiologically active gossypol-related compound could be, concurrently, measured. Therefore, a method capable of measuring gossypol-related compounds but not based on reaction with a coloring agent should be particularly useful as an alternative to the AOCS recommended procedure for free gossypol analysis.

A new quantitation system that eliminates through the use of second-derivative UV spectrophotometry (O'Haver and Green, 1976) the need for chromogenic reaction has been recently applied for the determination of total gossypol in cottonseed products (Botsoglou and Kufidis, 1990). The performance of this system on the determination of free gossypol in cottonseed and cottonseed meals is investigated in this study.

MATERIALS AND METHODS

Instrumentation. A Perkin-Elmer Model 512 double-beam UV–vis spectrophotometer equipped with 10-mm quartz absorption cells was used in this study. Derivative UV spectra were produced by electronic differentiation of the spectrophotometer output signal and monitored on a Perkin-Elmer Model 165 chart recorder. Electronic differentiation of the output signal was accomplished by a Perkin-Elmer Model 200-0507 derivative spectrum unit that permitted selection of six (1, 2, ..., 6) time constants (sensitivities). Since positive as well as negative signals were to be expected in derivative spectra, the recorder pen had to be set at 50% full-scale deflection before scanning. Second-derivative spectra were recorded in the wavelength range 260–340 nm at a scanning speed of 240 nm/min with monochromator slit set at 3 nm and time constant 5.

Chemicals. The solvent mixture used in the analysis was prepared by mixing 715 mL of ethanol, 285 mL of all-glass-distilled

0021-8561/91/1439-0478$02.50/0

water, 200 mL of peroxide-free diethyl ether, and 2 mL of glacial acetic acid. All chemicals were of ACS grade.

Gossypol stock solution was prepared by weighing ca. 12 mg of standard gossypol–acetic acid (89.62% gossypol by weight; Makor Chemicals, Ltd.) and adding solvent to give 50 mL. Aliquots of this solution were further diluted to give working solutions in the range 0.7–4 μg/mL. Stock and working solutions were prepared daily and protected from light throughout the analysis.

Extraction Procedure. An accurately weighed sample of ground (1-mm screen) cottonseed meal (ca. 1 g) or dehulled and ground (2-mm screen) cottonseeds (ca. 0.5 g) was transferred to a 250-mL glass-stopper Erlenmeyer flask. The bottom of the flask was covered with glass beads (4 mm in diameter), a volume (50 mL) of 70% aqueous acetone was added, and the flask was shaken for 1 h on a wrist action mechanical shaker. Flask content was filtered through Whatman No. 40 filter paper into another flask; the first 5 mL of the filtrate was discarded.

Hydrolysis and Cleanup Procedure. A 25-mL aliquot of the filtrate was pipetted into a 50-mL volumetric flask, and 1 drop of hydrochloric acid (0.05 mL) was added. The flask was placed in a 65 °C water bath, heated for 1 h, removed from the bath, and cooled to room temperature. Flask content was transferred to a 250-mL separatory funnel with two rinses, 3 mL each, of 70% aqueous acetone, and a 100-mL volume of distilled water acidified with 1 mL of hydrochloric acid was added in the funnel. The suspension formed was extracted with 50 mL of chloroform, and the lower organic layer was filtered through anhydrous sodium sulfate on Whatman No. 40 paper into a 100-mL glass-stopper round-bottom flask. Sodium sulfate and filter paper were rinsed with an additional 5 mL of chloroform, and the combined filtrates were evaporated to dryness by using, initially, a rotary vacuum evaporator at 35 °C and, finally, a nitrogen stream. The remaining residue was dissolved with 25 mL of the solvent mixture and stored in the flask until analyzed.

Second-derivative UV spectra were obtained by running samples against the solvent mixture according to prementioned conditions. Quantitation was based on measurement of the recorded D_1D_2 heights (Figure 1). When D_1D_2 heights were out of the recorder range, samples were further diluted with the solvent mixture. All of the sample solutions gave essentially unchanged values for gossypol on standing for periods of time up to 6 h. Calibration curves were constructed by plotting the D_1D_2 heights versus concentration for each of the prepared working solutions. The concentration of gossypol in samples was calculated by reference to calibration curve and multiplication by appropriate dilution factor.

RESULTS AND DISCUSSION

Typical ultraviolet and the corresponding second-derivative spectra of a cottonseed meal extract and a standard gossypol solution are illustrated in Figure 1. The absorbance differentiation $d^2A/d\lambda^2$, where A is the absorbance and λ is the wavelength, transformed the normal spectrum into a series of maxima and minima that were used to resolve and locate the analytical band. This differentiation enhanced the intensity of sharp features and diminished the effect of overlapping bands. Consequently, the distance in the ordinate direction between the characteristic maximum D_1 and the adjoining minimum D_2, which corresponded, respectively, to the inflection point and to the maximum of the conventionally recorded analytical band around 300 nm, can also be used for quantitative purposes (Botsoglou et al., 1985; Blanko and Sanchez, 1986; El-Yazbi et al., 1986; Kitamura et al., 1987). Regression analysis of the data obtained by running a series of working solutions showed the reponse to be linear within the range studied (Table I). Therefore, gossypol could be readily quantitated by reference to calibration curve [$y = 1.1 + 30.7x$, $r = 999$, where y represents the D_1D_2 height (millimeters) and x the concentration of gossypol (micrograms per milliliter).

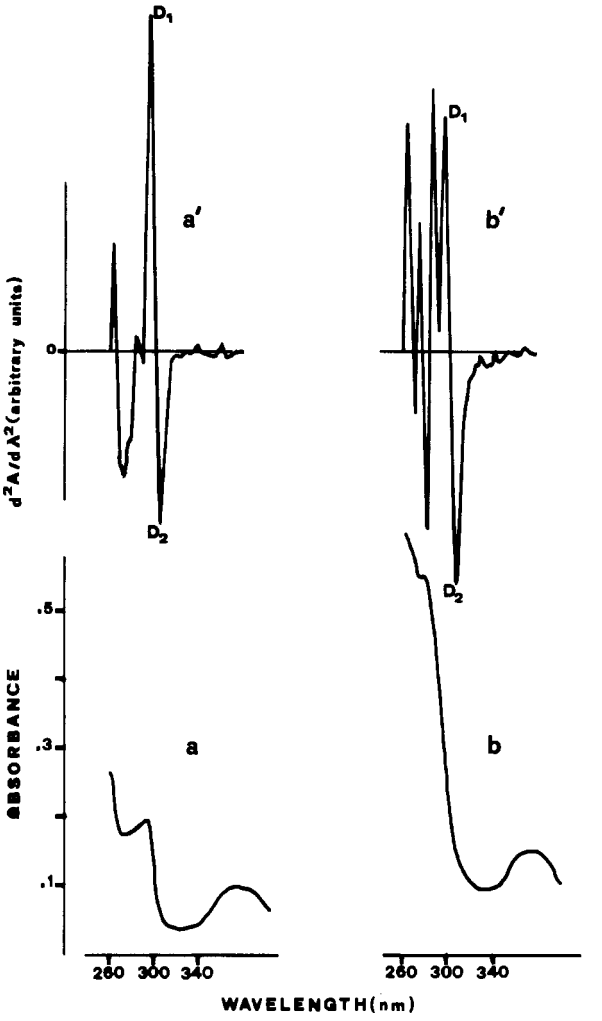

Figure 1. Ultraviolet (a, b) and the corresponding second-derivative (a′, b′) spectra of a standard solution of gossypol (a) and an extract of cottonseed meal (b).

Table I. Precision Data of Calibration Curve for Gossypol Determination by Second-Derivative Spectrophotometry

concn of gossypol in std soln, μg/mL	mean D_1D_2 height[a] (n = 5), mm	SD	rel SD, %
0.717	20.8	0.8	3.9
1.434	43.1	0.9	2.1
2.151	65.7	0.4	0.6
2.868	85.4	1.1	1.3
3.585	109.8	0.8	0.7

[a] Regression equation: $y = 1.1 + 30.7x$. Correlation coefficient $r = 0.999$.

The procedure used to extract free gossypol from cottonseed and cottonseed meals was that suggested by Pons and Guthrie (1949) and adopted by the American Oil Chemists' Society (AOCS, 1975). According to AOCS Method Ba 7-58, the term "free" gossypol defines gossypol and gossypol-related compounds that are soluble in 70% aqueous acetone under specified conditions. Because the definition of this term depends to a large extent on the solvent used for extraction, free gossypol in this study refers to data obtained by following the AOCS method; application of a different extraction system was not attempted.

The liquid–liquid partition process followed in the cleanup procedure was that reported by Schramm and Benedict (1958). By this process gossypol could be quantitatively transferred from aqueous acetone extracts to chloroform. However, most free gossypol in aqueous acetone extracts of cottonseed meals consists of the so-

Table II. Effect of Heating Cottonseed Meal Extracts with Hydrochloric Acid on Their Gossypol Content

time of heating, min	mean[a] concn of gossypol, ppm, in extracts[b] heated at 85 °C	75 °C	65 °C
5	290 (58.3)[c]		
10	382 (77.2)	297 (59.8)	
15	379 (76.5)		
20	335 (67.5)	359 (72.5)	
30		378 (76.4)	
40		344 (69.4)	319 (64.3)
50			359 (72.5)
60			380 (76.8)

[a] Duplicate analysis. [b] Unheated extracts were found to contain 225 ppm of gossypol. [c] Values in parentheses are the recorded D_1D_2 heights (millimeters). Sample extracts had been diluted to 75 mL with solvent mixture.

called "soluble-bound" forms that include complexes of gossypol with phosphatides, amino acids, and small peptides (Dechary, 1957; Martin, 1959; Matson et al., 1960). Therefore, conversion of these soluble-bound forms to gossypol was required. King et al. (1958) pointed out that the soluble-bound forms can be converted into gossypol by heating the aqueous acetone extracts for 1 h at 65 °C with 1% hydrochloric acid. Deacon (1968) reported that this conversion might also be effected by heating the cottonseed meal extracts for 10 min at 100 °C with dilute hydrochloric acid. Accordingly, the use of hydrochloric acid for the conversion of these compounds into gossypol for determination by second-derivative spectrophotometry was investigated.

Because of the known stability of gossypol in aqueous acetone, it was used as the solvent for hydrolysis of the soluble-bound forms. Duplicate aliquots (25 mL) of the aqueous acetone extract of a solvent-extracted cottonseed meal were heated with 1 drop of hydrochloric acid at 85 °C for 5, 10, and 15 min, at 75 °C for 10, 20, 30, and 40 min, and at 65 °C for 40, 50, and 60 min. The hydrolyzed mixtures were cooled, partitioned into chloroform, and analyzed by second-derivative spectrophotometry. The results, shown in Table II, indicate that the soluble-bound forms can be converted into gossypol by heating the aqueous acetone extracts with hydrochloric acid, but the hydrolysis cannot proceed for more than 35 min at 85 °C or 30 min at 75 °C without apparent loss in gossypol content. Hydrolysis for 60 min at 65 °C, 10 min at 85 °C, or 30 min at 75 °C worked equally well. However, examination of the corresponding second-derivative spectra showed that high-temperatures increased the intensity of the adjacent analytical bands. Therefore, the 60-min hydrolysis at 65 °C appears to be the best choice.

Chloroform extracts were evaporated to dryness at 35 °C since they contained acetone, which interferes with the UV spectrophotometric analysis (UV cutoff at 330 nm). Unattending evaporation at temperatures higher than 40 °C was avoided as low recoveries of gossypol could result.

Since there may have been interferences in extracted samples affecting the accuracy of the second-derivative determination, a standard addition method of analysis was evaluated. Nine of 12 samples from a screw-pressed cottonseed meal were spiked with standard gossypol at three fortification levels by using a gossypol–acetic acid solution in 70% aqueous acetone (1.35 mg of gossypol/mL), and all samples were submitted to free gossypol determination by the developed method. Least-squares and regression analyses of the data (Table III) based solely on the three-level spiking showed that the relationship between "added" (x) and "found" (y) gossypol was adequately described by a linear regression ($y = 214 + 0.894x$, $r = 0.996$). Since the intercept of this regression line, which actually is the value in parts per million that is predicted for the unspiked samples, was not different from the arithmetic mean of the unspiked samples (214 vs 206), it might be concluded that interferences were not present in extracted samples. The absence of interfering bands of other absorbing compounds permitted accuracy evaluation to be made by using the data from both the spiked and unspiked samples. Least-squares and regression analyses of these data showed that linearity was quite acceptable ($y = 208 + 0.912x$, $r = 0.998$). Therefore, the slope (0.912 ± 0.036) of this regression line could be used as an estimate of the overall recovery ($91.2 \pm 3.6\%$) in the proposed method.

Table III. Accuracy Data Based on Recovery of Gossypol Added to a Cottonseed Meal at Three Concentrations

spiking level	concn of gossypol added ($n = 3$), ppm	mean concn found, ppm ± SD
–	0	206 ± 4.4
1	135	330.7 ± 0.6
2	270	463.7 ± 9.3
3	405	572 ± 8.7

Table IV. Precision Data for the Determination of Free Gossypol by Second-Derivative UV Spectrophotometry

day	concn of gossypol found, ppm	mean value, ppm	SD	rel SD, %
1	366, 391, 415 363, 393, 406	389	20.9	5.4
2	380, 380, 366 397, 386, 383	382	10.1	2.6
3	405, 407, 377 402, 390, 400	397	11.4	2.9
	overall mean	389	15.3	3.9

Variance Estimates source	rel SD, %
within day	3.8
between day	1.1
overall	4.0

Table V. Free Gossypol Content of Cottonseed Products As Determined by Different Methods

type of product	concn of gossypol, ppm, found by AOCS Method Ba 7-58	derivative spectrophotometry	combined procedure[a]
1. screw-pressed meal (single-pressed)	624	549	561
2. screw-pressed meal (double-pressed)	315	299	309
3. screw-pressed meal (single-pressed)	1130	1060	1045
4. screw-pressed meal (double-pressed)	841	748	745
5. solvent-extracted meal	718	679	704
6. solvent-extracted meal	627	567	602
7. solvent-extracted meal	647	592	606
8. solvent-extracted meal	790	390	617
9. cooked cottonseed	9812	10139	9265
10. raw cottonseed	10424	10546	9644

[a] By hydrolyzing the aqueous acetone extract, partitioning it into chloroform, evaporating the solvent, and determining gossypol in the residue as dianilinigossypol according to AOCS method.

The precision of the method was studied by assaying, on each of three different days, six samples of a solvent-extracted cottonseed meal. To estimate the overall precision, the raw data (Table IV) were subjected to "analysis of variance and expected mean squares for the one way classification-balanced design" (Wernimont,

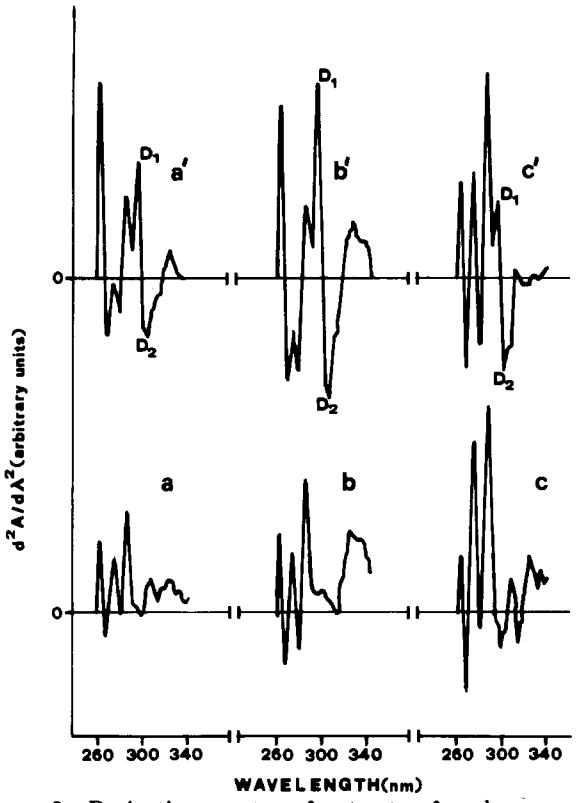

Figure 2. Derivative spectra of extracts of soybean meal, cornmeal, and barley meal alone (a, b, c, respectively) and spiked with 75 ppm (a'), 120 ppm (b'), and 76 ppm (c') gossypol.

1987). It was found that the overall precision was 4.0%, whereas the within-day and between-day precision values were 3.8% and 1.1%, respectively.

To validate the method, two cottonseed samples and several screw-pressed and solvent-extracted cottonseed meal samples were submitted to free gossypol analysis by the described procedure. Aliquots of the aqueous acetone extracts of all samples were treated also according to the recommended procedure except that the residues remaining in the flasks after chloroform removal were dissolved with aqueous acetone and analyzed by the AOCS method. Other aliquots of the same aqueous acetone extracts were analyzed directly by the official AOCS method. The results presented in Table V show that the proposed method gives values that vary between 88% and 95% of those obtained by the AOCS method in seven of the eight cottonseed meal samples analyzed. They also indicate that when the aniline procedure is applied to meal extracts that have been hydrolyzed and cleaned up, the agreement with the derivative method is remarkably good. Clearly, the hydrolysis–cleanup is losing ca. 8% of the contained gossypol. On the other hand, it appears that the proposed method gives values about 10% higher than the AOCS method when applied to seed. This difference could not be attributed to the effect of overlapping bands since the curves for seed extracts were quite similar to the one for standard gossypol (Figure 1). The considerably lower value found for sample 8 was not quite unexpected as, when the analysis was performed, part of the residue remaining in the flask after chloroform removal could not be dissolved in the solvent mixture. As this solvent mixture has been widely used in the past for free gossypol extractions (Smith, 1968), the inconsistency for sample 8 between the proposed and the AOCS method might be attributed to extraneous compounds derived from cottonseed processing, which could react with aniline to develop color and increase absorbance readings.

Applicability of the proposed method to glandless cottonseed and mixed feeds was not investigated. Soybean meal, cornmeal, and barley meals contain substances that interfere with gossypol analysis by reaction-based spectrophotometric methods (Storherr and Holley, 1954; Stipanovic et al., 1984). However, when these were submitted to free gossypol analysis, the derivative spectra obtained (Figure 2) were free of interferences. Recoveries of 87%, 96.3%, and 85% were noted for soybean meal, cornmeal, and barley meal, respectively when the samples were spiked with standard gossypol and resubmitted to analysis.

In conclusion, the results of the present study show that the proposed second-derivative spectrophotometric method is an efficient and reliable means of quantitating free gossypol at concentration levels normally expected for cottonseed and cottonseed meals. The analytical performances offered by the method make it an alternative to the AOCS recommended procedure. Although the method involves a multistep analytical scheme, one analyst can easily process eight samples in 3 h. The equipment needed is easily accessible as most modern UV spectrophotometers allow almost instant generation of derivative spectra. Therefore, the proposed method could also be useful to the industry for routine monitoring of free gossypol in cottonseed products.

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Received for review April 9, 1990. Revised manuscript received September 24, 1990. Accepted October 19, 1990.