

Determination of (+)-, (–)-, and Total Gossypol in Cottonseed by High-Performance Liquid Chromatography

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ABSTRACT: Gossypol, a pigment in cottonseed, is a polyphenolic, binaphthyl dialdehyde. Due to steric hindrance between the functional groups of the molecule at the bond connecting the two naphthyl rings, gossypol exists as (+)- and (–)-isomers. Gossypol is physiologically active with the (–)-isomer appearing to be more active and causing temporary infertility in males. It is thus important to know the amounts of isomers in livestock feeds. A quantitative high-performance liquid chromatography (HPLC) procedure was developed for the separation of (+)- and (–)-gossypol contained in cottonseed. This method involves derivatization of gossypol with (*R*)-(–)-2-amino-1-propanol followed by HPLC separation employing either a Phenomenex Prodigy (5 μ , ODS-3, 100 \times 3.2 mm) or a MetaChem Inertsil (5 μ , ODS-3, 100 \times 3.0 mm) reversed-phase column eluted with 80% acetonitrile and 20% 10 mM KH₂PO₄ adjusted to pH 3.0 with H₃PO₄ at 1.0 mL/min. The (+)- and (–)-gossypol-2-amino-1-propanol complexes eluted at roughly 1.4 and 2.6 min, respectively. It was found that gossypol from Upland (*Gossypium hirsutum*) seed was rich in the (+)-enantiomer, with the (+)- and (–)-enantiomers in a ratio of about 65:35, respectively, while gossypol from the seed of a Pima (*G. barbadense*) cultivar (S-6) was slightly richer in the (–)-enantiomer (46.8:53.2).

Paper no. J9171 in *JAOCs* 76, 1351–1355 (November 1999).

KEY WORDS: Barbadense, cottonseed, enantiomers, gossypol, *hirsutum*, HPLC, 6-methoxygossypol, Pima, polyphenol, Upland.

Cottonseed, a coproduct of cotton production, is either fed primarily to cattle as whole seed, or the oil is extracted to provide edible oil for human consumption and the meal is used as a feed supplement for livestock. However, cottonseed contains the polyphenol gossypol. Gossypol, and small quantities of several pigments contained in processed cottonseed meal with structures similar to gossypol, may exhibit adverse physiological properties (1). It can be toxic to all animals in sufficient quantity (1), but has been used, in small controlled doses as a male contraceptive in humans (2). Gossypol can also cause temporary male infertility in livestock. To maximize cottonseed's economic utilization, it is important to determine the biological activities of gossypol, its isomers, and

related compounds. Due to steric hindrance between the functional groups of the molecule at the bond connecting the two naphthyl rings (atropisomerism), gossypol has both (+)- and (–)-isomers, as shown in Figure 1. Proportions vary with the species and variety of cotton. In the seed of the commercially important varieties of Upland cotton (*Gossypium hirsutum*) grown in the United States, the predominant isomer is (+)-gossypol; whereas, in seed from the Pima (*G. barbadense*) varieties currently being grown (–)-gossypol tends to predominate (3). Pima cotton also contains small but quantifiable amounts of (+)- and (–)-6-methoxygossypol isomers (Fig. 1) and trace amounts of (+)- and (–)-6,6'-methoxygossypol isomers, while Upland seed contains very small amounts of these gossypol-like isomers (4). Pima cotton typically accounts for 2–3% of annual U.S. cotton production. It is primarily grown in California, with lesser amounts grown in Arizona, Texas, and New Mexico, whereas Upland cotton is grown throughout the cotton-growing belt. Although the toxic effects of gossypol on animals have been extensively studied (1,5), very little information is available on the comparative toxicity of its isomers. This is due to the lack of a suitable method for quantifying the isomers, and the unavailability of the individual isomers in sufficient quantities for animal studies. (+)-Gossypol was first crudely isolated in 1968 from the leaves and flowers of the tropical tree *Thespesia* using chemical means (6). However, it was not known until 1979 that (+)-gossypol does not exhibit antifertility activity in males or the reduction of body weight frequently observed with racemic gossypol (7). Thus, except for contraceptive activity and weight reduction, the differences in physiological properties between (+)- and (–)-gossypol have not been thoroughly investigated. Various high-performance liquid chromatography (HPLC) methods for separating gossypol enantiomers have been developed since 1968 (3,8–11). Although Cass (3) measured isomer ratios in many different cottonseed samples, he did not quantitate his results nor demonstrate the precision and accuracy of his method. The methods of Wu *et al.* (10) and Kim *et al.* (11), which used [*R*]-[–]-2-amino-1-propanol to convert gossypol to an anil, were developed for analysis of plasma and tissue. They are micro (4 mg maximum sample weight) methods and are not practical for analysis of cottonseed samples. This paper describes modification of the method of Kim *et al.* (11) to quantitatively determine (+)-,

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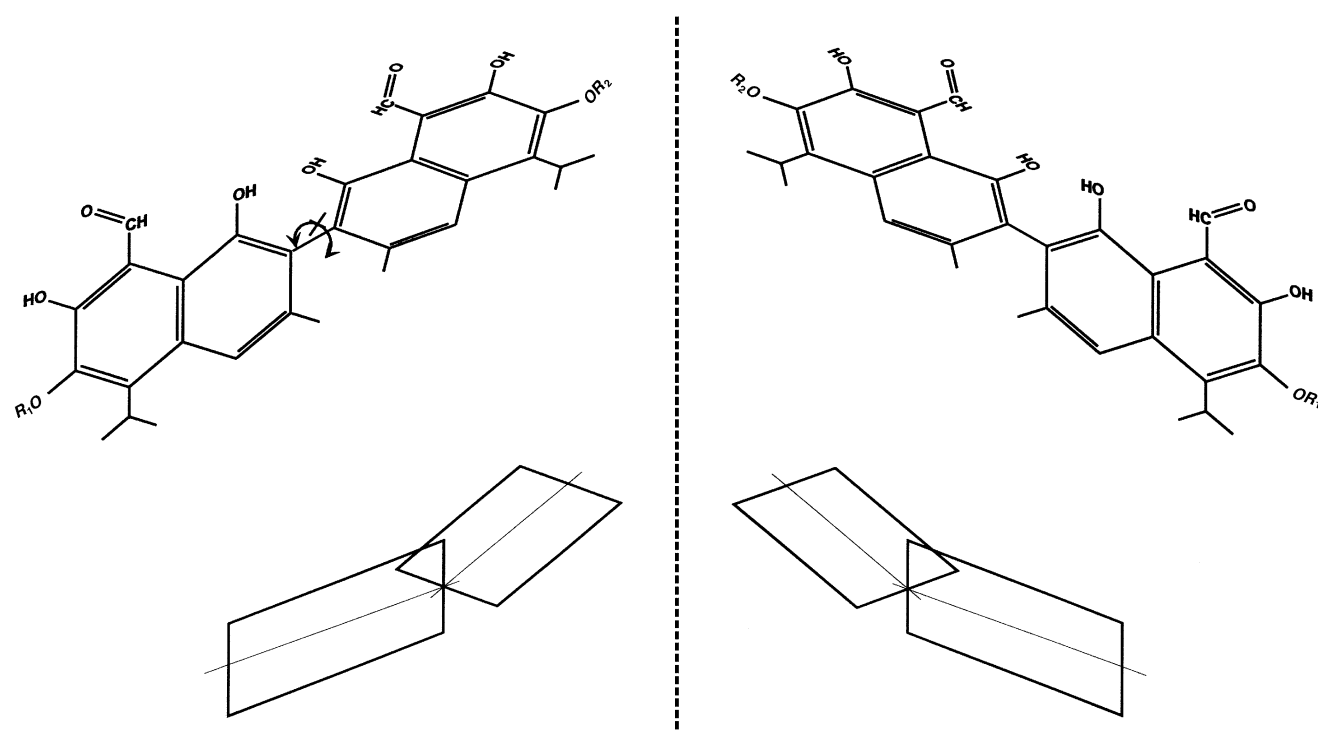


FIG. 1. Enantiomers of gossypol and other minor gossypol-like compounds in cottonseed. Gossypol, $R_1, R_2 = H$; 6-methoxygossypol, $R_1 = H, R_2 = CH_3$; 6,6'-dimethoxygossypol, $R_1, R_2 = CH_3$.

(-)-, and total gossypol (TG) contained in cottonseed obtained from different areas of the U.S. Cottonbelt.

EXPERIMENTAL PROCEDURES

Apparatus. The liquid chromatograph (LC) pump was an HP 1100 (Hewlett-Packard, Avondale, PA), operated isocratically with a mobile phase flow rate of 1.0 mL/min. The LC was fitted with either a Phenomenex, Inc. (Torrance, CA) Prodigy (5 μ , ODS-3, 100 \times 3.2 mm) or MetaChem Technologies Inc. (Torrance, CA) Inertsil (5 μ , ODS-3, 100 \times 3.0 mm) reversed-phase column. Samples were injected using a Rheodyne 1725 injector (Rheodyne Inc., Cotati, CA) equipped with a 20- μ L sample loop. A Hewlett-Packard 1040A scanning diode array detector set at 254 nm and a HP Chem Station (Pascal Series) were used to measure peak area (absorbance units, AU) on a valley-to-valley baseline basis.

Solvents and reagents. The mobile phase consisted of 80% acetonitrile (MeCN) and 20% 10 mM KH_2PO_4 buffer adjusted to pH 3.0 with H_3PO_4 . It was prepared by adding 200 mL of a solution containing 1.36 g/L KH_2PO_4 (reagent grade) and adjusted to pH 3.0 with H_3PO_4 (reagent grade) to 800 mL of MeCN (HPLC grade). Mobile phase was brought up to room temperature before use. Complexing reagent was made by mixing 2 mL of *d*-alaninol ([R]-[α]-2-amino-1-propanol) (Sigma Chemical Co., St. Louis, MO, 97% pure; or Lancaster Synthesis, Pelham, NH, 98% pure) and 10 mL glacial acetic acid (reagent grade) into a 100 mL volumetric flask, cooling to room temperature, and diluting to volume with *N,N*-dimethylform-

amide (spectrophotometric grade). Complexing reagent was stored in the refrigerator and brought to room temperature before use. Standard racemic gossypol solution was made by accurately weighing 38.0 mg of gossypol acetic acid (GAA) into a 25-mL volumetric flask, bringing to volume with complexing reagent, and mixing until all GAA was in solution. GAA produced in the Southern Regional Research Center's pilot plant from cottonseed gums was used as a source of racemate (12,13). GAA is very stable in comparison to pure gossypol, which is a strong antioxidant. The sample used in this study was over 98% pure after storage at $-6^\circ C$ for over 30 yr.

Standardization. Standard gossypol solutions of 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mL were pipetted into 20 \times 125 mm, round-bottom glass tubes fitted with natural polypropylene screw caps. Additional complexing reagent was pipetted to give a total volume of 4 mL in each tube. The tubes were tightly capped and heated in a 100°C dry bath for 30 min, cooled, and 16 mL of mobile phase was pipetted into each tube. After capping the tubes and thoroughly mixing the contents, the standards were filtered through Whatman No. 2 filter paper. A 5-mL aliquot of each standard was pipetted into a 10-mL volumetric flask, and the flasks were filled to volume with mobile phase. The 20- μ L injector loop was flushed and filled with standard. The standard was injected into the column and the total run time was 4 min. The (+)- and (-)-gossypol-aminopropanol (GA) isomer peaks eluted at 1.4 and 2.6 min, respectively. The (+)-GA isomer peak position was identified earlier by Kim *et al.* (11) using (+)-gossypol isolated from *Thespesia populnea*. The weights of pure gossypol

used (wt of GAA \times 0.8962, which assumes that the GAA is virtually pure) and the corresponding peak areas were used to calculate the linear calibration equation constants m (slope) and b (intercept) for each isomer:

$$\begin{aligned} \text{mg (+)-gossypol} &= m_{(+)} [(\text{+})\text{-peak area}] + b_{(+)} \\ \text{mg (-)-gossypol} &= m_{(-)} [(\text{-})\text{-peak area}] + b_{(-)} \end{aligned} \quad [1]$$

Total, (+)-, and (-)-gossypol sample analysis. Acid-delinted cottonseed samples from the 1996 crop were cracked in a Bauer mill (No. 148, The Bauer Bros. Co., Springfield, OH), and whole and cracked meats were separated out. The meats were then dried for 4 h at 82°C in a Blue M Touchmaster forced-draft oven (General Signal, Blue Island, IL). A 0.2-g ground sample of meats (Retsch Centrifugal Grinding Mill ZM 1, 1.0 mm screen, Brinkmann Instruments Inc., Westbury, NY) containing 1.6–3.5 mg of gossypol was accurately weighed into a 20 \times 125-mm sample tube. Four milliliters of complexing agent was added, the tube capped, and the gently mixed slurry was heated in a dry bath (100°C) for 30 min. The slurry was cooled for 30 min and 16 mL of mobile phase was added. After capping, the slurry was well mixed by repeated inversions and filtered through Whatman No. 2 filter paper. A 5-mL aliquot of filtered sample was pipetted into a 10-mL volumetric flask and the flask was filled to volume with mobile phase. The 20- μ L injector loop was flushed with sample and the sample was injected.

Gossypol recovery. Standard gossypol solutions of 0.5, 1.0, 2.0, 3.0, 3.5, and 4.0 mL, containing 1.3622 mg gossypol/mL, were pipetted into six 20 \times 125-mm round-bottom glass tubes each containing 0.2 g of the same milled meat sample. Additional complexing reagent was pipetted to give a total complex volume of 4 mL in each tube. The tubes were then treated as in the total, (+)-, and (-)-gossypol sample analysis.

Calculations. The (+)- and (-)-GA complex peak areas for the sample and the appropriate standardization m and b constants were used to calculate the percentages of (+)- and (-)-gossypol using the following formulas:

$$\% \text{ (+)-gossypol} = \frac{m_{(+)} [(\text{+})\text{-peak area}] + b_{(+)}}{\text{sample weight (g)} \times 10} \quad [2]$$

$$\% \text{ (-)-gossypol} = \frac{m_{(-)} [(\text{-})\text{-peak area}] + b_{(-)}}{\text{sample weight (g)} \times 10} \quad [3]$$

The (+) and (-) percentages of gossypol were then added together to obtain the percent total gossypol in each sample.

RESULTS AND DISCUSSION

Figure 2 is a chromatogram showing the derivatives of racemic gossypol with (*R*)-(-)-2-amino-1-propanol (GA). The (+)- and (-)-GA derivatives, essentially equal in area, eluted at 1.44 and 2.66 min, respectively. The small peak at 1.97 is an unknown impurity in the aminopropanol derivatization agent that has reacted with gossypol. It represents 2–3% of the

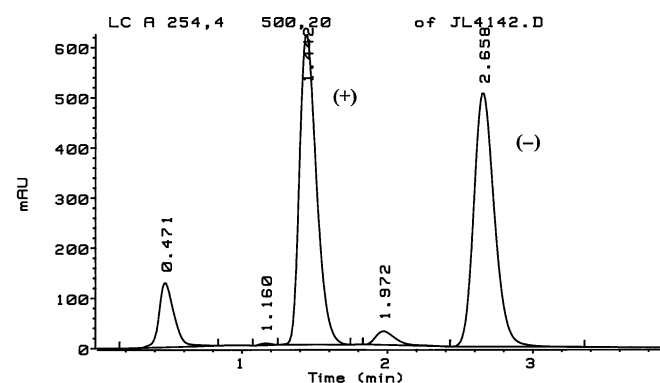


FIG. 2. High-performance liquid chromatography (HPLC) chromatogram of the (+) and (-)-gossypol-aminopropanol (GA) isomers produced by the reaction of (+)-2-amino-1-propanol with gossypol acetic acid racemate. The (+)-isomer eluted at 1.44 min and the (-)-isomer at 2.66 min. The peak eluting at 1.97 min is a reaction product of an aminopropanol impurity with gossypol. Chromatographic conditions as described in text.

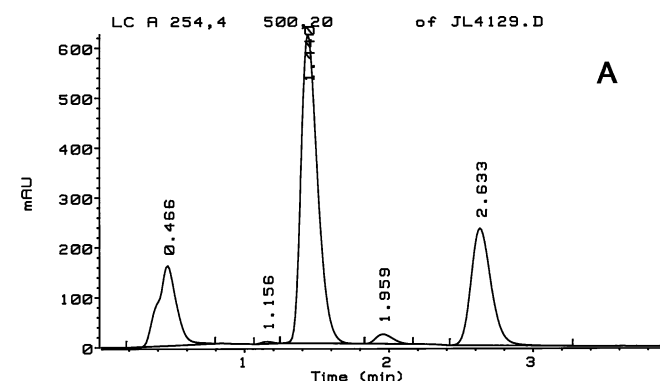


FIG. 3A. HPLC chromatograms of the (+) and (-)-GA isomers produced from milled Upland variety kernels. The (+)- and (-)-isomers eluted at 1.44 and 2.63 min, respectively. The peak eluting at 1.96 is a reaction product of an aminopropanol impurity with gossypol. Chromatographic conditions as described in text. See Figure 2 for abbreviations.

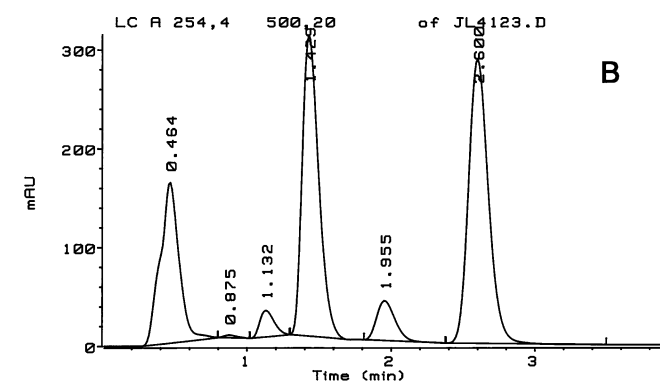


FIG. 3B. HPLC chromatograms of the (+) and (-)-isomers produced from milled Pima variety kernels. The (+)- and (-)-GA isomers eluted at 1.43 min and 2.6 min, respectively. The (+)- and (-)-methoxygossypol-aminopropanol complex peaks eluted at 1.13 and 1.96 min, respectively. Chromatographic conditions as described in text. See Figure 2 for abbreviations.

TABLE 1
Percent Total Gossypol and (+) and (–)-Isomers in Various 1996 Crop Upland and Pima Variety Cottonseed Meat Samples^a

| Variety | Total gossypol | | | | Isomers | | | |
|---------|----------------|---------------------|--------------------------|--------|-----------------------|---------|---------|--------|
| | HPLC (%) | SD ^b (%) | Ba 8-78 ^c (%) | SD (%) | HPLC ^d (%) | (+) (%) | (–) (%) | SD (%) |
| Upland | 0.919 | 0.015 | 1.044 | 0.023 | 1.066 | 67.5 | 32.5 | 0.2 |
| | 1.383 | 0.009 | 1.584 | 0.024 | 1.603 | 68.6 | 31.4 | 0.1 |
| | 1.586 | 0.024 | 1.858 | 0.024 | 1.837 | 69.7 | 30.3 | 0.2 |
| | 0.964 | 0.009 | 1.134 | 0.013 | 1.118 | 65.9 | 34.1 | 0.4 |
| | 0.854 | 0.011 | 0.993 | 0.018 | 0.991 | 61.9 | 38.1 | 0.2 |
| | 1.542 | 0.030 | 1.776 | 0.018 | 1.787 | 69.3 | 30.7 | 0.1 |
| Pima | 0.997 | 0.017 | 1.169 | 0.008 | 1.156 | 46.8 | 53.2 | 0.1 |

^aAll samples replicated 5X.
^bStandard deviation.
^cReference 16, AOCS method.
^dPercent gossypol calculated using linear least squares relationship of AOCS method Ba 8-78 to high-performance liquid chromatography (HPLC) method.

total (+) and (–) area, as would be expected based on the reported purity of the aminopropanol reagent. Although gossypol does not racemize easily, (+)-anhydrogossypol has been reported (14) to racemize at 180–200°C. Individual diastereoisomers of gossypol Schiff’s bases have also been reported to racemize in sunlight (15). However, none of these conditions existed in this method and no racemization of isomers was observed. The GA derivatives produced here degraded very slowly over time, but the degradation was not found to affect quantification after standing for 1 h after preparation.

Figure 3A is a chromatogram of the GA isomers from a 1996 crop of Upland cottonseed rich in the (+)-enantiomer with a percentage ratio of 67.6:32.4 for the (+)- and (–)-enantiomers, respectively. Figure 3B, a chromatogram produced from a seed sample from a 1996 Pima crop in El Paso, Texas, shows a slightly greater percentage of the (–)-gossypol enantiomer—53.1% compared to 46.9% for the (+)-enantiomer. Pima products, besides being higher in the (–)-enantiomer, also usually show a greater abundance of other gossypol-like compounds as compared to Upland seed products. The two significant peaks eluting at 1.13 and 1.96 min are 6-methoxygossypol-aminopropanol isomers. These two peaks, along with the 1.96 min peak at eluting on top of the aminopropanol impurity-gossypol complex peak, interfere with an accurate determination of TG in Pima seed samples. In order to cleanly resolve the 6-methoxygossypol-aminopropanol isomers, it was necessary to use 80% acetonitrile in the mobile phase. However, if Pima seed samples are not being analyzed, the acetonitrile ratio can be increased from 80 to 85%, which reduces sample analysis time for Upland seed samples to under 3 min.

Table 1 shows the percentages of TG and its (+) and (–) isomers found in Upland and Pima seed varieties. Three high-gossypol varieties also have the highest percentage of the (+)-isomer. The (+)-isomer in the Upland seed set varied from 61.9 to 69.7%, similar to the 61% reported by Cass *et al.* for an Upland variety (3). The precision of the HPLC method, as shown by the SD for the HPLC samples, replicated five times, is comparable to the SD found for the official AOCS method

samples. Table 1 also shows a significant difference between TG determined by the official AOCS spectrophotometric method (16) and the HPLC method described here. The consistently lower results for the HPLC method are expected because by definition the official AOCS method measures both gossypol and gossypol-like compounds, whereas the HPLC method can distinguish between gossypol and gossypol-like compounds. Although there is not an exact correspondence between the two, they are highly correlated, as can be seen in the table. A linear least squares relationship between the HPLC and AOCS methods gives a correlation factor $r^2 = 0.999$, with an SE of 0.012. This indicates that a calibration equation might be used to estimate AOCS TG from HPLC.

Table 2 shows the recovery of gossypol from seed samples spiked with various levels of GAA. Recoveries ranged from 98.9 to 99.8%

The results demonstrate that this new HPLC method can conveniently and quantitatively determine the isomers of gossypol in cottonseed. Because of the clean separation, it could probably be scaled up to provide researchers with a means of producing suitable quantities of gossypol isomers for medical research where gossypol compounds have shown promise in their action on viruses. Although the method worked well on cottonseed samples, when applied to screw-pressed and extracted meals, significant modifications, which will be reported upon in a future paper, were found to be necessary.

TABLE 2
Recovery of Gossypol from Spiked Cottonseed Meat Samples

| Gossypol added (mg) | Total (+),(–)-gossypol found ^a (mg) | Recovery ^a | |
|---------------------|--|-----------------------|-------|
| | | (%) | SD |
| 0 | 1.601 | — | 0.055 |
| 5.448 | 7.000 | 99.1 | 1.0 |
| 4.768 | 6.314 | 98.9 | 1.2 |
| 4.086 | 5.673 | 99.6 | 1.4 |
| 2.724 | 4.295 | 98.9 | 2.2 |
| 1.362 | 2.961 | 99.8 | 3.4 |
| 0.681 | 2.277 | 99.1 | 6.1 |

^aAverage of five replicates.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the National Cottonseed Products Association. J.L. Landry performed spectrophotometric and chromatographic analysis.

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[Received April 8, 1999; accepted July 20, 1999]