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Isolation of 6-Methoxy Gossypol and 6,6′-Dimethoxy Gossypol from *Gossypium barbadense* Sea Island Cotton

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6-Methoxy gossypol and 6,6′-dimethoxy gossypol were isolated from the seeds and root bark of a St. Vincent Sea Island variety of cotton (AZK-267, GRIN# PI 528406). Crude mixtures of gossypol and the methoxy compounds were obtained by extraction of the tissue with acetone and precipitation with acetic acid. After recrystallization, the preparations were treated with 3-amino-1-propanol to form gossypol Schiff's bases, which were separated by preparative reverse phase chromatography. The separated Schiff's bases were then hydrolyzed with acid, extracted into diethyl ether, concentrated, and precipitated with acetic acid. From the above procedure, both methoxy gossypol compounds were obtained as 1:1 molar acetic acid solvates. Each compound was prepared in sufficient amounts to determine its physical properties and begin testing for bioactivity. Light absorbance differed significantly for the di-3-amino-1-propanol derivatives of gossypol and the methoxy gossypol compounds at 254 nm. Relative response factors were developed, which can be used for determining or correcting analytical measurements of these methylated gossypol forms.

KEYWORDS: Cotton; cottonseed; gossypol; natural products; preparative HPLC; separation processes

INTRODUCTION

Gossypol [1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarbaldehyde] (**Figure 1**) is a polyphenolic terpene that is found within species of the Malvaceae family. It is most frequently encountered as a component of the cotton plant (*Gossypium* sp.), where it appears to have a role in protecting the plant against fungi and insects (*1*, *2*). Gossypol is known to be antinutritive or even toxic to some animals, which limits the use of cottonseed and cottonseed meal in feeding applications (*3*), but the compound also possesses a wide array of interesting biological activities, including activity as an anticancer (*4*, *5*) and antiviral (*6*, *7*) agent and as a male contraceptive (*8*, *9*).

In some cotton varieties, gossypol is known to exist partially as 6-methoxy and 6,6′-dimethoxy derivatives (**Figure 1**) (*10*, *11*). As part of a study on the seed distribution of the gossypol enantiomers in *Gossypium barbadense* varieties, Percy et al. (*11*) observed that some of the Caribbean accessions contained significant amounts of these methoxy gossypol derivatives. A review of their data identified a St. Vincent Sea Island variety (AZK-267, GRIN# PI 528406) as having a relatively high total gossypol level with a high degree of methylation.

Both 6-methoxy gossypol and 6,6′-dimethoxy gossypol have been reported in the literature as synthesis products (*12*, *13*).

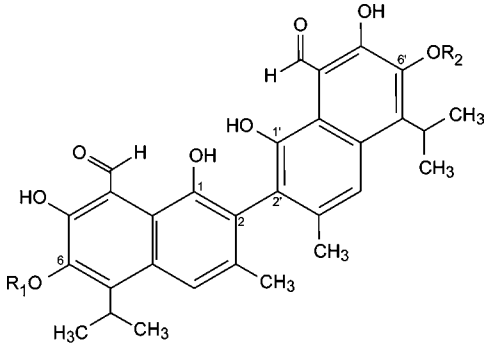

Figure 1. Structures of gossypol, 6-methoxy gossypol, and 6,6′-dimethoxy gossypol.

The procedures needed to prepare these derivatives include the demethylation of hexamethoxy gossypol with sulfuric acid to form a mixture of the dimethoxy and monomethoxy derivatives followed by separation and cleanup. The main argument for chemically preparing these compounds (*13*) was that their concentration in most cotton plant tissues is low. The identification of the Sea Island varieties as having very high concentrations of these natural products diminishes this argument. Regardless of source, bioactivity studies on these products are extremely limited (*14*).

In this study, we developed a preparative chromatographic technique to isolate the methoxy gossypol derivatives in

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sufficient amounts to determine their physical properties and to begin investigation of their biological activity. Compositional and physical properties and relative response factors of the recovered compounds are also reported.

MATERIALS AND METHODS

Greenhouse and Field Production. A St. Vincent Sea Island seed sample (~100 seeds) was donated by R. Percy (Western Cotton Research Laboratory, Agricultural Research Service). Seeds were initially planted in the greenhouse. Seeds recovered from the greenhouse plants were ginned to remove cotton fibers. The bulked seed was then planted for three successive growing seasons on a 15.4 × 30.8 m^2 (50 × 100 ft^2) plot at our research facility. Seeds (all three years) and root bark (two years) were recovered from the field plants.

Extraction of Seed and Root Bark. Cotton bolls were collected and ginned, and roots were cut from the plants. The root bark was stripped immediately after harvesting, as drying made separation of the bark difficult. Stripped bark was air-dried at room temperature. Seeds were cracked with a Bauer Bros. (Springfield, OH) 20.3 cm (8 in.) disk mill. Hulls were removed by sieving, and the dehulled seed was ground in a model 160Z Alpine Kolloplex (Augsburg, Germany) pin mill. The root bark was ground in a model SM 2000 Retsch (Haan, Germany) hammer/cutter mill.

Ground seed was first extracted overnight with petroleum ether to remove glycerides and was then re-extracted with acetone to rupture the pigment glands and solubilize the gossypol compounds. Root bark, which is largely devoid of oil, was extracted directly with acetone. The slurries were filtered through Whatman #4 filter paper, and the retained seed or bark was washed with additional acetone until the filtrates ran clear. The filtrates were concentrated in a rotary evaporator, and acetic acid was added to precipitate a crude gossypol fraction, which was separated by filtration and washed with hexane to remove acetone and acetic acid. The crude products, which contained all three gossypol compounds, were dried overnight under a vacuum to remove residual hexane. These fractions were recrystallized once from acetone and acetic acid, which improved the product color and improved the pressure stability during preparative liquid chromatography runs.

Analytical-Scale Chromatography. Analytical-scale high-pressure liquid chromatography (HPLC) was conducted by forming 3-amino-1-propanol Schiff's bases of the gossypol compounds as described by Hron et al. (*15*). Analyses were conducted on the roots and seed tissue, on the isolated crude and recrystallized gossypol fractions, and on the final separated products. To form the amine derivatives, a complexing reagent consisting of 3-amino-1-propanol (2 mL), glacial acetic acid (10 mL), and dimethylforamide (to give a total volume of 100 mL) was added to the sample and the mixture was heated to 100 °C for 30 min. Ratios of sample-to-complexing reagent varied depending on the expected range of gossypol compounds. For seeds and bark, 100 mg of sample was derivatized in 2 mL of the complexing reagent. For the gossypol fractions and final products, ~1 mg of sample was derivatized in the same volume of reagent.

Separation and detection of the Schiff's base derivatives were achieved with a Waters (Milford, MA) model 2695 pumping system, model 996 photodiode array detector, and a SGE (Austin, TX) 100 mm × 4.0 mm i.d., 5 μm, Inertsil ODS-2 cartridge column. An isocratic mobile phase was used consisting of 60/40 acetonitrile/phosphate buffer (10 mM, pH 3). Prior to analysis, the mixtures containing the amine derivatives were diluted and mixed with four volumes of mobile phase. An aliquot of this solution was then centrifuged to remove particles and injected onto the chromatograph. Injection volumes were 20 μL. Eluted compounds were detected with the photodiode array detector set at 254 nm. Spectra of the final products (as the 3-amino-1-propanol Schiff's bases) were recorded between 210 and 600 nm.

Although prior work (*11*) indicated that these chromatography conditions would detect and separate gossypol and the methoxy gossypol derivatives, HPLC−atmospheric pressure chemical ionization (APCI) mass spectroscopy was conducted on the root bark extract peaks to confirm their identities. For this, a Micromax (Manchester, United Kingdom) model MCX mass spectrometer was attached to the chromatography system after the photodiode array detector. The instrument was operated with a corona voltage of 3.5 kV and a cone voltage of 30 V, and both positive and negative ionization modes were monitored over a 250−750 *m*/*z* range. The complexing reagent and basic chromatographic conditions were used as described above, except that the phosphate buffer in the mobile phase was replaced with an ammonium formate buffer (10 mM, pH 3). This change had no significant effect on the elution of the gossypol compounds. The injection volume for this analysis was 40 μL.

Preparative-Scale Chromatography. For preparative separation, the recrystallized gossypol fraction was derivatized with 3-amino-1-propanol (in a 10 molar excess) in dimethylformamide/acetic acid/3-amino-1-propanol (78:10:12 v/v/v) by heating at 100 °C for 30 min. A Waters HPLC system consisting of a model 717 autoinjector, model 600 pump, and model 2487 ultraviolet−visible (UV/vis) detector was modified by installing a 2.4 mL sample loop and prep-scale detector cell. A Waters XTerra Prep MS 150 mm × 19 mm i.d., 5 μm, C18 OBD reverse phase column was used with an isocratic mobile phase consisting of 62/38 acetonitrile/phosphate buffer (10 mM, pH 3). The mobile phase was pumped at 18 mL/min. The compounds were detected at 254 nm.

The derivatized gossypol solution was diluted with an equal volume of mobile phase, and 2 mL aliquots were injected onto the preparative system. Each injection contained 42 mg of the recrystallized gossypol preparation. Initially, all three compounds were collected for each run. In later work, only the mono- and dimethoxy Schiff's bases were collected. Peaks were initially collected manually; later, they were collected with a programmable fraction collector.

Hydrolysis of Gossypol Schiff's Bases. Di-3-amino-1-propanol-gossypol was used to study the kinetics of removing the 3-amino-1-propanol groups. The hydrolysis was conducted in the elution solvent (i.e., in the preparative HPLC mobile phase) by adding phosphoric acid (0.5 mL acid per 10 mL) and heating at 50 or 70 °C for between 0.5 and 16 h. The reaction was followed by HPLC on an SGE 100 mm × 4.6 mm i.d., 5 μm, Inertsil ODS-2 reverse phase column with a 70/30 acetonitrile/phosphate buffer (10 mM, pH 3) mobile phase. Aliquots of the reaction solution (20 μL) were injected directly onto the column.

Product Recovery. After hydrolysis, each eluted fraction was concentrated in a rotary evaporator to remove most of the acetonitrile. The sample was then transferred to a separatory funnel and extracted with diethyl ether. The gossypol partitioned into the ether phase while the phosphoric acid, acetonitrile, and aminopropanol distributed between the phases. The aqueous phase was removed, and the ether phase was washed 3−4 times with equal volumes of water to remove the acid, solvent, and amine. The ether phase was then concentrated, and an equal volume of acetic acid was added to induce precipitation of the gossypol. Most of the remaining ether was then evaporated. The precipitate was washed with hexane to remove the acetic acid and stored under vacuum overnight to remove residual hexane.

Product Characterization. Carbon, hydrogen, and nitrogen values were measured by combustion methods (Galbraith Laboratories, Knoxville, KY) for all three products. Melting points were determined with a Gallenkamp (Leicester, United Kingdom) melting point apparatus.

The compositional analysis suggested that the recovered compounds were likely solvates containing acetic acid in a 1:1 molar ratio. To confirm this, the amount of acetic acid present in the samples was measured by gas chromatography. A model 5890 Series 2 plus Hewlett-Packard (Palo Alto, CA) gas chromatograph with a split injector and a flame ionization detector was fitted with a J & W Scientific (Folsom, CA) 15 m × 0.25 mm i.d., 0.1 μm, FFAP capillary column. Helium was used as the carrier gas at a linear flow rate of ~30 cm/s. The split ratio was set to 1:50. The inlet temperature was held at 200 °C (just above the melting temperatures of the three compounds), and the detector was set at 240 °C. The column temperature was programmed to start at 100 °C for 2 min and then to ramp to 170 °C at 10 °C/min. The amount of acetic acid found in the preparations was quantified by internal standardization (*16*). Propionic acid was used as the internal standard.

The absorbance spectrum of each compound in acetonitrile was determined on a Perkin-Elmer (Wellesley, MA) model EZ210 UV/vis spectrophotometer, and the spectrum of each compound as its 3-amino-

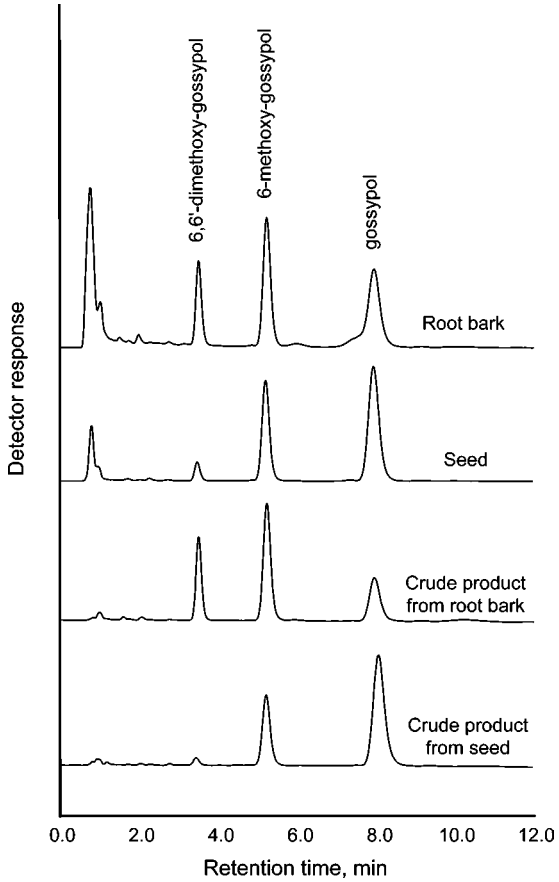

Figure 2. Chromatograms of the gossypol compounds from St. Vincent Sea Island root bark and seed and the crude gossypol mixtures obtained by acetone extraction of these materials. All samples were derivatized with 3-amino-1-propanol prior to chromatography.

1-propanol derivative was determined with the Waters photodiode array detector. Standard HPLC response curves were prepared by making a serial dilution of each compound in the 3-amino-1-propanol complexing reagent (*15*). Aliquots of these dilutions were heated to form di-3-amino-1-propanol derivatives, diluted with four volumes of mobile phase solvent, and injected onto the HPLC column. HPLC response factors (absolute and relative) for the pure compounds (i.e., after accounting for the weight of the acetic acid) were determined from the standard curves. The response factors were used to determine the levels and distribution of the methoxy derivatives in the seeds and root bark of the St. Vincent cotton plants. As a final check of the separation, the recovered compounds were analyzed by analytical-scale HPLC, again as 3-amino-1-propanol derivatives.

RESULTS AND DISCUSSION

Analytical-scale liquid chromatography was conducted on the seeds, root bark, and crude gossypol products extracted from these tissues by derivatizing with 3-amino-1-propanol (**Figure 2**). The root bark yielded three main component peaks but also contained a few minor contaminating peaks (not identified). Ground seeds yielded a "cleaner" chromatogram with fewer impurities. However, the seeds appeared to have significantly less of the methylated forms, especially 6,6′-dimethoxy gossypol. Both the seeds and root bark tissues yielded essentially identical chromatograms for the different years that tissue was collected.

Extraction of either the root bark or the defatted seeds with acetone followed by concentration and precipitation with acetic acid resulted in crude products that contained all three gossypol forms in roughly the same distribution as found in the parent material (**Figure 2**). The crude product obtained from the root bark was largely devoid of the contaminants found within the root bark itself (**Figure 2**).

HPLC-APCI mass spectroscopy on the product obtained from the root bark confirmed the identity of the three main chromatography peaks as dimethoxy gossypol, methoxy gossypol, and gossypol. In position ion mode, $[M + H]^+$ ions were observed corresponding to the diamino-propanol Schiff's base of each compound. For the gossypol peak, a *m*/*z* 633.4 ion was observed (633.7 calcd); for the methoxy gossypol peak, a *m*/*z* 647.5 ion was observed (647.8 calcd); and for the dimethoxy gossypol peak, a *m*/*z* 661.4 ion was observed (661.8 calcd). Also present in each spectra were fragments that corresponded to the loss of one or both of the 3-amino-1-propanol groups. The negative ion mode yielded complementary results.

A single recrystallization of the crude fractions, which were a greenish-yellow to olive green in color, from acetone and acetic acid, yielded much purer yellow products. Analytical-scale analysis of the recrystallized materials produced chromatograms essentially identical to the chromatograms for the nonrecrystallized samples (not shown). However, recrystallization was beneficial for preparative liquid chromatography, as it resulted in improved pressure stability during separation. Because the root bark appeared to have a greater fraction of the compounds present in methylated form, most of the preparative work was conducted with the recrystallized product obtained from the bark.

Initially, several attempts were made to separate the compounds by normal phase open-column chromatography. Acid-washed silica with a hexane/acetone mobile phase resulted in some separation of the products but with severe streaking of the gossypol along the column length. The streaking was minimized by forming Schiff's base gossypol derivatives and by preincubating the silica with an acetone solution of gossypol prior to packing of the column. After enough mobile phase was allowed to clear the column of unbound gossypol, separation of the methoxy compounds was achieved. However, the bound gossypol fraction degraded quickly (especially in the presence of light) and bled degradation products (reddish-brown in color) into the eluted fractions. In addition, the column capacity was much lower than expected. After several attempts to work around these problems, it was subsequently decided to try an approach similar to previously reported analytical and preparative methods that used reverse phase stationary phases to separate gossypol as Schiff's base derivatives. Matlin and coworkers, for example, used this approach with a chiral amine to separate and isolate (+)- and (−)-gossypol (*17*). Although the method requires derivatization and hydrolysis, the technique proved favorable for separating and recovering the methoxy gossypol derivatives.

Preparative-scale chromatography was achieved on a Waters XTerra C18 reverse phase column with acetonitrile/phosphate buffer mobile phase on 3-amino-1-propanol derivatized gossypol fractions (**Figure 3**). During typical collection runs, samples were injected repeatedly for 1−2 days yielding a total elution volume between 500 and 1000 mL for each peak. Each fraction was then treated with phosphoric acid to hydrolyze the Schiff's bases.

In separating the gossypol enantiomers by reverse phase liquid chromatography, Matlin et al. (*17*) used di-L-phenylalanine methyl ester−gossypol Schiff's bases. To recover the original compounds, they concentrated the eluted fractions, partitioned the Schiff's bases into diethyl ether, and hydrolyzed the

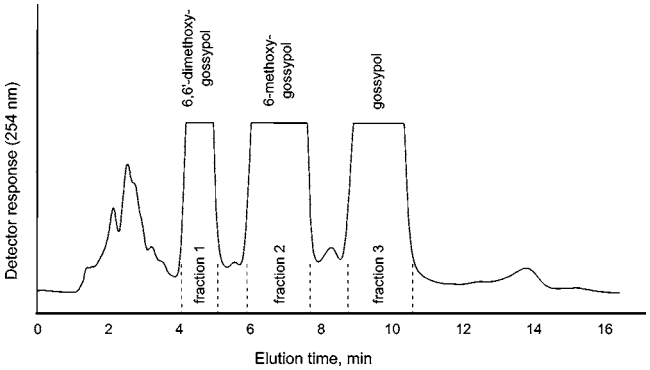

Figure 3. Preparative-scale chromatogram of the recrystallized gossypol fraction obtained from root bark. Compounds were treated with 3-amino-1-propanol to form gossypol Schiff's bases prior to chromatography.

complexes at −1 °C in a solution containing sulfuric acid, acetic acid, water, and ether. Chiral gossypol was insoluble in this solution and precipitated as the Schiff's bases were hydrolyzed. Because racemic gossypol forms inclusion complexes with many small molecules and the exact complex obtained is often sensitive to the crystallization conditions (*18*), we modified Matlin's procedure to favor precipitation of the compounds as acetic acid solvates. In our approach, the hydrolysis was conducted by adding phosphoric acid directly to the pooled elution volume, followed by the removal of the acetonitrile by rotary evaporation, extraction into ether, water washing, and precipitation of the compounds with acetic acid.

Di-3-amino-1-propanol-gossypol was used to study the hydrolysis of gossypol Schiff's bases in mobile phase (62/38, acetonitrile/phosphate buffer). The Schiff's base compound was prepared from purified gossypol acetic acid and was injected repeatedly onto the preparative column. The peak containing di-3-amino-1-propanol-gossypol was collected. After addition of phosphoric acid and heat, aliquots of the solution were injected directly onto an Inertsil reverse phase analytical column (**Figure 4A**). The distribution of products (**Figure 4B**) indicated that the hydrolysis followed a two-step pathway, first forming the mono-3-amino-1-propanol gossypol derivative and then forming gossypol. At 50 °C, the reaction was 70% complete at 5 h but complete if left overnight, i.e., 16 h (result not shown). At 70 °C, the reaction was essentially complete after 3 h (**Figure 4B**). An additional small peak was found to occur toward the end of the hydrolysis. This peak has not been identified, but it does not appear to separate with the products during the final crystallization step. On the basis of the results for di-3-amino-1-propanol-gossypol, hydrolysis of the methoxy gossypol Schiff's bases was conducted at 70 °C for 3 h with the same concentration of phosphoric acid.

From 2 days of preparative chromatography (42 injections taking ∼14 h) followed by recovery of the hydrolyzed products, ∼400 mg of 6,6′-di-methoxy gossypol and ∼600 mg of 6-methoxy gossypol were prepared. In this manner, several batches have been made over a three month period yielding sufficient sample to test the properties of the compounds and initiate some crystallization and biological studies. HPLC analysis of the final products from these collections showed that the separations were essentially complete (**Figure 5**).

Under the conditions used to prepare the final products (ether and acetic acid), gossypol forms a solvate with acetic acid in a 1:1 molar ratio. Carbon and hydrogen analyses of the methoxy compounds indicated that they were also likely 1:1 molar clathrates with acetic acid (**Table 1**). To confirm this, samples were subjected to gas chromatography to measure the amount

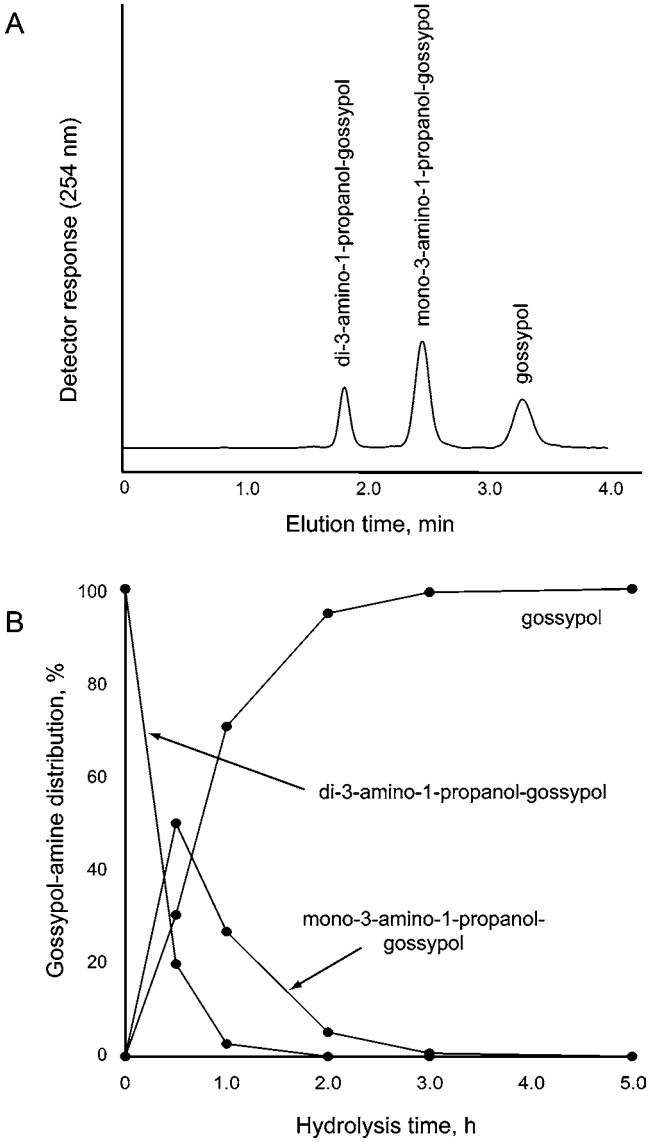

Figure 4. Gossypol Schiff's base hydrolysis products and hydrolysis kinetics at 70 °C. (**A**) Chromatogram of the hydrolysis products after addition of phosphoric acid for 0.5 h. (**B**) Distribution of hydrolysis products over time. The distribution is based on the assumption of the products having identical response factors at 254 nm. Complete conversion to gossypol is obtained after 3 h.

of acetic acid contained within the preparations. The measured acetic acid levels were equal to compound-to-acetic acid molar ratios of 1:1.05 for gossypol, 1:0.98 for 6-methoxy gossypol, and 1:1.08 for 6,6′-dimethoxy gossypol. The nitrogen content was below the detection limit (<0.5%), confirming the completeness of the hydrolysis.

The melting points for the three compounds differed significantly, with 6,6′-dimethoxy gossypol−acetic acid (1:1) having the lowest melting point and gossypol−acetic acid (1:1) having the highest melting point (**Table 1**). In the gossypol acetic acid (1:1) crystal form, the 6- and 6′-hydroxyl hydrogen atoms contribute to intermolecular hydrogen bonds (*20*). Assuming that all three compounds exist in the same basic crystalline structure, these hydrogen bonds cannot form if the 6- and 6′-groups are methylated. Hence, the lattice energies of these crystals would be expected to be lower, leading to the lower melting points. We are currently trying to prepare single crystals for diffraction studies to determine the molecular structures of these compounds.

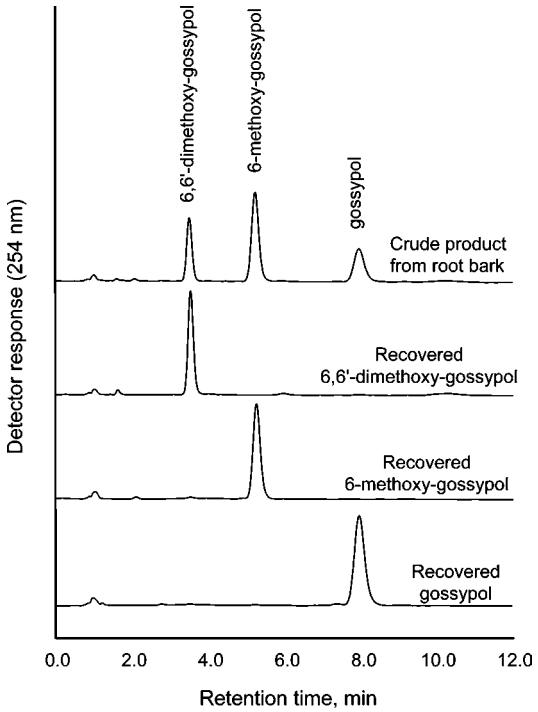

Figure 5. Chromatograms of the compounds obtained by extraction of St. Vincent Sea Island root bark and the compounds isolated by preparative chromatography. All samples were derivatized with 3-amino-1-propanol prior to chromatography.

Table 1. Physical Properties of Recovered Gossypol, 6-Methoxy Gossypol, and 6,6′-Dimethoxy Gossypol[a]

compound	melting point (°C)	carbon (%)[b]	hydrogen (%)[b]
gossypol	183.7 (182.4)[c]	66.3 (66.4)	6.1 (5.9)
6-methoxy gossypol	177.5	66.7 (66.9)	6.5 (6.1)
6,6′-dimethoxy gossypol	166.4	66.6 (67.3)	6.6 (6.3)

[a] All three compounds were isolated as acetic acid solvates in a 1:1 molar ratio. [b] Values in parentheses are expected values for 1:1 molar acetic acid solvates. For the pure compounds, expected carbon values are 69.9% for 6-methoxy gossypol and 70.3% for 6,6′-dimethoxy gossypol. [c] The value in parentheses is a measured value for an authentic sample of gossypol–acetic acid (1:1) isolated from cottonseed soapstock (*19*).

UV/vis absorbance spectra are similar for gossypol, 6-methoxy gossypol, and 6,6′-dimethoxy gossypol (**Figure 6A**). UV/vis absorbance spectra for the di-3-amino-1-propanol Schiff's bases, which are commonly used for the detection and measurement of gossypol, were less similar (**Figure 6B**). Most pronounced was the presence of distinct peaks at 266 and 350 nm in the 6,6′-dimethoxy gossypol spectrum that appear as shoulders in the gossypol and 6-methoxy gossypol spectra.

Most analytical procedures detect gossypol (again as the di-3-amino-1-propanol derivative) at 254 nm (*11*, *15*). This wavelength is a carryover from early procedures when 254 nm single wavelength detectors were common. Despite the similarities in the spectra of the di-3-amino-1-propanol-gossypol compounds, HPLC standard curves (**Figure 7**) and the corresponding response factors differed considerably at 254 nm. At least in part, these differences result because the absorbance for these compounds at 254 nm occurs on shoulders of the spectra maxima (**Figure 6**), where the responses can be very sensitive to small differences.

The amount and distribution of gossypol and methoxy gossypol forms for the St. Vincent Sea Island root bark and

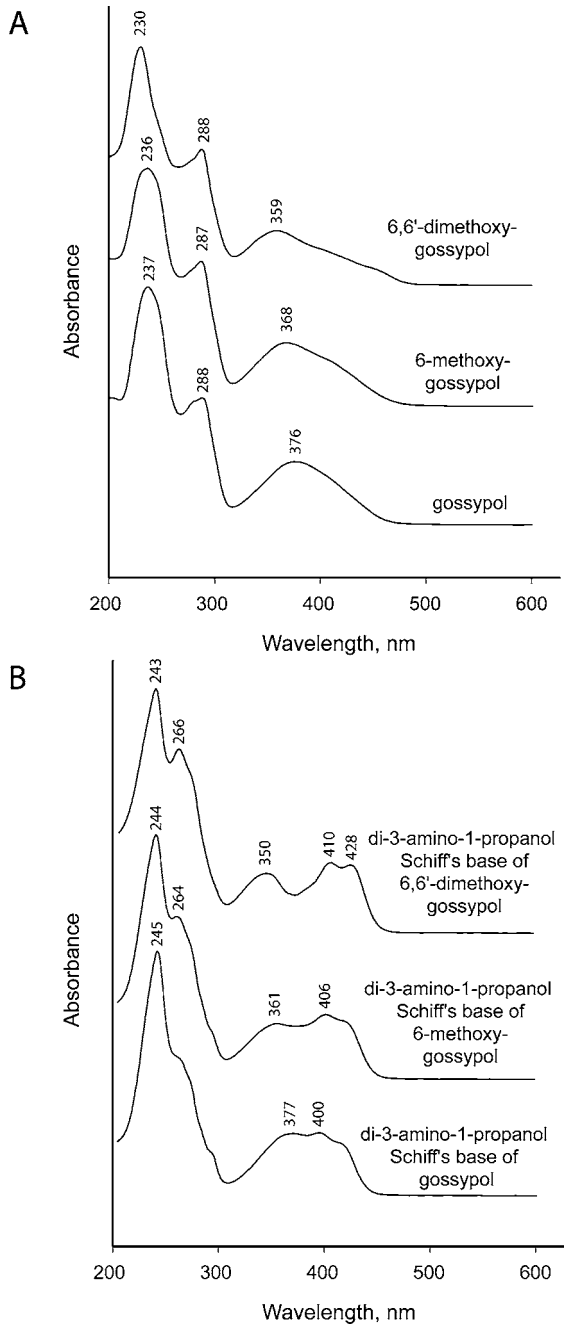

Figure 6. UV/vis absorbance spectra for gossypol and its methoxy derivatives. (**A**) Spectra for gossypol, 6-methoxy gossypol, and 6,6′-dimethoxy gossypol. (**B**) Spectra for the di-3-amino-1-propanol Schiff's bases of the same compounds.

seed calculated from the response factors indicate that, although the total amount of gossypol and its methoxy forms is less in the root bark than in the seed, the amount of the 6,6′-dimethoxy derivative is much greater in the root bark (**Table 2**). Because the bark is also easier to extract, it appears to be a better source of these gossypol derivatives.

Relative response factors were calculated from measured response factors derived from standard curves of both 6-methoxy and 6,6′-dimethoxy gossypol. Because gossypol is much easier to obtain, these relative response factors should be useful for correcting analytical measurements for the methoxy forms based on response factors derived for gossypol. From duplicate determinations, the relative response factor ($slope_{6MG}/slope_G$) for 6-methoxy gossypol was 0.80 ± 0.01. For 6,6′-dimethoxy gossypol, the relative response factor ($slope_{6DMG}/slope_G$) was

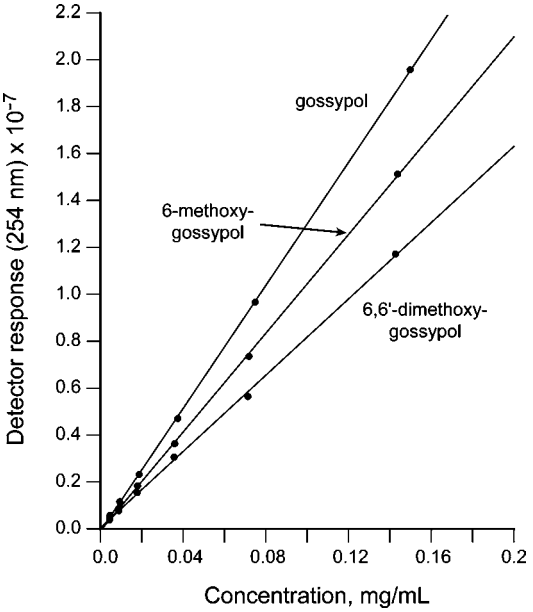

Figure 7. HPLC standard curves for the di-3-amino-1-propanol Schiff's bases of gossypol, 6-methoxy gossypol, and 6,6′-dimethoxy gossypol.

Table 2. Amounts and Distribution of Gossypol Compounds in the Seeds and Root Bark of St. Vincent Sea Island (AZK-267) Cotton

	dehulled seed[a]		root bark[a]	
compound	concn (%)	dist. (%)	concn (%)	dist. (%)
gossypol	0.90	52.0	0.34	25.2
6-methoxy gossypol	0.70	40.3	0.64	47.2
6,6′-dimethoxy gossypol	0.14	7.7	0.38	27.6
total gossypol compounds:	1.74		1.36	

[a] Dry weight basis.

0.62 ± 0.02. Both of these values are for the di-3-amino-1-propanol Schiff's bases. Multiplying these values by gossypol's response factor should provide a good estimate of the response factor for the methylated compounds. These factors, however, only apply for absorbance readings obtained at 254 nm and only for the 3-amino-1-propanol Schiff's base derivatives.

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