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HPLC preparation of the chiral forms of 6-methoxy-gossypol and 6,6′-dimethoxy-gossypol

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ABSTRACT

A concentrated mixture of gossypol, 6-methoxy-gossypol, and 6,6′-dimethoxy-gossypol was extracted from the root bark of St. Vincent Sea Island cotton with acetone. This extract was derivatized with *R*-(−)-2-amino-1-propanol to form diastereomeric gossypol Schiff's bases. Analytical-scale reverse-phase chromatography of these Schiff's bases produced six peaks, indicating separation of the enantiomeric forms of the three gossypol compounds. The elution order of the peaks was found to vary with the polarity of the mobile phase. The chromatography was scaled to a preparative level and was used to isolate each compound. After hydrolysis of the separated Schiff's bases, the original compounds were recovered by precipitation from solutions of diethyl ether, acetic acid, and water. Fifty injections yielded approximately 500 mg of each methoxy-gossypol enantiomer and 300 mg of each dimethoxy-gossypol enantiomer. Each compound was characterized for carbon and hydrogen content, optical rotation, UV–vis light absorption, and melting point. Standard curves were developed and were used to measure the concentration of each gossypol form in the root bark and dehulled seed of St. Vincent Sea Island cotton. In seed tissue, 48% of the gossypol compounds were methylated, and the (−)-optical form was found to be in a slight excess to the (+)-optical form (53–54%) for all three compounds. In root bark, 71% of the gossypol compounds were methylated, and the (+)-optical form was in excess to the (−)-optical form for all three compounds. However, in this tissue the extent of enantiomeric excess decreased with the degree of methylation, with 77% of the gossypol existing in the (+)-optical form and 59% of the 6,6′-dimethoxy-gossypol existing in the (+)-optical form.

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1. Introduction

Gossypol [1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarbaldehyde] (Fig. 1) is a polyphenolic terpene that is found in the cotton (*Gossypium* sp.) plant and a few related species. In cotton, gossypol is beneficial in that it appears to inhibit insect predation [1]. In cottonseed and cottonseed meal, the compound can be toxic, which limits the use of cottonseed and cottonseed meal as a feed ingredient [2]. Gossypol also has a wide range of potentially important biological activity, including inhibitory effects against viruses [3,4], protozoa [5], and cancer cells [6–8]. The compound also exhibits male contraceptive effects [9–11].

Some varieties of *G. barbadense* are known to contain significant amounts of *O*-methylated gossypol derivatives (i.e., methoxy-gossypol derivatives) (Fig. 1) [12,13]. We recently reported that a St. Vincent Sea Island cotton variety had >45% of its seed gossypol content and >70% of its root bark gossypol content as either 6-methoxy-gossypol (6-MG) or 6,6′-dimethoxy-gossypol (6,6′-DMG) [14].

The presence of hydroxyl and methyl substituents adjacent to the bridge bond on each gossypol naphthalene ring restricts the motion about this bond, resulting in a chiral axis. As a result, gossypol exists in two enantiomeric forms. Both forms are produced by the cotton plant, but in ratios that vary with cotton species, variety, and tissue [15–18]. Considerable work has established that the gossypol enantiomers have different anti-cancer and anti-viral activities [3,4,7,8], contraceptive effects [9,10], and animal toxicities [19].

Because the methylated gossypol derivatives have the same hydroxyl and methyl substituents adjacent to the binaphthalene bridge bond, these compounds would also be expected to exist as enantiomers. However, no attempt has been made to isolate the individual optical forms of these gossypol derivatives, and no information is available on their biological activity.

In cottonseed products and animal tissues, (+)-gossypol and (−)-gossypol are usually measured by forming diastereomeric Schiff's bases with a chiral amine (Fig. 2), which are then separated on an achiral reverse-phase stationary phase [20,21]. With *R*-(−)-2-amino-1-propanol as the amine, this technique is now a

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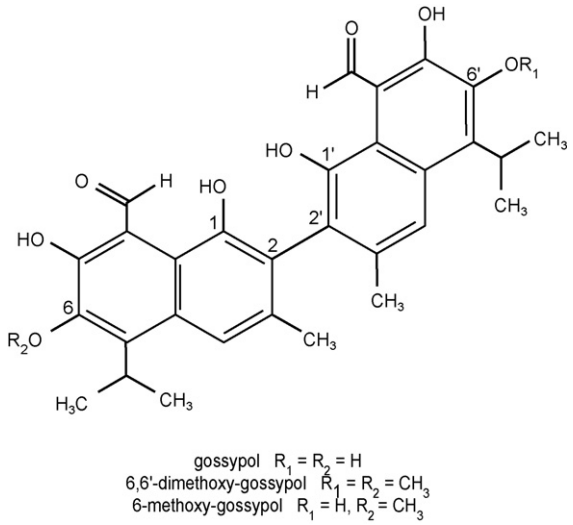

Fig. 1. Structure of gossypol, 6-methoxy-gossypol, and 6,6′-dimethoxy-gossypol.

Recommended Practice (Ba 8a-99) of the American Oil Chemists' Society [22]. With L-phenylalanine methyl ester as the amine, Maltin et al. used the approach to isolate the individual diastereomeric Schiff's bases of gossypol [23]. After hydrolysis of the bases, Maltin recovered gram quantities of each gossypol enantiomer.

In a previous report, we extracted the root bark tissue of St. Vincent Sea Island cotton with acetone to recover a dry extract highly enriched in gossypol and its two methylated derivatives [14]. This extract was then treated with 3-amino-1-propanol to make Schiff's base gossypol derivatives, which allowed for sharper peaks and baseline separation of gossypol and each methoxy derivative on a reverse-phase stationary phase. After collection of the peak fractions and hydrolysis of the Schiff's bases, 6-MG and 6,6′-DMG were recovered by precipitation from diethyl ether and acetic acid. Characterization of the products indicated that both compounds were acetic acid solvates in a 1:1 ratio [14]. At that time, the distribution of the optical forms in these preparations was not studied.

In this report, we extend our work on these compounds to separate and recover the individual enantiomers of 6-MG and 6,6′-DMG. This was achieved by substituting *R*-(−)-2-amino-1-propanol for 3-amino-1-propanol in the formation of the Schiff's bases. The resulting diastereomers were then separated by preparative-scale reverse-phase chromatography. After collection of the individual peaks, each Schiff's base was hydrolyzed to remove the amine, and the compound was recovered by precipitation from solutions containing diethyl ether, acetic acid, and water. The resulting products were characterized, and standard curves were developed and used to determine the amounts of the individual optical forms of gossypol and its methylated derivatives in the root bark and seeds of St. Vincent cotton and in the mixed extract of gossypol compounds obtained by acetone extraction of the root bark. Finally, the standard curves were used to determine the distribution of optical forms in the previously recovered acetic acid solvates of 6-MG and 6,6′-DMG [14].

2. Experimental

2.1. Preparation of seed and root bark tissue

St. Vincent Sea Island Superfine cotton (GRIN #PI 528406) was grown on site, and seed and root tissue were harvested from the plants. After ginning, the seed was cracked with a Bauer Bros. (Springfield, OH, USA) 20.3 cm (8 in.) disc mill. The hulls were separated by sieving, and the dehulled seed was cleaned by air classification. Any remaining hull pieces or foreign matter were removed by hand. Clean seed tissue was then ground with a Braun model MR 430-HC food chopper to pass a 20 mesh sieve, and the ground material was freeze-dried. Bark was peeled from the freshly recovered roots, dried at ambient conditions, ground in a Retsch (Haan, Germany) model SM 2000 hammer/cutter mill to pass a 4 mm Retsch screen, and freeze-dried.

2.2. Extraction of root bark

Root bark (100 g) was extracted with acetone (1.2 L) at room temperature for 2 h with mixing. The slurry was filtered over Whatman #4 filter paper, and the retained bark was washed with additional acetone until the solvent draining from the bottom of the root bark cake ran clear. The filtrate was then concentrated by rotary evaporation to a small final volume (∼8 mL). A half volume of acetic acid was added, and the combined solution was agitated overnight resulting in a precipitant. The precipitant was separated on Whatman #4 filter paper, washed with hexane to remove acetone and acetic acid, and then dried overnight under vacuum to remove residual hexane. This dry extract was recrystallized once from acetone and acetic acid and was washed and dried again. Previous analysis indicated that this material contained significant amounts of gossypol, 6-MG, and 6,6′-DMG [14].

2.3. Formation of Schiff's bases

Gossypol Schiff's bases were formed with *R*-(−)-2-amino-1-propanol (D-alaninol, CAS #35320-23-1) by the method of Hron et

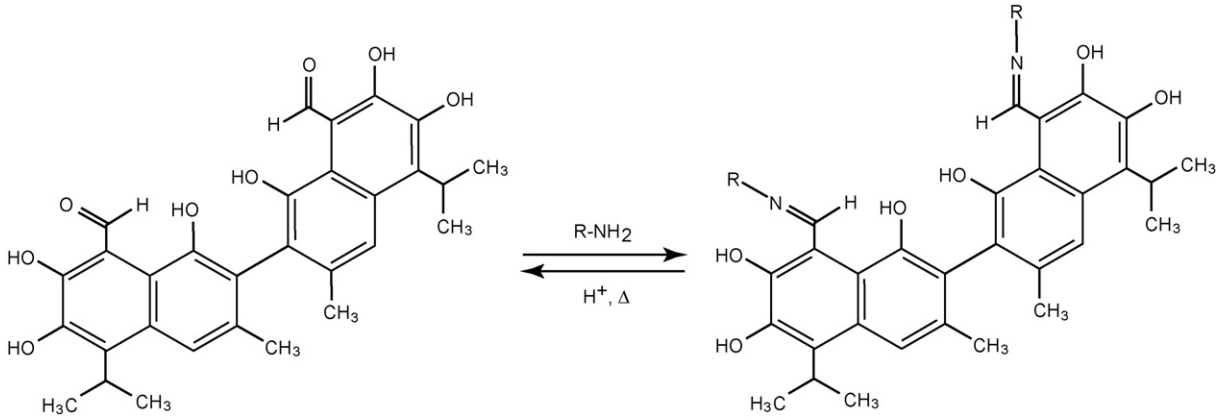

Fig. 2. Reaction of gossypol with an amine to form a diamino-gossypol Schiff's base. Heating in the presence of acid reverses the reaction.

al. [21]. For analytical work, a complexing reagent was made with 2 mL of amine, 10 mL of glacial acetic acid, and sufficient dimethylforamide (DMF) to yield a total solution volume of 100 mL. The complexing reagent was added to the sample (2 mL per milligram of expected gossypol compounds) and the mixture was heated to 95–100 °C for 30 min. After cooling to room temperature, the mixture was then diluted with four volumes of mobile phase and vortex mixed. For chromatography, an aliquot was centrifuged briefly (~12,000 × *g*, 5 min) to settle any particles, and the supernatant was taken for injection onto the chromatograph. For preparative work, the complexing reagent consisted of 6.2 mL of the amine, 10 mL of glacial acetic acid and sufficient DMF to yield 100 mL of total volume. Each 100 mg of gossypol extract was dissolved in 2 mL of this solution, corresponding to a 10-fold molar excess of the amine. The solution was heated to 95–100 °C for 30 min to accelerate the formation of the Schiff's bases. After cooling, the solution was diluted with an equal volume of mobile phase and filtered through a Millipore (Bedford, MA, USA) 0.45 μM pore diameter PVDF syringe filter prior to chromatography.

2.4. Analytical chromatography

Separation and detection of the Schiff's base derivatives was achieved with a Waters Corp. (Milford, MA, USA) model 2695 pumping system and model 996 photodiode array detector. An SGE, Inc. (Austin, TX, USA) Inertsil ODS-2 cartridge column (5 μm particles, 4.0 mm i.d. × 100 mm) was used for the stationary phase. The mobile phase was composed of acetonitrile and 10 mM phosphate buffer (pH 3). The buffer was prepared by dissolving 1.36 g of monopotassium phosphate in 1 L of Millipore (Bedford) filtered (18 ΩM cm) water. The pH of the solution was then adjusted to 3.0 by adding a few drops of phosphoric acid.

To study the effect of mobile phase on the chromatography, the mobile phase composition was varied between 85:15 (v/v) acetonitrile/phosphate buffer and 60:40 (v/v) acetonitrile/phosphate buffer. From this study, a mobile phase consisting of 60:40 (v/v) acetonitrile/phosphate buffer was selected for the analytical work. Injection volumes were 10 μL. The compounds were detected with the photodiode array detector at 254 nm. Root bark, dehulled seed, the recrystallized gossypol extract, the enantiomeric forms of 6-MG and 6,6′-DMG recovered by preparative chromatography (as described below), and the previous preparations of 6-MG and 6,6′-DMG [14] were analyzed with the method.

2.5. Preparative chromatography

A Waters HPLC system consisting of a model 717 autoinjector, model 600 pump, model 2487 ultraviolet–visible (UV–vis) detector and model WFC III fraction collector was modified by installing a 2.4 mL sample loop and a prep-scale detector cell. A Waters XTerra Prep MS C18 OBD reverse-phase column (5 μm particles, 19 mm i.d. × 150 mm) was used as the stationary phase. Mobile phase was pumped at 18 mL/min. To optimize the separation, the mobile phase composition was varied over the chromatographic run. Initially, the mobile phase consisted of 58:42 (v/v) acetonitrile/phosphate buffer, which was pumped for 17 min. At 17 min, the mobile phase was ramped linearly to 78:22 (v/v) acetonitrile/phosphate buffer over a 2-min period. This was maintained for 4 min, and then the mobile phase was ramped linearly back to the starting 58:42 (v/v) acetonitrile/phosphate buffer over the final 2 min of the run. The eluted compounds were detected at 254 nm.

Repeated 2 mL aliquots were injected onto the preparative system. Each injection contained 50 mg of the Schiff's base compounds formed from the root tissue extract. Four peaks were collected corresponding to the two 6-MG and two 6,6′-DMG Schiff's bases. The two peaks corresponding to the (+)- and (−)-gossypol Schiff's bases were not collected.

2.6. Product recovery

The amine portion of the complexes was removed as previously described for di-3-amino-1-propanol gossypol Schiff's bases [14]. Briefly, phosphoric acid was added to the recovered peak fractions (0.5 mL acid per 10 mL of collected peak volume), and the acidified solution was heated at 70 °C for 3 h with mixing. Each hydrolyzed fraction was then concentrated by rotary evaporation to remove most of the acetonitrile. The concentrate was transferred to a separatory funnel and diethyl ether was added, which partitioned the gossypol compound into the organic phase. Repeated water washings of the ether phase removed the remaining acid, acetonitrile, and amine. Most of the ether was then evaporated under a dry stream of nitrogen, and acetic acid was added to the concentrated solution. Water was then added in an amount sufficient to maintain a single phase solution but force the gossypol compound to precipitate. For the 6-MG products, a ratio of ether, acetic acid, and water was 6:2:10 (v/v/v). For the 6,6′-DMG products, a ratio of ether, acetic acid, and water was 6:5:20 (v/v/v). Each precipitant was collected over Whatman #4 paper and was water washed to remove traces of acetic acid. The products were then dried in the dark under vacuum.

2.7. Product characterization

For each compound, carbon and hydrogen levels were determined by combustion (Galbraith Laboratories, Knoxville, KY, USA). Melting points were determined with a Thomas Hoover (Philadelphia, PA, USA) Uni-melt capillary melting point apparatus and were uncorrected.

The UV–vis absorption spectrum (200–700 nm) of each compound was determined in acetonitrile with the Waters photodiode array detector. The UV–vis absorption spectrum of each compound as its *R*-(−)-2-amino-1-propanol Schiff's base was also determined in mobile phase (60:40 (v/v) acetonitrile/10 mM, pH 3 phosphate buffer).

Standard curves (detector response on the ordinate and compound concentration on the abscissa) were prepared by making a series of solutions of each methoxy-gossypol compound in complexing reagent [21,22]. Aliquots of these solutions were heated to form the Schiff's base derivatives, cooled to room temperature, and diluted with four volumes of 60:40 (v/v) acetonitrile/phosphate buffer mobile phase prior to HPLC analysis. Similar standard curves were made from racemic gossypol–acetic acid (1:1) for the (+)- and (−)-forms of gossypol. The curves were used to determine the levels and distribution of the optical methoxy derivatives in the seed and root bark of the St. Vincent cotton plants as well as the extract of gossypol compounds prepared from the root bark. The standard curves were also used to determine the relative amounts of the individual enantiomers in the previous isolations of the two methoxy-gossypol derivatives [14]. Relative response factors were calculated from the slopes of the standard curves.

3. Results

3.1. Analytical chromatography

Treating seed, root bark, or the root bark extract of gossypol compounds with *R*-(−)-2-amino-1-propanol and analyzing the resulting complexes by HPLC produced six peaks (Fig. 3). Treatment of the same materials with 3-amino-1-propanol yielded three peaks [14]. Chromatography of the same Schiff's bases formed with racemic gossypol–acetic acid (1:1) produced two peaks with elution times corresponding to the forth and sixth peaks from the

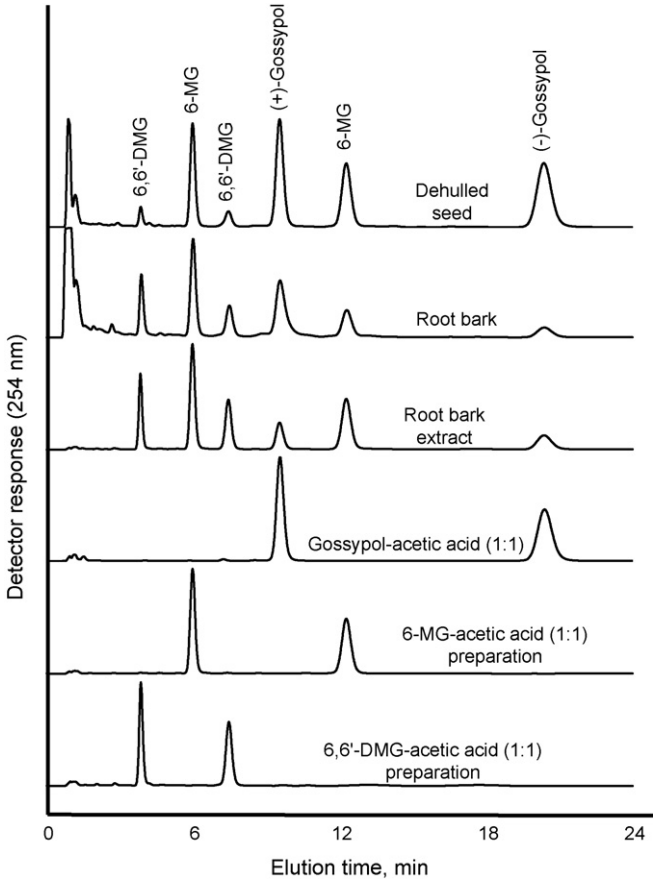

Fig. 3. Chromatograms of the gossypol Schiff's base compounds formed with *R*-(−)-2-amino-1-propanol from various St. Vincent Sea Island cotton plant tissues, including dehulled seed, root bark, and an acetone extract of the root bark. Also shown are comparable chromatograms for the *R*-(−)-2-amino-1-propanol Schiff's bases made with gossypol:acetic acid (1:1) and the previously obtained acetic acid solvates of 6-methoxy-gossypol (6-MG) and 6,6′-dimethoxy-gossypol (6,6′-DMG) [14].

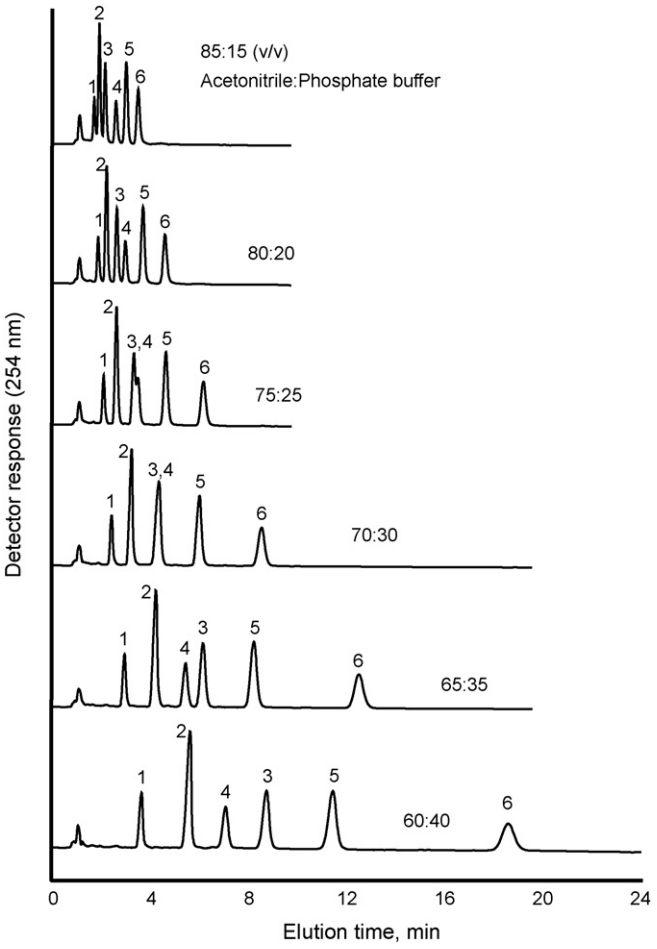

Fig. 4. Effect of mobile phase polarity on the elution of *R*-(−)-2-amino-1-propanol Schiff's bases of gossypol and its methoxy derivatives.

plant tissue chromatograms (Fig. 3). At these HPLC conditions [21], the first eluted gossypol peak is known to correspond to the (+)-gossypol diastereomer and the second peak is known to correspond to the (−)-gossypol diastereomer [21], which was confirmed by Schiff's base derivatization and chromatography of optically pure gossypol samples (not shown).

Chromatography of the previously obtained 6-MG preparation [14] also yielded two peaks corresponding to the second and fifth peaks in the plant samples (Fig. 3). Likewise, the previously isolated 6,6′-DMG preparation [14] yielded two chromatographic peaks with elution times corresponding to the first and third peaks in the plant samples (Fig. 3).

3.2. Influence of mobile phase polarity on methoxy-gossypol elution

To maximize column loading for the preparative-scale separation, elution of the six peaks in the root bark extract was studied with different ratios of acetonitrile and phosphate buffer in the mobile phase. At an acetonitrile-to-phosphate buffer ratio of 85:15 (v/v), all six peaks elute quickly (Fig. 4). By increasing the percentage of buffer in the mobile phase, the peaks eluted more slowly. As the mobile phase became more polar, the relative rates of elution of the third and fourth peaks changed (Fig. 4). These peaks were well separated when the mobile phase consisted of an 85:15 (v/v) ratio of acetonitrile and phosphate buffer, but began to co-elute when the buffer volume was increased to 25–30% of the mobile phase volume. Further increasing the proportion of buffer in the mobile phase separated the peaks but in the reverse elution order (Fig. 4). With a 65:35 (v/v) acetonitrile/phosphate buffer, baseline separation was again achieved for all six compounds, although the third and fourth peaks eluted closely. At a 60:40 (v/v) acetonitrile/phosphate buffer, marked separation was found for all six compounds. From the analytical chromatography of the gossypol acetic acid standard and the previously analyzed 6,6′-DMG sample (Fig. 3), the two peaks changing elution order were the Schiff's bases of (+)-gossypol (peak 3; Fig. 4) and one of the optical forms of 6,6′-DMG (peak 4; Fig. 4).

3.3. Preparative chromatography

After the derivatization with the chiral amine, 2 mL aliquots containing 50 mg of the mixed gossypol extract were injected repeatedly onto the preparative HPLC system (Fig. 5). Eluted fractions were collected for the peaks corresponding to the four methylated gossypol Schiff's bases. Maintaining an initial low ratio of acetonitrile-to-phosphate buffer in the mobile phase allowed for baseline separation of the first five peaks. Increasing the acetonitrile concentration at 17 min hastened the elution of the sixth peak (which was not collected) and shortened the overall run time. Typically, 40–50 injections were completed over a 2-day period, producing about 500–1000 mL of elution volume for each peak.

Hydrolysis of the Schiff's bases was achieved by addition of phosphoric acid and heating at 70 °C for 3 h [14]. Following hydrol-

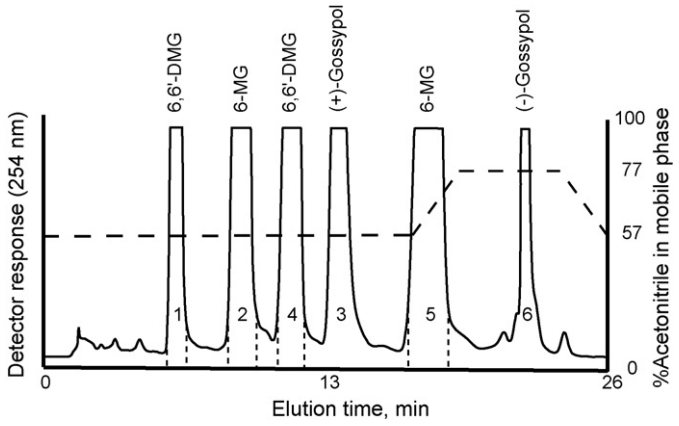

Fig. 5. Preparative separation of gossypol, 6-methoxy-gossypol (6-MG) and 6,6′-dimethoxy-gossypol (6,6′-DMG) Schiff's bases formed with *R*-(−)-2-amino-1-propanol. Peaks labeled 1, 2, 4, and 5 were collected. (Peaks are labeled to be consistent with Fig. 4.)

ysis, the acetonitrile was removed by rotary evaporation until some precipitation of the products was observed to occur. Diethyl ether was then added, which partitioned the gossypol product into the organic phase. After water washings of the ether phase to remove residual acid, amine, and acetonitrile, the ether was evaporated to a low volume. Acetic acid was then added to allow for the addition of water to the ether solution without phase separation. The water addition resulted in rapid precipitation, and further evaporation of the ether essentially forced the entire product to precipitate. Because the 6,6′-DMG products were less prone to precipitate (i.e., more soluble in ether) than the 6-MG products, proportionally more acetic acid and water were used to affect the recovery of these compounds.

From two days of repeated runs, approximately 500 mg of each 6-MG optical form and 300 mg of each 6,6′-DMG optical form were prepared. Analytical chromatography of each product indicated that the compounds were essentially free of the other gossypol compounds present in the initial mixture (Fig. 6).

3.4. Compound characterization

Carbon and hydrogen analyses of each compound were found to be in good agreement with expected results (Table 1) and, unlike the prior preparations [14], the chiral products were not solvates. The enantiomer pairs had essentially the same carbon and hydrogen compositions, melting points, and UV–vis absorption peaks (Table 1). The specific molar rotations were also similar in absolute value but opposite in sign.

As expected, the UV–vis absorption spectra of the enantiomers for each methylated gossypol compound also were identical (Fig. 7).

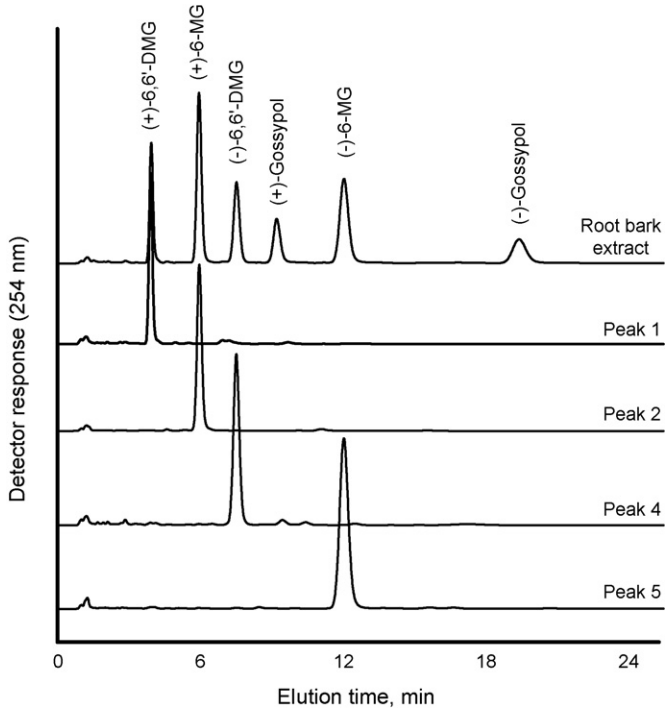

Fig. 6. Chromatography of the isolated optical forms of 6-methoxy-gossypol (6-MG) and 6,6′-dimethoxy-gossypol (6,6′-DMG) after reaction with *R*-(−)-2-amino-1-propanol. A chromatogram of the *R*-(−)-2-amino-1-propanol Schiff's bases of the gossypol compounds from the acetone extract of St. Vincent Sea Island cotton root bark is also shown. Peak numbering is as given in Figs. 4 and 5 and Table 1.

Only small differences were apparent in the spectra among the methylated and non-methylated gossypol forms (Fig. 7). The spectra of these compounds were essentially the same as the corresponding spectra from the previous isolations of 6-MG and 6,6′-DMG [14]. Some minor peak shifts were observed, but these differences were likely the result of the different instruments used to obtain the data.

More differences were observed among the spectra of the *R*-(−)-2-amino-1-propanol Schiff's base complexes of the methylated compounds (Fig. 8). The most notable differences were the shoulder peak at ~265 nm, which became more pronounced, and the shoulder peak at ~370 nm, which shifted to lower wavelengths, in the spectra of the methylated compounds compared with the spectra of gossypol (Fig. 8). Despite the diastereomeric nature of these compounds, the spectra of the diastereomers of each methylated form were almost identical. These spectra were also similar to the spectra previously reported for the corresponding non-diastereomeric 3-amino-1-propanol Schiff's bases [14].

Table 1
Physical properties of isolated optical forms of 6-methoxy-gossypol and 6,6′-dimethoxy-gossypol

Elution peak[a]	Compound identity[b]	Melting point (°C)	Carbon[c] (%)	Hydrogen[c] (%)	Specific molar rotation $[\alpha_D]^{25d}$ (°)	λ_{max}[d] (nm)	λ_{max}[e] (nm)
1	(+)-DMG	180	70.4 (70.3)	6.34 (6.27)	+298	230, 288, 359	242, 265, 350, 410, 428
2	(+)-MG	176	69.9 (69.9)	6.09 (6.06)	+344	236, 287, 368	244, 264, 361, 406
4	(−)-DMG	179	70.2 (70.3)	6.32 (6.27)	−302	230, 288, 359	242, 266, 350, 409, 428
5	(−)-MG	174	70.0 (69.9)	6.12 (6.06)	−346	236, 287, 368	244, 264, 361, 406

[a] See Fig. 5.
[b] MG, 6-Methoxy-gossypol; DMG, 6,6′-dimethoxy-gossypol.
[c] Expected values in parentheses.
[d] In acetonitrile.
[e] As Schiff's base complexes with *R*-(−)-2-amino-1-propanol, in mobile phase (60:40 acetonitrile:phosphate buffer, pH 3).

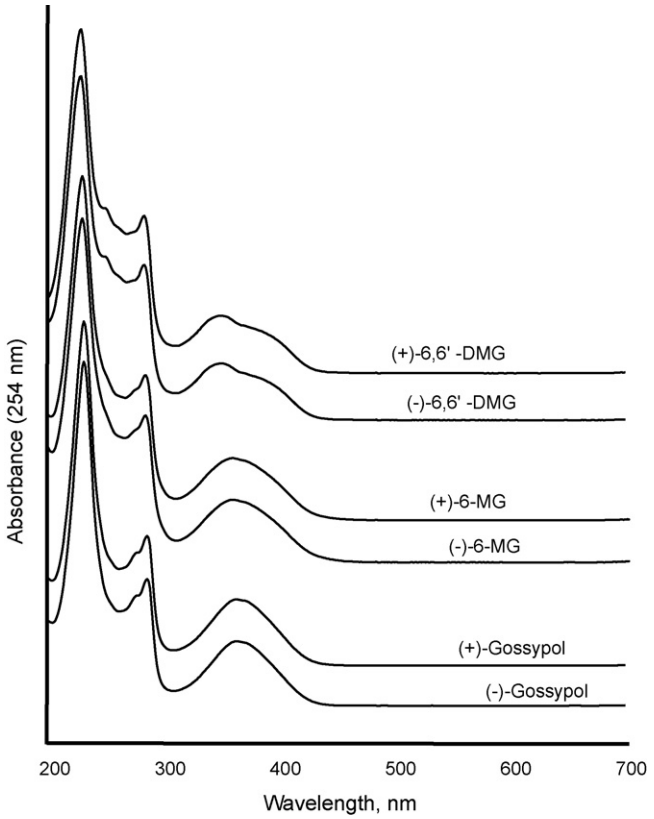

Fig. 7. UV–vis spectra (200–700 nm) for the optical forms of gossypol, of 6-methoxy-gossypol (6-MG) and 6,6′-dimethoxy-gossypol (6,6′-DMG) in acetonitrile.

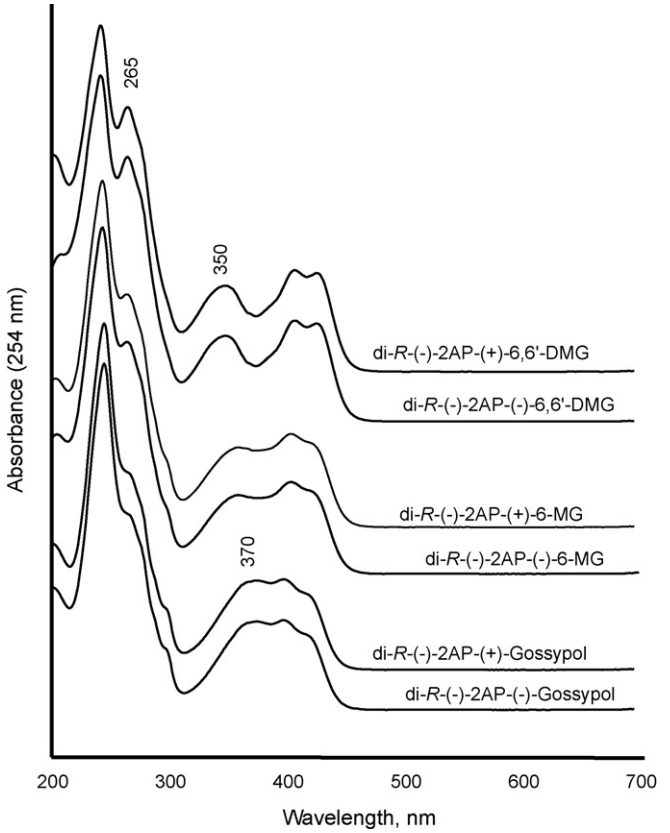

Fig. 8. UV–vis spectra (200–700 nm) for the optical forms of the R-(−)-2-amino-1-propanol diastereomeric Schiff's bases of gossypol, of 6-methoxy-gossypol (6-MG) and 6,6′-dimethoxy-gossypol (6,6′-DMG) in 60:40 (v/v) acetonitrile–phosphate buffer (10 mM, pH 3).

3.5. Quantification of methoxy-gossypol compounds in St. Vincent Sea Island cotton

Standard curves were prepared for the *R*-(−)-2-amino-1-propanol Schiff's bases of each methylated gossypol derivative at 254 nm. Standard curves for the same Schiff's base of gossypol were also prepared from racemic gossypol–acetic acid (1:1). For each compound, the responses were linear over the tested concentration range (0.01–0.22 mg/mL). Within each series of optical forms, the slopes of the standard curves increased in the order 6,6′-DMG < 6-MG < gossypol. Among each pair of optical forms, the slopes of the standard curves differed only modestly.

The concentration of each compound in the seed and root bark of St. Vincent Sea Island cotton was determined from the standard curves (Table 2). For the seed and root bark tissues, the total concentration of gossypol compounds was lower than the amounts previously reported for this variety [14]. Because gossypol concentration in seed can vary significantly year-to-year and the samples

Table 2
Concentrations of the optical forms of gossypol, 6-methoxy-gossypol and 6,6′-dimethoxy-gossypol in the seed and root bark of St. Vincent Sea Island cotton

Compound[a]	Dehulled seed			Root bark			Recrystallized acetone extract from root bark		
	Concentration[b,c] (%)	%(+)-[d]	Distribution[e] (%)	Concentration[b,c] (%)	%(+)-[d]	Distribution[e] (%)	Concentration[b,c] (%)	%(+)-[d]	Distribution[e] (%)
(+)-Gossypol	0.312	47.3		0.210	77.1		7.38	51.8	
(−)-Gossypol	0.374			0.063			6.89		
			52.0			28.8			15.9
(+)-6-MG	0.249	46.5		0.272	67.1		23.4	52.5	
(−)-6-MG	0.286			0.134			21.2		
			40.6			42.9			49.7
(+)-6,6′-DMG	0.046	45.5		0.157	58.6		14.5	47.0	
(−)-6,6′-DMG	0.051			0.111			16.4		
			7.4			28.3			34.4
Total	1.32			0.95			89.8		

[a] 6-MG, 6-Methoxy-gossypol; 6,6′-DMG, 6,6′-dimethoxy-gossypol.
[b] Dry weight basis.
[c] Standard curves (detector area = slope (concentration) + intercept, R^2) for (+)-gossypol, 1.227×10^9 (concentration) − 1.626×10^5, $R^2 = 0.9999$; for (−)-gossypol, 1.201×10^9 (concentration) − 1.956×10^5, $R^2 = 0.9999$; for (+)-MG, 1.016×10^9 (concentration) − 1.396×10^5, $R^2 = 0.9981$; for (−)-MG, 0.960×10^9 (concentration) − 0.734×10^5, $R^2 = 0.9999$; for (+)-DMG, 0.841×10^9 (concentration) − 0.767×10^5, $R^2 = 0.9884$; for (−)-DMG, 0.764×10^9 (concentration) − 0.178×10^5, $R^2 = 0.9954$.
[d] Percent of the (+)-optical form for each compound.
[e] Distribution of methylated and non-methylated gossypol forms (summing over both enantiomers).

prepared for this work were from a more recent crop year, this difference was not unusual.

The ratio of the (+)- and (−)-optical forms for each compound varied between the root bark and seed tissues. In seed tissue, the (−)-form was present in a slight excess (53–54%) for each compound. For root bark tissue, the (+)-form was in excess for all three compounds, but the percentage of (+)-gossypol decreased with the extent of gossypol methylation. (+)-Gossypol was 77% of the gossypol fraction; (+)-6-MG was 67% of the 6-MG fraction; and (+)-6,6′-DMG was 59% of the 6,6′-DMG fraction. Combining the enantiomers of each compound, the distribution of each of the gossypol forms was very similar to the distributions previously reported in both the seed and root bark tissues [14].

The standard curves were also used to quantify the gossypol compounds present in the root bark extract that was used as the starting material for the preparative separation. Measurement of the individual gossypol compounds in the extract accounted for 89% of the extract mass, and the optical forms of each gossypol compound were present in about equal amounts. The enantiomeric excess of each of the three compounds was 3.6% for (+)-gossypol, 5.0% for (+)-6-MG, and 6.0% for (−)-6,6′-DMG.

From the slopes of the standard curves, relative response factors were calculated based on the standard curve of the compound of interest and the standard curve for the same optical form of gossypol (e.g., $\text{slope}_{(-)\text{-MG}}/\text{slope}_{(-)\text{-G}}$). These relative response factors were 0.68 for (+)-6,6′-DMG, 0.66 for (−)-6,6′-DMG, 0.83 for (+)-6-MG and 0.82 for (−)-6-MG. These ratios indicate that while the spectra of these compounds appear similar (Fig. 8), there are some differences in the relative specific absorptivities among the *R*-(−)-2-amino-1-propanol Schiff's bases of gossypol and its methylated derivatives at 254 nm.

4. Discussion

Analytical chromatography of the seed, root bark and the acetone extract of the root bark of St. Vincent Sea Island cotton yielded three principal peaks when the materials were treated with 3-amino-1-propanol [14]. APCI-mass spectrometry and subsequent isolation of these peaks confirmed their identities as the di-3-amino-1-propanol Schiff's bases of gossypol, 6-methoxy-gossypol, and 6,6′-dimethoxy-gossypol [14]. By treating the same tissues with *R*-(−)-2-amino-1-propanol, six peaks are formed, which suggests that resolution of the individual optical forms was occurring for each compound. As similar peak doubling was also observed with the previously obtained 6-MG and 6,6′-DMG preparations (Fig. 3), this confirmed that these compounds existed as enantiomers and that both optical forms were present in the seed and root bark of St. Vincent Sea Island cotton.

The chromatographic separation of diastereomeric gossypol Schiff's bases (formed with a chiral amine) is a well known phenomenon that is used for the HPLC measurement of gossypol's optical forms in cottonseed and related products [20–22]. The large elution time differences that occur for the gossypol diastereomers make this system an impressive example of how chiral derivatization can be used to affect enantiomer separation. Several conformational features of the gossypol molecule likely contribute to produce this result. These include the atropic isomerism of the gossypol backbone, the existence of multiple reactive aldehyde groups, and the perpendicular orientation of the naphthalene rings that places these reactive groups a distance away from the chiral axis. This combination of factors results in a substantial difference in the molecular "foot print" of the individual gossypol diastereomers, even when the diastereomers are formed with relatively simple chiral amines.

Optical rotation values (Table 2) enabled the assignment of the enantiomeric form to each compound (Fig. 6). From these results, the (+)-enantiomer of each methylated gossypol compound (as its di-*R*-(−)-2-amino-1-propanol Schiff's base) was found to elute before the corresponding (−)-enantiomer. This is the same elution order as for the optical forms of gossypol. For each methylated gossypol form, the elution order (dextrorotary form before the levorotary form) was unaffected by mobile phase conditions. However, increasing mobile phase polarity did change the order of elution of the *R*-(−)-2-amino-1-propanol Schiff's base peaks for (+)-gossypol and (−)-6,6′-DMG (Fig. 4). Consequently, some care should be used in making these peak assignments in samples containing mixtures of these compounds.

The methoxy-gossypol Schiff's bases eluted more rapidly than the gossypol Schiff's base, which suggests that the methylation is disrupting hydrophobic interactions between the non-polar stationary phase surface and the gossypol binaphthalene rings. This appears contrary to the expectation that the substitution of a less polar methoxy group for a more polar hydroxyl group would increase elution times during reverse-phase chromatography. The more rapid elution of the methylated forms likely relates to the planar shape of the naphthalene rings. In gossypol crystal structures [24,25], the 6-position hydroxyl group forms an intra-molecular hydrogen bond with the adjacent hydroxyl group at the 5-position, effectively orienting this hydroxyl group within the plane of the naphthalene ring. This hydrogen bond would also be expected to exist in relatively non-polar environments, e.g., as would exist near the chromatographic stationary phase. In the methylated compounds, steric effects force the methyl groups to reside outside of the naphthalene ring planes, a conformational shift that is observed in the crystal structure of 6,6′-DMG:acetic acid (1:1) [26]. This loss of planarity at the ends of the naphthalene rings likely interferes with the strength of the hydrophobic interactions that occur between the rings and the octadecylsilyl moieties of the stationary phase and leads to faster elution of the methylated forms.

Regarding the diastereomeric 6-MG Schiff's bases, four chromatographic peaks might have been expected to occur, as the presence of only one methyl group makes each half of the dimeric gossypol backbone distinct. Nevertheless, we observed no resolution of these distinct compounds, even varying the mobile phase polarity over a wide range. This lack of separation likely occurs because the site of methylation lies very close to the extended chiral axis of the molecule. This positioning makes it unlikely that these compounds can be resolved by any chiral derivatization of the aldehyde moieties. Chiral derivatization with a compound that involves the *para*-orientated phenolic hydroxyl groups, which only exists on one side of this molecule (Fig. 1), might provide the discrimination needed to separate these forms.

Although it is not uncommon to see small peaks of methoxy-gossypol in the gossypol chromatograms of Pima cottonseeds [21], most *G. barbadense* cotton varieties have relatively low levels of the methylated gossypol forms. The St. Vincent Sea Island variety was chosen for this work as it was known to have high relative concentrations of 6-MG and 6,6′-DMG. Although the seed tissue had a greater total amount of gossypol compounds, root bark tissue was used for the preparative work as it had a greater fraction of its gossypol compounds in one of the two methylated forms (Table 1).

The distribution of the gossypol enantiomers varied considerably in St. Vincent Sea Island cotton tissues. Seed had slightly more (−)-gossypol (52.7%) than (+)-gossypol (47.3%). A small excess of (−)-gossypol is frequently observed in the seed for *G. barbadense* varieties [13,15,16] and has been previously reported for this variety [13]. In the root bark, (+)-gossypol is in considerable excess relative to (−)-gossypol. That the root bark and seed tissue differ in their ratio of optical forms suggests that there are some differences in the dimerization of the hemi-gossypol compounds among tissues

of the same variety. Differences in the ratio of the optical forms of gossypol in tissues have also been reported by Stipanovic et al. [18], who found that *G. hirsutum* var. mare-galante cotton varieties have an enantiomer excess of (+)-gossypol in both seed and root tissues, but that the excess was markedly less in the root material than in the seed material, which is opposite the trend observed here.

Tissue differences were also apparent in the distribution of the optical forms of the *O*-methyl derivatives. In the seed tissue, the ratio of optical forms was essentially the same for gossypol and its two methylated derivatives (i.e., all three gossypol compounds have a slight excess of the (−)-optical form). In the root bark, the measured enantiomeric excess of the (+)-optical form decreased in the order of gossypol > 6-DMG > 6,6′-DMG. Hence, methylation appeared to influence the distribution of gossypol enantiomers in the root tissue but not in the seed tissue, which also indicates that there are differences in the mechanisms of gossypol synthesis in the different tissues. It would be of interest to see if a similar trend exists within other cotton varieties producing significant levels of the methoxy-gossypol compounds.

The concentrated extract that was used as starting material for the separations was prepared by acetone extraction of the root bark followed by concentration and precipitation of the compounds with acetic acid. For seed without significant levels of the methoxy-gossypol derivatives, this procedure would be expected to yield gossypol as a racemate and equimolar acetic acid solvate, i.e., gossypol–acetic acid (1:1). In this process, any enantiomeric excess of one optical form is left uncrystallized in the mother liquor. A similar partitioning appears to occur when significant amounts of the methylated gossypol derivatives are also present. For each compound, the extract was found to contain a much reduced level of enantiomeric excess compared with the enantiomeric excess in the root bark substrate. Hence, in the presence of acetic acid, each gossypol derivative appears to precipitate as a racemate with most of the enantiomeric excess left in solution. On a molar basis, the enantiomeric excess of the combined (+)-optical forms was 1.0%, much less than the enantiomer excess of any of the individual compounds (3.6–6.0%), which suggests that gossypol molecules of one methylated form can become occluded within the crystal lattice of another methylated form. The recently determined molecular structure of 6,6′-DMG:acetic acid (1:1) [26] also suggests this possibility, as methylation did not affect how the gossypol molecules pack into these crystal lattices.

The total concentration of gossypol and its methylated derivatives in the recrystallized extract was ~89.8%, which is similar to the concentration of gossypol in gossypol:acetic acid (1:1), i.e., 89.62%. If all three gossypol compounds exist in the extract as equimolar acetic acid solvates, then a mixed preparation would be expected to contain between 89.6 and 90.1% gossypol compounds (and between 9.9 and 10.4% acetic acid) depending on the amounts of the various forms in the mixture. Although we have not tried to determine the amount of acetic acid in the recrystallized extract, this agreement suggests that the three compounds are all present in the extract as acetic acid solvates and that the recrystallized extract is otherwise largely free of contaminants.

The total percentage of the two methylated gossypol compounds was notably higher in the extract (84.1%) compared with the root bark (71.2%), indicating that the methylated forms were concentrated during the extraction process (Table 2). This accumulation is likely related to the different degrees of enantiomeric excess that exist for these compounds in the root bark. Because gossypol has the greatest difference in its enantiomeric ratio in this tissue (Table 2) and the compounds precipitated in roughly equimolar amounts, more (+)-gossypol is left unrecovered in solution. Consequently, less total gossypol is recovered compared with the methylated compounds leading to the higher percentages of the methylated forms in the extract.

There is now considerable evidence that preparations of 6-MG and 6,6′-DMG made by separating Schiff's bases formed with 3-amino-1-propanol are racemic. As discussed above, the extract prepared from the root bark contains nearly racemic amounts of each compound. Hence, nearly equal amounts of the individual enantiomeric forms of each compound would have been separated during preparative chromatography and would have been present during the final precipitation step. From our standard curves, quantitative analysis of these preparations confirmed that similar amounts of each enantiomer were present in these products. From these determinations, the enantiomeric excess of the (+)-optical form in the 6-MG preparation was 0.04%, and the enantiomeric excess of the (−)-optical form in the 6,6′-DMG preparation was 0.3%. Given that some experimental error exists in the preparation of these standard curves, these determinations are within expected limits for racemic products. Hence, precipitation of either 6-MG or 6,6′-DMG from solutions containing both optical forms in the presence of acetic acid tends to result in racemic acetic acid solvates.

Because gossypol–acetic acid (1:1) is readily available but the methylated derivatives are not, relative response factors (detector response of a compound relative to the detector response of a standard) were developed to be used to correct values of the methoxy compounds determined "as gossypol." Despite the similarity of the *R*-(−)-2-amino-1-propanol Schiff's base absorption spectra with the previously reported absorption spectra for the 3-amino-1-propanol Schiff's bases, small differences exist in these spectra that are reflected in these factors. For 6-MG, the relative response factors for the (+)- and (−)-optical forms were similar to each other and are only slightly greater than the relative response factor determined for the racemic acetate of 6-MG when complexed with 3-amino-1-propanol (0.80 [14]). For 6,6′-DMG, the relative response factors of the (+)- and (−)-optical forms were 7 and 10% higher, respectively, than the relative response factor for the racemic compound derivatized with 3-amino-1-propanol (0.62 [14]), indicating that there are relative differences in the specific absorptivity of these Schiff's base complexes at 254 nm. Hence, these relative response factors apply only when the same complexing amine and detector wavelength are used in the analytical chromatography. Although some care is needed in applying these factors, determining a concentration of one of these compounds as if it were the same optical form of gossypol and dividing through by these factors should yield a reasonable estimate for the concentration of the corresponding methylated compound.

5. Summary

The chiral forms of 6-MG and 6,6′-DMG have been separated and isolated by preparative chromatography of Schiff's base diastereomers formed with *R*-(−)-2-amino-1-propanol. Starting from an acetone extract of St. Vincent Sea Island cotton root bark, separation was achieved on a C18 reverse-phase stationary phase. The diastereomers were hydrolyzed with phosphoric acid and were recovered from solutions of diethyl ether, water, and acetic acid. Physical properties of the products were determined. Standard curves were prepared and the concentration of these compounds in St. Vincent Sea Island cotton plant tissues was determined.

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