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Determination of Gossypol Enantiomer Ratio in Cotton Plants by Chiral Higher-Performance Liquid Chromatography

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A celulose tris(3,5-dimethylphenylcarbamate) coated onto APS silica (Nucleosil, particle size, 7 μm; pore size, 500 Å) was used under a reversed-phase condition to measure the enantiomeric ratios of gossypol enantiomers in cottonseeds, flowers, and roots in a number of cultivars samples of different *Gossipium* species. While unidimensional chromatography was used for measuring the enantiomeric ratio of all the samples of *G. hirsutum*, *G. mustelinum*, and in the seeds of *G. barbadense*, multidimensional chromatography was necessary for the analysis of samples of roots and flowers of *G. barbadense*. In the latter case, an ODS Hypersil column was used in the first dimension for sample clean up, and the enantiomers were resolved on the second dimension by the chiral column. As expected, all the seed samples of *G. hirsutum* and *G. mustelinum* showed the (*P*)-(+)-enantiomer in excess, whereas the seeds of *G. barbadense* showed the (*M*)-(−)-enantiomer. However, (*P*)-(+)-gossypol was found in enantiomeric excess in three samples examined of roots and in one of flower of *G. barbadense.* These results are discussed in this paper.

KEYWORDS: Chiral chromatography; enantiomeric ratio; cottonseed; cotton roots; cotton flowers

INTRODUCTION

Gossypol (**Figure 1**) is a dimeric sesquiterpene isolated from the cotton plant and other members of the *Malvaceae* family. It is an axial chiral compound due to restricted rotation about the internaphthyl bond (*1*). It is known worldwide for its ability to inhibit sperm maturation (*2*−*3*), but in addition to this, a number of interesting biological activities have been reported and may be useful as an antineoplastic agent (*4*−*8*). In all cases, the (*M*)-(−)-gossypol was found to be more active than the (*P*)-(+)-enantiomer indicating that it is the more biologically active compound (*2*−*8*).

However, the analysis of the enantiomeric ratios of gossypol in different *Malvaceae* species has showed that (*P*)-(+)-enantiomer is found in enantiomeric excess in cottonseeds except for *G. barbadense*, which shows a modest excess of the (*M*)-(−)-enantiomer (*9*−*13*).

The methods employed for measuring enantiomeric ratios of gossypol in all cases reported involve derivatization to form a diastereoisomeric mixture with separation on an achiral C18 column (*9*−*13*).

Suitable conditions for the direct resolution of gossypol enantiomers, using a chiral carbohydrate carbamate phase under reversed-phase conditions, have been developed and used for isolation of multimilligram quantities of gossypol enantiomers but have not yet been used for assessing the enantiomeric ratio of gossypol in cotton plant (*14*−*15*).

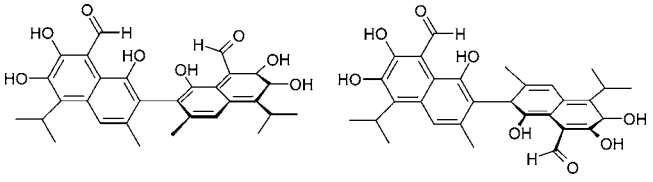

Figure 1. Enantiomers of gossypol.

The importance of measuring the enantiomeric excess of gossypol enantiomers in the cotton plant is well-established (*9*−*13*, *16*), and a number of papers that deals with this information have been published such as the papers in the use of cottonseed meal in diets of animals (*17*−*20*).

This work reports the ratio of gossypol enantiomers in seeds, flowers, and roots in a number of cultivar samples of different *Gossipium* species measured by direct chiral HPLC.

MATERIALS AND METHODS

The silicas used had the following properties: Hypersil (Shandon, U.K.; particle size, 5 μm; pore size, 120 Å) and Nucleosil (Macherey-Nagel, Germany; particle size, 7 μm; pore size, 500 Å). The Nucleosil silica was aminopropylated according to the reported procedure (*21*).

Isocyanates used were from Aldrich. Solvents were Chromar HPLC grade from Mallinckrodt Baker (St. Louis, MO). The filter paper used was analytical grade from Quimibras (Rio de Janeiro, Br.) Cellulose used was Avicel from Merck. HPLC dead times (t_o) were estimated by using acetonitrile.

(±)-Gossypol acetic acid used, as a standard, was the one isolated from cottonseeds as described in the literature (*14*). The (−) and (+)

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Table 1. Enantiomeric Excess of Gossypol in Flowers, Roots, and Seeds of Cotton

		gossypol (%)		enantiomeric excess (%)		
species/variety	sample (cultivar)	(–)	(+)	(–)	(+)	sample
G. hirsutum						
Marie-galante	Moco CNPA 5 M	15	85		70	cotoon seeds[a]
		3	97		94	roots[a]
		7	93		86	flowers[b]
Marie-galante	Moco CNPA 4M	6	94		88	cotton seeds[a]
	Moco CNPA 4 M	8	92		84	roots[b]
	Veludo	13	87		74	roots[b]
	Emparn-2	5	95		90	roots[b]
Latifolium	CMPA 7H	36	64		28	cotton seeds[a]
	CMPA 7MH	37	63		26	cotton seeds[a]
	HG 1845	42	58		16	cotton seeds[b]
	Precoce 2	37	63		26	cotton seeds[b]
	IAC 17	40	60		20	cotton seeds[a]
G. barbadense						
Brasiliense tussac	R.b. bag (86-40)	50	50			cotton seeds[a]
	R.b. bag (84-31)	67	33	34		cotton seeds[a]
	Quebradinho	22	77		55	roots[c]
	Pima A	30	70		40	roots[c]
	CNPA 98/01	25	75		50	roots[c]
		39	61		22	flowers[c]
G. mustelinum						
	Selvagem	35	65		30	cotton seeds[a]
		6	94		88	roots[a]
		16	84		68	flowers[b]

[a] Mobile phase: CH_3CN: 0.01 mol L^{-1} K_2PO_4 adjusted to pH 3.0 with H_3PO4 (55:45) at 1.0 mL min^{-1}, detection at 254 nm. [b] Mobile phase: CH_3CN: 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40) at 1.0 mL min^{-1}, detection at 254 nm. [c] Multidimensional analysis.

Table 2. Time Events for the Switching of Columns and of Mobile Phases

time (min)	pump	event	valve position
0.00–11.50	pump 1 (eluent A)[a]	sample cleanup by C18 column	1
	pump 2 (eluent B)	conditioning of the chiral column	
11.50–15.00	pump 1 (eluent A)	gossypol is transferred to the chiral column	2
14.50–35.00	pump 2 (eluent B)	analysis of the gossypol enantiomers	1
	pump 1 (eluent A)	conditioning of C18 column	

[a] Eluents: pump 1: (A) CH_3CN: 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (65:35 v/v); pump 2: (B) CH_3CN: 0.01 mol L^{-1} KH_3PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40 v/v), flow-rate: 1.0 mL min^{-1}, λ 254 nm. Sample: roots and flowers: CNPA 98/01 (*G. barbadense*) was transferred to the analytical column during 11.50–15.00 min. For the root Pima A sample, the switching time was 13.00–16.00 min, while for the roots of the quebradinho sample, the switching time was from 11 to 15.00 min.

separated enantiomers were obtained as described previously (*15*). The elution order was determined by injection of each enantiomer.

Cotton seeds, flowers, and roots were donated by the Centro Nacional de Pesquisa do Algodão, EMBRAPA (Campina Grande, Brazil), which holds voucher specimens of all the seeds, flowers, and roots referred to in this study.

Two analytical HPLC system were used: system 1 consisted of two Shimadzu (Kyoto, Japan) LC-10AD pumps, a Rheodyne 7125 injector fitted with a 20 μL loop, and either a SPD-10AV UV−vis or a photodiodo array SDP-M10Avp detector with a CBM 10A interface. System 2 consisted of two Shimadzu LC-10ATvp pumps (Kyoto, Japan), with one pump having a valve FCV-10ALvp for selecting solvent, an auto sampler model Sil10ADvp, a SPD-10Avp UV−vis detector, and a SCL10Avp interface. A sample valve HPLC 7000 Nitronic EA (Sulpelco, St. Louis, MO) was used for the automated column switching. Data acquisition in system 1 was performed using CLASS-LC 10 software and on system 2 using CLASS-VP software.

The chiral columns were prepared as described elsewhere (*14*, *21*) and consisted of cellulose tris(3,5-dimethylphenylcarbamate) coated onto APS-Nucleosil silica (7 μm particle size and 500 Å pore size) (20% w/w, 150 × 4.6 mm i.d.). The octadecylsilane column used (150 × 4.6 mm i.d.) (C18-Hypersil, 5 μm particle size, and 120 Å pore size) was packed by the ascending slurry method, using methanol for the preparation of the slurry (50 mL) and also for the packing. The packing was carried out at a pressure of 7500 psi.

The chiral columns were evaluated in the normal mode of elution, as described elsewhere, before it was eluted in the reversed-phase mode (*21*). This was achieved by elution of 2-propanol followed by acetonitrile, using approximately 200 mL of each solvent, before using the selected mobile phase CH_3CN: 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40 or 55:45 v/v). For the ODS column, the same type of solvent, in a different percentage (65:35 v/v), was used as the mobile phase.

Standard solutions of (±)-gossypol (0.01 mg mL^{-1}) were analyzed in all chromatography runs as a control to the method.

Determination of Enantiomer Ratios of Gossypol in Cotton Seeds, Flowers, and Roots Using Unidimensional Chromatography. Ground room-temperature dried cotton seeds, flowers, or roots (5.0 g) were extracted with ether (50 mL) for 30 min, then the extracts were filtered through a filter paper, and the solvents evaporated to dryness under reduced pressure. The residues were redissolved in acetonitrile and filtered through a nylon membrane (25 mm; 45 μm Corning), and then an aliquot (25 μL) was analyzed on the chiral column with CH_3CN 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40 or 55:45 v/v) as the eluent using HPLC system 1. The flow rate was 1.0 mL $min.^{-1}$ For the samples of cottonseeds, UV spectra were recorded in the range of 230−290 nm using a photodiodo array, and the enantiomers were detected at 290, while a UV−vis detector at 254 nm was used for the samples of roots and flowers.

Determination of Enantiomer Ratios of Gossypol in Flowers and Roots Using Multidimensional Chromatography. The room temperature dried cotton flowers and roots were grinded, and after that the samples (5.0 g) were extracted with ether (50 mL) for 30 min. The extracts were filtered through a filter paper before the solvents were

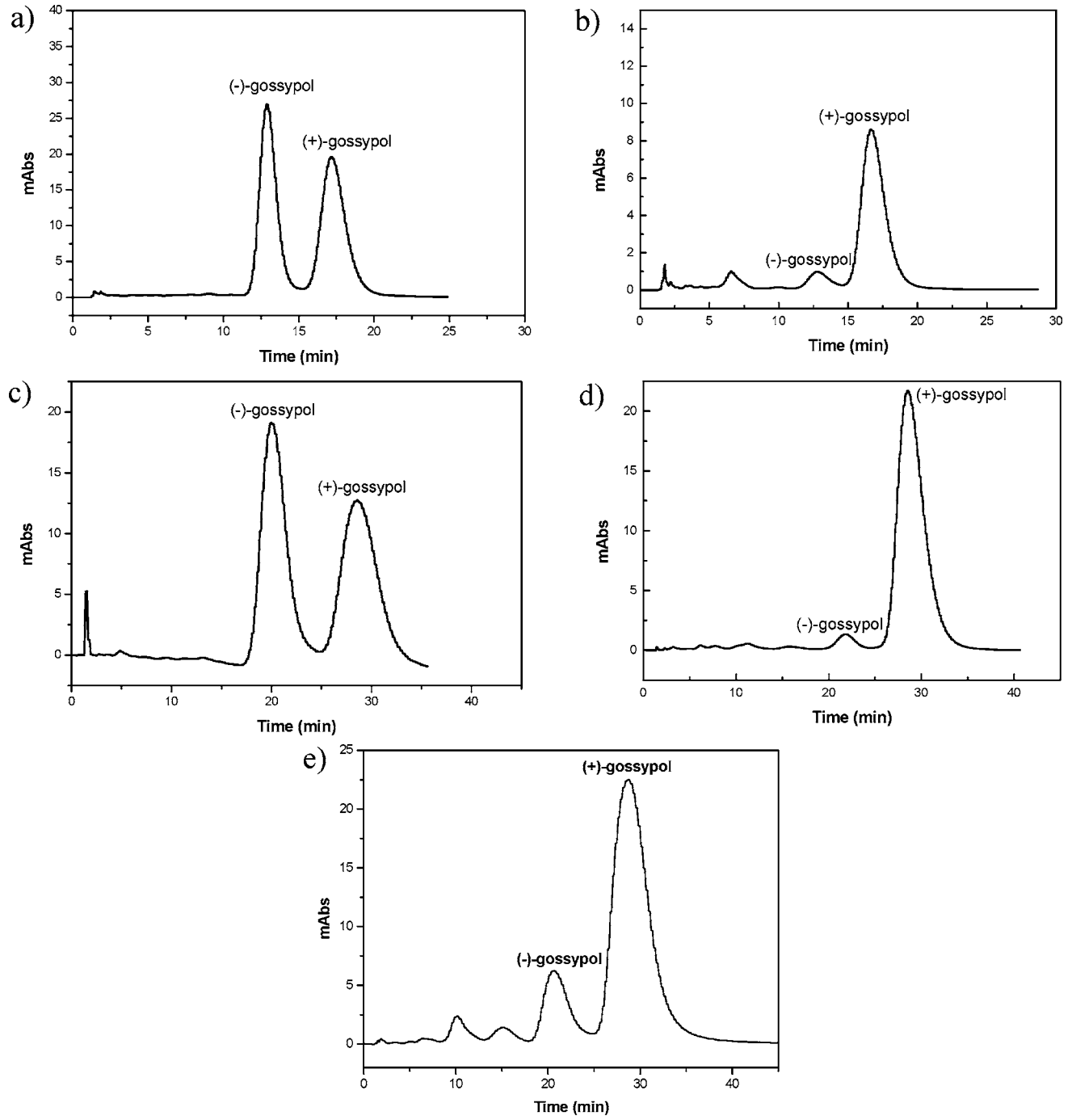

Figure 2. Enantioresolution of gossypol enantiomers on celulose tris(3,5-dimethylphenylcarbamate) coated onto Nucleosil (500 Å, 7 μm) (20% w/ w) using CH_3CN: 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40 v/v) as mobile phase for panels **a** and **b** while CH_3CN: 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (55:45) was used for panels **c**–**e**. Flow rate used was 1.0 mL min^{-1} with detection at 254 nm: (**a**) racemic gossypol standard sample; (**b**) flower, Moco CNPA 5M sample; (**c**) racemic gossypol standard sample; (**d**) root, Moco CNPA 5M sample; and (**e**) cotton seed, Moco CNPA 5M.

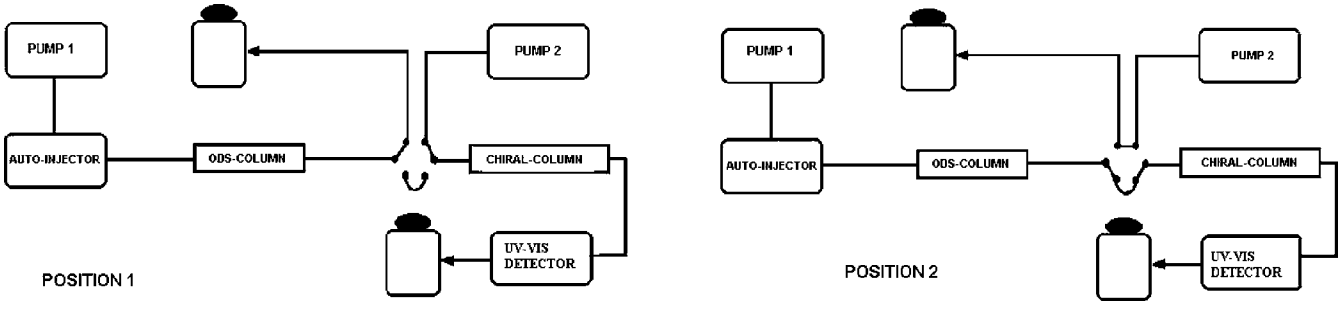

Figure 3. Schematic diagram of the column-switching system.

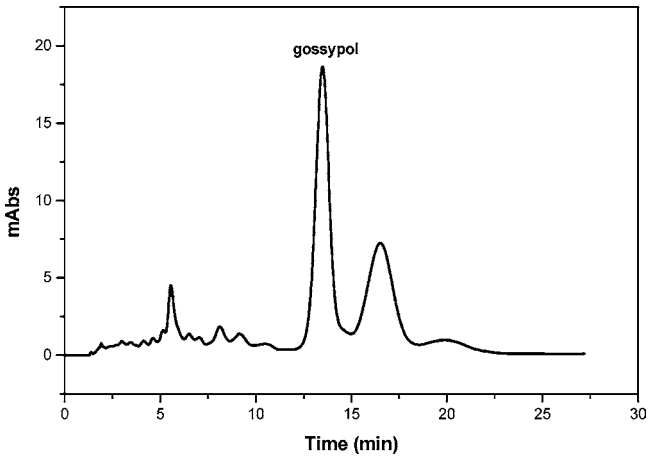

Figure 4. Typical chromatogram to determine the time program for switching between achiral and chiral columns. Sample: root (*G. barbadense*) CNPA 98/01.

evaporated to dryness under reduced pressure. The residues were redissolved in acetonitrile and filtered using a nylon membrane (25 mm; 45 μm Corning), and an aliquot (20 μL) was analyzed using HPLC system 2. The ODS column and CH_3CN 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (65:35 v/v) as the mobile phase was used in the first dimension for extraction and clean up. The polysaccharide column using CH_3CN 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40 v/v) as the eluent was used for the enantioseparation in the second dimension. The conditions used are described in **Table 2**.

The flow rate used was 1.0 mL min^{-1} in both columns, and the enantiomers were detected at 254 nm.

RESULTS AND DISCUSSION

The evaluation of the relative toxicity of gossypol enantiomers to plant pathogens has been neglected until recently. Stipanovic and collaborators measured the ratio of gossypol enantiomers in cottonseed, and roots of seedlings germinated from seed that had been treated with *T. virens* and compared with untreated controls. The (−)-gossypol was found in minor percentage in control and treated roots, but levels of this enantiomer were somewhat higher in roots from treated seeds (*23*). This work demonstrates the importance of measuring the ratio of the gossypol enantiomers in different parts of the cotton plant.

Having previously developed an efficient method for direct separation of gossypol enantiomers (*14*−*15*), the use of this method for measuring the enantiomeric ratio of gossypol in seeds, flowers, and roots of different species of cotton was evaluated and optimized and is now described.

The species selected *G. hirsutum*, *G. mustelinum*, and *G. barbadense* were the same used before when the enantiomeric ratios of gossypol were measured in cottonseeds by derivatization with a chiral amine to form Schiff's bases (*9*). Sixteen cultivar samples were examined. All the samples of *G. hirsutum* and *G. mustelinum* and the seeds of *G. barbadense* were evaluated by unidimensional chromatography with cellulose tris-

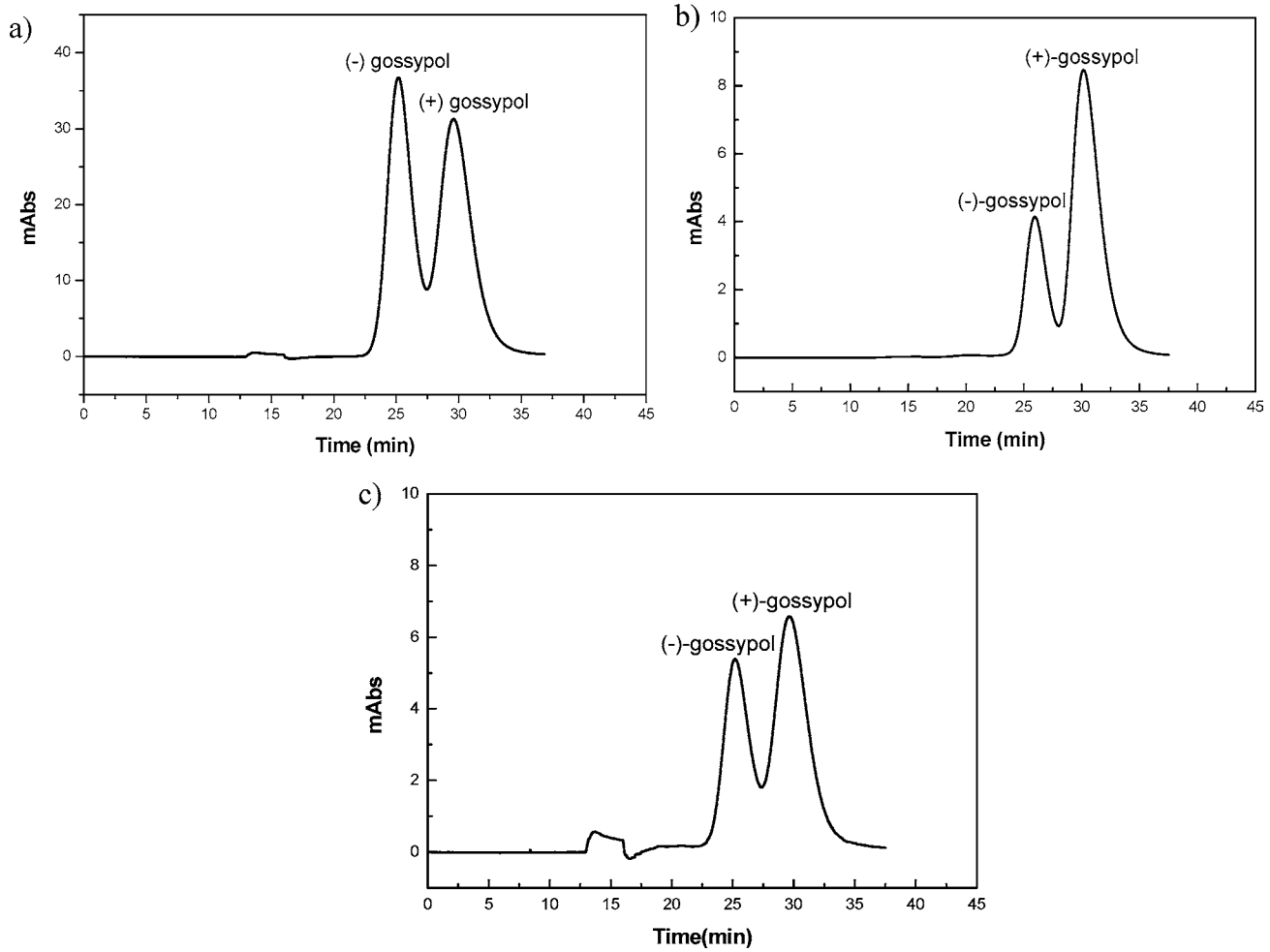

Figure 5. Enantioresolution of gossypol on celulose tris(3,5-dimethylphenylcarbamate) coated onto Nucleosil (500 Å, 7 μm) (20% w/w) using multidimensional chromatography. Conditions as stated in **Table 2** (**a**) racemic gossypol standard sample, (**b**) root, CNPA 98/01 sample, and (**c**) flower, CNPA 98/01 sample.

(3,5-dimethylphenylcarbamate) as the chiral stationary phase, and the mobile phases used are described in **Table 1**.

All the seeds samples of *G. hirsutum* and *G. mustelinum* showed the (+)-enantiomer in excess. Higher percentages were observed in the samples of the variety Marie Galante when compared with the latifolium. These results are in agreement with the previously published work (*9*).

The e.e. of the (+)-enantiomer was also observed in the samples of flowers and roots of these two species, while the comparison of the e.e. of (+)-gossypol in the roots, flowers, and seeds of samples of one cultivar showed differences with a higher percentage of (+)-enantiomer in the roots. This was observed with samples of two cultivars evaluated: Moco CNPA 5M and Selvagem (**Table 1**).

The chromatograms shown in **Figure 2** exemplify the separation obtained at the established conditions.

As expected, the (*M*)-(−)-gossypol was found in enantiomeric excess in one of the two samples of seeds of *G. barbadense* examined (*9*−*11*, *16*).

For measuring the enantiomeric ratio of gossypol in the roots and flower samples of *G. barbadense*, the use of multidimensional chromatography was necessary. This was due to the overlapping of other terpenes with one of the enantiomer's chromatographic band.

An ODS column was used in the first dimension for separating gossypol from the other components of the mixture, and after transferring, the enantiomers were resolved on the second dimension by the chiral column. **Figure 3** illustrates the HPLC system used, and the chromatographic events are listed in **Table 2**.

To determine the elution profile and retention times of gossypol in the sample cleanup procedure, the ODS column was first directly connected to the UV detector. **Figure 4** illustrates a typical chromatogram used for estimating the time necessary for transferring the gossypol fraction from the ODS column to the chiral column. Sample cleanup was made with CH_3CN 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (65:35 v/v) as mobile phase at 1.0 mL min^{-1} delivered by pump 1 (position 1, **Figure 3**). After the cleanup time (**Table 2**), the switching valve redirected the flow from the waste to the analytical chiral column (position 2, **Figure 3**). Gossypol was eluted onto the chiral column between the times listed in **Table 2** for each type of sample. The chiral analyses were performed using CH_3CN 0.01 mol L^{-1} KH_2PO_4 adjusted to pH 3.0 with H_3PO_4 (60:40 v/v) as mobile phase at 1.0 mL min^{-1} but delivered by pump 2 (position 1, **Figure 3**). The ODS column was conditioned by pump 1, while the separation was carried out on the chiral column. The enantiomeric separations obtained at those conditions are exemplified by the chromatogram shown in **Figure 5**.

While it is well-established that the (*M*)-(−)-gossypol is found in enantiomeric excess in the seeds of cultivars of *G. barbadense* (*9*−*11*, *16*), the (*P*)-(+)-enantiomer was found in enantiomeric excess (40−55%) in three samples examined of roots and in one of flowers (22%) of *G. barbadense*. These results are in agreement with previous results obtained when the enantiomeric ratios of cotton roots and flowers of *G. barbadense* were measured by the Schiff's base method (*9*). This is an important issue to be pursued in the investigation of the cotton plant resistance to pathogens (*23*, *24*).

The chiral methods described in this work are practical and should be conveniently used for assessing the enantiomeric ratios of gossypol in different cultivars samples of cotton in replacement of the indirect methods normally used.

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LITERATURE CITED

(1) Freedman, T. B.; Cao, X.; Oliveira, R.; Cass, Q. B.; Nafie, L. A. Determination of the Absolute Configuration and Solution Conformation of Gossypol by Vibrational Circular Dichroism. *Chirality* **2003**, *15*, 196−200.

(2) Waites, G. M. H.; Wang, C.; Griffin, P. D. Gossypol: reasons for its failure to be accepted as a safe, reversible male antifertility drug. *Int. J. Androl*. **1998**, *21*, 8−12.

(3) Segal, S. J. *Gossypol: a potential antifertility agent for males*; Plenum Press: New York, 1985.

(4) Bushunow, P.; Reidenberg, M. M.; Wasenko, J.; Winfield, J.; Lorenzo, B.; Lemke, S.; Himpler, B.; Corona, R.; Coyle, T. Gossypol treatment of recurrent adult malignant gliomas. *J. Neuro. Oncol.* **1999**, *43*, 79−86.

(5) Shelley, M. D.; Hartley, L. Groundwater PW, Fish RG. Structure−activity studies on gossypol in tumor cell lines. *Anti-Cancer Drugs* **2000**, *11*, 209−216.

(6) Van Poznak, C.; Seidman, A. D.; Reidenberg, M. M.; Moasser, M. M.; Sklarin, N.; Van Zee, K.; Borgen, P.; Gollub, M.; Bacotti, D.; Yao, T. J.; Bloch, R.; Ligueros, M.; Sonenberg, M.; Norton, L.; Hudis, C. Oral gossypol in the treatment of patients with refractory metastatic breast cancer: a phase I/II clinical trial. *Breast Cancer Res. Treat.* **2001**, *66*, 239−248.

(7) Benz, C. C.; Keniry, M. A.; Ford, J. M.; Townsend, A. J.; Cox, F. W.; Palayoor, S.; Matlin, S. A.; Hait, W. N.; Cowan, K. H. Biochemical correlates of the antitumor and antimitochondrial properties of gossypol enantiomers. *Mol. Pharmacol.* **1990**, *37*, 840−847.

(8) Zhang, M.; Liu, H.; Guo, R.; Wu, X.; Li, B.; Roller, P. P.; Wang, S.; Yang, D. Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells. *Biochem. Pharmacol.* **2003**, *66*, 93−103.

(9) Cass, Q. B.; Tiritan, E.; Matlin, S. A.; Freire, E. C. Gossypol enantiomer ratios in cotton seeds. *Phytochemistry* **1991**, *30*, 2655−2657.

(10) Jaroszewski, J. W.; Strom-Hansen, T.; Hansen, S. H.; Thastrup, O.; Kofod, H. On the botanical distribution of chiral forms of gossypol. *Planta Med*. **1992**, *58*, 454−458.

(11) Cass, Q. B.; Tiritan, M. E.; Bassi, A. L.; Degani, A. L. G.; Freire, E. C. Comparison of the enantiomeric ratio of gossypol in the roots, flowers, and seeds of various samples of cotton plants. Presented at the 18th Annual Meeting of the Brazilian Chemical Society, 1995**,** PN 107.

(12) Hron, R. J., Sr.; Kim, H. L.; Calhoun, M. C.; Fisher, G. S., Determination of (+)-, (−)-, and total gossypol in cottonseed by high-performance liquid chromatography. *J. Am. Oil Chem. Soc.* **1999**, *76*, 1351−1355.

(13) Schmidt, J. H.; Wells, R. Evidence for the presence of gossypol in malvaceous plants other than those in the cotton tribe. *J. Agric. Food Chem.* **1990**, *38*, 505−508.

(14) Cass, Q. B.; Bassi, A. L.; Matlin, S. A. First direct resolution of gossypol enantiomers on a chiral HPLC phase. *Chirality* **1999**, *11*, 46−49.

(15) Cass, Q. B.; Oliveira, R. V. Separation of multimilligram quantities of gossypol enantiomers on polysaccharide-based stationary phases. *J. Liq. Chromatogr. Relat. Technol.* **2002**, *25*, 819−829.

(16) Zhou, R. H.; Lin, X. D. Isolation of (−)-gossypol from natural plant. *Contraception* **1988**, *37*, 239−245.

(17) Santos, J. E. P.; Villasenor, M.; Robison, P. H.; DePeters, E. J.; Holmberg, C. A. Type of cottonssed and level of gossypol in diets of lactating dairy cows: plasma gossypol, health, and reproductive performance. *J. Dairy Sci*. **2003**, *86*, 892−905.

(18) Bailey, C. A.; Stipanovic, R. D.; Ziehr, M. S.; Haq, A. U.; Sattar, M.; Kubena, L. F.; Kim, H. L.; Vieira, R. M. Cottonseed with a high (+)- to (−)-gossypol enantiomer ratio favorable to broile production. *J. Agric. Food Chem*. **2000**, *48*, 5692−5695.

(19) Mena, H.; Santos, J. E. P.; Huber J. T.; Simas, J. M.; Tarazon, M.; Calhount, M. C. The effects of feeding varying amounts of gossypol from whole cottonseed and cottonseed meal in lactating dairy cows. *J. Dairy Sci*. **2001**, *84*, 2231−2239.

(20) Sullivan, J. L.; Huber, J. T.; Haper, J. M. Performance of dairy cows fed short staple, pima, and cracked pima cottonseed and feed characteristics. *J. Dairy Sci*. **1993**, *76*, 3555−3561.

(21) Matlin, S. A.; Tiritan, M. E.; Crawford, A. J.; Cass, Q. B.; Boyd, D. R. HPLC with carbohydrate carbamate chiral phases: Influence of chiral phase structure on enantioselectivity. *Chirality* **1994**, *6*, 135−140.

(22) Cass, Q. B.; Degani, A. L. G.; Cassiano, N. M. The use of a polysaccharide-based column on multimodal elution. *J. Liq. Chromatogr. Relat. Technol.* **2000**, *23*, 1029−1038.

(23) Puckhaber, L. S.; Dowd, M. K.; Stipanovic, R. D.; Howel, C. R. Toxicity of (+)- and (−)-gossipol to the plant pathogen, *Rhizoctonia solani*. *J. Agric. Food Chem*. **2002**, *50*, 7017−7021.

(24) Martin, G. S.; Liu, J.; Benedict, C. R.; Stipanovic, R. D.; Magill, C. W. Reduced levels of cadinane sesquiterpenoids in cotton plants expressing antisense (+)-D-cadinene synthase. *Phytochemistry* **2003**, *62*, 31−38.

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