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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF TERPENE ALDEHYDES IN COTTON

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SUMMARY

A high-performance liquid chromatographic method has been developed for the analysis of terpene aldehydes in cotton plant material. This represents an improvement over previous methods, providing quantitative analysis for all the major terpene aldehydes using a minimum of plant material and preparation time.

INTRODUCTION

Terpene aldehydes (TAs) in cotton are generally found in pigment glands located throughout the plant[1,2]. The TAs most frequently found in appreciable quantities in the glands have been identified as (Fig. 1) gossypol (I), 6-methoxygossypol

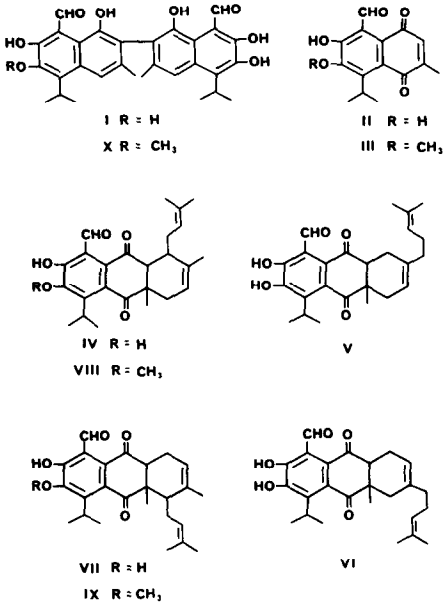

Fig. 1. Structures of TAs. See text for nomenclature.

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(X), 6,6'-dimethoxygossypol (not isolated for this study), hemigossypolone (II), 7-methoxyhemigossypolone (III), and heliocides H1 (IV), H2 (V), H3 (VI), H4 (VII), B1 (VIII) and B4 (IX)[3]. The TAs have been implicated in the resistance of cotton to insect[4,5] and pathogen attack[6], and have been suggested as possible byssinosis agents[7]. Gossypol has also been studied as having spermicidal activity[8].

High-performance liquid chromatographic (HPLC)[9,10] and gas–liquid chromatographic (GLC)[11,12] methods have been developed for the analysis of gossypol, but not the other TAs. A colorimetric method based on the formation of a yellow Schiff base upon reaction of the TAs with aniline[13] or *p*-anisidine[14] is generally used today, but lacks specificity, giving only total TA content. NMR[15] and GLC[16] methods have been developed for the analysis of the individual TAs, but a number of factors limits their uses in the routine analysis of plant material. The NMR method lacks sensitivity and requires an NMR spectrometer and the GLC method requires partial purification and derivatization of the TA extract. A sensitive, quantitative procedure that is simple and quick is still very much needed to analyze for the TAs in cotton plants in order to study their diverse biological activities in cotton pests and to study their health and pharmacological effects in man. We report such an HPLC procedure here.

EXPERIMENTAL*

Chromatography was performed on an IBM 9533 liquid chromatograph with a Rheodyne 7125 injector, a Waters 450 variable-wavelength detector, and a Hewlett-Packard 3390A reporting integrator. All solvents used were of HPLC-grade and were obtained from various manufacturers. HPLC columns were obtained from IBM, filters from Millipore, Biobeads S-X2 from Bio-Rad, Silic-AR CC-7 from Mallinckrodt, and thin-layer chromatography (TLC) plates from Whatman. 18-Crown-6 ether and *p*-bromophenacyl bromide were obtained from Aldrich.

Isolation and preparation of standards

Hemigossypolone (HGO), 7-methoxyhemigossypolone (MHGO), and heliocides H1, H4, B1, and B4 were isolated from glanded Pima S-4 flower buds (squares) as follows:

The squares were freeze-dried and ground into a fine powder with a Sorvall Omni-mixer. The powder was extracted with hexane–ethyl acetate–acetic acid (500:500:1) by ultrasonification with a Branson sonifier. The extract was concentrated under vacuum, methanol added, and the solution filtered. The filtrate was concentrated and chromatographed on Biobeads S-X2, eluting with ethyl acetate. The fractions were monitored for TAs by TLC using cyclohexane–chloroform–methanol (6:3:1) (solvent A) and silica gel plates (film thickness 250 μm). The fractions containing TAs were combined, concentrated, and chromatographed on a Silic-AR CC-7 column, eluting with hexane and 1% acetic acid. HGO crystallized out upon elution and was recrystallized from cyclohexane. The fractions containing B1 and B4 were

* Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

combined and concentrated, as were those containing H1, H4 and MHGO. B1 and B4 were separated by repeated preparative TLC using hexane with silica gel plates (1000 μm), each was recrystallized from hexane. Heliocides H1, H4 and MHGO were isolated by repeated preparative TLC using solvent A and silica gel plates (1000 μm), each was recrystallized from hexane. Heliocides H2 and H3 were prepared from the Diels-Alder reaction of HGO and myrcene[17] followed by preparative TLC using hexane–ethyl acetate–acetic acid (90:10:0.5) and silica gel plates (1000 μm). 6-Methoxygossypol (MG) was isolated from Pima S-4 glanded flower petals. The petals were extracted as above, the extract concentrated, and the material precipitated by hexane collected. MG was isolated from this precipitate by preparative HPLC on a 250 × 9.0 mm I.D. cyano column, eluted with hexane–acetonitrile–methanol–acetic acid (100:5:7.5:2). Each of the TAs separated was identified by NMR. Gossypol (G) was obtained as the acetic acid complex from the USDA (Southern Regional Research Center, New Orleans, U.S.A.).

The internal standard (*p*-bromophenacyl undecanoate) was prepared from *p*-bromophenacyl bromide and methyl undecanoate. Methyl undecanoate was saponified with potassium hydroxide and methanol, the pH adjusted to 8 with hydrochloric acid, and methanol removed under vacuum. A solution of 0.25 *M* *p*-bromophenacyl bromide in acetonitrile containing 0.0125 *M* 18-crown-6 ether was added to the potassium undecanoate, the mixture heated, filtered, and the *p*-bromophenacyl undecanoate crystals collected the next day.

HPLC analysis was conducted isocratically on a 5-μm C_{18} column (250 × 4.5 mm I.D.) with an ultrasonically degassed solvent system composed of acetonitrile–water–dimethylformamide (DMF)–methanol–acetic acid–phosphoric acid (55:25:20:5:2:0.06, v/v). Analyses were carried out at flow-rate of 1.0 ml/min for 26 min and 1.8 ml/min for the next 14 min. Peaks were detected by UV monitoring at 270 nm.

PROCEDURE

Analysis of standards

An amount of three mg of each TA was weighed out and dissolved in 10 ml ethyl acetate. Aliquots of 1.0, 0.8, 0.6, 0.4 and 0.2 ml were taken in triplicate and the ethyl acetate removed under a stream of nitrogen. Each aliquot was diluted with 100 μl of the internal standard solution (50 mg *p*-bromophenacyl undecanoate in 10 ml DMF), filtered through a 0.5-μm fluoropore filter, and 10-μl portions injected for analyses.

Analysis of plant material

Cotton plant parts were dissected, freeze-dried, and ground into a fine powder. Triplicate 100-mg samples were weighed out into 12-ml conical graduated centrifuge tubes. Each sample was extracted by ultrasonification with 10 ml of hexane–ethyl acetate–acetic acid (500:500:1) and made up to 12 ml with the same solvent. At least 98% of the total TAs was removed from the plant material with the first extraction, therefore a second extraction was not considered necessary. The samples were centrifuged and a 10-ml aliquot taken from each and the solvent removed under vacuum. Internal standard solution (100–500 μl) was added to each sample and 10-μl injections of the filtered samples analyzed by HPLC.

For purposes of comparison, 2.5 g of plant material was analyzed by using the method of Waiss *et al.*[15].

RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of the standard terpene aldehydes. Excellent separation is obtained among HGO, MHGO, G, MG, B4 and B1. The compounds H1, H2, H3 and H4, while not baseline separated, are resolved enough to provide adequate quantitation. The less polar (methoxylated) analogues of the TA pairs (MHGO/HGO, MG/G, B1/H1 and B4/H4) eluted later as would be expected on a reversed-phased system. Figs. 3–6 show the TAs present in very high gossypol (VHG) flower buds, Stoneville 7A bracts, Pima S-4 bracts and Pima S-4 flower petals. Plant material samples were spiked with standards to confirm terpene aldehyde identification. These data support the observation[3] that the *Gossypium barbadense* lines of cotton (Pima S-4) contain the methoxylated counterparts, while the *Gossypium hirsutum* lines (VHG and Stoneville 7A) do not.

A response factor for correlating the amount of TA to the internal standard was calculated in the usual way[18]. Table I lists the response factors and their standard deviations and the capacity factors for each TA. The response factors were constant in the range tested (6–30 μg TA injected onto column). The TAs were detectable down to 10 ng per injection, but the response factors were not constant at this low range.

The amount of TA in the plant material was calculated as follows:

$$\%\ (\text{dry wt.}) = \frac{(\text{area TA})}{(\text{area IS})} \times \frac{(\text{wt. IS})}{(R_F)} \times (1.2) \times \frac{1}{(\text{wt. powder})} \times (100)$$

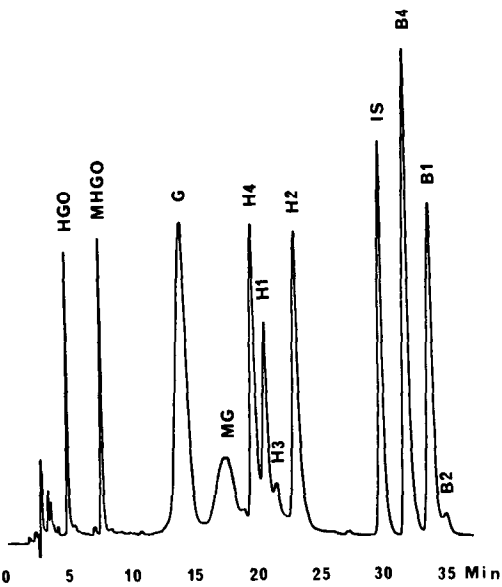
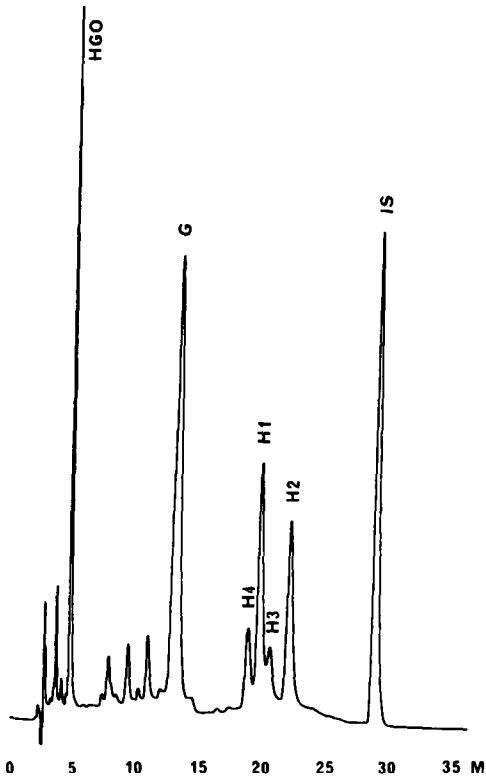


Fig. 2. Chromatogram of standard TAs.



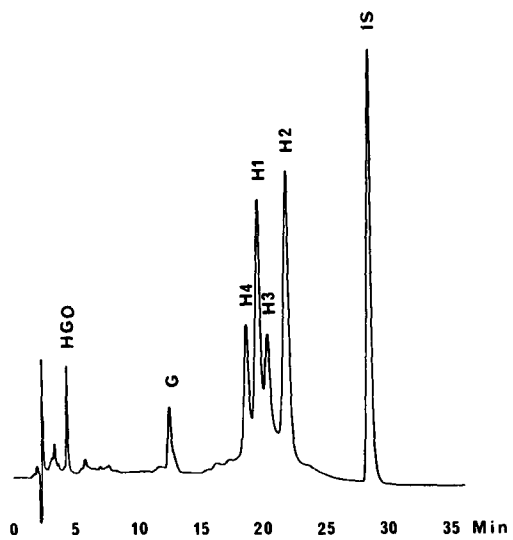


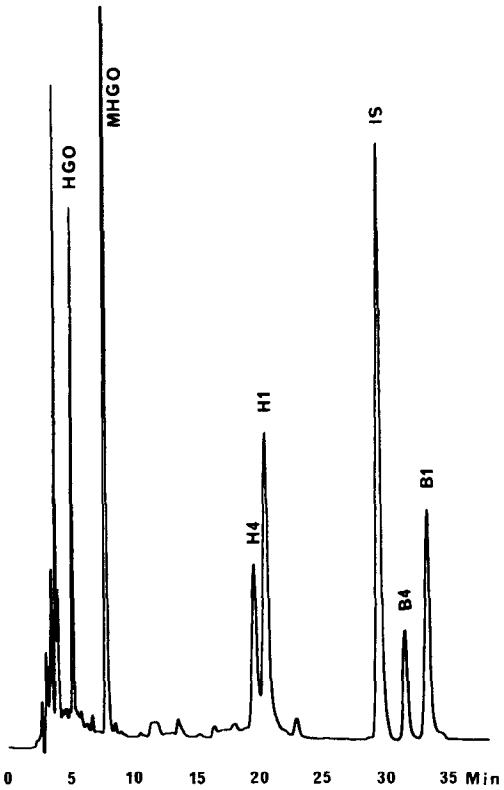
Fig. 3. Chromatogram of VHG flower buds.
Fig. 4. Chromatogram of Stoneville 7A bracts.

The areas of the TA and internal standard (IS) peaks were determined by the integrator. R_F is the response factor; wt. IS is the amount of internal standard added in mg. The factor 1.2 converts the amount of TA in the 10 ml aliquot back to the entire 12 ml aliquot. Wt. powder is the weight in mg of freeze-dried plant powder used.

A comparison of the percent dry weights of the individual TAs obtained by the HPLC and NMR methods is given in Table II. The levels calculated by HPLC are in good agreement, considering the instability of these compounds, with those detected by NMR, which had previously yielded highly comparable results with the aniline colorimetric procedure. Levels of 0.01–0.001% which could be detected by HPLC using 100 mg of plant material could not be detected by the NMR procedure using 2.5 g of plant material. TA contents of less than 0.006% were quantified by the HPLC procedure using 1.0 g of plant material, while 20 g of plant material with partial clean-up of the extract was required to detect the compounds by NMR.

CONCLUSION

This HPLC method has many advantages over any present method used to analyze for terpene aldehydes in plant material. All commonly found TAs were separated and were quantified with relatively small amounts of plant material, making



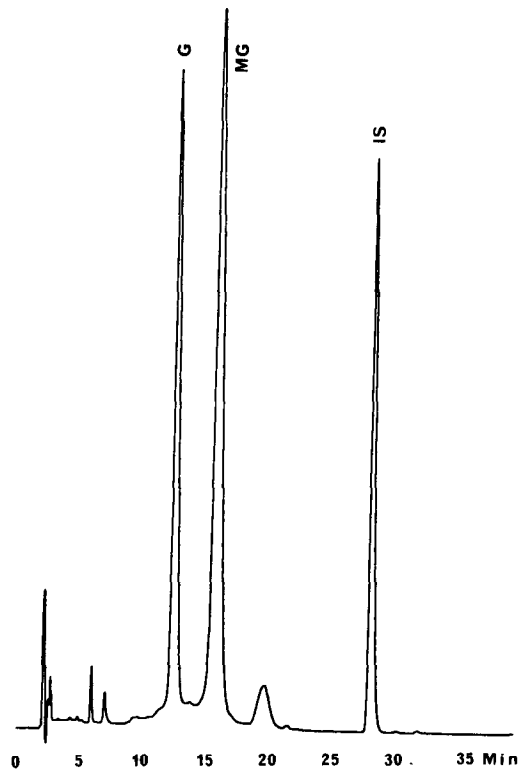


Fig. 5. Chromatogram of Pima S-4 bracts.

Fig. 6. Chromatogram of Pima S-4 flower petals.

TABLE I

CAPACITY AND RESPONSE FACTORS OF TAs

Terpene aldehyde	*Capacity factor (k')*	*Response factor*	*Standard deviation*
HGO	1.2	3.67	0.17
MHGO	2.4	4.48	0.33
G	5.1	5.72	0.13
MG	6.7	5.10	0.30
H4	8.1	6.33	0.18
H1	8.3	8.67	0.18
H3	8.5	5.63	0.17
H2	9.0	8.66	0.35
IS	12.6	–	–
B4	13.1	3.46	0.11
B1	14.0	3.54	0.08

TABLE II

PERCENT (DRY WT.) TAs IN COTTON BY HPLC AND NMR METHODS

HPLC values are the average of three runs with standard deviations not exceeding 5%.

Cotton variety		*HGO*	*MHGO*	*G*	*MG*	*H1*	*B1*	*H4*	*B4*	*H2*	*H3*
VHG	% By HPLC	0.24	–*	0.86	–	0.18	–	0.07	–	0.16	0.09
Flower buds	% By NMR	0.28	–	1.01	–	0.23	–	0.13	–	(0.24 Total)	
Stoneville	% By HPLC	0.01	–	0.01	–	0.05	–	0.03	–	0.07	0.04
7A bracts	% By NMR	0.002	–	**	–	0.06	–	0.03	–	0.09	0.03
Pima S-4	% By HPLC	0.10	0.19	0.006	0.008	0.08	0.13	0.06	0.07	–	–
Glanded bracts	% By NMR	0.04	0.10	**	**	0.08	0.12	0.04	0.05	–	–
Pima S-4	% By HPLC	0.01	0.02	0.41	0.93	0.001	0.01	0.001	0.003	–	–
Glanded petals	% By NMR	**	**	0.38	0.76	**	**	**	**	–	–

* Not detected.

** Detected, but not quantitated with 20 g plant material.

it 10–20 times more sensitive than the NMR procedure. This procedure is nearly as sensitive as the GLC procedure[15], but clean-up and derivatization of the extract is not required. We are currently using this HPLC method to study the role of TAs in the molecular basis of host plant resistance in cotton.

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