CHROM. 5050

GAS–LIQUID CHROMATOGRAPHY OF GOSSYPOL*

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SUMMARY

Extraneous peaks resulting from the gas–liquid chromatography of gossypol trimethylsilyl derivatives are shown to be the products of incomplete silylation of the gossypol molecule. A comparison of methods for the treatment of cotton root extracts prior to chromatography is presented and the quantitative determination of gossypol as its trimethylsilyl ether is discussed.

INTRODUCTION

Gossypol (2,2'-bis(8-formyl-1,6,7-trihydroxy-5-isopropyl-3-methylnaphthalene) and related pigments are naturally occurring substances found primarily in specialized glands of the cotton plant. The toxicity of gossypol to a wide variety of organisms has been investigated and its role in disease resistance demonstrated[1]. As part of a program concerning the biochemical nature of cotton resistance to nematodes it was of interest, therefore, to determine the gossypol content of cotton roots of various cotton varieties. A recent publication[2] has dealt with the gas–liquid chromatographic (GLC) determination of gossypol. According to this report, the highly reactive silyl donor N,O-bis(trimethylsilyl) acetamide (BSA) was a suitable agent for the preparation of the trimethylsilyl derivative of gossypol. Upon GLC, however, such a derivative yielded two shoulder peaks which the authors attributed to the occurrence of gossypol in three tautomeric forms. Evidence was cited in support of this proposal, that purification of the TMS-gossypol derivative by column or thin-layer chromatography did not remove the shoulder peaks. It was also stated that silylation in different solvents reduced the shoulder peaks in certain instances and varied individual peak size in others. Tetrahydrofuran (THF) was a solvent reported to result in the production of at least two shoulder peaks.

During the course of the present investigation it was noted that the occurrence of shoulder peaks was related to the ratio of BSA to gossypol and that a single peak could be produced by the addition of adequate amounts of BSA to the reaction mixture. This report presents evidence which indicates that the extraneous peaks arise from incomplete silylation of the gossypol molecule. A procedure for the partial purification of gossypol prior to silylation is also given and the quantitative determination of gossypol in cotton roots discussed.

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METHODS AND MATERIALS

Gossypol acetic acid[3], a primary reference standard, containing 89.62% gossypol by weight was dissociated by solution in dilute sodium carbonate and the gossypol recovered according to the method of PONS *et al.*[4]. The resulting product was used without further purification.

GLC analyses were performed on a Varian Aerograph chromatograph equipped with a flame ionization detector. A 46 × 0.21 cm (I.D.) stainless-steel column packed with 3% SE-52 on 100–120 mesh Gas-Chrom Q (Applied Science, State College, Pa.) was operated isothermally at 250 or 255° with a carrier (nitrogen) flow of 46 ml/min. Injections were made directly on the column, with the injector portion of the column maintained at 275°.

Standard solutions for quantitative analysis and determination of detector response (Fig. 1) were prepared by dissolving quantities of gossypol in carbon disulfide, sufficient to provide a known volume of solution upon addition of the BSA. For

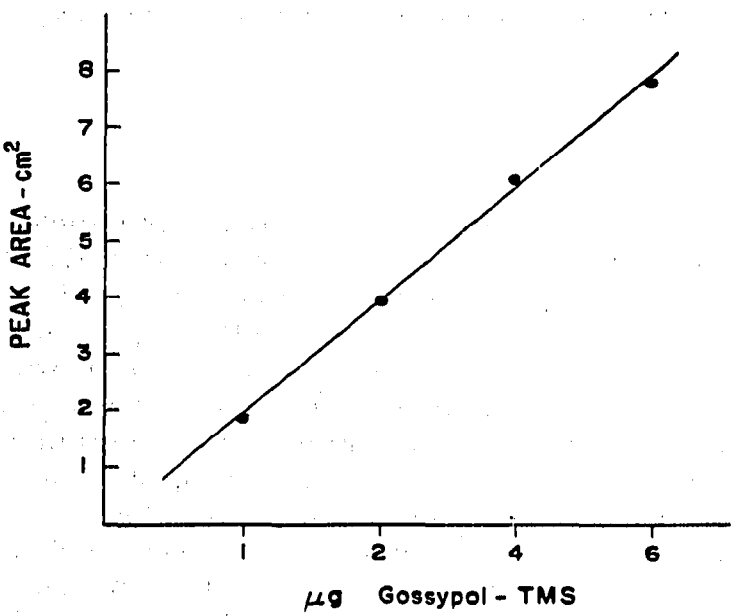


Fig. 1. Detector response and calibration curve for gossypol-TMS.

example, to make a solution containing 1 mg/ml of gossypol, 1 ml of CS_2 containing 1 mg of gossypol was evaporated under N_2 in a tared glass vial and the residue redissolved in 0.6 ml of carbon disulfide. To this was added 0.4 ml BSA (Pierce Chemical Company, Rockford, Ill.), the vial flushed with N_2, sealed and held at 50° for 30 min. One microliter aliquots were taken directly from the reaction mixture for injection.

Peak areas were estimated by triangulation, and standard curves prepared by plotting peak area against weight or molarity of gossypol. Unknown quantities of gossypol were determined by measuring peak areas and comparing them directly with those of the standard curve. A new standard curve was prepared for each analysis although a deviation of less than 3% was noted between analyses.

IR spectra were obtained in CS_2 on a Perkin-Elmer InfraCord equipped with NaCl micro-cavity cells (Barnes Engineering, Stamford, Conn.).

EXPERIMENTAL

The influence of BSA concentration on the production of extraneous peaks was determined by weighing 10 mg quantities of gossypol into tared glass vials and adding 1 ml of THF and 15–360 μl of BSA. The vials were capped with teflon-lined lids, shaken and held at 50° for 20 min prior to analysis.

Alternatively, 10 mg of gossypol were reacted with 30 μl of BSA in 1 ml of THF and the mixture incubated at 40°. At regular intervals, following addition of the BSA, 1 μl samples were subjected to GLC analysis.

Partially silylated gossypol was prepared by dissolving 120 mg of gossypol in 2 ml of THF and 125 μl of BSA. After standing at room temperature for 30 min, the THF and BSA were removed at 80° under high vacuum. The last traces of BSA were removed by dissolving the product in 5 ml of anhydrous carbon tetrachloride which was then removed under vacuum at 80°. This process was repeated three times and the resulting residue taken up in a small amount of carbon tetrachloride. Unsilylated gossypol was removed by sweep-distillation at 275° and the partially silylated material collected in glass U-tubes at —80°. The product obtained by this procedure had a GLC retention time corresponding to that of peak c in Fig. 2, and a GLC purity of 95%. Gossypol-TMS corresponding to peak a (Fig. 2) was obtained in 99.5% purity in a fashion similar to that just described except that the reaction was carried out in carbon disulfide, 1 ml of BSA added and the reaction mixture, under N_2, heated at 40° for 30 min in a sealed glass vial.

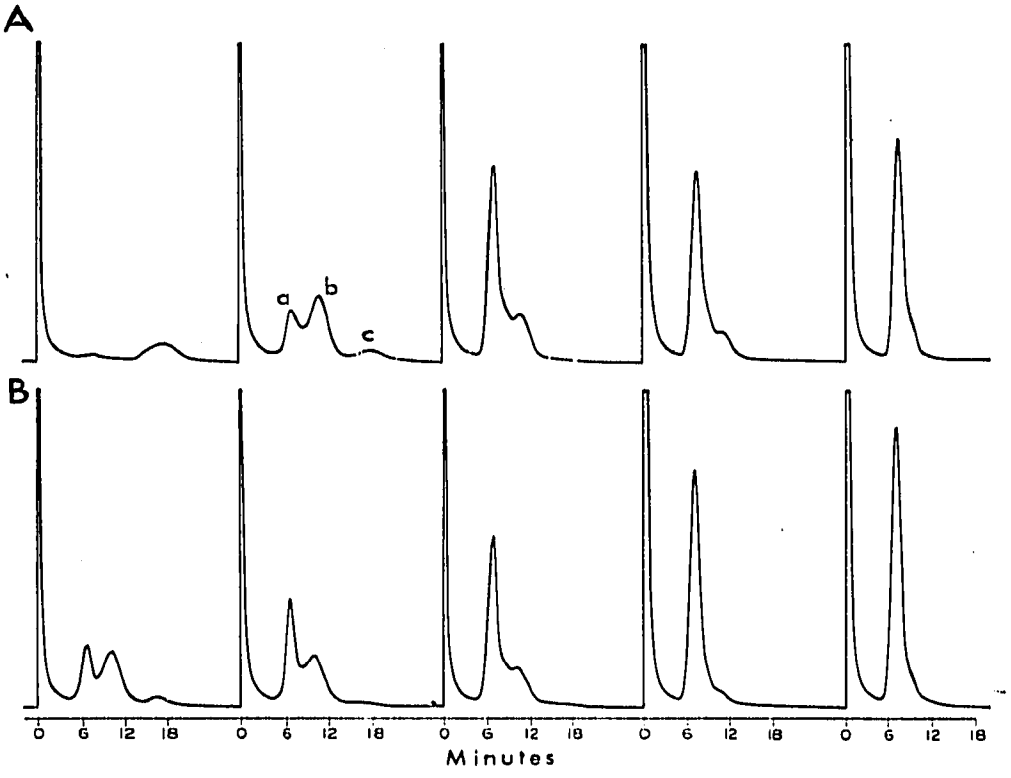


Fig. 2. (A) Chromatograms produced following the reaction of gossypol with 15, 30, 60, 120 and 360 μl of BSA. (B) Chromatograms produced at time intervals of 0, 30, 60, 180 and 300 min following the addition of 30 μl of BSA to 10 mg of gossypol in 1 ml of THF.

Plant root materials were prepared for GLC analysis of gossypol following a cleanup procedure suggested by BELL[5]. Fresh roots (30–50 g) were mascerated in a high-speed blendor in 300 ml of re-distilled acetone. The extract was filtered once through a Whatman No. 2 filter, prewashed with acetone, and the filtrate rinsed with an additional 200 ml of acetone. The acetone and most of the water were then removed under vacuum at 50° and the residue dissolved in 200 ml of ether. The ether solution was washed once with 100 ml of water at pH 6.5 and extracted with 200 ml of 0.5% sodium borate. After a single extraction of the borate phase with 100 ml of ether, it was acidified to pH 2 with 6 *N* HCl and re-extracted with 3 portions (100 ml) of ether. Traces of HCl were removed by washing the combined ether extracts once with 200 ml of water, drying over anhydrous sodium sulfate and evaporating to dryness at 30° under vacuum. The residue was silylated as described above for standard gossypol and 1 μl aliquots of the reaction mixture injected directly.

In an effort to reduce sample preparation to a minimum, crude acetone extracts were dried as before and the residue was silylated in CS_2 without further purification. Relative recovery of gossypol from cotton roots was estimated for both techniques by supplementing 5 g quantities of fresh roots with 5 mg of authentic gossypol. Four separate analyses were performed for each method using unsupplemented crude extracts as controls.

RESULTS

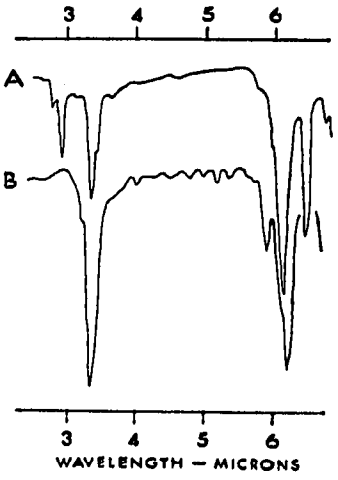
BSA concentration

Concentrations of BSA less than 36 μl per 1 mg of gossypol resulted in the production of more than one GLC peak (Fig. 2A). When 10 mg of gossypol were treated with 15 μl of BSA, a chromatogram was produced in which the major component (peak c) had a retention time of 2.5 relative to gossypol-TMS (peak a). At 30 μl of BSA to 10 mg of gossypol, the major component (peak b) had a retention time of 1.8 in relation to gossypol-TMS. Of the total peak area, 35% was represented by gossypol-TMS (peak a) and less than 10% by peak c. Increasing concentrations of BSA increased the area under peak a and reduced peaks b and c, accordingly. Since the total peak area at the highest BSA concentration exceeded that at 30 μl BSA by almost 20%, it was assumed that a portion of the gossypol in the latter case remained unsilylated or insufficiently silylated to pass through the GLC column.

Sampling at increasing time intervals following the introduction of a minimal amount of BSA produced a series of chromatograms (Fig. 2B) similar to those obtained by adding increasing amounts of BSA to the reaction mixture. In addition to gossypol-TMS, injections made soon after the addition of BSA resulted in the appearance of peaks b and c. When the reaction period was increased, the area under these peaks decreased accompanied by a proportionate increase in peak a (gossypol-TMS).

Infrared analyses

IR spectra (Fig. 3A) of material comprising peak c showed absorption bands at 2.8 and 6.2 μ corresponding to the oscillations of free phenolic hydroxyl groups and carbonyl groups respectively. The presence of the band at 2.8 μ is thus indicative of the incomplete silylation of the gossypol molecule since such bands do not exist in the



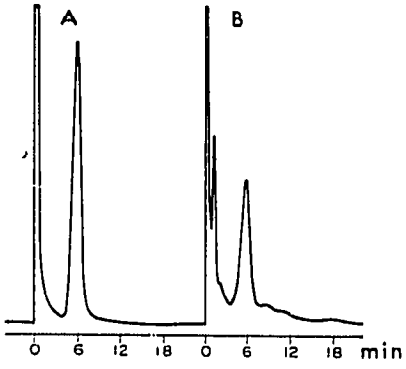


Fig. 3. IR spectra of (A) partially silylated gossypol (peak c) and (B) gossypol-TMS (peak a).

Fig. 4. Chromatograms of gossypol-TMS extracted from cotton roots. (A) borate purified prior to silylation, (B) silylation of crude extract.

spectra of gossypol hexamethyl ethers. Accordingly, spectra of fully silylated gossypol (Fig. 3B) showed no absorption at 2.8 μ (OH) and increased absorption at 3.42 μ (CH_3). Partial silylation of gossypol also resulted in the appearance of a strong absorbtion band at 6.4 μ which could not be explained on the basis of the aldehydic structure of gossypol or its TMS derivative.

Cotton roots

Acetone extracts of cotton roots subjected to borate–ether purification gave rise to a single symmetrical peak upon GLC of the TMS reaction mixture (Fig. 4A). Non-purified extracts also produced a well-defined gossypol-TMS peak, accompanied, however, by an elevated baseline and several ill-defined peaks (Fig. 4B). It was not determined whether these additional peaks arose from incomplete silylation of the native gossypol or if they represented other compounds of sufficient volatility to pass through the GLC column. Heating the reaction mixture at 40° for 3 h did not reduce their proportion relative to gossypol-TMS. Relative recoveries of gossypol from cotton roots by silylation of the crude acetone extract and extracts partially purified by treatment with 0.5% sodium borate are presented in Table I. It was noted that in order to silylate completely gossypol in supplemented acetone extracts, it was necessary to add BSA in excess of the amount considered adequate for purified samples. Less than 120 μl per mg gossypol resulted in the appearance of shoulder peaks corresponding to peaks b and c in Fig. 2.

DISCUSSION

The occurrence of gossypol and certain of its derivatives in one or more tautomeric forms has been proposed on the basis of IR and chemical studies[6]. These studies have shown that at least some derivatives exist primarily in one form. Thus, while the dimethyl ether of gossypol is said to have the aldehyde tautomeric form, the hexamethyl ether of gossypol is thought to exist in the quinoid form and hexaacetates in

the hemiacetal form. In previous GLC studies it has been suggested that the appearance of more than one peak following injection of the TMS derivative supports the occurrence of such tautomers. The present investigation shows that at least one of the peaks, formed when THF is used as the reaction solvent, results from incomplete silylation of the free phenolic hydroxyl groups. No free phenolic hydroxyl stretching absorption in the IR spectrum was observed when gossypol was silylated under optimum conditions. If it were possible to separate, by GLC, all of the products of incomplete silylation, more than three peaks might be expected. The asymmetry of peak a (Fig. 2) suggests the presence of additional peaks incompletely resolved under the conditions of analysis. Gossypol showed less of a tendency to form partially silylated products in CS_2 than in THF. Furthermore, GLC flame ionization detectors are relatively insensitive to carbon disulfide with the result that less interference from the injection solvent can be expected. For these reasons, carbon disulfide is a more suitable solvent than THF for the silylation of gossypol. Should THF be the solvent of choice, however, it is necessary to use at least 40 μl of BSA per mg of gossypol to effect complete silylation.

Partial purification of the acetone extracts with a solution of sodium borate resulted in 40–56% losses of the gossypol added to fresh cotton roots. Failure to recover higher percentages can be explained in part by the presence, in cotton roots, of powerful emulsifiers. These substances make it difficult to obtain good two-phase separations of gossypol-containing organic solvents washed with aqueous solutions. The addition of methanol serves to break these emulsions, but also may cause gossypol to be partially distributed in the discarded phase.

Although silylation of crude acetone extracts of cotton roots resulted in an increased base line and the appearance of additional peaks (Fig. 4B) (probably not gossypol-TMS related), considerably higher recoveries of added gossypol were observed (Table I). Also, reduced sample manipulation resulted in greater analytical precision. For cotton roots, this procedure is preferred.

TABLE I

RECOVERY OF GOSSYPOL FROM COTTON ROOTS

$$\% \text{ recovery} = \frac{\text{wt. of gossypol recovered} - \text{wt. of indigenous gossypol}}{\text{wt. of gossypol added}} \times 100.$$

Expt. No.	*Recovery (%)*	
	Acetone extract	*Borate treated extract*
1	92.4	44.0
2	92.8	59.4
3	91.8	54.6
4	92.4	59.6
Mean ± S.E.	92.4 ± 0.2	54.4 ± 3.7

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REFERENCES

1 A. A. BELL, *Phytopathology*, 59 (1969) 1119.
2 P. K. RAJU AND C. M. CATER, *J. Am. Oil Chemists' Soc.*, 44 (1967) 465.
3 C. L. HOFFPAUIR, J. A. HARRIS AND J. P. HUGHES, *J. Assoc. Offic. Agr. Chem.*, 43 (1960) 329.
4 W. A. PONS, JR., J. POMINSKI, W. H. KING, J. A. HARRIS AND T. H. HOPPER, *J. Am. Oil Chemists' Soc.*, 36 (1959) 328.
5 A. A. BELL, Personal communication, 1970. Present address: U.S.D.A., A.R.S., Texas A & M, College Station, Texas.
6 A. L. MARKMAN AND V. P. RZHEKHIN, *Gossypol and its Derivatives*, translated from Russian by L. MARKIN; Israel Program for Scientific Translations, Jerusalem, 1968.