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Gas-Liquid Chromatography of Trimethylsilyl Derivatives of Abscisic Acid and Other Plant Hormones[1]

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Abstract. **This paper describes a new method for qualitative and quantitative assay of abscisic acid and other acidic plant hormones, such as indoleacetic acid and the gibberellins, by the gas-liquid chromatography of their trimethylsilyl derivatives. Interfering substances in plant extracts were largely removed by preliminary column chromatography with carbon-celite and elution of the abscisic acid with 60 % acetone, permitting direct determination of abscisic acid by gas-liquid chromatography using a flame ionization detector. (A level of 0.65 mg/kg fr wt was found.) This method enables measurement of amounts of abscisic acid as low as 0.025 μg. In impure samples collected by gas-liquid chromatography the abscisic acid recovered could be measured quantitatively by use of its ultraviolet absorption maximum at 260 mμ.**

Investigations of plant hormones have usually had to depend upon separate bioassays for the detection and measurement of each hormone. Bioassays have limited reliability, both qualitatively and quantitatively, and this limits the scope of investigations which can be undertaken. Chemical assays have limited value because the hormones in plants are present in extremely small amounts and other compounds are generally present which interfere with chemical measurements. For a chemical method to have substantial value it should have both the ability to separate the hormone from interfering compounds and to quantitatively measure the amount present. Since gas-liquid chromatography (GLC) offered these possibilities, work was instituted to adapt it for plant hormone analysis.

The first investigators to utilize GLC for the separation of plant hormones were Ikegawa *et al.* (9) in 1963. They were able to separate the methyl esters of gibberellins 1 through 9 but worked only with pure compounds. Stowe and Schilke (18), Grunwald *et al.*, (7), and DeMoss and Gage (5) have reported similar results with indole auxins. Dedio and Zalik (4) applied GLC analysis to a partially purified cabbage extract and were able to show the presence of an indole but did not identify it further. Powell (15) obtained peaks corresponding to IAA (from maize) and indoleacetonitrile (from cabbage). Koizumi *et al.*, (11) have extended this to give a quantitative measure of IAA from mung bean seedlings. Pryce *et al.*, (16) have used GLC for the identification of gibberellins in bean seed extracts and Sitton *et al.*, (17) have determined gibberellin precursors in roots by GLC of thin layer purified extracts. Cavell *et al.*, (3) have succeeded in identifying several gibberellins from crude and purified acid fractions of the seeds of *Phaseolus*.

For the plant hormones which are free acids, volatile derivatives must be made prior to chromatographic analysis. Brook *et al.*, (2) compared the use of diazomethane with and without using trifluoroacetic anhydride for preparing derivatives of indoles and Dedio and Zalik (4) used both diazomethane and BF_3methanol to prepare methyl esters. Cavell *et al.*, (3) prepared both the methyl ester by using diazomethane, and also the trimethyl silyl ether of this ester using hexamethyl disilazane and trimethylchlorosilane. Most workers (2, 3, 4, 7, 15, 16, 18) used diazomethane for preparation of the methyl esters; but because of its highly explosive nature many investigators have hesitated to use this reagent. A safer method involves the preparation of silyl derivatives as reported by Sweeley *et al.*, (19). Gamborg (6) has used silyl derivatives in the separation of organic acids from enzymatic reactions, and thus silylation methods appear suitable for acidic plant hormones without prior esterification.

This paper reports experiments on new derivatives of abscisic, gibberellic and indoleacetic acids prepared by using the reagent bis(trimethylsilyl) acetamide (BSA) (Klebe *et al.*, 10). Gas-liquid chromatographic analysis of these derivatives appears to offer a method for quantitative analysis of the extracted hormones with a minimum of preliminary purification.

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Materials and Methods

The preparation of the trimethylsilyl (TMS) derivatives was rapid and simple. The sample, whether pure crystalline material or dried plant extracts, was placed in a 3 ml test tube and 0.1 ml BSA was added to it. The test tube was then capped and allowed to stand at least 30 minutes before its contents were analyzed by GLC.

The chromatographic instruments used were 2 Varian Aerographs. Analytical work was accomplished on a Model 204 equipped with a flame ionization detector, a linear temperature programmer, and a Model 650 Aerograph hydrogen generator. This system employed a 5 foot by one-eighth inch stainless steel column with nitrogen carrier gas flow rate of 25 ml/min, a hydrogen flow rate of 25 ml/min, and an air flow rate of 300 ml/min. A preparative system utilized an Aerograph A-90P3 with a thermal conductivity detector, a 5 foot × one-fourth inch stainless steel column, and helium carrier gas flow rate of 25 ml/min. All columns were preconditioned at 300° overnight. The column packing consisted of 5 % SE 30 or 5 % QF-1 coated on 60/80 mesh, acid washed, DMCS treated Chromosorb W.

The pure standards used were as follows: gibberellic acid (GA_3) from K & K Laboratories, lot 13098, indoleacetic acid (IAA) from Nutritional Biochemical Corporation; synthetic (racemic) abscisic acid (ABA) obtained from the Shell Development Company, and a mixture of ABA and its trans isomer (t-ABA) obtained from the Research Division of the Reynolds Tobacco Company.

Plant extracts were obtained by grinding 10, 5-day-old cotton fruits (approx 25 g) in 100 ml of 80 % acetone. The bolls had been frozen on dry ice in the field and stored at —20° until 5 minutes before extraction. After the ground material had set in the 80 % acetone for 6 hours, the 80 % acetone was filtered, evaporated to an aqueous residue, acidified to pH 2 and extracted twice with ethyl acetate. The acidic ethyl acetate was combined and extracted twice with 5 % sodium bicarbonate. The pH of the combined bicarbonate was adjusted to 2 and it was extracted twice with ethyl acetate. The ethyl acetate was dried to yield the plant acids. If further purification was desired, the extract was placed on a 2 cm diameter column containing a mixture of 6 g celite and 3 g carbon (Darco activated carbon, grade G-60, Atlas Chemical Industries), and 25 ml of deionized water were passed through. The active material was then eluted by passing 200 ml of 60 % acetone in water through the column.

Chromatography with the flame ionization instrument consisted of injecting 3 μl of the silylated extract in BSA while the column was isothermal at 60° with the injector temperature at 180 to 190° and the detector temperature at 190°. The column was kept at 60° for 6 minutes before a linear temperature program was begun, with a rate of increase of either 12.8° per minute or 9.1° per minute. When the maximum temperature, 220°, was reached, isothermal conditions were maintained for the duration of the run.

Chromatography with the thermal conductivity instrument consisted of injecting 25 μl of the silylated extract in BSA with the column temperature at 75°, the injector temperature at 150°, and the detector temperature at 220°. The column was left at 75° until all of the unreacted BSA reagent had eluted from the gas chromatograph. The rheostat was then turned up to give a non-linear temperature increase to a maximum of 260°. The region desired was collected in glass capillary tubes, sealed, and stored in a refrigerator in the dark.

Samples collected as above were used for obtaining infrared spectra by removing the sample with a syringe from the freshly opened tube and placing it in a microcavity sodium chloride cell for analysis utilizing a Beckman IR8 instrument. If the sample was very small it was transferred with methylene chloride, and the latter was evaporated before scanning the spectrum. For measurement of mass spectra, sections of the tube containing the sample were directly inserted in the sample chamber of a Varian M66 mass spectrometer. The ultraviolet spectra were determined in 0.005 N ethanolic sulfuric acid in a 1 cm path length cell using a Beckman DBG spectrophotometer. The sample was removed from the collecting tube by rinsing with the above solvent. The biological activity of the collected samples was determined by using the cotton explant abscission bioassay (Addicott *et al.*, 1; Lyon and Smith, 12).

Results and Discussion

Preliminary GLC studies using the liquid phase SE 30 showed no movement of either ABA or GA_3 through the column at temperatures up to 300°. However, the TMS derivatives could be successfully chromatographed on the flame ionization instrument with retention times as given in table I.

Since no results of reactions between BSA and

Table I. *Retention of TMS Derivatives on Flame Ionization Instrument*

Time given is from point of injection and includes 6 minutes isothermal operation and then programmed temperature at 12.8°/minute.

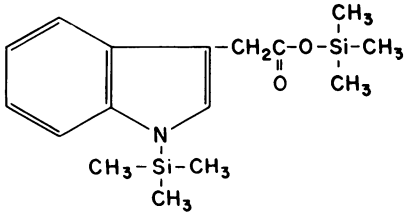
Compound	Retention time QF-1	SE 30
	min	*min*
IAA-TMS	14.8	15.3
ABA-TMS	17.6	16.8
tABA-TMS	18.4	17.6
GA-TMS	20.2	21.5

Table II. *Mass Spectra and Infrared Spectra of TMS Derivatives*

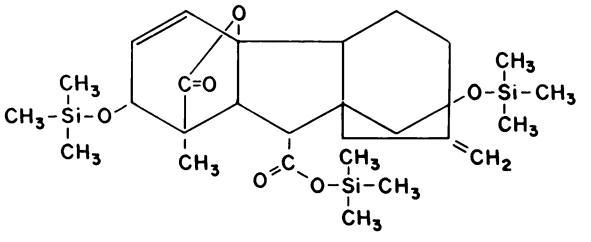
Compound	Mol wt: Parent peak	Mol wt: Original hormone	Mol wt: Added	Bond stretch: Present	Bond stretch: Absent
IAA-$(TMS)_2$	319.1	175.2	143.9	TMS ester	OH,NH COOH
GA_3-$(TMS)_3$	562.4	346.4	216.0	TMS ester, lactone	OH,NH,COOH
ABA-$(TMS)_2$	408.2	264.3	143.9	TMS ester	OH,NH,COOH

plant hormones have been published, the nature of the derivatives was first characterized. Accordingly, the TMS derivatives were chromatographed on the thermal conductivity instrument, collected, and then examined by infrared and mass spectroscopy. The results of these analyses are presented in table II. Since each TMS group adds 72 mass units to the molecular weight, 2 TMS groups had been added to IAA and ABA and 3 groups to GA_3. The lack of any O-H or N-H stretching frequencies in the IR spectra of these TMS derivatives coupled with the mass spectral data leads to the proposal of the structures in figure 1 as the actual structures of the derivatives. Points of interest are the replacement of the hydrogen atom of the indole ring with TMS, the reaction with the tertiary hydroxyl of ABA, and that the lactone bridge of GA_3 remained intact. The formation of the silyl ether of the tertiary hydroxyl of ABA is an indication of the greater silylating power of BSA as compared to some other silylating reagents since MacMillan and Pryce (13) reported that this ether is not formed when a mixture of hexamethyl disilazane and trimethylchlorosilane is added to methyl abscisate or its relative methyl phaseate. While the reaction with the NH group of the indole ring does occur and goes to completion, this reaction appears to proceed at a slower rate than the reaction with the carboxyl group. This is in agreement with recent work of Grunwald *et al.*, (8) using BSA on indoles. Thus, if IAA was chromatographed soon after the BSA was added, or if insufficient BSA was used, 2 peaks were seen from a pure preparation of IAA. If sufficient BSA was present, the first IAA peak completely disappeared within 30 minutes after the addition of the BSA. The stability of these derivatives was borne out by their ability to be chromatographed, recovered, stored, and further analyzed.

IAA-TMS



GA_3-TMS



ABA-TMS

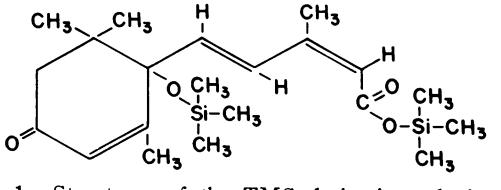


FIG. 1. Structures of the TMS derivatives obtained from the reaction of BSA with samples of pure IAA, GA_3, and ABA.

Of prime interest was the retention of biological activity by the chromatographed derivative since any chemical method will at first need to be correlated with existing data on the biological effects of the hormone. When the TMS derivatives were bioassayed the results (fig 2) were in agreement with what would be expected from the original hormones: in the cotton explant bioassay when the hormones are at high concentration (0.1–10 μg/abscission zone) GA_3 moderately promotes abscission, ABA strongly promotes abscission, and IAA both completely inhibits abscission and stimulates petiole epinasty (Addicott *et al.*, 1). Since the TMS derivatives have a similar effect on the explants as the unsilylated hormones it is probable that the hormones had been regenerated either by the warm agar in which the samples were prepared or by the explants themselves. BSA had no effect in the bioassay (from 0.005–0.15 μl/abscission zone). Abscisic acid has a characteristic absorption in the ultraviolet spectrum as shown by Milborrow (14), and when the ABA-TMS derivative was dissolved in 0.005 N ethanolic sulfuric acid, the characteristic absorption for pure ABA, with a maximum at 260 mμ, was observed.

Thus, GLC analysis of the TMS derivatives of plant hormones represents a potentially useful research tool, which overcomes most of the past difficulties with the application of gas chromatographic techniques to plant extracts. The method provides

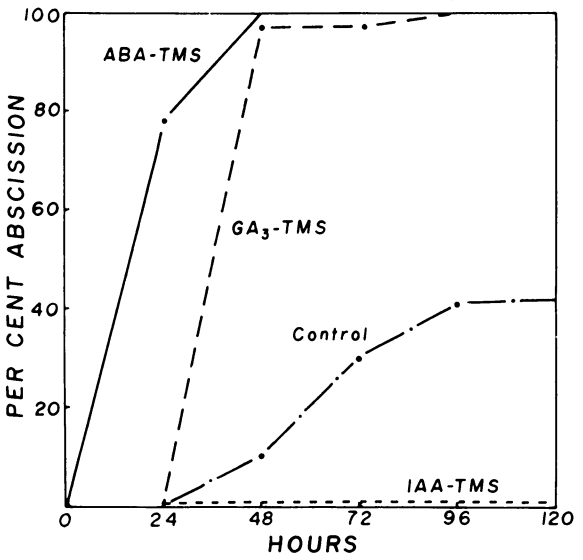


FIG. 2. Time course curves of abscission responses to TMS derivatives of ABA, GA_3, and IAA in the cotton explant bioassay. Each curve is based on 40 abscission zones. Petiole epinasty occurred within 24 hours after the IAA-TMS treatment.

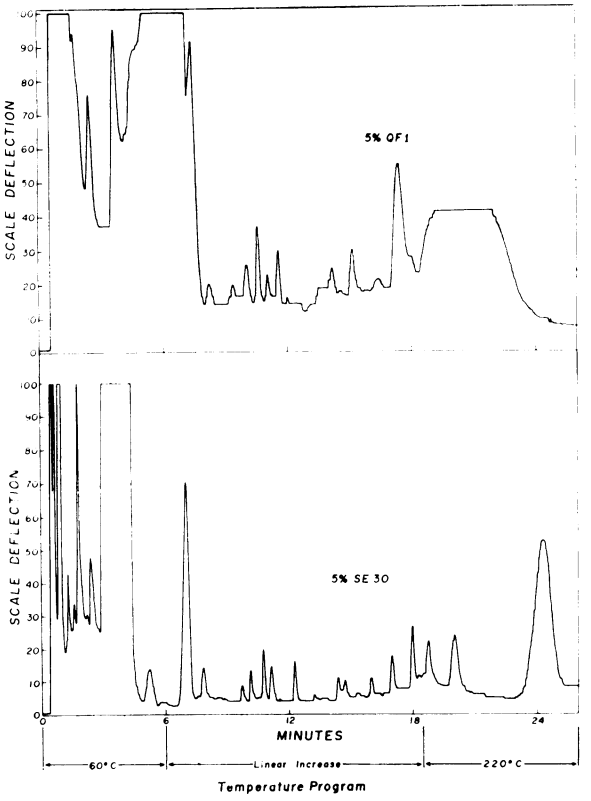


FIG. 3. Chromatograms obtained from injection of 0.5 μl (0.5 % of the total sample) of the silylated crude acid fraction of 10 cotton fruits. The linear temperature increase was at a rate of 12.8°/minute. Attenuation was $\times 64$ with a flame range setting of $\times 1$.

easily prepared derivatives, which (i) show no rearrangements, (ii) are stable, (iii) separate well in gas chromatography, and (iv) have easily regeneratable biological activity. The versatility of the method can also be extended by the use of a simple preliminary sample purification.

The successful application of the method is illustrated in the following measurement of the level of ABA in developing cotton fruit. A TMS derivative of the unpurified acid fraction of an 80 % acetone extract of ground cotton fruits was analyzed using the GLC techniques, and the chromatograms seen in figure 3 were obtained. There was no visible peak in either of these which co-chromatographs with ABA due to an unknown compound which was present in much greater concentration than ABA. This compound appeared close to where ABA is expected, and masked any ABA that was present. Gas-liquid chromatography was, therefore, applied in 2 different methods for the measurement of ABA. In the first method the crude acid derivatives were chromatographed on the thermal conductivity instrument and the region corresponding to ABA-TMS was collected. This was then dissolved in 0.005 N ethanolic sulfuric acid and its absorption spectrum from 320 to 220 mμ was obtained, which showed an ultraviolet maximum at 260 mμ. In addition the recovered region stimulated abscission, and when rechromatographed on the flame ionization instrument showed a shoulder peak which co-chromatographed with pure ABA-TMS. Thus, this use of GLC of the TMS derivative provided a valuable preparative step in the purification of ABA in small extractions. In the second method GLC was used to give a direct measurement of ABA following 1 additional purification, in which the crude acids were applied to a small carbon-celite column and the ABA and various other compounds were eluted with 200 ml of 60 % acetone. This fraction was dried and the TMS derivative prepared and chromatographed on SE 30 and on QF-1. The results, as seen in figure 4, show a peak which co-chromatographs with abscisic acid in both systems. Thus, direct measurement of abscisic acid in extracts of small amounts of plant material is possible.

A comparison of the amounts found by these 2 methods is shown in table III. The amount determined from the UV absorption at 260 mμ was only 20 % of that determined from the peak area of the carbon column purified sample. An injection of 20 μg of Reynolds ABA+t-ABA was collected and measured by determining UV absorption at 260 mμ. The amount present was 3.9 μg or 20 % of that injected. Thus a considerable loss occurs in the chromatography and collection of the sample but the percent loss appears to be constant. A similar percent recovery was obtained from extracts with higher amounts of ABA. Thus the method can be used for comparative studies of levels of ABA. When the loss has been calculated the absolute levels can be determined as well. This high loss does

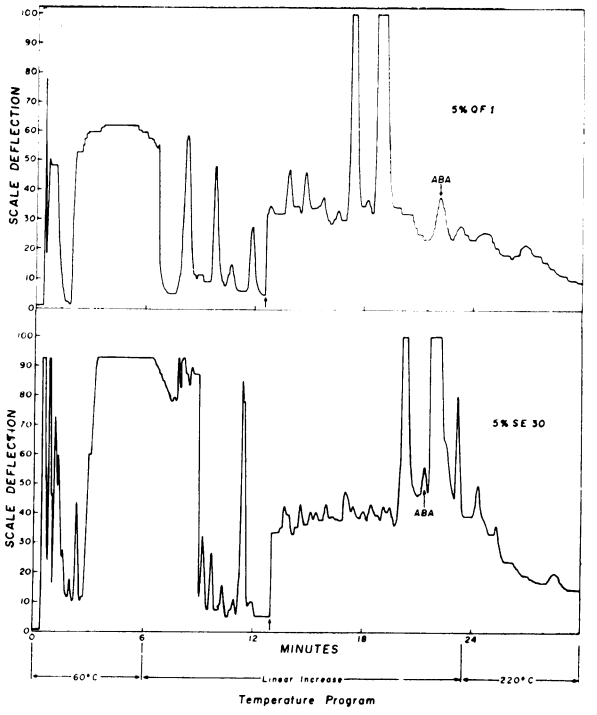


FIG. 4. Chromatograms obtained from injection of 3 μl (3.0 % of the total sample) of the silylated, carbon purified, acid fraction of 10 cotton fruits. The linear temperature increase was at a rate of 9.1°/minute. Attenuation was at ×64 up to the arrow and at ×16 after the arrow with a flame range setting of ×1 during the whole run.

Table III. *Level of Abscisic Acid in 5 Day Old Cotton Fruit*

Method	Actual amt measured	mg/kg fr wt
GLC peak[1]	0.38 μg	0.65
UV absorption[2]	0.61 μg	0.13

[1] GLC peak from 3 μl of silylated sample.
[2] UV absorption was measured on material recovered from 25 μl of silylated sample.

however inject a certain level of uncertainty into the measurement and requires frequent calibration with known amounts to determine whether the percent recovery has remained constant. The direct measurement of the peak areas of carbon purified plant extracts thus appears to be a better method for determination of ABA. The levels determined with this measurement while again probably not wholely quantitative, are very useful in a comparative study and give a good first approximation of the actual levels in the plant.

The level of 0.65 mg/kg fresh weight is within the range of that determined by Milborrow (14) for several other plants by his racemate dilution method. While perhaps not as accurate as the racemate dilution method, the GLC analysis permits a determination with less preliminary purification and with equipment more readily available to most laboratories.

This GLC method enabled direct chemical assay of abscisic acid with a minimum of preparation. It appears readily adaptable to the other acidic hormones in plant extracts, although a method of purification other than a carbon column must be used for indolic compounds. The method makes feasible the processing of large numbers of small samples with faster and more specific measurements than can be provided by bioassay. To measure changes of 2 or more hormones in the same tissue using bioassays would require multiple extractions, separate purifications and individual measurements which make such a project very laborious and in some cases not feasible. This GLC method however offers the possibility of following the level of 2 or more hormones simultaneously.

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