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Note

Silylation technique for plant growth regulators

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Derivatization of plant growth regulators to assist in the determination of their presence and concentration in various plant tissues by gas–liquid chromatography (GLC) has been widely applied[1–6]. Methylation and silylation are the most commonly used means of derivatization for this group of compounds. For purposes of identification GLC–mass spectrometry (MS) of the resulting derivatives is the method of choice in many instances[1,6–9].

Our studies of plant growth regulators in citrus have been primarily concerned with abscisic acid (ABA) and the gibberellins. Since it was necessary to perform a concurrent analysis of these compounds, the usual derivatization methods had to be modified. The silylation technique we developed offers some advantages and overcomes shortcomings associated with other methods.

EXPERIMENTAL

Reagents and materials

The growth regulator standards used were four gibberellins (GA_1, GA_3, GA_5, GA_9) and synthetic, mixed isomers of *cis*- and *trans*-ABA. Bis(trimethylsilyl) acetamide (BSA), Tri-sil and Tri-sil/BSA-Formula P from Pierce (Rockford, Ill., U.S.A.) were used as the silylating reagents. Diazomethane was used for methylation. Reagent grade acids were used as catalysts.

Apparatus

A Hewlett-Packard 5830A gas chromatograph was equipped with a dual hydrogen flame detector and a Hewlett-Packard 18850A GC terminal. Dual glass columns (0.9 m × 0.2 cm I.D.) were packed with 3% OV-17 on Chromasorb W HP (100–120 mesh).

The chromatographic conditions were as follows: injection temperature, 250°; detector temperature 275°; column temperature, 125° for 4 min, then increased at 3°/min to 245°; carrier gas (nitrogen) flow-rate, 40 ml/min. Identification of the GLC peaks was performed by a Hewlett-Packard 5992A GLC–MS system. The ionization beam energy was 70 eV while the accelerating voltage was 3 kV.

Procedure

Aliquots of solutions of growth regulator standards were taken to dryness

under a stream of nitrogen in reacti-vials and then reacted with the different derivatizing reagents. The silylating reagents were used at temperatures ranging from ambient to 98° for varying periods of time. Individual acids, such as citric, ascorbic, oxalic, and trichloracetic, were added in different amounts to those reagents in some instances. Methylation was accomplished by reacting diazomethane for 30 min at ambient temperature.

The procedure finally accepted for silylation was the following: 0.5 ml of oxalic acid in ethyl acetate (10 mg/ml) was added to the evaporated standards and taken to dryness under a stream of nitrogen, then 0.5 ml BSA was placed in the reacti-vial, capped and heated for 5 min at 50°. This was done to cause reflux action down the sides of the vial and to assure that the sample was solubilized. It was then held at ambient temperature for 30 min.

RESULTS AND DISCUSSION

There are references in the literature[10–12] where the gibberellins as well as ABA are simply methylated using diazomethane. However, this is not as desirable for the gibberellins because (a) there is a broadening of the peaks and a lack of definitive separation[5] and (b) for GLC–MS work the fragmentation pattern of the methyl esters is less clearly diagnostic of structure than that of their trimethylsilyl ethers[13].

Previous workers[14,15] have used BSA with mild conditions to silylate several growth regulators, including gibberellins, simultaneously. Our findings reveal that, with these conditions, all of the gibberellins cannot be completely silylated in all positions, *i.e.*, the trimethylsilyl esters of the carboxyl groups and the trimethylsilyl ethers of the hydroxy groups. Even the use of higher temperature conditions and longer periods of time did not achieve complete silylation with BSA for some gibberellins, *e.g.*, GA_1 and GA_3. It was determined that one or more of the hydroxy positions were difficult to silylate.

If a proper and efficient GLC column is not used the incomplete silylation may not be obvious because all of the peaks are not apparent. When, for instance, a glass column (1.8 m × 0.4 cm I.D.) packed with 3% OV-210 on Chromasorb W HP (80–100 mesh) was used with GA_3 that was incompletely silylated, only one distinct peak and a few not very obvious humps were obtained. However, by switching to the column referred to under Experimental and keeping all other conditions the same, three definite peaks were found. It was determined by GLC–MS that these additional peaks were due to partially silylated GA_3 and not foreign substances. Such GLC data without positive identification could lead to misinformation as to the number and identity of compounds present in a plant sample. Table I shows the potential number of peaks with partial silylation of a limited mixture of growth regulators and some of the possible confusion. This overlap in elution time could be even more complex with a greater number of standards.

Experimental observations indicated that the presence of acid promoted better silylation. Poole[16] states that the reaction rate leading to silyl ether formation can be increased by the addition of an acid catalyst; none being more successful than trimethylchlorosilane (TMCS). Schneider *et al.*[17] found that BSA + 10% TMCS worked well on silylating several gibberellins.

TABLE I

ELUTION TIMES FOR SILYLATED AND PARTIALLY SILYLATED GROWTH REGULATORS

Gas chromatographic conditions: Column, 3% OV-17 on 100–120 mesh Chromsorb W (0.9 m × 0.2 cm I.D.); injection temperature, 250°; column oven temperature, 125° for 4 min; detector temperature, 275°; rate of temperature increase, 3°/min to 245°; nitrogen flow-rate, 40 ml/min.

Compound	*Number of positions*		*Elution time (min)*
	Available	*Silylated*	
cis-ABA	2	2	24.0
trans-ABA	2	2	27.0
GA_9	1	1	28.9
GA_5	2	2	31.8
		1	34.1
GA_1	3	3	34.4
		2	37.0
		1	37.6
GA_3	3	3	35.3
		2	38.3
		1	39.0

Tri-sil/BSA, Formula P was tried without success, however the use of heat may have contributed to its failure. Tri-sil, which is similar in composition to the hexamethyldisilizane, trimethylsilyl chloride and pyridine combination, used by Cavell *et al.*[6] to form the trimethylsilyl ethers of the methyl esters of the gibberellins, was successful. Fifteen minutes at room temperature produced complete silylation, however upon standing for a longer period of time or with the addition of heat, there was a degradation of *cis*- and *trans*-ABA while the gibberellins remained stable. This could have been caused by the evolution of hydrochloric acid from trimethylchlorosilane due to the presence of moisture and consequently a very low pH.

Several organic acids were used in conjunction with BSA. The greater the dissociation constant the shorter the period of time and the less heat was necessary for complete silylation of the gibberellins. The use of either citric or asorbicc acid was beneficial, but the reaction rate was still much too slow. When trichloracetic acid was used, the reaction rate at ambient temperature was extremely rapid, but breakdown products of the gibberellins and ABA were observed. Again, excessively low pH conditions were probably present.

The best results were obtained by using BSA plus oxalic acid, which needed only 30 min at ambient temperature for complete silylation. The ABA's as well as the gibberellins were stable upon standing with or without the addition of heat. The oxalic acid was also silylated, but at a slower rate than any of the plant growth regulators. Therefore, after performing as a catalyst, it was gradually eliminated as an acid by being reacted and thus a prolonged low pH condition was avoided.

It was also determined that, when oxalic acid was used with actual plant tissues, it effected a more complete solubilization of the sample to be silylated and gave higher recovery values for fortified samples.

Schneider *et al.*[17] found that the trimethylsilyl ether trimethylsilyl esters of the

gibberellins yielded as distinct and sharp peaks as did the trimethylsilyl ether methyl esters. GLC and MS of these trimethylsilyl ether and ester gibberellin have been found to be of great value, especially since they can be collected by preparative gas chromatography into aqueous ethanol which hydrolyzes them for bioassay[18].

Therefore this technique not only provides complete silylation which avoids the possibility of extraneous peaks, but it also greatly enhances the detection sensitivity by producing larger single peaks. It also produces the trimethylsilyl ether trimethylsilyl esters that afford the best possibilities for identification.

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