Journal of Chromatography, 192 (1980) 429–433

CHROM. 12,609

Note

Comparative study of derivatisation procedures for the quantitative determination of the auxin, phenylacetic acid, by gas chromatography

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(First received October 12th, 1979; revised manuscript received December 12th, 1979)

Phenylacetic acid (PAA) has been shown to occur as a constituent of many higher plants[1–3]. It has also been isolated and chemically identified from the brown alga, *Undaria pinnatifida*[4]. Since this acid has been shown to exhibit auxin-like growth-promoting activity in wheat coleoptile and pea stem tissues[4,5], in tobacco callus[6] and in roots of pea seedlings[7], quantitative estimates of the level of PAA in higher plant tissues are clearly important for assessing the potential role of this substance as a natural growth regulator. Beyond the several demonstrations of its occurrence in plants, few investigations have been made dealing with the quantitative determination of PAA; indeed, only one paper is presently available reporting the quantitation of PAA in several plant tissues[3].

It is possible that the failure of some investigators to achieve quantitative measurement of PAA in plant extracts arises from difficulties that can be encountered during the derivatisation of this substance for gas–liquid chromatographic (GLC) analysis. The commonly used derivatisation techniques for other acidic growth-regulating components of higher plants, such as 3-indolylacetic acid (IAA), abscisic acid (ABA) and gibberellic acid (GA_3), are not adequate for reliable quantitative determination of PAA. The standard method employed for derivatisation of these carboxylic acids is to form the methyl ester by passing gaseous diazomethane for 2–4 min through an ethereal solution of the sample, according to the procedure described by Schlenk and Gellerman[8]. The solution is then concentrated to dryness and the residue redissolved in a known, small volume (100–500 μl) of an appropriate solvent for injection into the gas chromatograph. The methyl esters of IAA, ABA and GA_3 produced by this procedure are stable and can be easily detected and quantitatively measured by GLC. Although PAA is also readily methylated by this technique, the methyl ester is extremely volatile and is easily lost in the flow of nitrogen gas during the methylation procedure, especially when the diazomethane treatment is longer than 1 min. Any remaining ester may be lost during concentration of the final reaction mixture prior to GLC analysis.

In earlier work in this laboratory on PAA quantification in shoot and leaf extracts using GLC analysis[7], the above problems were largely overcome by a combination of the following changes to the standard diazomethane derivatisation procedure; namely, a larger sample of plant extract was used for each methylation, the gaseous diazomethane step was allowed to proceed for only 30 sec and finally, the

reaction mixture was concentrated to dryness slowly overnight using a warm sand-bath rather than employing a rapid steam of air or nitrogen gas. Using this modified procedure, consistent results were obtained for PAA levels in several aliquots from the same plant extract, but since the number of aliquots of each extract derivatised was normally five, the complete analysis of many extracts for PAA using this procedure was necessarily slow. Clearly, a faster, more stable and therefore more reliable method for derivatising PAA in plant extracts is required.

Recently, pentafluorobenzyl esters have been used for the successful GLC analysis of chlorophenoxyalkyl acids present in soil extracts[9,10] and we therefore decided to investigate the suitability of this form of esterification for the quantitative determination of PAA in plant extracts by GLC. At the same time, a modified diazomethane methylation procedure for PAA has been developed and the GLC results obtained with this method and from that employing pentafluorobenzylation are compared with those obtained using the standard gaseous diazomethane procedure for derivatising PAA.

EXPERIMENTAL

Reagents and chemicals

PAA was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). The pentafluorobenzyl bromide was obtained from Pierce (Rockford, Ill., U.S.A.). The N-methyl-N-nitroso-*p*-toluenesulfonamide for generation of diazomethane was obtained from Sigma (St. Louis, Mo., U.S.A.). All solvents used were of analytical reagent grade.

Standards

Standard samples of PAA were prepared in 1-μg, 10-μg and 1-mg amounts. Three replicate samples of each amount were derivatised using each of the three different derivatisation procedures.

Derivatisation procedures

Standard methylation. The standard derivatisation method used was formation of the methyl ester by passing diazomethane through a solution containing the authentic compound dissolved in 10% methanol in diethyl ether. The diazomethane qas was bubbled through the solution for 2 min, or until a yellow colour was observed[8]. The reaction mixture was then evaporated to dryness under a gentle stream of nitrogen and the residue redissolved in a known small volume of ethyl acetate. Several aliquots of this solution were analysed by injection into the gas chromatograph.

Modified methylation. A solution of diethyl ether containing 10% methanol was saturated with diazomethane by passing the gas through it for approximately 10 min or until a dark yellow colour was observed. This solution was then added, in appropriate volumes, to septum capped vials containing the standard PAA samples. After several minutes, aliquots of each reaction mixture were injected into the gas chromatograph without any prior evaporation and resolubilisation in ethyl acetate.

Pentafluorobenzylation. The pentafluorobenzyl ester of PAA (PFB-PAA) was prepared by a slight modification of the method described by De Beer *et al.*[11]. An excess of 1% pentafluorobenzyl bromide in acetone (0.5 ml) and 10 mg anhydrous

potassium carbonate were added to the crystalline samples of PAA and the mixtures were heated in a Pierce reacti-therm heating module at 60° for 90 min. Each reaction mixture was then evaporated to dryness under a gentle stream of nitrogen. A 1-ml volume of glass distilled water was added to solubilize the dry residue and this aqueous fraction was then extracted three times with ethyl acetate. The combined ethyl acetate fractions were evaporated under a gentle stream of nitrogen and the residue redissolved in a known small volume of ethyl acetate in readiness for injection into the gas chromatograph.

Gas chromatography

All samples were analysed in a Pye Series 104 gas chromatograph using a glass column (2 m × 2 mm I.D.) packed with 3% OV-17 on Chromosorb W (80–100 mesh). Nitrogen was used as the carrier gas at a flow-rate of 20 ml/min and flame ionisation detectors (FIDs) were employed. The methylated derivatives of PAA were chromatographed at a column temperature of 115°, while the pentafluorobenzylated derivatives were analysed at 180°. All derivatised samples were prepared in such a way that a 1-μl aliquot would contain 20 ng PAA.

RESULTS AND DISCUSSION

The relative peak responses obtained from GLC analysis of 1-μl aliquots, each equivalent to 20 ng PAA, taken from the different sized samples of PAA derivatized by each of the three esterification procedures are presented in Table I. The average response obtained from several aliquots of the three samples of PFB-PAA was taken as the 100% response value and the other values were calculated on this basis. Typical scans obtained during GLC of equilavent aliquots taken from each of the 10 μg samples of PAA derivatized by the three different procedures are shown in Fig. 1. The results in Table I show that no detectable product was obtained when 1- or 10-μg amounts of PAA were derivatized by the standard methylation procedure, but a single product peak was detected when a 1-mg sample was employed. The losses observed when this derivatization procedure is used with small amounts of material appear to be due to the volatility of methyl-PAA (Me-PAA). In the standard methylation procedure, the ethereal reaction solution was evaporated to dryness under a gentle stream of nitrogen and the residue resolubilised in ethyl acetate. It is this concentration step where the majority of the volatile Me-PAA is lost. If evaporation is very gentle, such as when the vial is left overnight so that the reaction mixture will evaporate slowly, then losses are not nearly so great and recoveries can be consistent. Based on our experience with the standard diazomethane method, it is easy to understand how some investigators could use this procedure for methylating a relatively large sample of authentic PAA (*e.g.*, 1.0–2.0 mg) and obtain a distinct peak on the scan when the sample was analysed in the gas chromatograph. However, when an acidic plant extract was methylated by the same procedure, since the sample would likely contain much smaller amounts of PAA (< 200 μg), the volatility losses incurred during derivatization and concentration of the reaction mixture could well result in little or no Me-PAA being found when aliquots were analysed by GLC.

These volatility problems led to the modified methylation procedure described in the Experimental section in which losses due to the high volatility of Me-PAA are

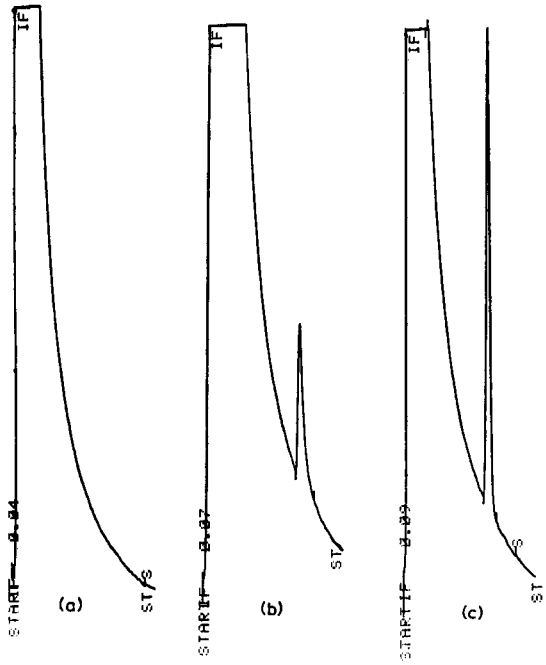

Fig. 1. Chromatograms obtained with 1-μl aliquots, equivalent to 20 ng PAA, from 10-μg samples of phenylacetic acid derivatised either by the standard methylation procedure (a), the modified methylation procedure (b) or the pentafluorobenzylation procedure (c).

TABLE I

COMPARATIVE PERCENTAGE RESPONSE OF THE FLAME IONIZATION DETECTOR TO Me-PAA AND PFB-PAA

The 100% value was determined from the average peak-area response obtained with PFB-PAA, since this ester gave the largest consistent response.

Method of esterification	*Amount of PAA derivatised*		
	1 μg	*10 μg*	*1 mg*
Standard methylation	0	0	42
Modified methylation	66	53	51
Pentafluorobenzylation	95	104	101

avoided. In this procedure, a 10% solution of methanol in ether which has been previously saturated with diazomethane is added to a septum sealed vial containing the PAA sample for derivatisation. This procedure results in almost instantaneous methylation of PAA and the methyl ester produced can be readily detected when 1-μl aliquots of the reaction mixture are analysed in the gas chromatograph. The data in Table I show that when 1-μg, 10-μg and 1-mg samples of PAA were methylated by this modified diazomethane procedure and then analysed by GLC, consistent detector responses were obtained from identical aliquots from the 10-μg and 1-mg samples, but similar aliquots from the 1-μg sample yielded approximately a

26% larger detector response, as calculated from the increased peak area using a Hewlett-Packard integrator system. This inconsistency is due to the high volatility of ether used as the injection solvent. The small volumes of ether required to give a 1-μl injection containing 20 ng Me-PAA from the 1-μg preparation results in sufficient solvent evaporation within the vial to cause significant change in the sample concentration. The use of very small vials, or larger solvent volumes, would reduce this problem. This non-gaseous method of methylation appears then to be useful for the determination of small amounts of PAA, provided careful standardisation of the derivatisation technique is achieved.

The results obtained from GLC analysis of 1-μg, 10-μg and 1-mg samples of PAA derivatised by the pentafluorobenzylation indicate that this method of derivatisation yielded consistent detector responses with aliquots from all three sample sizes. The FID response to this PAA derivative was found to be approximately twice that obtained with the methyl ester (Table I, Fig. 1). The increased detector response to PFB-PAA is likely due to the increased molecular weight of this derivative passing through the FID. If an electron capture detector is employed, as was used by Makita *et al.*[12] in the analysis of phenolic acids as PFB esters, this would greatly enhance GLC sensitivity to PFB-PAA. Thus, on the basis of the present results, we conclude that PFB esterification is a consistent derivatisation technique for the GLC analysis of nanogram quantities of PAA.

It should be emphasized that although this paper reports two derivatisation procedures which will yield reliable quantitative results in the GLC analysis of nanogram amounts of authentic PAA, it does not necessarily follow that these methods will be adequate for the quantitative determination of nanogram amounts of PAA in a plant extract. However, preliminary investigations in our laboratory indicate that both the non-gaseous diazomethane procedure and pentafluorobenzylation are reliable derivatisation methods for the quantification of both PAA and IAA in plant extracts. Pentafluorobenzylation is the more sensitive procedure since electron capture detection can also be employed, in addition to FID, during gas chromatography and so allow for a higher level of selectivity by the GLC.

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