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Communication

A Highly Sensitive Method for Quantitative Determination of Abscisic Acid[1]

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ABSTRACT

An abscisic acid derivative was formed by reaction with pentafluorobenzyl bromide which allowed highly sensitive detection by gas-liquid chromatography with electron capture detection. In comparison to the methyl ester derivative, the pentafluorobenzyl derivative of abscisic acid was four times more sensitive to electron capture detection and was stable at room temperature in the presence of ultraviolet light. Derivatization was rapid and the molecular weight of the new compound was confirmed by gas-liquid chromatography-mass spectrometry.

Abscisic acid is a plant growth regulator that plays a role in abscission, stomatal closure, fruit ripening, seed and bud dormancy, and senescence. GLC is the most commonly used method for quantitative determination of ABA. It must be derivatized before GLC detection because of its low volatility. Formation of ABA-ME[3] is most often used and provides for high sensitivity quantification (7). Diazomethane is used to produce the methyl ester derivative, but it is an extremely hazardous material to handle (6). It is potently antigenic, highly explosive, and the ABA derivative is photolabile. Several other derivatives have been used for GLC determination. The ethyl ester (5), *p*-nitrobenzyl ester (8), and trimethyl-silyl derivative (1) are several that have been used with varying success.

Pentafluorobenzylation by extractive alkylation has recently been described for GLC-ECD of IAA (2), sulfonamides (3), and inorganic anions (9). PFB derivatives give very high electron capture responses and high volatility. Reaction times are usually rapid and resulting compounds are relatively stable. The procedure used in this work employs tetrabutylammonium hydrogen sulfate to furnish the counter ion and ethyl acetate as the organic phase. The resulting compound has good stability, volatility, and increased quantitative detectability compared to previously described methods.

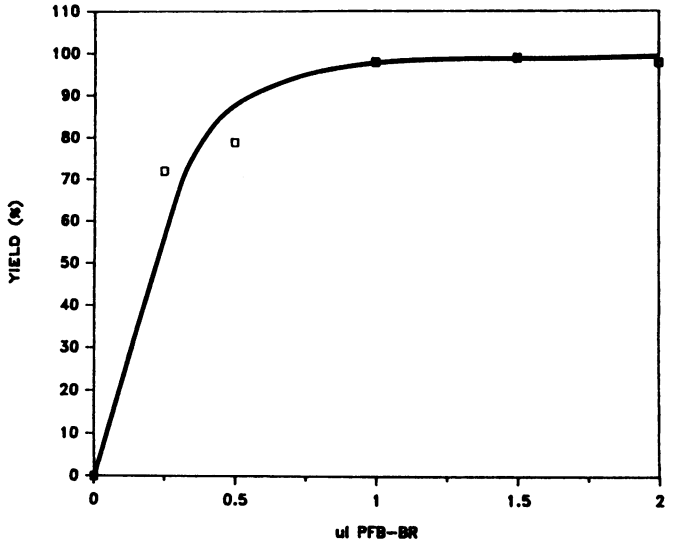

FIG. 1. Amount of PFB-Br required for derivatization of 10 μg ABA. Reactants: 0.1 M TBA-HS in 0.2 M NaOH (50 μl), 10 μg ABA, and 1 ml ethyl acetate, and the stated volume of PFB-Br.

MATERIALS AND METHODS

Diazomethane was prepared according to Schlenk and Gellerman (6) using an ethereal diazomethane generator (Wheaton Scientific, Millville, NJ). Standard ABA-ME was synthesized from ±*c,t*-ABA (Sigma). Methanol was used for the organic solvent and standard ABA-ME in silicone coated vials was stored at −1°C in darkness.

PFB esters of ABA were prepared in a fume hood by dissolving ±*c,t*-ABA in 1 ml ethyl acetate and shaking for 30 min at 25°C with 1 μl PFB-Br (Pierce, Rockford, IL) and 50 μl of 0.1 M TBA-HS in 0.2 M NaOH. Working in a fume hood is required because PFB-Br is a strong lachrymator. The organic phase was dried under N_2 and redissolved in 1 ml spectro grade benzene.

Free ABA (excluding other chemical forms) was extracted and purified from carrot somatic embryos according to a modified method described by Hubick and Reid (4). GLC was performed in a Varian 2100 gas chromatograph equipped with ^{63}Ni ECD. Samples (1 μl) used for data shown in Figure 2 were injected into a 1.85 m × 4 mm i.d. glass column packed with 2% EPON 1001 coated on 80 mesh AW-DMCS Chromasorb W. Column, injec-

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[3] Abbreviations: ABA-ME, methyl ester of abscisic acid; ABA-PFB, pentafluorobenzyl ester of ABA; *c,t*-ABA, *cis,trans* isomer of ABA; *t,t*-ABA, *trans,trans* isomer of ABA; PFB-Br, pentafluorobenzyl bromide; TBA-HS, tetrabutylammonium hydrogen sulfate; ECD, electron capture detection.

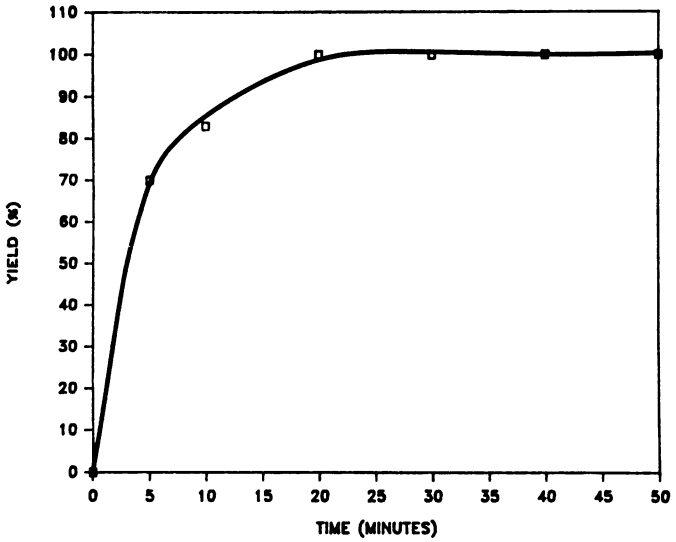

FIG. 2. Time course for derivatization. A 10 μg sample of ABA in 1 ml ethyl acetate was reacted with 1 μl PFB-Br and 50 μl of 0.1 M TBA-HS in 0.2 M NaOH at 25°C.

Table I. *Stability of c,t-ABA-ME and c,t-ABA-PFB to UV Light and Storage at 25°C*

Compound	Integrator Counts at Hours of UV Light:			
	1	2	4	120
c,t-ABA-ME	3,104	2,315	2,062	1,628
c,t-ABA-PFB	12,782	12,569	12,861	12,714

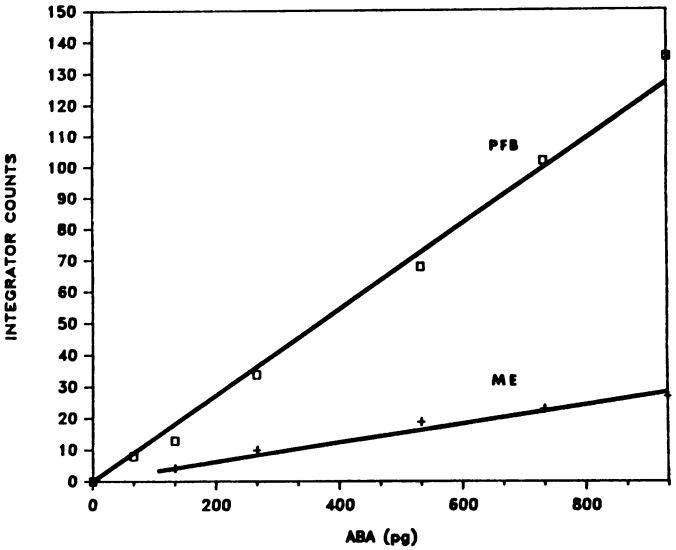

FIG. 3. Standard curve of ABA-ME and ABA-PFB. (Reactants: known weights of standard ABA in 1 ml ethyl acetate reacted with 1 μl PFB-Br and 50 μl of 0.1 M TBA-HS in 0.2 M NaOH.)

tion, and detector temperatures were 220, 260, and 270°C, respectively. All other samples were analyzed using a 1.85 m × 2 mm i.d. glass column with 3% OV-17 on 80 mesh Gas Chrom Q with filtered ultrapure N carrier gas at a flow rate of 60 ml/min. Column, injection, and detector temperatures were 220, 250, and 290°C, respectively. Tracings were recorded on a Varian A-25 strip chart recorder and the peak areas were calculated with an Autolab 6300 digital integrator.

For further characterization of ABA-PFB, GLC-MS was used.

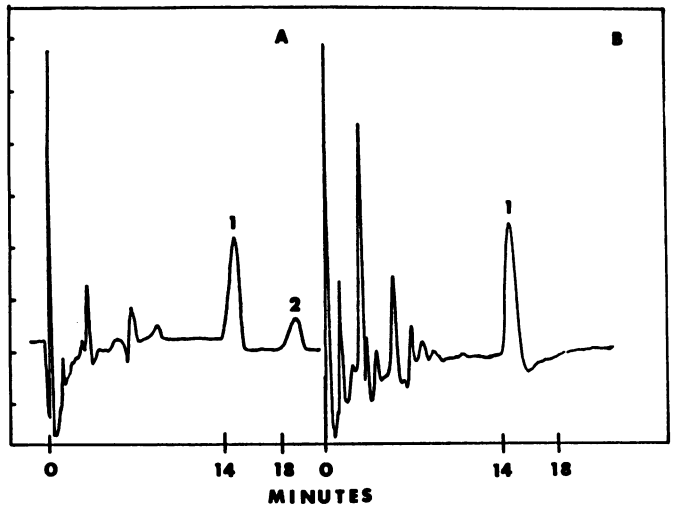

FIG. 4. Gas chromatograms for determination of ABA-PFB. A, Standard ABA-PFB. B, Authentic plant sample. 1, *c,t*-ABA and 2, *t,t*-ABA.

Mass spectra of derivatized extracts were recorded by a Finnigan model 4021 GLC-MS equipped with a 1.85 m × 2 mm i.d., 3% OV-17 glass column and programmed between 200 and 250°C at 5°C per min. Ionization was 70 eV with a source temperature of 200°C. The scan rate was 0.95 scan per s.

RESULTS

All of the available *c,t*-ABA was derivatized by the addition of 7.0 μmol PFB-Br to the derivatization mix (Fig. 1). Ten μg/g fresh weight of ABA is in the upper range of physiologically active concentration of the hormone in plant material. Amounts of reactant used above 1 μl per 10 μg ABA resulted in fouling of the electron capture detector.

Figure 2 presents the time course for derivative formation of ABA-PFB from 10 μg ABA in 1 ml ethyl acetate, 1 μl PFB-Br, and 50 μl of 0.1 M TBA-HS in 0.2 M NaOH. Shaking the mixture at 25°C for 20 min was sufficient for maximal derivatization.

Both of the derivatives, ABA-ME and ABA-PFB, were stable for 3 weeks at −1°C in darkness. One μl aliquots sampled weekly had less than 2% deviation as measured in integrator counts. The PFB ester was also stable when exposed to UV light at room temperature for 5 d. The ABA-ME was converted to a 50:50 mixed *c,t*- and *t,t*-ABA isomer by 4 h in UV light provided by a long wave UV lamp (Table I), and over 40% loss of integrator counts had occurred by 5 d.

Retention times of the *c,t*- and *t,t*-ABA isomers of ABA-ME and ABA-PFB were compared. *t,t*-ABA was synthesized by conversion of *c,t*-ABA in 4 h of UV light before derivatization. The isomers of ABA-PFB were retained on-column about 4 times longer than that of ABA-ME. Retention times for *c,t*-ABA-ME, *t,t*-ABA-ME, *c,t*-ABA-PFB and *t,t*-ABA-PFB were 2.75, 4.95, 12.92, and 18.52 min, respectively.

The standard curve for ABA-ME was linear in the range of 0.01 to 1 ng per 1 μl injection. Quantitation of ABA-PFB was linear from 0.001 to 1 ng. The PFB ester increased sensitivity 4 fold over the methyl ester derivative (Fig. 3).

A gas chromatogram of a PFB derivative of ABA extracted from carrot somatic embryos is presented in Figure 4. The two standard isomers were effectively resolved within 19 min and no other compounds co-eluted with naturally occurring *c,t*-ABA shown in the chromatogram of the authentic plant sample. This fact was confirmed by GLC-MS. Figure 5 presents the mass spectra of the PFB derivatized ABA standard and ABA extracted

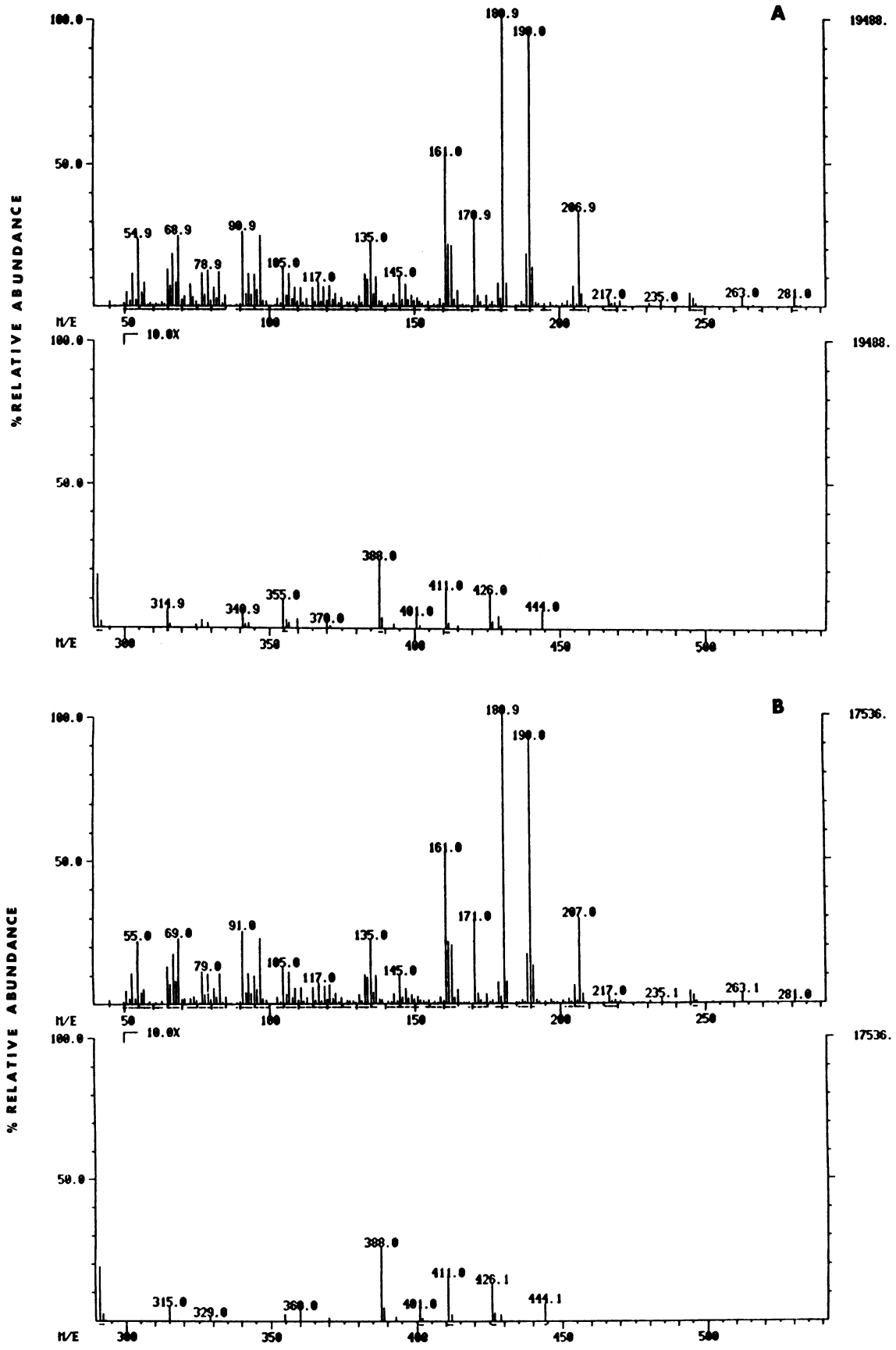

FIG. 5. Mass spectra of the PFB esters of standard ABA (A) and ABA extracted from carrot somatic embryos (B).

from carrot somatic embryos. The mol wt of ABA-PFB was confirmed at 444 mass units.

DISCUSSION

Pentafluorobenzylation of ABA offers several important advantages over methylation. The reaction is fast, about 20 min, in comparison with the time for diazomethane preparation from Diazald, which is more than 8 h using a Wheaton generator under recommended conditions. Sensitivity of detection by electron capture is increased four fold and allows for detection and quantitation at lower ABA concentrations. More importantly, the derivative is stable at room temperature and resists isomerization by stabilizing the double bonds of ABA. The procedure is also cost efficient at less than $0.01 per sample (based on reagent cost) and no special equipment is needed.

The agent forms derivatives of many compounds that become detectable by GLC-ECD because of increased sensitivity, but at the same time causes a loss of selectivity. Because of this loss, interfering GLC peaks could present a problem in quantitation if purification procedures are not stringent. Further purification of the phytohormone may be needed before derivatization if increased sensitivity of detection is required.

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