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MICROSCALE PREPARATION OF PENTAFLUOROBENZYL ESTERS

ELECTRON-CAPTURE GAS CHROMATOGRAPHIC DETECTION OF INDOLE-3-ACETIC ACID FROM PLANTS

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SUMMARY

A microscale method is described for the preparation of the pentafluorobenzyl ester of an organic acid, indole-3-acetic acid, using α-bromopentafluorotoluene as the esterifying agent and the volatile base, N-ethyl-piperidine. The resultant reaction mixture may be used directly for gas chromatography employing an electron-capture detector, or greater sensitivity and selectivity can be attained by negative ion chemical-ionization gas chromatography–mass spectrometry. The method is applicable to assay of indole-3-acetic acid in resinous plant material such as olive leaves.

INTRODUCTION

Pentafluorobenzyl (PFB) esters of organic acids are convenient for gas chromatographic (GC) analysis using an electron-capture detector (ECD)[1–3]. Previously described methods for preparation of PFB esters utilized large reaction volumes, an inorganic water-soluble base such as K_2CO_3, and partitioning the derivative into a water-immiscible solvent or further purification. Such procedures precluded microscale derivatization and also resulted in poor recoveries of a labile aromatic compound such as indole-3-acetic acid (IAA). We describe a microscale procedure for the preparation of PFB esters and illustrate its use in the analysis of IAA from plant material. Purification subsequent to derivatization is not usually required, but for those cases requiring purification a C_{18} reversed-phase high-performance liquid chromatographic (HPLC) procedure is described.

The N-heptafluorobutyryl and N-trifluoroacetyl derivatives of IAA have been utilized[4,5] but these required a second step for derivatization of the carboxyl group and reaction yields were low owing to sensitivity to trace amounts of water[6]. Trichloroethyl esters of IAA have also been used, but adaptation to assay of IAA has

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not been described[7]. The derivatization procedure we report results in a compound sufficiently volatile for GC analysis and measurable by an ECD. In addition, the method provides quantitative yields of the PFB ester without anhydrous conditions and using reagents stable to normal storage conditions.

MATERIALS AND METHODS

Reaction conditions

Optimal derivatization conditions were established by monitoring the reaction using silica gel 60 (E. Merck, Darmstadt, G.F.R.) thin-layer chromatography (TLC) (chloroform–methanol–water, 85:14:1) and visualization of indoles with Ehmann's reagent[8]. Derivatization was carried out in 300-μl actinic glass "Microflex" (Kontes, Vineland, NJ, U.S.A.) tubes in a block heater at 60°C.

Plant material

Young olive leaves (*Olea europaea* L. cv. Manzanillo) (15 g) were ground within 15 min of collection in acetone–water (70:30) containing the isotope-labeled IAA. The resultant homogenate was filtered, the residue reextracted by grinding in an additional volume of acetone–water (70:30), and the combined filtrates freed of acetone *in vacuo*. The sample was made to pH 2.5 with sulfuric acid and extracted three times with chloroform. The combined organic phases were extracted with 1 *N* $NaHCO_3$, brought again to pH 2.5 and reextracted three times with chloroform. The organic phases were pooled, dried over anhydrous granular Na_2SO_4, filtered, reduced to near dryness, and dissolved in methanol for chromatography on a Varian LC-5020 high-performance liquid chromatograph equipped with a guard column of 20–40-μm RP-8 and an analytical Varian MicroPak MCH-10 column. Eluent was monitored at 254 nm and 1-ml samples collected. IAA was eluted isocratically using methanol–water–acetic acid (25:75:5) as solvent with a flow-rate of 2 ml/min. Recently we have found with acidic plant extracts that good separation can be obtained using methanol–water–tetrahydrofuran (25:75:5), thus eliminating problems associated with the removal of acetic acid. Samples containing IAA were pooled, reduced in volume *in vacuo*, transfered to a "Microflex" tube and reduced to dryness under a stream of nitrogen. The sample was dissolved in 50 μl of redistilled acetone and 1 μl of N-ethylpiperidine (Pfaltz & Bauer, Flushing, NY, U.S.A.) added followed by 5 μl of α-bromopentafluorotoluene (Aldrich, Milwaukee, WI, U.S.A.). After reaction for 45 min at 60°C, the sample was either diluted with acetone for analysis by GC–ECD or chromatographed by C_{18} reversed-phase HPLC with isocratic elution with methanol–water (70:30).

The above procedure served for assay of "free" IAA. When it was desired to assay free plus ester and amide-linked IAA[9] it was necessary to hydrolyze the extract with 7 *N* NaOH for 3 h at 100°C prior to extraction and purification as previously described[9]. In experiments utilizing GC–ECD, 4.6 nmol (0.26 μCi) of [2-^{14}C]IAA (Radiochemical Centre, Amersham, Great Britain) was added to 15 g fresh weight of plant material as internal standard. For samples to be assayed by GC–selected ion monitoring–mass spectrometry (GC–SIM–MS) 7.2 μg of [4,5,6,7-$^2H_4$]IAA[10] was added as internal standard in addition to the [2-^{14}C]IAA.

Preparation of standards

PFB ester of IAA, for use as a standard, was synthesized using 2.9 mmol (0.5 g) of IAA and 4.8 mmol each of N-ethyl-piperidine and α-bromopentafluorotoluene. The product was dissolved in diethyl ether and partitioned against 0.1 *M* $NaHCO_3$, column chromatographed on silica gel 60 (solvent as for TLC), recrystallized from ethanol–water (70:30), and dried *in vacuo* over P_2O_5 for three days. The resultant light yellow needles (m.p. 128–130°C), absorbed at 219 nm ($\log \varepsilon = 4.51$) and at 280 nm ($\log \varepsilon = 3.75$). Color production with Ehmann's reagent was identical to that of an equimolar amount of IAA[8,11]. Further characterization of the standard was by GC–MS.

Gas–liquid chromatography

For electron-capture detection a Packard Model 419 equipped with a ^{63}Ni ECD was used. Operating conditions were injector port and detector at 270°C, column oven 250°C and nitrogen as carrier gas at 30 ml/min through a 1.3 m × 4 mm glass column packed with 1% OV-17 on Gas-Chrom Q (Supelco, Bellefonte, PA, U.S.A.). The detector was operated in pulse mode with 10-μsec period and 10-μsec width. For MS work a 10 ft. × 2 mm glass column packed with 3% SP2250 on 80–100 Supelcoport (Supelco) was used coupled to a Hewlett-Packard 5985a GC–MS instrument. Temperature was programmed from 200–260°C at 10°C/min after a 3-min isothermal hold and He carrier gas was at 30 ml/min. Some studies also utilized a flame-ionization detector with a Hewlett-Packard 402 and a 4 ft. × 2 mm 3% OV-17 on Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) glass column held isothermally at 225°C with nitrogen as carrier gas at 30 ml/min.

RESULTS AND DISCUSSION

Mineral salts are incompatible with HPLC and GC, thus requiring the substitution of a volatile organic base for the K_2CO_3 employed by earlier workers[1–3]. Table I compares the efficacy of K_2CO_3 and a variety of organic bases. Highest yields were obtained using N-ethyl-piperidine, and as little as 0.5 μg/ml was sufficient to permit quantitative derivatization as judged by TLC (Table II). The use of 2.5 μl (a large excess) of α-bromopentafluorotoluene was also necessary for quantitative derivatization (data not shown). Routinely, 5 μl of α-bromopentafluorotoluene and 1 μl of N-

TABLE I

COMPARISON OF VARIOUS ORGANIC BASES WITH K_2CO_3

Reaction contained 50 μg IAA, 50 μl α-bromopentafluorotoluene and the indicated base.

Base	*Yield (%)*
0.5 *M* K_2CO_3 (50 μl in water)	0
0.25 K_2CO_3 (100 μl in acetone–water, 50:50)	90
Pyridine (50 μl)	2
Piperidine (50 μl)	1
N-Ethyl-piperidine (50 μl)	100
2-Ethyl-piperidine (50 μl)	5

TABLE II

AMOUNT OF N-ETHYL-PIPERIDINE REQUIRED IN DERIVATIZATION REACTION

All reactions contained 5 μl α-bromopentafluorotoluene, 50 μg IAA and 50 μl acetone.

Volume of N-ethyl-piperidine	*Yield (%)*
1.0 nl	10
5.0 nl	10
10.0 nl	15
50.0 nl	60
0.1 μl	70
0.5 μl	100
1.0 μl	100
5.0 μl	100
10.0 μl	100
50.0 μl	100

ethyl-piperidine were added to 50 μl of acetone containing up to 0.3 μmole of organic acid giving a molar ratio of reagent:base:reactant of 110:24:1. For larger amounts of organic acids the amount of α-bromopentafluorotoluene and N-ethyl-piperidine can be doubled while retaining the same volume of solvent.

Derivatization of 50 μg of IAA in 50 μl of acetone with 5 μl of α-bromopentafluorotoluene and 1 μl of N-ethyl-piperidine as a function of time is shown in Fig. 1. In the first 30 sec, without heating, the reaction goes to 60% of completion and, with heating at 60°C, a near quantitative yield is obtained in 45 min. TLC of the reaction mixture after 45 min showed no unreacted IAA and this procedure would detect as little as 25 ng of unreacted IAA[8] in the 10 μl spotted. Thus, the reaction proceeds to, at least, 99.75% completion. Quantitative yields were further established by reversed isotope dilution analysis[12]. A 10-μl aliquot of a [^{14}C]IAA-containing reaction mixture

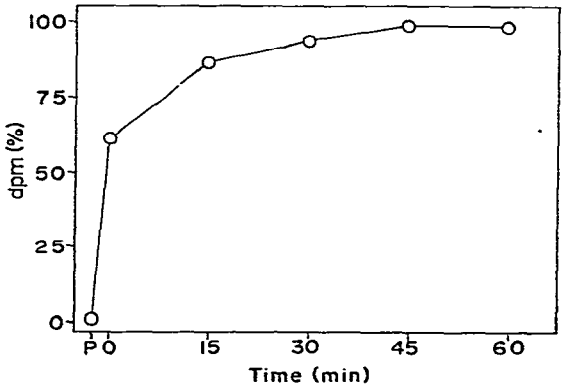


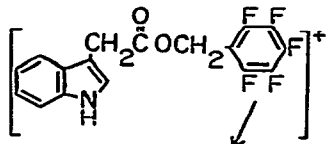
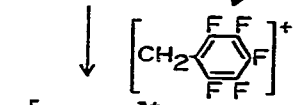
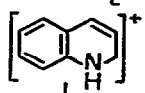
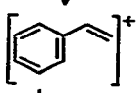
Fig. 1. Time course of derivatization. A 50-μg amount of IAA plus 100 nCi [^{14}C]IAA was derivatized at 60°C. At the indicated time 5 μl was removed and added to a vial containing 1 ml diethyl ether and 1 ml 100 m*M* $KHCO_3$. Data are percentage of dpm found in the upper phase. Data point indicated by "P" was sampled prior to the addition of reagent and the zero time point was taken within a few seconds after derivatization reagent was added.

was added to 1 mg of IAA-PFB and purified by TLC and C_{18} reversed-phase HPLC. The specific activity of the purified product as determined by UV extinction and liquid scintillation counting indicated that 97% of the label co-chromatographed with the authentic standards. Thus, only 3% of the IAA is not recovered as the derivative and this includes unrecovered counts due to: (1) radiolabeled impurities in the [^{14}C]IAA, (2) less than quantitative derivatization and (3) degradation during derivatization.

The identity of the reaction product was established by combined GC–MS on a Hewlett-Packard 5985a instrument using electron-impact ionization at 70 eV and positive-ion detection. The mass fragmentation pattern is given in Table III and shows fragments expected of both a pentafluorobenzyl ester and of a 3-substituted indolealkanoic acid.

TABLE III

POSITIVE ION 70 eV ELECTRON-IMPACT FRAGMENTATION PATTERN FOR INDOLE-3-ACETIC ACID PENTAFLUOROBENZYL ESTER OBTAINED WITH A HEWLETT-PACKARD 5985a GAS CHROMATOGRAPH–MASS SPECTROMETER

m/z	*Abundance (%)*	*Fragment*
355 (M^+)	3.0	
181	8.7	
130	Base peak	
103	7.1	
77	7.9	

Analysis of IAA isolated from olive leaves by GC–ECD of the C_{18} HPLC purified derivatization mixture is shown in Fig. 2. The IAA peak is well separated from contaminant peaks. Direct injection of the diluted reaction mixture, without HPLC, yields a similar chromatogram except that the solvent peak is longer and additional small peaks appear before the IAA (Fig. 3). If specific activity is to be determined prior to the GC step, then the post-derivatization HPLC step provides assurance that underivatized IAA and most degradation products are not counted. However, in our work with olive leaves the values obtained with or without this step

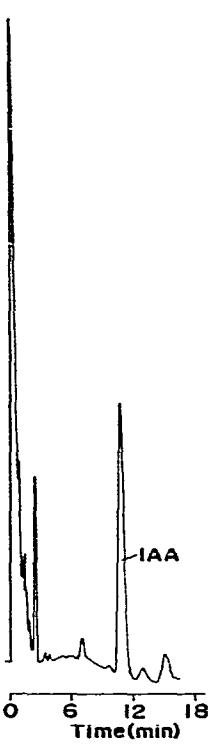


Fig. 2. GC–ECD of a sample from olive leaves purified as described in text and using the post derivatization C_{18}-HPLC step. Based on external standardization, peak represents 133 pg of IAA injected as its PFB ester.

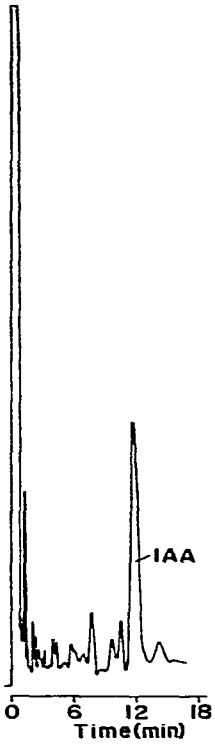


Fig. 3. GC–ECD of a sample from olive leaves purified as described in text. Sample was injected after dilution to 10 ml with acetone and without further purification. Peak represents 119 pg of IAA injected as its PFB ester.

were similar. Most accurate quantitation is provided by using the double standard method previously described[13].

GC–MS was used to validate the assay of IAA as the PFB ester in two ways: first, a GC–SIM–MS assay was compared with the above assay on two identical aliquots of the same lot of olive leaves. The first aliquot was analyzed by an ECD and the second aliquot was prepared for GC–SIM–MS by adding 7.2 μg of [$^2H_4$]IAA to the homogenate and using post-derivatization HPLC to reduce the mass of material entering the mass spectrometer. The MS assay was that described by Magnus *et al.*[10] except that the ions at m/z 130 and 355 were monitored for the naturally occurring IAA and ions at 134 and 359 were monitored for the ^{2}H-labeled internal standard. The ratios of ion intensities at 130:134 and 355:359 were used for quantitation. As is seen in Table IV the two methods agree well and the values obtained by GC–ECD for corn seed agree with previous reports. The differences observed are within those expected for biological variation.

A repetitive scan analysis of the samples used for GC–SIM–MS showed no extraneous fragment ions greater in quantity than 2% of base peak in both the alkali-hydrolyzed and non-hydrolyzed samples thus providing assurance as to the identity and composition of the GC peaks.

A further advantage of the halogenated derivative is that it permits assay by

TABLE IV

AMOUNT OF NATURALLY OCCURRING INDOLE-3-ACETIC ACID AS DETERMINED BY GC–ECD AND BY OTHER ISOTOPE DILUTION METHODS

Plant material	*Amount of IAA μg/g fresh weight*	*Analysis procedure*
Olive leaves (non-hydrolyzed)	1.2	GC–ECD
	1.9	GC–SIM–MS
Olive leaves (7 *N* NaOH treated)	2.6	GC–ECD
	3.2	GC–SIM–MS
Olive callus tissue (non-hydrolyzed)	0.2	GC–ECD
(7 *N* KOH treated)	0.9	GC–ECD
Sweet corn kernels (non-hydrolyzed)	0.8	GC–ECD
	0.5–1.0	Ref. 9
	1.3	Ref. 14

negative ion chemical-ionization MS (Fig. 4), thus increasing the sensitivity attainable by GC–SIM–MS. Negative-ion spectra using either ammonia or methane as reagent gases were obtained using a Finnigan 4000 instrument equipped with pulsed positive/negative-ion detection. These results are presented in Table V. Using GC–SIM–MS negative-ion chemical-ionization MS and ammonia as reagent gas permitted the detection of 5 pg of IAA as its PFB ester.

The procedure described for the formation of PFB esters is rapid, simple to use, results in high yields, and should be adaptable to study a variety of organic acids

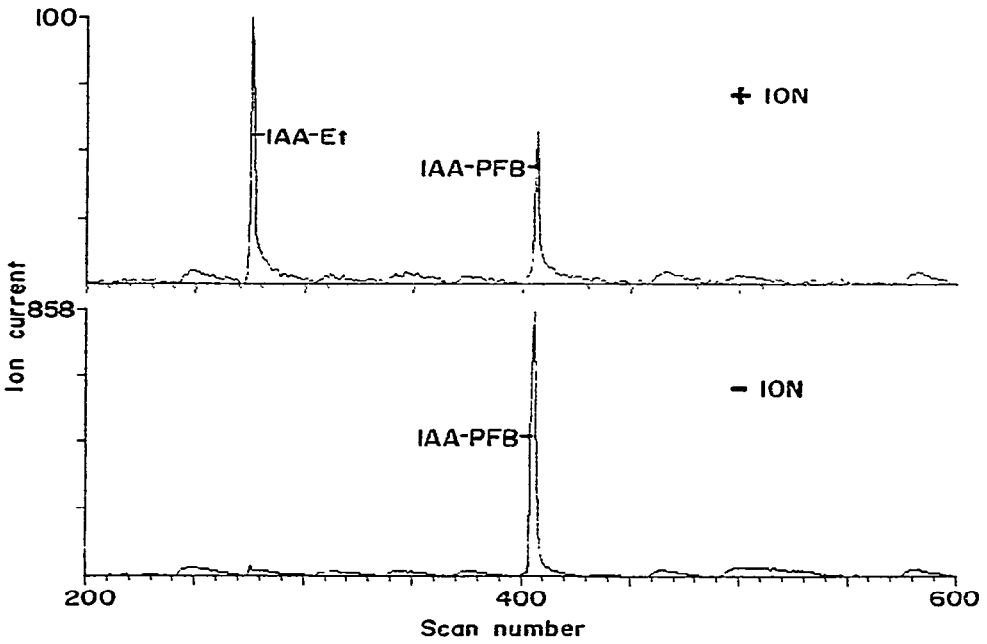


Fig. 4. Pulsed positive/negative-ion ammonia chemical-ionization total ion chromatogram obtained on a Finnigan 4000 instrument. An injection of 100 ng IAA ethyl ester (IAA-Et) and 100 ng IAA pentafluorobenzyl ester (IAA-PFB) in 1 μl chloroform was made onto a 20 m SE-54 J & W quartz capillary. Injector was at 250°C, oven at 130°C for 2 min then 15°C/min to 280°C. Injection was splitless for 0.8 min then split 40:1. Scans shown were taken from 4.7 min (scan 200) to 14 min (scan 600).

TABLE V

NEGATIVE-ION CHEMICAL-IONIZATION FRAGMENTATION FOR INDOLE-3-ACETIC ACID PENTAFLUOROBENZYL ESTER OBTAINED ON A FINNIGAN 4000 CAPILLARY GAS CHROMATOGRAPH–MASS SPECTROMETER AND USING THE INDICATED REAGENT GAS

Reagent gas	*Fragment ion (m/z)*	*Relative abundance (%)*
Methane	335	1.5
	174	100
Ammonia	335	1.5
	196	15
	174	100

(*cf.* refs. 1 and 15). The use of N-ethyl-piperidine allows great variation in the amount of base used and owing to its volatility under GC conditions, it does not interfere with analysis. The applicability of the method to a highly resinous and fatty tissue such as olive leaves, suggests its general suitability for study of organic acids and plant hormones which are present only in minute amounts.

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