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Note

High-performance gas chromatographic method for the estimation of the indole-3-acetic acid content of plant materials

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Gas chromatography has long been used as a method for estimating the indole-3-acetic acid (IAA) content of plant materials. In most previous studies flame ionization or electron-capture detectors have been used. While flame-ionization detection (FID) has been used with some success, this detector is neither highly sensitive nor selective. Electron-capture detection (ECD) is highly sensitive. However, ECD selectivity is not as advantageous as that offered by nitrogen–phosphorus detection (NPD). NPD has a high sensitivity and a selectivity, detecting only those compounds that contain nitrogen or phosphorus, which is highly advantageous in the analysis of indolic compounds[1]. For the present study a split-column high-performance gas chromatographic (HPGC) method for the analysis of IAA and other indolic compounds was developed. The procedure analyzed bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatives on a gas chromatograph equipped with two nitrogen–phosphorus detectors. Increased assurance of the identity of the compounds being detected was provided through the use of an injection splitter to deliver sample to two high-resolution capillary columns (one moderately polar and one non-polar). The NPD selectivity coupled with the high-resolution capability of the capillary columns reduced the number of steps required for sample preparation.

EXPERIMENTAL

Plant materials

Pea (*Pisum sativum*) cv. Alaska and soybean (*Glycine max*) cv. Tracy were used. Inocula were *Rhizobium leguminosarum* RP 212-13 (Kalo Agricultural Chemical, Columbus, OH, U.S.A.) and *Bradyrhizobium japonicum* I-110, a substrain of 3I1b110 (ref. 2). Seed surface treatment, germination, inoculation, and greenhouse growth conditions have been described in detail elsewhere[3]. Briefly, soybean seedlings were germinated and inoculated on agar plates before being transferred to greenhouse hydroponic pots, two seedlings per pot. The pots contained 8 l of 1/5 strength Hoagland's mineral nutrient medium made nitrogen free by replacing the nitrate salts with chloride salts. Pea seedlings were treated similarly except that they were grown in a growth chamber at 25°C (day) and 20°C (night) on a 12-h photoperiod. Growth chamber light was supplied at 400 μE m^{-2} s^{-1} photosynthetic photon flux density by three 400-W Lucalox, three 400-W mercury vapor, and six 100-W (100A21/TS) incandescent bulbs (all General Electric).

Extraction

Nodules were harvested at 0°C and extracted by a method modified from Bandurski and Schulze[4]. All steps were carried out at room temperature with minimum light. Nodules, 1.5 to 6 g, were mixed with acetone and water in a ratio of 1:7:2 (w/v/v) and homogenized at room temperature. For quantitative purposes an internal standard, indole-3-propionic acid (IPA), 50 μg g^{-1} fresh weight of nodule, was included. The homogenate was extracted by allowing the samples to stand for 24 h in the dark at room temperature. After extraction, solids were removed by centrifugation, acetone removed by evaporation under a hood draft, and volumes adjusted to 9 ml with glass distilled water. For bound IAA determinations, 1 ml of the sample was mixed with 1 ml of 14 *N* sodium hydroxide and heated to 95°C for 3 h under nitrogen. The hydrolyzed sample was then adjusted to pH 2.5 and extracted with diethyl ether and 1 *N* sodium hydrogen carbonate as described below for free IAA. For free IAA determinations, the remaining 8-ml sample was adjusted to pH 2.5 with 12 *N* sulfuric acid and extracted three times with 5–8 ml of diethyl ether. Next, the IAA was extracted from the ether by washing three times with 5–8 ml of 1 *N* sodium hydrogen carbonate, pH 8.4. The sodium hydrogen carbonate washings were combined and adjusted to pH 2.5 with sulfuric acid and the IAA extracted back into ether. The ether was evaporated under a stream of dry nitrogen and the samples resuspended in 200 μl of dry methanol and again dried under nitrogen. Next, the samples in open vials were placed in a desiccator under vacuum and held for 4 h to remove any remaining water.

Derivatization and chromatography

Dried samples were placed in a glove bag under a nitrogen atmosphere, mixed with 0.5 ml of BSTFA, capped, removed from the glove bag and sonicated in a sonic bath for 10 min, and heated on a dry block for 8 h at 95°C. Silylated samples were concentrated under a stream of nitrogen and injected into a Hewlett-Packard high-performance gas chromatograph, equipped with a cool on-column injector, a 1 m × 0.32 mm I.D. precolumn, a 1:1 injection splitter, two 25 m × 0.32 mm I.D. columns, and two nitrogen–phosphorus detectors. Columns were cross-linked methylsilicone (Hewlett-Packard) and RSL-300 polyphenylmethylsiloxane (Alltech). Carrier gas was helium at a flow-rate of 1.6 ml min^{-1}. Detectors were operated at 18 V.

RESULTS AND DISCUSSION

The HPGC system was capable of resolving and detecting pmole amounts of BSTFA silylated indole compounds. This was demonstrated by injecting a mixture containing 1.4 pmole (250 pg) each of four derivatized synthetic indole compounds into the gas chromatograph (Fig. 1). Under the conditions used here a minimum detectable limit, three times noise, of 0.4 pmole for IAA was indicated. Smaller amounts may be detectable. With NPD, sensitivity increases with higher bead voltages and in the present study a rather low voltage was used. Though a higher voltage would increase both noise and baseline drift. Also, greater sensitivity could be obtained by eliminating the split column since this reduces by half the amount of sample reaching each detector, but it was felt that the loss in sensitivity was more than compensated for by the increased confidence in sample identity. With NPD, 1–10 pg

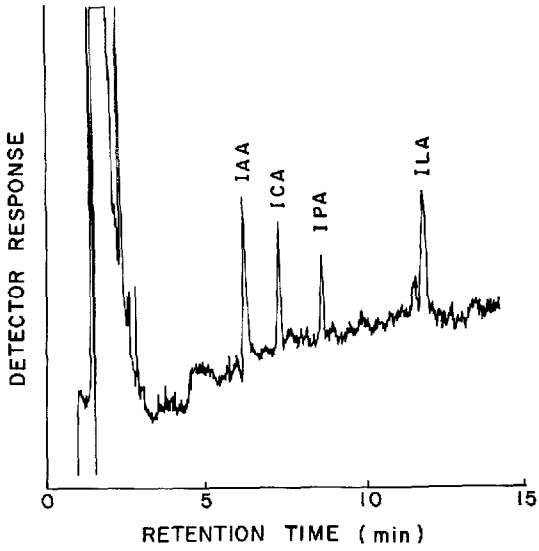

Fig. 1. Gas chromatography of BSTFA-derivatized synthetic indole compounds. A mixture of silylated IAA, indole-3-carboxylic (ICA), indole-3-propionic (IPA), and indole-3-lactic (ILA) acids (*ca.* 1.4 pmole each) was injected. This chromatogram was obtained with the cross-linked methyl silicone column. The column oven was run isothermal at 210°C.

of nitrogen (or phosphorus) content is generally required before a compound can be detected[5]. A 0.4-pmole sample of IAA would contain about 6 pg of nitrogen. This would suggest that only small gains in sensitivity are possible without detector improvements.

The IAA content of a 1.5-g sample of senescing pea nodules was estimated using this HPGC system. A peak with a retention time equal to that of the IAA standard and corresponding to about 88 ng g^{-1} fresh weight of plant material was detected (Fig. 2). The addition of derivatized synthetic IAA to derivatized pea nodule extract increased the size of the suspected IAA peak of both columns, giving further evidence that the suspect peak was IAA (data not shown).

The amount of IAA present in the nodule material was determined by using IPA as an internal standard. Small amounts of IPA have been detected in plant materials[6,7]. Thus, before using IPA as the internal standard nodule material was assayed for endogenous compounds having retention times similar to that of IPA. The amount of such compounds detected was less than 1% of the amount of internal standard used and therefore amounts were not sufficient to interfere with the use of IPA as an internal standard.

The NPD selectivity was necessary for the analysis of IAA in the nodule samples. IAA loss during extraction and sample cleanup is often a major problem[8,9] and it was felt that better IAA recovery would result from reducing the number of steps involved in this process. Sample preparation usually involves solvent partitioning and a chromatographic step, such as thin-layer or liquid chromatography before analysis by GC or high-performance liquid chromatography (HPLC)[8,10]. For this study solvent partitioning alone was used for sample cleanup prior to BSTFA sily-

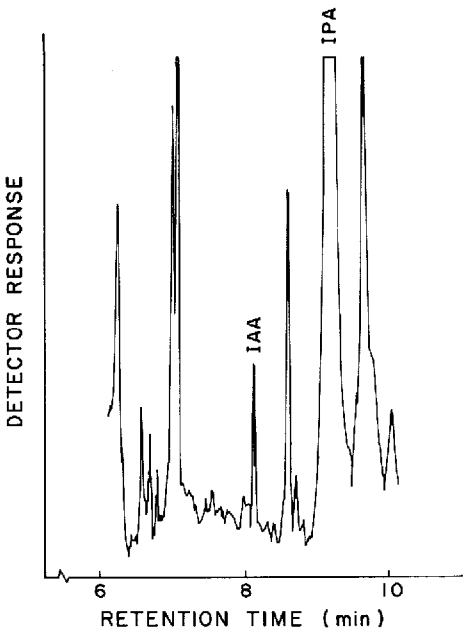

Fig. 2. BSTFA-derivatized free IAA extracted from senescing pea nodule. IPA was the internal standard. Nodules were harvested when plants were 6 weeks old. The column oven was programmed from 80 to 240°C (25° min^{-1}) and then run isothermal. For other conditions, see text.

lation and GC. Because of this shortened preparation procedure a large number of contaminants were present in the samples. Predictably, many of these contaminants did not contain nitrogen or phosphorus and were not detected by NPD. Thus use of NPD greatly reduced the number of peaks present in the chromatogram and made it much less difficult to identify the IAA peak. When FID was used, it was generally found that so many contaminants were present on the chromatograms that the IAA peak could not be identified.

The HPGC system was also used to estimate the amount of indole, IAA, indole-3-pyruvic acid, and indole-3-lactic acid present in the culture fluid of *Bradyrhizobium japonicum*. Fewer interfering compounds were present in culture fluid extracts than in plant nodule extracts and analysis could be conducted with either NPD or FID.

To evaluate the method further an IAA spike study was conducted in which known amounts of IAA were added to plant samples before extraction and analysis. The results of this study (Table I) show a good correlation between the amount of IAA added to the plant material and the amount of IAA estimated to be present in that plant material.

In conclusion, the main features of the HPGC system described are as follows. First, as was indicated earlier, the selectivity of NPD and of the high-resolution capillary columns used reduced the amount of sample preparation required. This reduced the time required for (and thus likely reduced IAA loss during) sample preparation. Solvent partitioning and GC analysis were the most laborious steps involved

TABLE I

RECOVERY OF IAA ADDED TO SOYBEAN NODULE MATERIAL

Nodules were harvested when plants were 8 weeks old f.wt. = Fresh weight.

Treatment	*Measured IAA (ng g^{-1} f.wt.)*
Nodule	60 ± 20
Nodule + 200 ng IAA g^{-1} f.wt.	280 ± 28
Nodule + 1000 ng IAA g^{-1} f.wt.	1005 ± 14

in the procedure. The time for GC analysis is in the range of 15 to 35 min per sample, the time required depending upon the method of injection and temperature program used. Second, assurance of compound identity is high. The selectivity of the detector coupled with the use of a split injector to deliver sample to two capillary columns with an efficiency of more than 65 000 plates each coupled with the use of a selective detector gives very high assurance of the identity of the compound(s) being assayed. Third, the sensitivity of the system is about equal to that generally reported for HPLC methods employing fluorescence or electrochemical detection[11]. Though with care better sensitivities are possible with HPLC using fluorescent detection[12], it has been suggested that the extra preparation required to obtain these improved sensitivities is too extensive for routine analysis[13]. HPGC systems coupled to mass spectrometry (MS) are also more sensitive and provide exceptional assurance of sample identity[14]. However, the expense of a GC–MS system is to high for many laboratories. The HPGC system used here cost much less than GC–MS and less than most HPLC systems.

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