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MICROSCALE ISOLATION TECHNIQUE FOR QUANTITATIVE GAS CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS OF INDOLE-3-ACETIC ACID FROM CHERRY (*PRUNUS CERASUS* L.)*

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SUMMARY

A rapid minicolumn technique is described that has been used for the isolation and mass spectral quantification of indole-3-acetic acid from cherry, a difficult to analyze tissue. The method used the resin XAD-7 to trap impurities. Samples were further purified on an amino minicolumn and then by high-performance liquid chromatography on a C_{18} column. After methylation, the samples were analyzed by selected ion monitoring gas chromatography–mass spectrometry. [$^{13}C_6$]indole-3-acetic acid was employed as an internal standard for isotope dilution calculations.

INTRODUCTION

Various techniques have been used to attempt to quantify indole-3-acetic acid (IAA) in plant tissues. By far the most accurate and certain technique is by use of stable isotope dilution and gas chromatography–mass spectrometry (GC–MS) detection[1–3]. In order to allow routine analysis of IAA by this technique it is necessary to have rapid methods for prepurification of plant extracts prior to the GC–MS analysis. Methods utilizing solvent partitioning, open columns, or minicolumn techniques followed by high-performance liquid chromatography (HPLC) have previously been described[2–6]. We have found the application of such techniques to be effective with a large number of different plant materials, however attempts to isolate

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IAA from vegetative tissues of cherry by such methods did not result in samples of sufficient purity for direct analysis by GC–MS. In this report we describe a simple and effective prepurification method for use on this difficult plant material. The method is based on the selective absorption properties of XAD-7 resin[4] and on the use of amino-ion exchange minicolumns. Techniques that are described are effective for analysis of free IAA and IAA released from ester and amide conjugates after selective base hydrolysis[2,5].

MATERIALS AND METHODS*

Extraction and purification

Cherry shoots (2–5 g) (*Prunus cerasus* L. cv Montmorency) were ground in propanol–0.2 *M* imidazole buffer, pH 7.0 (65:35) with a Polytron or Ultra Turrex homogenizer. Alternatively, freeze-dried material was ground with a Cyclone sample mill (UD Corp., Boulder, CO, U.S.A.) mechanical grinder and then suspended in propanol–imidazole buffer (65:35). About 100 ng/g fresh weight $^{13}C_6$-IAA was added during the extraction as an internal standard[2] along with *ca.* 30 000–50 000 dpm/g ^3H-IAA (29 mCi/m*M*, Amersham). After overnight extraction, to allow for isotope equilibration, the insoluble material was removed by centrifugation at 3000 *g* for 5 min. An amount of the extract equal to about 0.5 g (1.5 ml) was diluted to 10 ml with water. The extract was passed over a 3-ml bed volume column of XAD-7 (Mallinckrodt). The material passing through the column was collected and applied to a preconditioned Baker 10 SPE 2°-amino minicolumn. The minicolumn was then washed with 2 ml each of water, hexane, ethyl acetate, acetonitrile and then the IAA was eluted with 2% acetic acid in methanol[6]. The acidic methanol was removed *in vacuo* using a two-stage vacuum pump and a micro rotary evaporator. Immediately after drying, the sample was resuspended in 100 μl methanol–water (50:50) and injected onto a 125 mm × 4.6 mm I.D. Whatman ODS-3 HPLC column. The HPLC column was protected by a Whatman Co:Pell precolumn and elution was isocratic with acetonitrile–water (20:80) containing 1% acetic acid. Flow-rate was 1 ml/min. Fractions containing the radioactive tracer were pooled, reduced to dryness (as above), and methylated using diazomethane[7]. Analysis by GC–MS was on a Hewlett-Packard 5992 quadrupole machine with an open-split interface and was essentially as described[2,6] except that the dwell time for each ion was 100 ms. Ions monitored were m/z 130, 136, 189 and 195. GC was on an 11 m × 0.32 mm I.D. Chrompack CPSil 19 CB fused-silica wall-coated open-tubular capillary column using splitless injection. Ultrapure helium was the carrier gas and flow was at 1 ml/min. The injection temperature was 110°C and was held for 1 min. Following the isothermal hold, the temperature was programmed at 16°C/min. Under these conditions the methyl ester of IAA had a retention time of 8.6 min.

Fractions of the initial extract that were to be analyzed for free plus ester or total IAA, were hydrolyzed under basic conditions after evaporation to the water phase[2,5]. Free plus ester IAA was determined by hydrolysis using 1 *M* sodium hy-

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droxide for 1 h at 25°C and total (free plus ester plus amide) IAA was determined following 7 *M* sodium hydroxide hydrolysis at 100°C for 3 h under nitrogen. These conditions have previously been shown to quantitatively hydrolyze a number of different conjugates of IAA of the appropriate bond (ester or amide). In addition, the amide conjugates are not hydrolyzed to any detectable level by the 1 *M* sodium hydroxide treatment[2,5].

Following hydrolysis the samples were neutralized to pH 7 with hydrochloric acid. The neutralized sample (2–4 ml) was then diluted with water to approximately 20 ml and applied to a 3-ml bed volume column of Whatman standard grade cellulose powder. This column was attached on top of a Baker 10 SPE 2°-amino column such that the eluate passed through both columns in series. IAA voided both columns and was collected. The pH of the eluate was adjusted to 2.5 and then the eluate was applied to a 3-ml bed volume column of XAD-7. The IAA was then eluted twice with 2.5 ml of methanol and this was reduced to dryness *in vacuo*. HPLC, methylation, and GC–MS analysis were done as with the samples for analysis of free IAA.

Column preparation

XAD-7 (Mallinckrodt, 3416) was prepared by Soxhlet extraction with diethyl ether overnight. Following extraction the resin was washed with methanol and then stored as a methanol slurry until used. The slurry (3 ml) was poured into a 5 ml polypropylene syringe and then washed with water prior to use.

The 2°-amino Baker 10 SPE columns were preconditioned by washing with 2 ml each of hexane, ethyl acetate, acetonitrile, methanol, water and, finally, with 0.2 *M* imidazole buffer until the eluate was pH 7.0.

The HPLC column was slurry packed at 580 bars using 1.25 g ODS-3 5-μm resin (Whatman) in 20 ml ethylene glycol–methanol (50:50) and with methanol as the packing solvent. Prior to injection of the plant extract, the HPLC column was conditioned by washing extensively with methanol followed by equilibration in the running solvent for at least 15 min at 1 ml/min.

RESULTS AND DISCUSSION

Woody plant materials present special problems for homogenization and extraction of plant hormones and their conjugates. Homogenization of fresh material using a blender resulted in low measured values of both free and conjugated forms of IAA. This probably resulted from poor equilibration of the isotope label with the woody tissue and poor extraction of the conjugates (Table I). More complete homogenization was obtained by use of the Polytron, however the level of ester IAA detected was lower and the level of free IAA was higher than in the freeze-dried sample. This result suggests that ester IAA may have been hydrolyzed in the fresh material during the time required for homogenization. A similar increase in the amount of free IAA measured was noted by Sundberg when using buffer or methanol extraction of IAA from woody tissues of Scots pine[8]. The higher overall amount of IAA (greater isotope dilution) obtained and the lower level of free IAA relative to conjugates suggests that freeze-drying and grinding prior to extraction with an aqueous organic solvent is the preferred method for sample preparation with this material.

TABLE I

EFFECT OF HOMOGENIZATION TECHNIQUE ON LEVELS OF IAA AND IAA-CONJUGATES MEASURED BY ISOTOPE DILUTION ANALYSIS OF SHOOTS OF CHERRY

	ng IAA/g fresh weight		
	Blender/fresh shoots	*Polytron/fresh shoots*	*Mill/freeze-dried shoots*
Free IAA	25	92	56
Ester-linked IAA	34	10	72
Amide-linked IAA	0	26	96
Total IAA	59	128	224

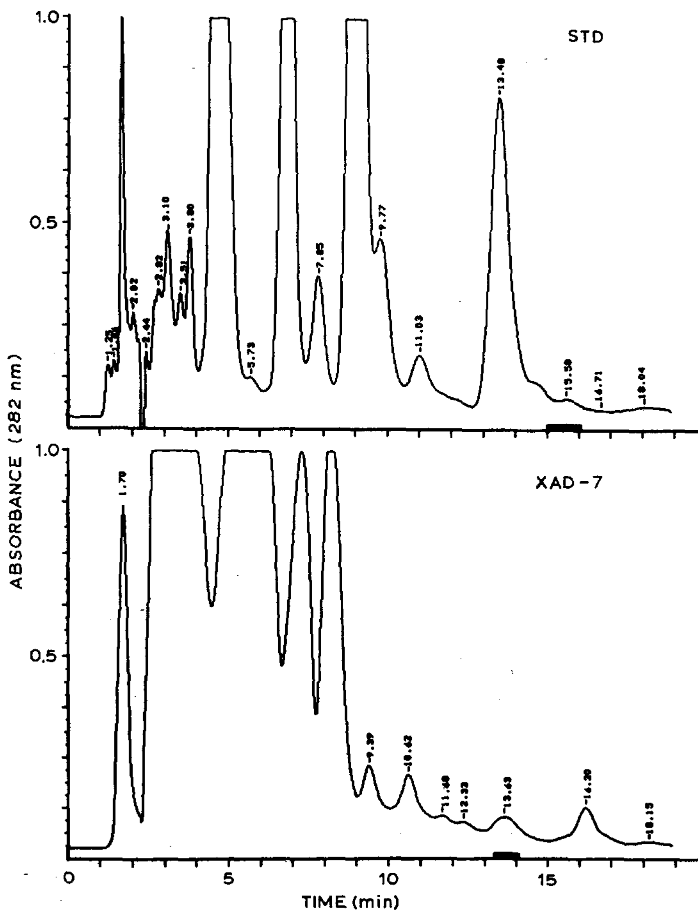

Fig. 1. Elution profile from reversed-phase HPLC of cherry stem extracts purified using the standard amino minicolumn technique[6] (upper chromatogram) and using the XAD-7 procedure (lower chromatogram). Samples were for free IAA analysis (no hydrolysis). The dark bar on the abscissa indicates the retention time of the ^{3}H-IAA standard.

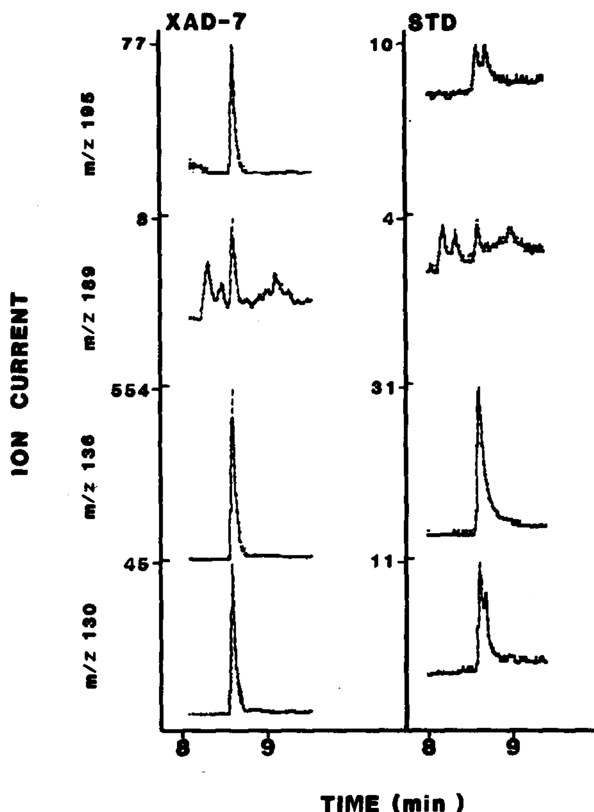

Fig. 2. Selected ion GC–MS chromatograms from free IAA samples purified from cherry stem extracts using the standard amino minicolumn technique[6] (right tracings) and the XAD-7 technique (left tracings).

Our standard minicolumn method[6], which has proven effective on a variety of seed and soft vegetative tissues, was utilized in preliminary studies of cherry. For determination of free IAA, this standard method was based on extraction in buffered solvent and an ion exchange step. For conjugate analysis selective hydrolysis was followed by desalting on a C_{18} minicolumn and then by ion exchange. Other than as indicated, the two methods are the same. Recovery of free IAA by use of the XAD-7 based procedure increased from about 35–40% to nearly 50%, even though the XAD-7 procedure uses an additional minicolumn step in the prepurification. The HPLC chromatogram shows less A_{282} absorbing material eluting near IAA in the sample prepurified using XAD-7 relative to the standard protocol (Fig. 1). GC–MS analysis of samples that were prepurified without XAD-7 had major contaminant peaks interfering with ions at m/z 130 and 195, making quantitation difficult (Fig. 2). In comparison, the XAD-7 procedure resulted in both higher recovery and a selected ion chromatogram free of interference. The quantitative GC–MS technique used for the final determination was accurate to within 4% of the amount of IAA extracted[2] and selected ion peaks resulting from as low as 50 pg of IAA methyl ester were sufficient for accurate quantitation when using the instrumentation described.

The technique described for use of XAD-7 differs from previously described XAD-7 methods in several ways[4,8–10]. Most significant is, that for free IAA analysis the XAD-7 was used to trap out impurities but was not used to retain the phytohormone. In the higher salt solution obtained following hydrolysis, the IAA was retained by the XAD-7. The earlier work describing use of XAD-7[4,8–10] did not address the problem of isolation of IAA released following basic hydrolysis. Our use of simple XAD-7 minicolumns allowed for more rapid and convenient analysis than possible with slurry or open-column techniques[4,8–10], yet was still effective for preparation of these samples for HPLC and GC–MS analysis.

The procedure for IAA analysis described in this report allows the rapid isolation and quantitation of IAA from woody tissue of cherry, a difficult to analyze tissue. This procedure has thus increased the range of plant materials where the precision and certainty inherent in quantitative mass spectrometry of IAA[2,6] can be easily applied. These procedures, or variations of these procedures, should also be applicable to other woody plant tissues of interest. These results underline the need to verify analytical methods that rely on detection systems less selective than GC–MS, before such methods are applied to new and unknown plant tissues.

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