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A Microscale Technique for Gas Chromatography-Mass Spectrometry Measurements of Picogram Amounts of Indole-3-Acetic Acid in Plant Tissues[1]

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A microscale technique has been developed for routine quantifications of picogram amounts of indole-3-acetic acid (IAA) in plant tissues by combined gas chromatography-mass spectrometry. Low- and high-resolution selected-ion-monitoring and selected-reaction-monitoring mass spectrometry techniques were compared for selectivity and precision. The best selectivity was obtained with selected-reaction-monitoring analysis, and 1-mg samples containing 500 fg of IAA could be analyzed accurately with this method. This technique was used to investigate the IAA distribution pattern along the longitudinal axis of tobacco (*Nicotiana tabacum* [L.]) leaves. In young, developing leaves an increase of endogenous IAA from the leaf tip to the base of the leaf was observed, whereas the level of IAA was uniform along this axis in mature leaves.

Ever since the identification of IAA as the major auxin in plants, there has been continuous development of methods for measuring its endogenous concentrations in plant tissues (Sandberg et al., 1987). Measuring IAA involves the detection and quantification of minute amounts of analyte in a plant extract that is a complex, multicomponent mixture. An accurate estimate of IAA content can be obtained only if the sample has been purified to remove substances that would otherwise interfere with the analysis. In such circumstances it is common practice to use methodology that offers high selectivity and that tends to be associated with low limits of detection. Procedures with high selectivity have the advantage that many impurities go undetected and therefore do not interfere with the analysis. The emphasis on purification is thus less severe than with nonselective procedures.

Several methods have been described by which picogram amounts of IAA can be detected, e.g. HPLC with fluorescence detection (Crozier et al., 1980) and immunological techniques (Maldiney et al., 1986). However, when applied to plant extracts, these methods still require purification to ensure that the detected response is accurate, being caused only by IAA. Although procedures are available to check the accuracy of HPLC fluorescence- and immunoassay-based estimates of IAA levels (Sandberg et al., 1985; Cohen et al., 1987), there is little evidence in the literature of those procedures being used on a routine basis (Neill and Horgan, 1987; Hedden, 1993; Crozier and Moritz, 1995).

The use of combined GC-MS is much more practical and is now employed widely. GC-MS provides precise measurements of small amounts of IAA, with the accuracy of each estimate being checked by comparing response ratios of several characteristic fragments (Cohen et al., 1986). However, as with other methods, several purification steps are needed if accurate quantitative estimates of IAA in plant extracts are to be obtained (Rivier, 1986; Vine et al., 1987; Chen et al., 1988; Dunlap and Guinn, 1989). By using a double-focusing HR mass spectrometer, the selectivity of the analysis increases and overcomes some of the problems caused by interfering substances. SRM is another method for increased selectivity in MS analysis with multianalyzer instruments (Gaskell and Millington, 1978). This technique involves detection of daughter ions originating from specified, metastable parent ions, resulting in an extremely high specificity that makes the procedure well suited for analysis of trace compounds in biological samples (e.g. Chess and Gross, 1980; Thorne and Gaskell, 1985; Dobson et al., 1994; Moritz and Olsen, 1995).

We have evaluated different GC-MS techniques for quantification of IAA in semi-purified plant extracts using a double-focusing HR mass spectrometer. A microscale method involving a limited amount of sample preparation work is described that allows accurate measurements of low picogram levels of IAA in extracts from 1 mg or less of tobacco (*Nicotiana tabacum* [L.]) leaf tissue.

MATERIALS AND METHODS

Plant Material

Nicotiana tabacum (L.) cv SR1 plants were grown in a greenhouse at a day temperature of 22°C, a night temperature of 17°C, and a RH of about 70%. The photoperiod was 18 h and consisted of natural daylight extended with light from metal halogen lamps (Osram 1043 [München, Germa-

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Abbreviations: HR, high resolution; LR, low resolution; SIM, selected ion monitoring; S/N, signal-to-noise ratio; SRM, selected reaction monitoring.

ny] HQI-TS 400 W/DH) giving a quantum flux density of 150 μmol m^{-2} s^{-1}. When not stated otherwise, the experimental material consisted of mature, fully elongated, vigorous leaves from 2-month-old tobacco plants. Leaf discs were punched out between the major lateral veins with 10- and 2.5-mm-diameter cork borers, giving a sample weight of approximately 10 and 1 mg ± 50 μg, respectively (Mettler AE160, AB Hugo Tillquist, Spånga, Sweden).

Extraction and Purification

The leaf discs were placed in an Eppendorf tube and homogenized in liquid nitrogen with a conical metal pestle that was connected to an electric drill. Five-hundred microliters of 0.05 M Na-phosphate buffer, pH 7.0, containing 0.02% (w/v) of sodium diethyldithiocarbamate as antioxidant and 50 or 250 pg of [$^{13}C_6$]IAA (>99% enrichment; Cambridge Isotope Laboratories, Woburn, MA) as an internal standard was added to the Eppendorf tube, and the sample was extracted under continuous shaking for 1 h at 4°C. After extraction, the pH was adjusted to approximately 2.6 with 1 M HCl, and the sample was slurried with 35 mg of Amberlite XAD-7 (Serva, Heidelberg, Germany) for 30 min. After removal of the buffer, the XAD-7 was washed with 2× 500 μL of 1% acetic acid before being slurried with 2× 500 μL of dichloromethane for 2× 30 min. The combined dichloromethane fractions were reduced to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY).

MS

The samples were methylated with ethereal diazomethane and thereafter trimethylsilylated with 25 μL of acetonitrile and 25 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% (v/v) trimethylchlorosilane (Pierce) at 70°C for 15 min. After evaporation in a Speed Vac concentrator, the samples were dissolved in heptane.

The samples were injected splitless by a Hewlett-Packard 7673 autosampler into a Hewlett-Packard 5890 gas chromatograph equipped with a 25 m × 0.25 mm i.d. fused silica capillary column with a chemical bond 0.25-μm SE-30 stationary phase (Quadrex, New Haven, CT). The injector temperature was 270°C and the column temperature was held at 60°C for 2 min, then increased by 20°C min^{-1} to 200°C and by 4°C min^{-1} to 260°C. The column effluent was introduced into the ion source of a JEOL JMS-SX/SX102A tandem mass spectrometer. The mass spectrometer was used in the single MS mode. The interface and the ion source temperatures were 260°C. Ions were generated with 70 eV at an ionization current of 300 μA.

LR and HR SIM measurements were performed using accelerating voltage switching from 10 kV. The dwell time was 50 ms, and *m/z* 202.105, *m/z* 208.125, *m/z* 261.118, and *m/z* 267.137 were recorded. HR analysis at a resolution of 5,000 and 10,000 was done with perfluorokerosene as a reference compound, with *m/z* 218.9856 as lock mass.

In the SRM mode, the acceleration voltage was 10 kV, the precursor ions were selected by magnetic switching, and the daughter ions were selected by simultaneous switching of the magnetic and electrostatic fields. The dwell time was 100 ms, and the reactions *m/z* 261.118 to *m/z* 202.105 and *m/z* 267.137 to *m/z* 208.125 were recorded. Xenon was used as the collision gas.

In all of the MS modes, calibration curves were recorded from 10 to 100 pg of IAA with 50 pg of [$^{13}C_6$]IAA as the internal standard. All data were processed by the JEOL MS-MP7000D data system.

Analysis of Precision

The precision is defined as the relative SD of the integrated area ratio between *m/z* 202.105 and *m/z* 208.125. It was estimated for: (a) GC-MS analyses in the different MS modes, by replicate measurement of standard mixtures consisting of 10 and 100 pg of IAA together with 50 pg of [$^{13}C_6$]IAA as internal standard; (b) GC-MS analyses of plant extracts by using a sample containing 10 pooled extracts that was analyzed nine times by SRM; and (c) the whole quantification procedure, including sample preparation, extraction, purification, and SRM analyses, by homogenizing a tobacco leaf in liquid nitrogen, dividing it into 10 10-mg aliquots that were processed and quantified individually.

RESULTS AND DISCUSSION

The isotope-dilution technique for quantitative analysis of organic compounds in mixtures was introduced by Rittenberg and Foster (1940). This method has the great advantage that it compensates for losses during sample preparation and subsequent analysis by the addition of stable or radioactive isotopes as internal standards. With the access to heavy isotopes, analysis can be performed by GC-MS with both high accuracy and high precision. Quantification by isotope-dilution GC-MS is based on the response ratio between selected masses for the native compound and the added internal standard. As has been discussed by Colby et al. (1981), the amount of internal standard relative to the native compound influences the reliability in quantification by isotope dilution. This is due to several factors, but of particular importance is the degree of isotopic substitution in the internal standard and the difference in masses between the native compound and the internal standard. Although it is possible to calculate the limits of response ratios when using a labeled internal standard, in practice the response of native versus labeled compounds should be within the range of the calibration curve. The [$^{13}C_6$]IAA that was used as internal standard in the present study is ideal for IAA quantifications due to the high degree of isotopic substitution and the large difference in mass from the native IAA.

To evaluate the usefulness of different GC-MS techniques for IAA quantification in the low picogram range, calibration curves were obtained by analyzing 50 pg of [$^{13}C_6$]IAA mixed with different amounts of 10 to 100 pg of native standard (Fig. 1). Within this range good linearity was obtained for all techniques, with correlation coefficients between 0.997 and 0.999. The high linearity of the calibration curves is explained by the fact that there are six

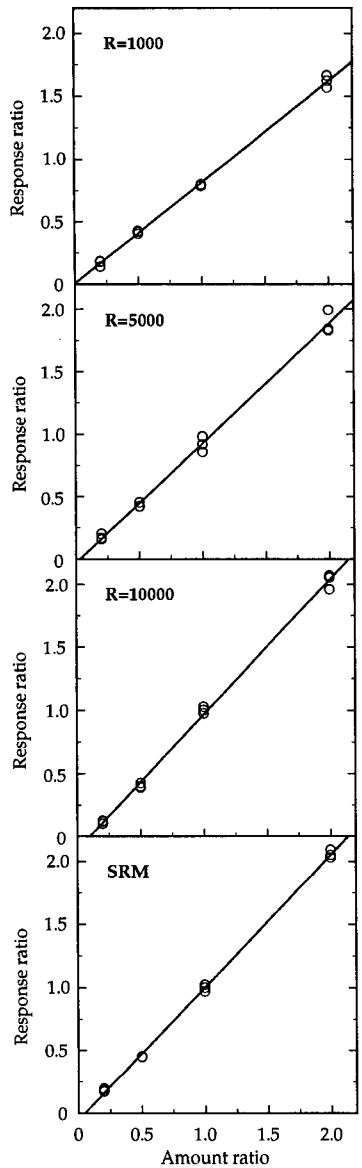

Figure 1. Calibration curves obtained with different MS methods after triplicate analysis of the methyl trimethylsilyl derivatives of 10 to 100 pg of IAA with 50 pg of [$^{13}C_6$]IAA as the internal standard.

^{13}C atoms in the internal standard, which minimizes the effect of the natural isotope abundances on the linearity of the calibration curves (Pickup and McPherson, 1976; Colby and McCaman, 1979). We also investigated the precision of peak-ratio response for the different MS techniques by replicate analyses of 10 and 100 pg of native standard together with 50 pg of [$^{13}C_6$]IAA (Table I). Generally, if the calibration curve covers a broad range, the SD over the range varies considerably, with increased SD values being associated with increased ion-abundance ratios. This is reflected in the generally higher precision for the 100-pg samples. Among the different MS techniques, SRM showed the highest precision with 6.2 and 1.6% for the 10- and 100-pg samples, respectively. This indicates a better S/N for SRM than for the other techniques.

Table I. *Precision of IAA analysis by different MS modes*

Standard mixtures containing 10 and 100 pg of IAA together with 50 pg of [$^{13}C_6$]IAA were analyzed as methyl trimethylsilyl derivatives, and the relative SD for the integrated area ratio between *m/z* 202.105 and *m/z* 208.125 for five replicate injections was calculated.

GC-MS Mode	Relative SD for Measured Amount	
	10 pg	100 pg
SIM	%	
R = 1,000	12.4	4.1
R = 5,000	12.5	5.9
R = 10,000	9.7	4.7
SRM	6.2	1.6

Having established a GC-MS methodology with both acceptable precision and sensitivity for IAA analysis, we wanted to develop a microscale technique for IAA analysis in plant tissues (Fig. 2). The aim was to set up a protocol with a minimum of sample preparation, avoiding time-consuming procedures, such as evaporation and tube-to-tube sample transfer. Phosphate buffer was chosen as extraction medium. Although a neutral buffer solution is an effective extractant for IAA, it has to be used with care because of the possible hydrolysis of IAA ester conjugates to IAA during extraction. Such unwanted conversions are minimized by a short 1-h extraction time, which is shown to be enough for establishing isotope equilibrium and is only a problem in tissues containing extremely high levels of IAA-ester conjugates, such as *Zea mays* seedlings (Sundberg, 1990). Buffer extraction has the advantage over organic solvents that it results in extracts less contaminated with nonpolar substances such as pigments and lipids. The

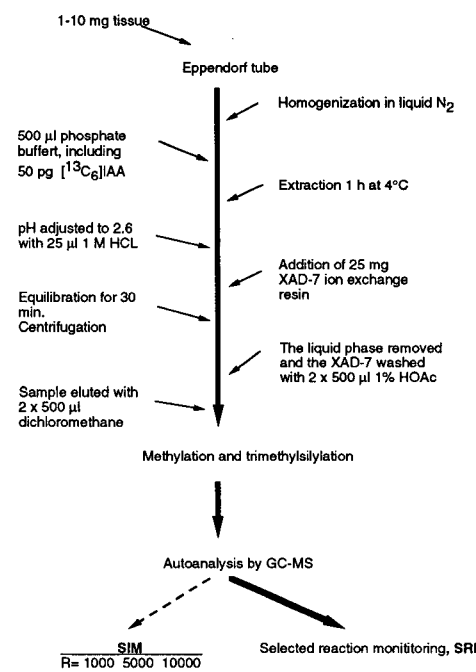

Figure 2. Protocol for extraction and purification of IAA from milligram amounts of plant tissue.

use of buffer as an extractant also allowed us to add Amberlite XAD-7 resin directly to the extraction medium. Amberlite XAD-7 acts primarily through ion-exchange mechanisms, and its usefulness in purification of plant extracts for hormone analyses is well documented (Sandberg et al., 1987). After removal of the buffer, IAA was eluted from the XAD-7 resin with dichloromethane and the samples were evaporated to dryness. The recovery for the whole procedure was estimated by the use of radiolabeled IAA to be about 70% (data not shown).

To evaluate the specificity and the practical value of the different MS methods, aliquots of a 10-mg plant extract were processed as outlined in Figure 2, excluding the addition of internal standard, and samples containing approximately 5 to 10 pg of IAA were analyzed (Fig. 3). From the results it can be concluded that LR-SIM is not suitable for analysis of the semi-purified extracts because of interfering substances chromatographing near the analyte. By increasing the resolution, the abundance of interfering substances is decreased. However, in the very HR range of 10,000 the absolute sensitivity is not sufficient, which reduces the advantage of the improved selectivity. The selectivity in the SRM mode was found to be excellent, producing mass chromatograms with only one major peak. In further analyses, the applicability of the HR (R = 5,000) and SRM modes were evaluated for 1-mg tobacco leaf samples containing very low amounts of IAA. An aliquot corresponding to approximately 500 fg of IAA was analyzed, and the obtained mass chromatograms showed a low S/N for R = 5,000, resulting in imprecise quantifications (Fig. 4). In contrast, the SRM data showed a high S/N, although the absolute intensity is lower than in the HR mode. Taken together, the SRM method was found to be the most suitable technique for accurate quantifications of the semi-purified extracts containing very small amounts of IAA.

For interpretation of quantitative data and for optimizing experimental design, an estimate of the error associated with the quantification of the experimental material under investigation is valuable. For tobacco leaf tissue, the precision related to the GC-MS step was 6.2%, whereas the whole quantification procedure involved a relative SD of 17%. This additional variation reflects weighing and pipetting errors. It should be pointed out, however, that the precision estimated here is related to the nature of the analyzed tissue, i.e. the amount of endogenous IAA in relation to other substances that will contribute to the level of noise and cannot be regarded as a general figure. However, considering the extremely low concentration of endogenous IAA in mature tobacco leaves, we find that the result is acceptable.

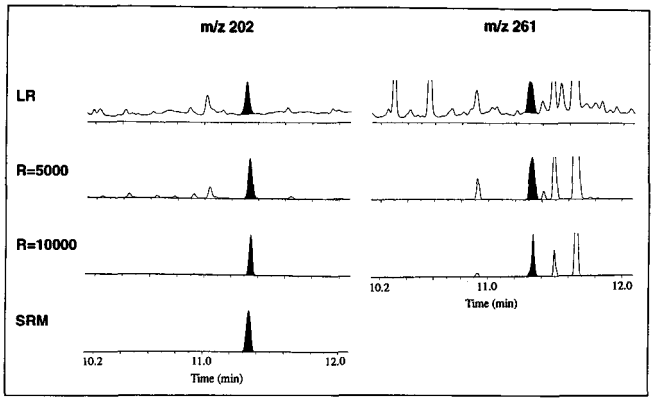

Figure 3. Mass chromatogram traces of IAA-methyl trimethylsilyl detected in extracts from 10 mg of plant tissue by LR-SIM, HR-SIM (R = 5,000 and 10,000), and SRM.

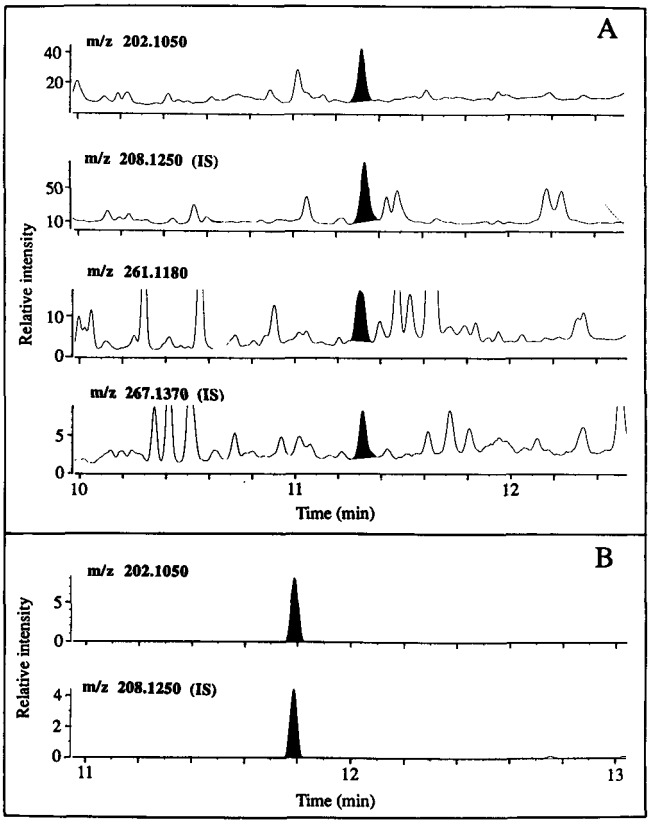

Figure 4. Mass chromatogram traces of IAA-methyl trimethylsilyl and [$^{13}C_6$]IAA-methyl trimethylsilyl detected by HR-MS (R = 5000) (A) and SRM (B) after extracting 1 mg of plant tissue with 50 pg of [$^{13}C_6$]IAA as internal standard (IS).

A reliable technique for hormone measurements in small amounts of plant tissue opens new perspectives for investigations on distribution patterns of these substances. This was illustrated by investigating the concentration variations in endogenous IAA along the longitudinal axis of developing and mature tobacco leaves (Fig. 5). In young, developing leaves an increase in IAA levels from tip to base was found. This pattern correlates with the pattern of leaf growth, i.e. a basipetal cessation of both cell division and cell expansion (Steeves and Sussex, 1989), and might reflect the rate of IAA synthesis, which is generally believed to be associated with young expanding shoots and leaves. A similar distribution pattern was found for cytokinins in developing leaves of *Capsicum annuum* L., sweet pepper (Ulvskov et al., 1992), suggesting a role for both auxin and cytokinin in the regulation of patterned leaf growth. The more uniform distribution of IAA in older leaves reflects a static stage in which growth is more or less completed.

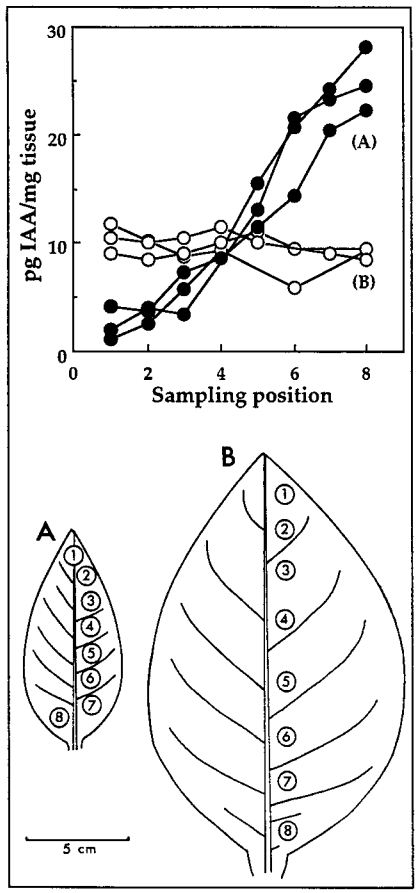

Figure 5. Levels of free IAA (pg/mg fresh weight) along the longitudinal axis of three developing, approximately 11-cm-long (A) and three mature, fully elongated, approximately 21-cm-long (B) tobacco leaves. Leaf discs, 10 mm in diameter, were sampled as illustrated.

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