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Determination of indole-3-pyruvic acid levels in *Arabidopsis thaliana* by gas chromatography–selected ion monitoring-mass spectrometry

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Abstract

A rapid and simple method is described for the determination of indole-3-pyruvic acid (IPA) levels in *Arabidopsis thaliana* by gas chromatography–selected ion monitoring-mass spectrometry (GC–SIM-MS). The method includes derivatization of IPA with hydroxylamine in the crude extract, followed by ethyl acetate partitioning, solid-phase extraction with C_{18} resin, reversed-phase high-performance liquid chromatography (HPLC), and GC–SIM-MS. Three derivatizing reagents were tested; these were pentafluorobenzylhydroxylamine, pentafluorophenylhydrazine, and hydroxylamine. Hydroxylamine proved to be the most useful, as the IPA-oxime was easiest to purify from plant extracts and was the most stable. IPA was quantified in *Arabidopsis* seedlings ranging in age from 5 to 12 days; levels varied from 4 to 13 ng/g, peaking at 7–9 days. © 1998 Elsevier Science B.V.

Keywords: *Arabidopsis thaliana*; Derivatization, GC; Indolepyruvic acid; Pentafluorophenylhydrazine; Pentafluorobenzylhydroxylamine

1. Introduction

Indole-3-acetic acid (IAA) is a key regulator of growth and development in higher plants. IAA is an endogenous plant compound, the biosynthesis of which has remained undefined despite decades of study [1,2]. As a result of extensive biochemical analysis, several pathways for IAA biosynthesis have been postulated and a number of possible intermediates have been identified [2]. An important component to the study of IAA biosynthesis is the accurate quantification of these putative intermediates and the rigorous establishment of precursor–product relationships. Much of the recent progress in our understanding of IAA biosynthesis has come from the use of stable isotopes in conjunction with MS, for both in vivo labeling studies and for the quantification of IAA and its precursors. This type of analysis has revealed that IAA is derived either from tryptophan or a precursor to tryptophan, and that multiple IAA biosynthetic pathways coexist in a single plant [2,3].

Indole-3-pyruvic acid (IPA) (Fig. 1a) is an intermediate in microbial IAA biosynthesis, and the pathway is well defined [4]. IPA is also thought to be an intermediate in IAA biosynthesis in a broad range of plant species; however, there is less quantitative evidence available for plants. While IPA has been identified in several plant species, mass spectral verification has only been carried out with tomato [5] and pea root nodules [6]. Isotope-labeling studies have demonstrated the capacity of IPA to act as a precursor to IAA in tomato [7], but enzymatic

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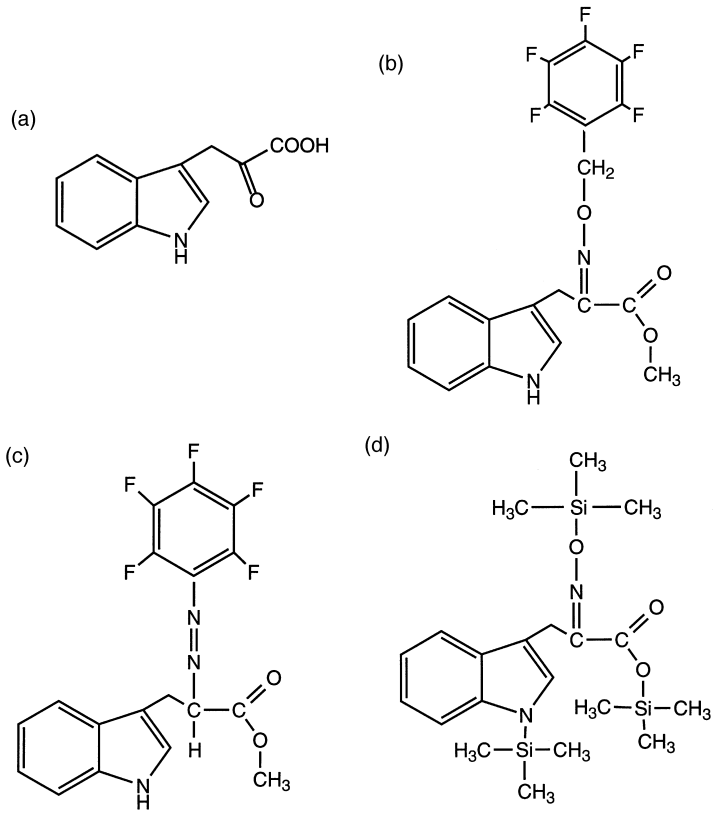

Fig. 1. The chemical structures of (a) IPA, (b) methylated pentafluorobenzyl oxime of IPA (IPA-PFB), (c) methylated pentafluorophenylhydrazone of IPA (IPA-PFPH), and (d) silylated oxime of IPA (IPA-oxime).

activities responsible for the inter-conversion of IPA to IAA have only been partially characterized [8].

In order to determine whether IPA was an intermediate in IAA biosynthesis in *Arabidopsis*, we first needed to verify that IPA was a native compound of this plant. IPA is very unstable and degrades in solution to IAA and other compounds within a matter of days. Cooney and Nonhebel [5] described a method for the quantification of IPA in tomato that generated the pentafluorobenzyl oxime of IPA (IPA-PFB), as seen in Fig. 1b. In our hands this method was not satisfactory for *Arabidopsis*, due to low recoveries of IPA-PFB. We therefore developed derivatization methods for IPA using either pentafluorophenylhydrazine (PFPH) or hydroxylamine, as seen in Fig. 1c,d. We found that hydroxylamine was the more useful derivatizing agent, as a single reaction product formed that was relatively easy to purify for subsequent GC–selected ion monitoring (SIM)-MS analysis.

2. Experimental

2.1. Chemicals

A methanolic IPA (Sigma, St. Louis, MO, USA) solution (1 mg/ml) was made just prior to use. Solvents (HPLC grade) were from VWR (Bridgeport, NJ, USA). [$^2H_5$]Tryptophan (indole-d5, 98%) was a product of Cambridge Isotope Labs. (Woburn, MA, USA). L-Amino acid oxidase (EC 1.4.3.2) was supplied by Boehringer Mannheim (Indianapolis, IN, USA), and catalase (from human erythrocytes, CAS 9001-05-2) was obtained from Sigma. Pyridine (anhydrous), PFPH, pentafluorobenzylhydroxylamine

(PFB), hydroxylamine, diethyldithiocarbamic acid (DIECA), and 2,6-di-*tert*.-butyl-4-methylphenol (BHT) were obtained from Aldrich (Milwaukee, WI, USA). *N*,*O*-Bis(trimethysilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Alltech (Deerfield, IL, USA).

2.2. Plant material

Aseptically grown *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia was used as a source of plant tissue. Seeds were surface sterilized [9] and sown onto plant nutrient medium with 0.5% sucrose [10] solidified with 0.7% ultrapure agar (US Biochemicals, Cleveland, OH, USA). Seedlings were grown under continuous illumination (100 μE m^{-2} s^{-1}; 1 einstein (E)=1 mol of photons) from cool white fluorescent lights at room temperature. Tissue was harvested from 5 to 12 days after sowing, and then weighed, frozen in liquid nitrogen and stored at −80°C.

2.3. Instrumentation and columns

Solid-phase extraction was carried out with disposable C_{18} columns (0.5 mg PrepSep, Fisher, Pittsburgh, PA, USA). HPLC was performed on a Varian Model 5000 LC system equipped with a UV detector set at 278 nm. The reversed-phase C_{18} column (Ultracarb 5 ODS [30], 50×4.6 mm) was obtained from Phenomenex (Torrance, CA, USA). GC–SIM-MS analysis was carried out with a Hewlett-Packard Model 5890 GC system fitted with a DB5MS fused-silica capillary column (30 m×0.25 mm I.D.) supplied by J&W Scientific (Folsom, CA, USA). The GC system was coupled to a Hewlett-Packard 5972 MS. Integration was performed using Hewlett-Packard Chemstation software.

2.4. Synthesis of [$^2H_5$]IPA

Just prior to use (or not more than 2 days before) $^2H_5$-labeled IPA was synthesized according to the method described by Cooney and Nonhebel [5], with the following modifications. [$^2H_5$]Tryptophan (88 μg) was reacted with 0.025 units of L-amino acid oxidase and 10^{-6} units of catalase in 0.4 ml phosphate buffer (pH 7) in the dark for 90 min at room temperature. The sample was brought to pH 3 and extracted with ethyl acetate (equilibrated with 10 m*M* L-ascorbic acid). The organic phase was removed to a new tube and dried to residue by rotary evaporation. The sample was dissolved in 50% methanol, 50% water, and subjected to HPLC in order to purify [$^2H_5$]IPA. The mobile phase was a linear gradient: solvent A (10% acetonitrile, 1% acetic acid) and solvent B (100% acetonitrile), from 0 to 50% solvent A in 20 min. A single peak with a retention time of 11.5 min was observed by monitoring UV absorbance. After derivatization (see below), GC–MS was used to confirm that this fraction contained [$^2H_5$]IPA. In order to determine the yield of [$^2H_5$]IPA, a standard curve was generated from the UV absorption profiles of known amounts of unlabeled IPA that had been subjected to the same HPLC conditions described above.

2.5. IPA derivatization

2.5.1. Pentafluorobenzyl oxime of IPA (IPA-PFB) and pentafluorophenylhydrazone of IPA (IPA-PFPH)

IPA was reacted with an excess (1000- to 3000-fold) of either PFB or PFPH in methanol. The samples were flushed with nitrogen (less than 0.5 ppm O_2) and sealed. A variety of incubation conditions were tried; 50°C for 90 min was optimal for generating IPA-PFB, while 45 min on ice was optimal for generating IPA-PFPH. The reactions were quenched by diluting the samples 10-fold with acetone. The samples were further diluted with acetic acid (to a final concentration of 1–5% acetic acid) and passed through disposable C_{18} columns that had been preconditioned with methanol, water, and 1% acetic acid. IPA derivatives were eluted with methanol. The samples were brought to residue under a stream of nitrogen, then dissolved in 75% methanol, 1% acetic acid, 24% water, and subjected to HPLC purification on a C_{18} column. For purification of IPA-PFB, the mobile phase was methanol and 1% acetic acid (3:1) and the flow-rate was 0.5 ml/min. For purification of IPA-PFPH, a linear gradient was established between solvents A and B (see Section 2.4) from 30 to 45% solvent A in 30 min. The flow-rate was 1 ml/min. The eluents were passed

through a UV detector set at 278 nm. Fractions corresponding to major peaks were dried using rotary evaporation, resuspended in methanol, methylated with ethereal diazomethane [11] and analyzed by GC–MS. In this manner, the HPLC retention times of IPA-PFB and IPA-PFPH were determined to be approximately 7.8 and 13.2 min, respectively.

2.5.2. Oxime of IPA (IPA-oxime)

IPA (in acetonitrile) was reacted with a 25-fold excess of hydroxylamine (in anhydrous pyridine). The sample was sealed and incubated on ice for 30 min then brought to residue with a stream of nitrogen, dissolved in 50% methanol, 50% water and subjected to HPLC. The mobile phase was a linear gradient: solvent A and solvent B, from 0 to 50% solvent A in 20 min. The major UV absorbing peak (with an HPLC retention time of 10.5 min) was collected, brought to residue by rotary evaporation, dissolved in ethyl acetate, transferred to small amber vials, and dried under a stream of nitrogen. Immediately prior to GC–MS, the sample was silylated by adding 30 μl of BSTFA+1% TMCS and incubating at 45°C for 30 min. Silylated samples were injected directly into the GC–MS system.

To generate the oxime of endogenous *Arabidopsis* IPA, 4–5 g of frozen tissue from 5–12-day-old seedlings were ground to a fine powder in liquid nitrogen with mortar and pestle. Glass beads (150–212 μm, Sigma) were added to aid with tissue disruption. Five ml of extraction buffer (35% 200 m*M* imidazole, pH 7, 65% acetonitrile) per gram of tissue, along with DIECA (1 mg per 5 ml extraction buffer), BHT (45 μg/ml extraction buffer), and 50–100 ng [$^2H_5$]IPA (as an internal standard) were added to the powder. Twenty mg of hydroxylamine (in pyridine) per gram of plant tissue were added to the crude extract and incubated on ice for 30 min. The sample was centrifuged for 10 min in a clinical centrifuge to pellet tissue debris. The supernatant was transferred to a new tube, the pellet resuspended in extraction buffer and centrifuged again. This was repeated twice, and the supernatants were pooled. After removing the solvent by rotary evaporation, the remaining aqueous solution was brought to pH 3 and then extracted with ethyl acetate (equilibrated with 10 m*M* L-ascorbic acid). The organic phase was transferred to a new tube and brought to residue by rotary evaporation. The sample was dissolved in 1% acetic acid and transferred to a disposable C_{18} column that had been preconditioned with methanol, distilled water, and 1% acetic acid. IPA-oxime was eluted with methanol, concentrated by rotary evaporation, made 50% with water and subjected to HPLC twice. The mobile phase was a linear gradient between solvent A and solvent B from 0 to 50% solvent A in 20 min for the first HPLC run, and from 10 to 40% solvent A in 15 min for the second HPLC run. The retention times of IPA-oxime were 10.5 and 8.3 min, respectively. Samples were dried, redissolved in ethyl acetate, transferred to small amber vials, dried under a stream of nitrogen gas and silylated as above.

2.6. GC–MS analysis

For GC–MS analysis of silylated IPA-oxime, the injector and initial oven temperatures were 280 and 140°C, respectively. After 1 min, the oven temperature was increased to 280°C at a rate of 30°C/min and held there for 10 min. The He carrier flow-rate was 26 cm/s. For silylated IPA-oxime, ions with m/z 227, 300, 345, and 434 were monitored, and for silylated [$^2H_5$]IPA-oxime, ions with m/z 232, 305, 350, and 439 were monitored. The correction factor, R (as defined by Cohen et al. [12]), for the natural abundance of heavy isotope, was determined by first calculating the ratio of ion abundances for a major fragment ion of silylated IPA-oxime (m/z 300) to the abundances of adjacent ions (m, $m+1$, $m+2$, $m+3$, $m+4$). Similarly, the ratio of ion abundances of the corresponding $^2H_5$-labeled fragment ion (m/z 305) to the abundances of adjacent ions (m, $m-2$, $m-1$, $m+1$, $m+2$) was determined as shown below. The ratio of ion abundances for m/z 300 was divided by the ratio of ion abundance for m/z 305 to yield R which was used in the isotope dilution equation shown in Section 3.2.

$$\frac{m/z\ 300}{(m/z\ 300 + m/z\ 301 + m/z\ 302 + m/z\ 303 + m/z\ 304)} = 0.534$$

and

$$\frac{m/z\ 305}{(m/z\ 303 + m/z\ 304 + m/z\ 305 + m/z\ 306 + m/z\ 307)} = 0.548$$

$$R = \frac{0.534}{0.548} = 0.974$$

3. Results and discussion

3.1. Comparison of IPA derivatives for the analysis of IPA in Arabidopsis

Since IPA is unstable in solution, measuring IPA directly in plant tissue is problematic and derivatization is necessary for reliable quantification. Wightman and Cohen used dinitrophenylhydrazine (DNPH) to generate the dinitrophenylhydrazone of IPA in order to identify IPA in mung bean seedlings [13]. They used TLC and IR for confirmation, after isolating IPA-DNPH from several hundred grams of tissue [13]. Badenoch-Jones et al. obtained mass spectral confirmation for the endogenous presence of IPA in pea root nodules [6], and Cooney and Nonhebel used PFB to stabilize IPA for mass spectral quantification in tomato [5]. Initially, we used PFB to derivatize IPA. Based upon the A_{278} profile of the HPLC eluent, we determined that IPA-PFB was not a major product (data not shown) and, therefore, PFB was not used for derivization of IPA in *Arabidopsis* crude extracts. Of the other two reagents, PFPH and hydroxylamine, we found that IPA-oxime was the easiest to purify from *Arabidopsis* crude extracts in reasonable yields. Fig. 1b–d shows the structures of methylated IPA-PFB, methylated IPA-PFPH, and silylated IPA-oxime.

Methylated IPA-PFPH was analyzed by GC–SIM-MS by monitoring the molecular ion (m/z 397) and fragment ions m/z 130 and 155 (data not shown). We found that methylated IPA-PFPH was only stable for 3–4 days at −80°C, and this derivative isolated from *Arabidopsis* had significant contaminants at m/z 130 and 155. The presence of contaminants in samples was compounded by poor GC separation. We were unable to eliminate this contamination by further purification of the sample prior to GC–MS, or by changing the GC parameters and, therefore, did not use PFPH in further experiments.

The full scan spectrum (from m/z 200 to 550) of a silylated mixture of IPA-oxime and [$^2H_5$]IPA-oxime is shown in Fig. 2. Silylated IPA-oxime yielded a molecular ion at m/z 434 and major fragment ions at m/z 227, 300, and 345. Silylated [$^2H_5$]IPA-oxime yielded a molecular ion at m/z 439 and major fragment ions m/z 232, 305 and 350.

In order to determine the utility of each of the

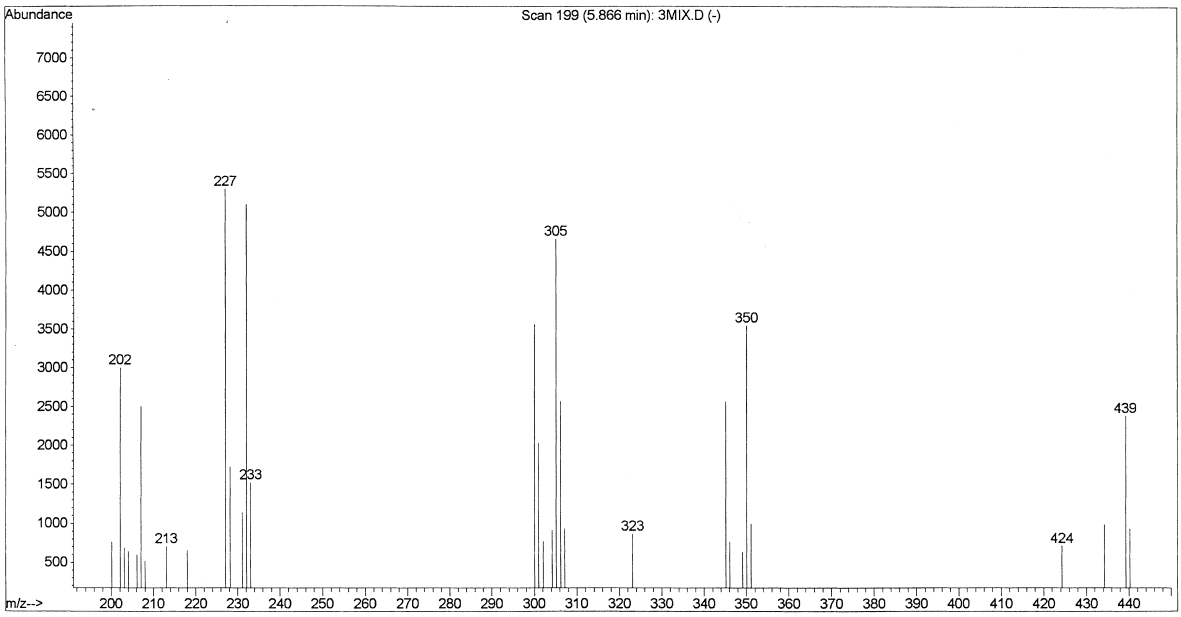

Fig. 2. Full scan spectrum from m/z 200 to 550 of a silylated mixture of [$^2H_5$]IPA-oxime and unlabeled IPA-oxime.

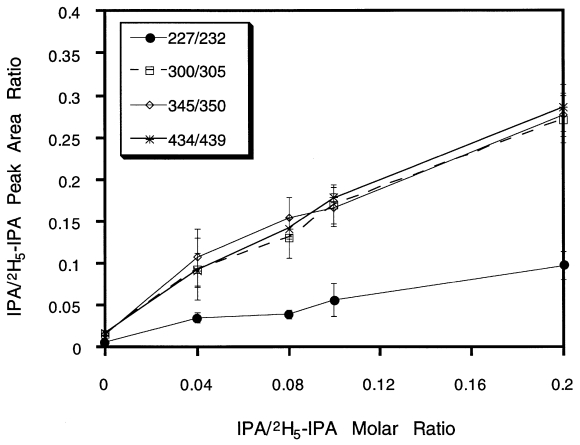

Fig. 3. Calibration plot of molar ratios of unlabeled IPA to [$^2H_5$]IPA against the peak area ratios derived from the molecular ions and major fragment ions. The amount of [$^2H_5$]IPA was held constant and unlabeled IPA was varied. Samples were prepared in triplicate.

deuterium-labeled ions for use in quantification of endogenous IPA, we generated a calibration plot (Fig. 3). Mixtures of [$^2H_5$]Trp and unlabeled Trp (in which [$^2H_5$]Trp was kept constant and unlabeled Trp was varied) were used to synthesize IPA which was subsequently derivatized (with hydroxylamine), silylated, and analyzed by GC–MS. Fig. 3 shows the plot of the molar ratios of IPA to [$^2H_5$]IPA versus the peak area ratios (of the silylated oximes) for these mixtures. Ideally, the slope should be close to 1, indicating that the peak area ratio obtained from GC–MS analysis is the same as the molar ratio. The observation that the slope was greater than 1 for the molecular ions (m/z 434 and 439) and fragment ions of m/z 300, 305, 345 and 350 indicated that deuterium was being lost during mass spectral fragmentation. This is also apparent in Fig. 2; the ratio of $^2H_5$-labeled ion abundance to unlabeled ion abundance decreased with the mass of the fragment ions. While deuterium was lost during mass spectral fragmentation, the relationship between molar ratio and peak area ratio for these three ion pairs was linear over a range of molar ratios tested (from 0.04 to 0.2, Fig. 3). Therefore, when the molar ratio of IPA to [$^2H_5$]IPA is 0.04–0.2, the labeled compound is a reliable internal standard and abundances of the ions with m/z 305, 350 and 439 can be used in the calculation of endogenous IPA. The slope for the ion pair with m/z 227 and 232 varied significantly from that of the other three ion pairs and was not used for the quantification of endogenous IPA.

Mass spectral identification of IPA in *Arabidopsis* is shown in Figs. 4 and 5. Fig. 4 is a full scan spectrum (from m/z 200 to 550) of silylated oximes of endogenous *Arabidopsis* IPA and [$^2H_5$]IPA (the internal standard) extracted from 7-day-old seedlings. Fig. 5 is a selected ion chromatogram of a silylated mixture of IPA-oxime and [$^2H_5$]IPA-oxime extracted from 12-day-old seedlings. Ions at m/z 300 and 434 are a major fragment ion and molecular ion of the silylated IPA-oxime, respectively; ions at m/z 305 and 439 are a major fragment ion and molecular ion of silylated [$^2H_5$]IPA-oxime, respectively. The GC retention time of the $^2H_5$-labeled fragment ion and molecular ion were virtually identical but slightly less than for the corresponding fragment ion and molecular ion of endogenous IPA (Fig. 5). Consequently, integration is from the left-most edge of the [$^2H_5$]-labeled ion peak to the right-most edge of the endogenous ion peak.

3.2. Quantification of IPA in Arabidopsis

IPA levels in *Arabidopsis* were calculated based upon a modification of the following isotope dilution equation [13]:

$$Y = \left(\frac{C_i}{C_f} - 1\right) \cdot \frac{X}{R} \cdot \frac{1}{FM}$$

where Y is the amount of IPA in the tissue; C_i is the initial ratio of [$^2H_5$]IPA to the total of [$^2H_5$]IPA and unlabeled IPA (assigned a value of 1). C_f is the final ratio of [$^2H_5$]IPA to the total of [$^2H_5$]IPA and unlabeled IPA (based upon the abundance of ion with m/z 300). C_f is the ratio of the abundance of the ion with m/z 305 to the sum of the abundance of ions with m/z 305 and 300. X is the amount of [$^2H_5$]IPA that was added as the internal standard (quantified by HPLC). R is the correction factor that accounts for the natural abundance of $m+1$, which was 0.974 in the case of the fragment ion with m/z 300. FM is the fresh mass (in grams) of the tissue. Based upon this equation, the levels of IPA in seedlings ranging in age from 5 to 12 days were calculated and are shown in Table 1. The levels of

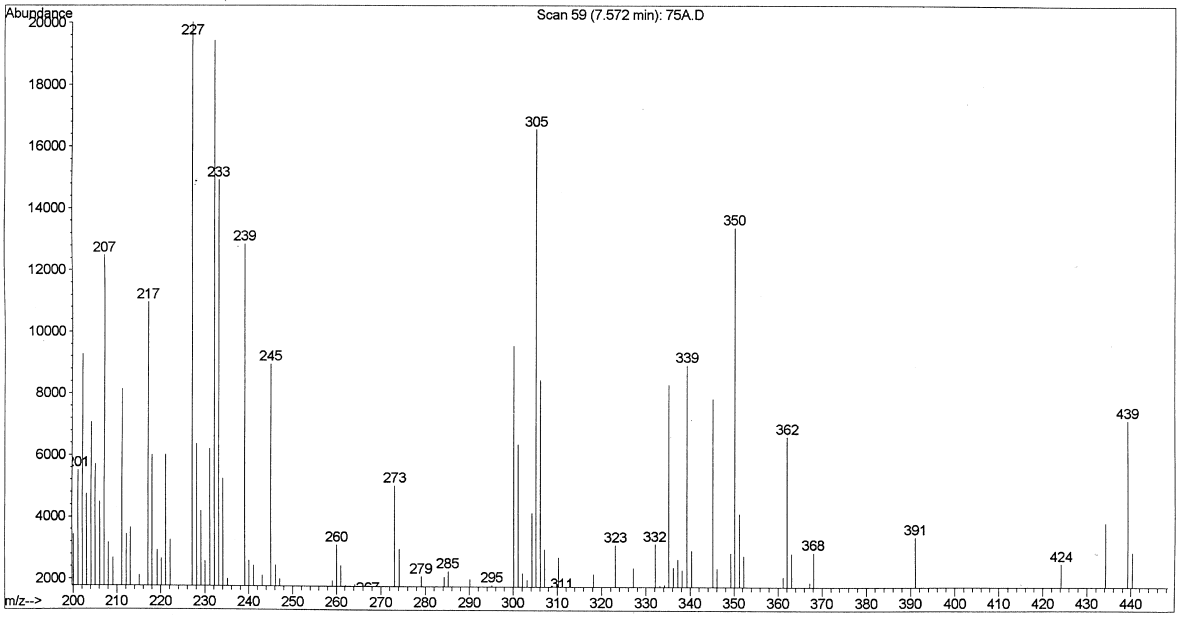

Fig. 4. Full scan spectrum from m/z 200 to 550 of silylated [$^2H_5$]IPA-oxime (internal standard) and the oxime of endogenous IPA isolated from 7-day-old *Arabidopsis* seedlings.

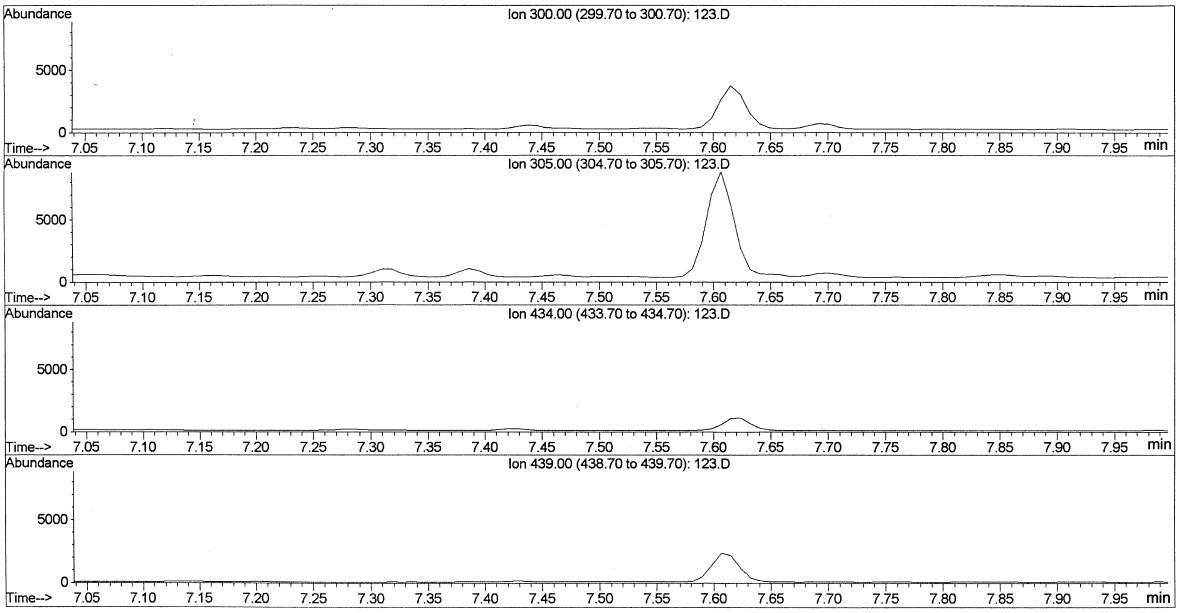

Fig. 5. Selected ion chromatogram of a silylated [$^2H_5$]IPA-oxime and the oxime of endogenous IPA isolated from 12-day-old seedlings. Selected ions are the molecular ions with m/z of 439 and 434, respectively, and the fragment ions with m/z of 305 and 300, respectively.

Table 1
Endogenous IPA levels in *Arabidopsis* seedlings

Age of seedling (days)	IPA (ng/g FM)
5	5.73±1.20
7	13.08±0.95
9	11.89±2.69
12	3.85±0.92

Values are given in ng/g fresh mass±the standard error from three independent samples.

IPA ranged from 4 to 13 ng/g FM, similar to the values obtained by Cooney and Nonhebel for tomato [5]. These values are also within the same order of magnitude as those for free IAA in tissue of the same age ([14]; Tam and Normanly, unpublished data).

We have found that hydroxylamine is a suitable reagent for derivatization of IPA, and the product, IPA-oxime, is comparatively easy to isolate from *Arabidopsis* crude extracts for subsequent GC–SIM-MS analysis. Now that we have established that IPA is a native compound in *Arabidopsis*, we can apply this method to stable isotope labeling studies in order to establish whether IPA is an intermediate in IAA biosynthesis.

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