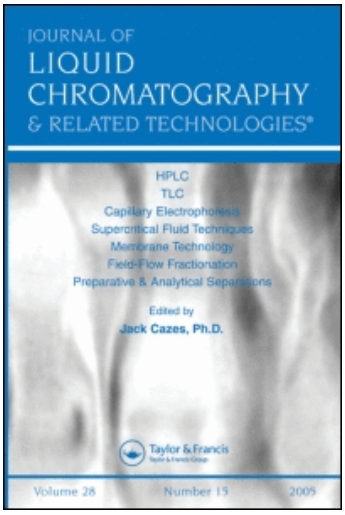

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QUANTITATION OF INDOLE-3-ACETIC ACID BY LC WITH ELECTROCHEMICAL DETECTION IN ETIOLATED HYPOCOTYLS OF *LUPINUS ALBUS*

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ABSTRACT

The objective of this work was to develop a simple procedure to determine the level of the plant hormone indole-3-acetic acid (IAA) in etiolated lupin hypocotyls using liquid chromatography with amperometric detection. A C18 reversed-phased column was used with a potential of +0.85V applied to the carbon electrode. We used two different auxin extraction procedures from lupin hypocotyl and similar quantities of indole-3-acetic were obtained. The method was capable of determining indole-3-acetic acid with a limit of quantification of 2.2 pg. The identity of the indole-3-acetic acid in lupin was confirmed by GC-MS. The content of this hormone in three zones of hypocotyl (apical, central, and basal) was estimated, and a concentration gradient: apical > central >

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basal, was observed with mean values of 657 ng IAA/g FW, 261 ng IAA/g FW, and 145 ng IAA/g FW, respectively.

INTRODUCTION

Plant tissues contain many indolic compounds. Indole-3-acetic acid (IAA) and their related-metabolites are of physiological significance. IAA, the most important auxin, is a phytohormone, which plays an important role in aspects of plant development, such as: cellular growth, rooting, vascular differentiation, apical dominancy, tropisms, partenocarpy, etc.(1)

Since the days of the use of bioassays to determine auxin activity, the methods of detection and quantitation of indolic compounds have evolved considerably. Currently, the most used methods are the radioimmunoassay, ELISA and gas-liquid chromatography with mass spectrometry detection (GC-MS).(2-4) High performance liquid chromatography coupled to a fluorescence detector is also widely utilized, because it offers excellent sensitivity and selectivity, and in contrast to GC-MS, the derivatization of samples is not required. In whichever chromatographic technique, the utilization of internal standards labelled with heavy or radioactive isotopes is essential for the precise correction of data due to the losses that occur during the isolation and purification steps. Another technique used for the determination of indolic compounds is liquid chromatography/electrochemistry (LC-EC). In spite of its excellent sensitivity, electrochemical detection coupled to LC is rarely used to determine indolic compounds in plant material.

In this paper, we present the development of a method to the determination and quantitation of free indole-3-acetic acid in etiolated hypocotyls of lupin (*Lupinus albus* L.) by LC-EC. Also, the spatial distribution of this plant hormone in different zones of the hypocotyl was studied.

EXPERIMENTAL

Reagents

Indole-3-acetic acid (IAA) was purchased from Aldrich Chemical Co (Madrid, Spain). Radioactive indole-3-acetic acid (5-^{3}H-IAA, specific activity 926 GBq mmol^{-1}), obtained from Amersham Int. (Buckinghamshire, UK), was used as internal standard to evaluate the losses during the isolation and purification steps of plant extracts. Scintillation cocktail, Ecoscint H, was obtained from National Diagnostics (Atlanta, USA). All solvents and buffers were HPLC-quality.

Plant Material

Seeds of *Lupinus albus* L.(cv. Multolupa) were kindly provided by Dr. J.M. Pozuelo from the Centro de Ciencias Medioambientales-CSIC (Madrid, Spain). Seeds were soaked in water for 24 h and grown in damp vermiculite at 25 ± 1°C in darkness. Etiolated uniform 7-day-old seedlings (65 ± 5 mm in length) were taken and the root and the cotyledon separated. The IAA distribution study was carried out with hypocotyls cut into three equal sections of 20 mm in length.

Extraction and Purification of IAA

Two different auxin extraction methods were used. The first procedure (Extr-1*)* was developed from that of reference (3) with some modifications. Briefly, lupin hypocotyls (1 g) were homogenised with an Ultra-Turrax T25 (IKA, Germany) in 50 mM sodium phosphate buffer (pH 8.0) containing 5 μM butylated hydroxytoluene (BHT) as antioxidant. The homogenate was maintained during 15 h at 4°C in darkness with minimal shaking, in order to ensure complete extraction of IAA. Afterwards, it was filtered under vacuum through a 0.45 μm nylon filter. The initial purification was done in two steps by solvent-partitioning using ethyl acetate and 50 mM sodium phosphate buffer (first at pH 8.0 and second at pH 3.0), as shown in Figure 1.

The two organic phases (~ 12 mL each) were evaporated under vacuum. Dry residue was redissolved in 1 mL of mobile phase, filtered (0.2 μm), and analysed by LC-EC. In the second extraction procedure used (Extr-2) (see Figure 1), etiolated hypocotyls (1 g) were cut into sections (3 mm) and, without homogenization, were submerged in vials containing 12 mL ethyl acetate with BHT (5 μM), for 15 h at 4°C in darkness with minimal shaking. The sections were then discarded and the solvent evaporated under vacuum. Dry residue was redissolved in 1 mL of mobile phase, filtered (0.2 μm) and analysed by LC-EC.

Analysis by LC-EC

Samples were analyzed using a Beckman System Gold liquid chromatographic instrument with programmable injector, thermostatted samples (4°C), and column (30°C), diode array detector and a Hewlett-Packard electrochemical detector with thermostatted cell (30°C), glassy carbon-PTFE support working electrode, and a solid state Ag/AgCl reference electrode. Samples (10-60 μL)

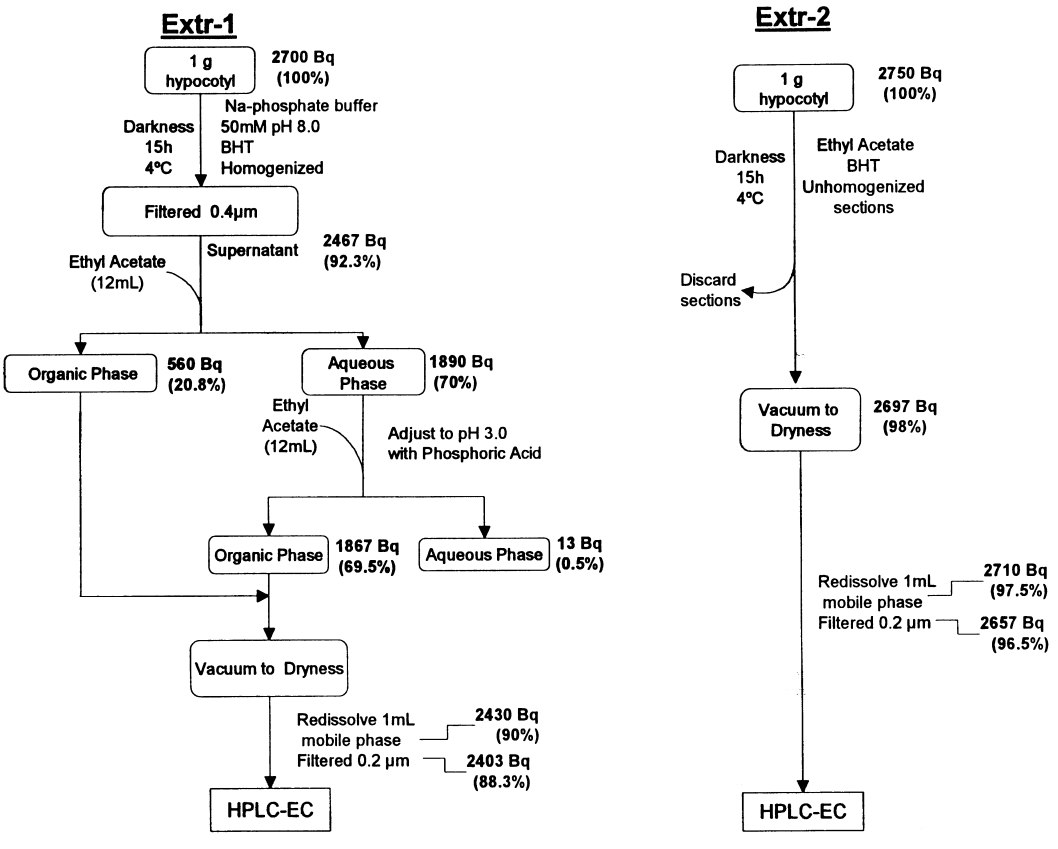

Figure 1. Extraction procedures of IAA from etiolated lupin hypocotyls. For each procedure (Extr-1 and Extr-2), the different steps prior to the LC-EC analysis of extracts are shown. To evaluate the losses in each step, the radioactivity present due to ^{3}H·IAA, used as internal standard (quantitative data in Bq) and in parenthesis the percentage with respect to the initial radioactivity, are shown.

were injected onto a C18 RP-ODS-Spherisorb column (250 x 4.5 mm, 5 μm particle size). A single mobile phase consisting of water:acetonitrile:glacial acetic acid (78:20:2) (pH 3.0) was used to elute IAA at a flow-rate of 0.7 mL/min. Mobile phase also contained 1 mM KCl according to the recommendation by this reference electrode. The electrochemical detector was set at a fixed voltage of +0.85 V on the most sensitive scale (0-50 nA). To preserve the electrode, we used a 2-position valve that permitted us to discard the first 15 min of elution, avoiding "excessive contamination" of the working electrode. At this elution time (15 min), we closed the valve and the IAA peak was detected with a retention time (Rt) of 27.5 min.

Analysis by GC-MS

IAA standard solutions, and collected and pooled fractions from each extraction procedure (Extr-1 and Extr-2) were analyzed by gas-liquid chromatography with mass spectrometry (70 eV, GC-MS).

We collected the IAA peak (Rt=27.5 min) of successive injections, taking fractions from 25.0 to 29.0 min in each sample. These collected fractions (2.8 mL each one; total volume 14-16 mL) were dessicated after the exchange of the mobile phase by ethyl acetate. Dry samples were derivatized to obtain the trimethylsilyles of IAA (TMS-IAA)(5) and analyzed by GC-MS. Also, samples were re-injected in the LC-EC to check the purity. Samples were introduced in a Hewlett-Packard 5890 chromatograph with an HP-5 MS (30 m x 0.25 mm) column using a flow of helium at 1 mL/min. A temperature program of 250 to 280°C at 10°C/min was used. TMS-IAA was made in 100 μL pyridine with 100 μL hexamethyldisilazane and 50 μL trimethylchlorosilane. TMS-IAA identification was done by comparing the mass spectra of samples with the standard IAA mass spectrum and, also, using the selective ion monitoring mode of the instrument.

Extraction Yield

To evaluate the losses of IAA during the extraction and purification procedures, tritiated-IAA (5-^{3}H-IAA) was used as internal radioactive label. In both procedures, an exact amount of ^{3}H-IAA (~1700 Bq) was added at the start and, in each purification step, aliquots (50 μL) were transferred to vials containing 10 mL scintillation cocktail (see Figure 1). The radioactivity present was estimated in a LKB-Rackbeta model 1211 liquid scintillation counter (Turku, Finland).

RESULTS AND DISCUSSION

Chromatographic System

Figure 2A shows a representative chromatogram of a standard solution of IAA using the electrochemical detector coupled to the LC. IAA eluted at 27.5 min. An oxidizing potential of +0.85 V was selected, but previously, we studied the response of IAA at different potentials. Figure 2B shows the voltammogram of IAA made in the interval +0.2 to +1.0 V, in increments of 0.1 V. The response of the electrode is significant beyond +0.4 V. The maximum response of IAA is reached at +0.85 V using water:acetonitrile:glacial acetic acid (78:20:2) (pH 3.0) as the mobile phase. Selecting +0.85 V as the voltage for the electrochemical detector, a

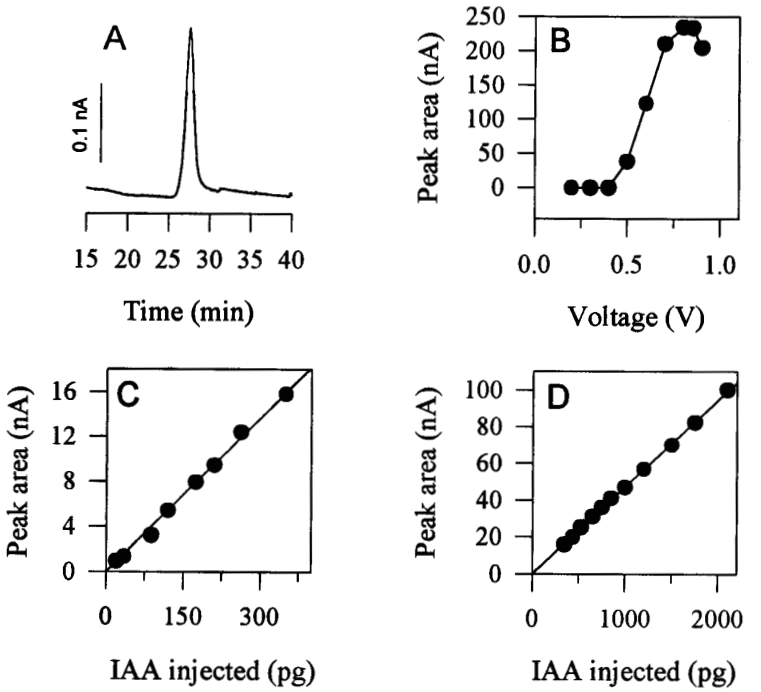

Figure 2. Chromatographic analysis of standard IAA. Panel A, Representative chromatogram of standard IAA (0.5 ng) registered at +0.85 V with the electrochemical detector coupled to LC; Retention time= 27.5 min; Panel B, Voltammogram of standard IAA. Panel C, Calibration curve of standard IAA using HPLC-EC at +0.85V in the range 20-350 pg ($y=0.045 \cdot x$; $r^2=0.993$; n=6). Panel D, Calibration curve of standard IAA in the range 350-2100 pg ($y=0.047 \cdot x$; $r^2=0.998$; n=6) under similar conditions.

calibration curve of signal response, versus IAA standard concentration injected onto the LC, can be made (Figure 2C and 2D). Excellent linearity appears across the two concentration ranges assayed: from 20 to 350 pg (Fig. 2C) and from 350 to 2100 pg (Fig. 2D). The limit of detection (LOD) and the limit of quantification (LOQ) were obtained according to the recommended guidelines.(6) Thus, LOD was calculated at a signal-to-noise ratio of 3 and LOQ at a signal-to-noise ratio of 10. In the present method, using the calibration curve in Fig. 2C, LOD and LOQ values of 0.66 and 2.2 pg, respectively, were calculated. Using the diode array detector (at 215 nm) the limit for IAA is around 10 ng (data not shown). These data are better when compared with those of other authors, where a limit of ca. 50 pg has been established for the electrochemical detector and the same amount for the fluorimetric detector coupled to LC under normal conditions.(3,7) However, the detection of as little as 1 pg using a fluorimetric detector(8) and as little as 0.9 fg using capillary electrophoresis with amperometric detection(9) have been reported.

IAA Quantitation in Lupin Hypocotyls

Two procedures for IAA extraction and purification have been developed (Figure 1). The procedure Extr-1 was more complicated, since a tissue homogenisation, filtration, and two solvent-partitioning steps were used. In contrast, Extr-2 procedure was simpler and tissue homogenization was not necessary. After the respective steps, dry extracts were redissolved in mobile phase, filtered, and different volumes were injected into the LC-EC. Figure 3 shows a represen-

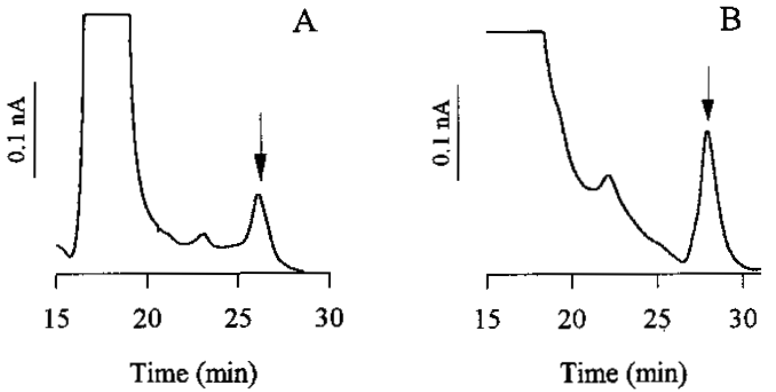

Figure 3. Chromatographic analysis of endogenous IAA. Representative chromatograms of lupin hypocotyl samples using each extraction procedure: Extr-1 (Panel A) and Extr-2 (Panel B). Arrows show the IAA peak (R_t= 27.5 min).

tative chromatogram of a hypocotyl sample from each extraction procedure. The peak with a retention time of 27.5 min (similar to the IAA standard, Fig. 2A) suggests that it is free endogenous IAA. In both extraction procedures, a similar chromatographic profile was observed. If a known amount of standard IAA was added to a hypocotyl sample, the peak at 27.5 min was increased proportionally to the quantity of IAA added, showing that this peak was probably the free IAA present in hypocotyls. We have checked that the peak of Rt=27.5 min is totally degraded by the enzymatic system IAA-oxidase. Also, we have not detected any interference of the biological matrix in the signal intensity compared to IAA standard injections.

To verify the nature of the peak at 27.5 min present in the HPLC-EC analysis, we obtained its mass spectrum by GC-MS of the trimethylsilyl-derivatives of IAA (TMS-IAA). In successive injections, the fractions containing endogenous IAA from hypocotyl samples were collected at the outlet of the electrochemical detector. After the exchange of the mobile phase by ethyl acetate and dessication, dry extract was derivatized to obtain the TMS-IAA and analyzed by GC-MS. The standard IAA and IAA samples from hypocotyls had identical mass spectra. Also, the identification was confirmed using the selective ion monitoring mode. The representative ions (*m/z*) of TMS-IAA: 319 (M+), 304, 247, 232, 202, and 130 (quinolinium ion) appear, demonstrating unequivocally the identity of the IAA, in agreement with other authors.(3,10)

Measurement of the radioactivity present in each purification step, using ^{3}H-IAA as internal standard, permitted us to evaluate the loss from each procedure, revealing a losses of 11.7 ± 0.6% in the procedure Extr-1 and of 3.5 ±0.2% in the case of Extr-2 (Fig. 1). Thus, for the precise quantitation of endogenous IAA present in hypocotyls, the respective integrated area of peaks were correlated with the calibration curve (Fig. 2C or 2D) and the values were corrected using the percentage losses in each extraction procedure.

The analysis of whole lupin hypocotyls shows that the level of IAA in this organ (~ 65 mm) was around ~350 ng IAA/g FW. Generally, in hypocotyls, three zones appear differentiated by their growing potential. The IAA distribution in the three zones of hypocotyls is shown in Table 1. An IAA concentration gradient appears following the sequence: apical > central > basal. In Table 1 it can also be seen that the quantitation of IAA is very similar using either procedure Extr-1 or Extr-2. The IAA gradient indicates that the highest IAA level appear in the apical zone, which is next to the cotyledon area. IAA is biosynthesized in the transition tissue (meristem) between the cotyledons and the apical hypocotyl zone. Thus, IAA is transported from the biosynthesis tissue to the whole hypocotyl, diminishing its concentration still further. This spatial distribution of IAA is in accordance with their growing potential: the zones with highest growth rate are also the zones with highest endogenous IAA.(11).

Table 1. Quantitation of Endogenous IAA by LC-EC in the Three Zones of Etiolated Lupin Hypocotyls Using Both Auxin Extraction Procedures

	Endogenous Free IAA ($ng \cdot g\ FW^{-1}$)		
	Extraction Procedure		
Hypocotyl Zone	Extr-1	Extr-2	Mean
Apical	651 ± 40[1]	663 ± 36	657
Central	252 ± 10	270 ± 20	261
Basal	148 ± 6	142 ± 18	145

[1]Mean value ± SE (n=6).

To conclude, the method presented for the estimation of free IAA using an LC-EC is easy and rapid to use, and can estimate low picogram levels of IAA, sensitivity similar to that achieved using fluorescence detection. Of the two procedures used for IAA extraction and purification, Extr-2 procedure is clearly advantageous because tissue homogenization is not necessary avoiding vacuum-filtration and solvent-partitioning steps. For etiolated lupin hypocotyls, Extr-2 procedure is recommended and the LC-EC method can measure IAA in as little as 50 mg of tissue. At present, we are adapting the method to measure endogenous IAA in green plant material, such as leaves, petioles, etc. in which additional interference due to photosynthetic pigment may perhaps be observed.

ACKNOWLEDGMENTS

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