# A Simple Procedure for Purification and Determination of Indoleacetic Acid by Gas Chromatography

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**Abstract:** A simple procedure for the purification of indoleacetic acid (IAA) prior to Gas Chromatography (GC) and Gas Chromatography - Mass Spectrometry (GC-MS) analyses was developed using insoluble polyvinylpyrrolidone (PVP) in the column. Moreover instead of using radioactive internal standard, non-radioactive indolebutyric acid (IBA) was used in the estimation of IAA. The new technique was found adequate for the purification of IAA for subsequent analysis by GC.

**Key Words:** GC, PVP, IBA, IAA.

## Introduction

Techniques for identification and quantitative analysis of the plant hormone IAA have been improved by utilization of High Performance Liquid Chromatography (HPLC) and GC-MS. McDougall *et al.* [7] use proper internal standards, for example ($^{13}C_6$) IAA [3, 7 & 10]. Solvent extraction, concentration and solvent partitioning have also been used for IAA purification. Bandurski and Schuize (1974, 1977) made quantitative studies of the IAA content of a number of plant tissues and based on the alkali lability of the IAA compounds, they were classified as free, ester and peptidic forms.

Thin layer Chromatography (TLC) is simple technique and IAA can be detected [10]. However, TLC cannot quantify the amount of IAA derived from plant tissue. HPLC is considered one of the best method of IAA determination [9]. Kuraishi et al. (1983) used HPLC in combination with spectroflourometry as the versatile HPLC detector. This method of analysis does not require the derivatization of IAA However, HPLC cannot identify IAA. On the other hand GC makes IAA identification easier by the use of GC-MS.

In this study, an attempt was made to simplify the method particularly the purification technique for IAA assessment. Insoluble polyvinylpyrrolidone (PVP) was used in the column (15x2 cm) for the purification of IAA. Moreover, instead of using radioactive internal standard non-radioactive indolebutyric acid (IBA) was used in the estimation of IAA.

## Materials and Methods

### *Plant Materials*

Seeds of radish *(Raphanus sativus),* corn *(Zea mays),* rice *(Oryza sativa)* and wheat were used in the investigation. Seeds were washed thoroughly with deionized water and germinated for 5 days at 30°C. The germinated seeds were transferred to plastic buckets, placed in polyethylene containers filled with a culture solution and grown in a glass-house under natural light conditions for several weeks.

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**Extraction of IAA**

Fifty grams of fresh materials from the top growth of plants were homogenized with methanol in a blender and 50μL of IBA was added to the extract as the internal standard. After filtration, methanol was removed from the extract under reduced pressure at 40°C. The aqueous residue was hydrolyzed 1) for I h in 1 M NaOH at room temperature for IAA (free + ester), and 2) for 3 h in 7 M NaOH at 100°C for IAA (free + ester + peptidic). For hydrolysis at 7 M NaOH IAA, the solution was prepared in a beaker and poured into a flask, and then the flask was placed on a heating mantle provided with a gas inlet and outlet tube and the flask was heated to 100°C. The $N_2$ gas was passed through the hydrolysis mixture. In both cases, the hydrolysate was then placed into an ice bath and adjusted to pH 2.2 with concentrated phosphoric acid and extracted two times with dichloromethane.

The dichloromethane was reduced in volume to 100 mL and extracted 2 times with 250 mL of 1M $NaHCO_3$ and centrifuged for 5 min at 3,000 rpm.The centrifuged sample was adjusted to pH 2.2 with concentrated phosphoric acid and extracted 3 times with dichloromethane. The dichloromethane phase was collected into a beaker containing anhydrous $Na_2SO_4$ to remove water. The dichloromethane phase was filtered and evaporated to dryness under reduced pressure at 40°C to get a dichloromethane soluble acidic fraction (Fig. 1).

**Purification Technique for IAA**

***Purification by insoluble polyvinylpyrrolidone (PVP) column***

The dichloromethane-soluble acidic fraction was dissolved in a small volume and applied into a PVP column (15x2cm), which was equilibrated with a mixture of 0.2M $Na_2HPO_4.12H_2O$ and 0.1 M citric acid solutions (pH 8.0) and then eluted with the same mixture. The first 60mL of the eluted fraction was discarded and the following fraction (Suspected IAA and IBA as internal standard) were collected up to 300 mL. The fraction was acidified to pH 2.2 with phosphoric acid and extracted 3 times with dichloromethane. The dichloromethane extract was collected and combined in a beaker containing anhydrous $Na_2SO_4$. After filtration the dichloromethane was evaporated to dryness under reduced pressure at 40°C to get a purified extract.

**Determination of IAA by GC**

For the quantitative estimation of IAA, 50μL of 1000 ppm indole butyric acid (IBA) solution as internal standard and 0.02% butylated hydroxytoluene (BHT) to protect against oxidative degradation of IAA were added to a sample weighing 50 g. Five microliters of the methylated final extract was applied to a GC (Shimadzu, Model GC-7A); for 1 M NaOH-labile IAA, column: SP-2401 (uniport, HPO 3x300 cm), temperature: 210°C, carrier gas: He 50mL/min, detector: flame thermoionic detector (FTD); for 7M NaOH-labile IAA, column: ULBON HR-1701 (0.53 x 15m), temperature: 180°C, He: 50mL /min detector: FTD.

The content of IAA was calculated from the peak area of IAA and compared with that of IBA as internal standard using a chromatopac (Shimadzu, model C-RIA).

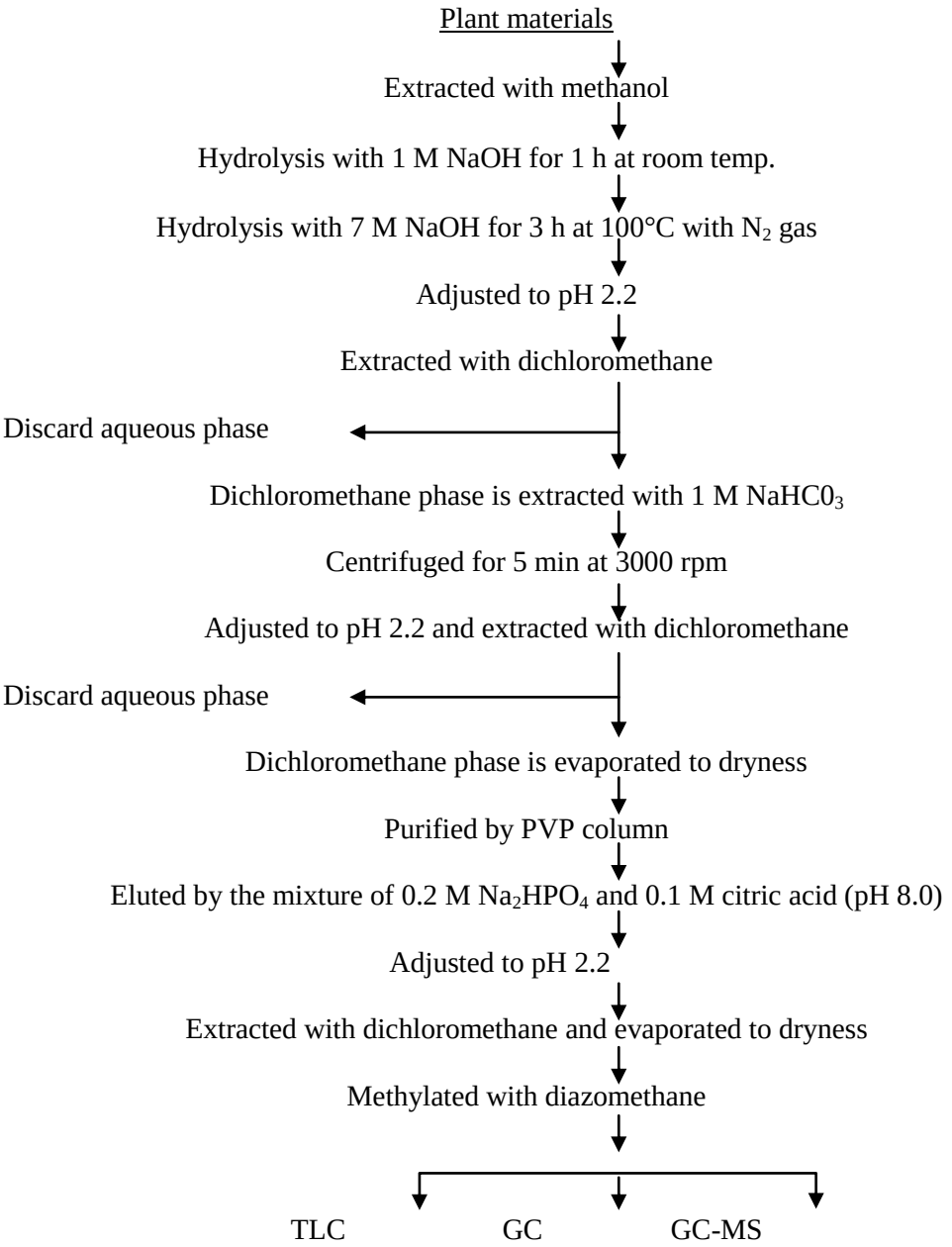


***Fig. 1:*** *Extraction, purification, identification and determination of IAA.*

**Identification of IAA by GC-MS**

The GC-MS analysis of methylated IAA was performed with a Hitachi GC-MS, Model G-300 and M-2000 AM. The GC conditions were as follows: column =Neutra Bond-ll; temperature=190-220°C. Before analysis, the purified sample was methylated with a

diazomethane ether solution. Five micro liters of the methylated final extract were subjected to GC-MS and compared with the methylated authentic IAA.

**Recovery of IAA and IBA**

The quantitative determination of the amount of IAA in the plant tissue was made by the IBA as internal standard in the plant tissue. Three experiments were carried out in order to measure the recovery of IAA and IBA during purification process through PVP-column (Fig. 2). $NaH_2PO_4$. $2H_2O$ was used in these experiments instead of plant extract because of its pH similarity. About 2g of $NaH_2PO_4$ $2H_2O$ was dissolved in deionized water to which authentic IAA and IBA (50μL each of 1,000 ppm) were used. The procedure is same as discussed above.

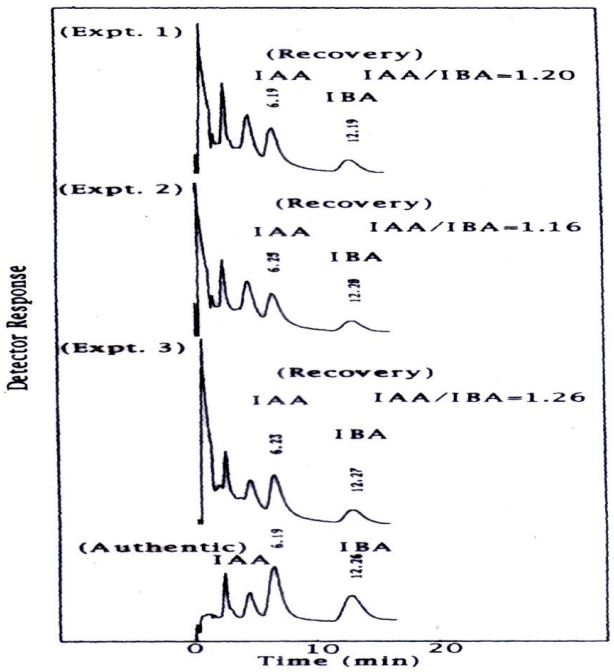


***Fig. 2 :*** *Gas chromatogram of the recoveries of IAA and IBA after undergone PVP-column purification process in 3 experiments and authentic IAA and IBA*

**Results**

Four Chromatograms showing IAA and IBA as detected by GC are shown in figure 2. Retention times of peaks of IAA and IBA of three experiments coincided completely with that of authentic IAA and IBA. The retention times of peaks of IAA and IBA for three experiments were 6.19, 6.25, 6.23 and 12.19, 12.28, 12.27 while authentic IAA and IBA were 6.19 and 12.26 minutes, respectively. The putative methylated IAA in radish shoots was identified by gas chromatography-mass spectrometry (GC-MS) and the results are shown in figure 3 and 4. The gas chromatogram in figure 3 shoots the peak of putative

IAA and internal standard IBA from radish shows and authentic IAA and IBA. The peak of putative IAA from radish shoots coincided with the peak of authentic IAA with the same retention time. The mass spectra (Fig. 4) of the putative IAA from radish shoots showed high peaks at 189 and 130 m/e. These peaks were identical with those of the authentic IAA. The peak at 189 m/e is the parent peak of the methylated IAA and the peak at 130 m/e is the base peak of the methylated IAA. These results indicate the presence of IAA in radish shoots.

The recovery of IAA through PVP column was based from the following formula:

$$\% \text{ Recovery of IAA} = \frac{\text{peak area of PVP sample of methylated IAA}}{\text{peak area of standard methylated IAA}} \times 100$$

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$$\% \text{ Recovery of IAA} = \frac{\text{Area of PVP sample of methylated IBA}}{\text{Area of standard of methylated IBA}} \times 100$$

The recovery of IAA for 3 (three) experiments were 63.4%, 46.9%, 52.8% and for IBA 50.3%, 38.9%, 45.5%, respectively. The percentage of recovery of IAA was higher than that of IBA. The ratios of the recoveries of IAA was higher than that of IBA. The ratios of the recoveries of IAA/IBA were 1.26, 1.20 and 1.16 respectively and the average was 1.21. So the estimation of IAA in our study was calculated as follows:

Results obtained from the experiments ÷ 1.21.

**Discussion**

Several experiments were conducted to evaluate the purification technique of IAA determination. Extraction of IAA and purification techniques of acid fraction was done by insoluble polyvinylpyrrolidone (PVP) Column. A discussion to the results obtained are presented in the paper.

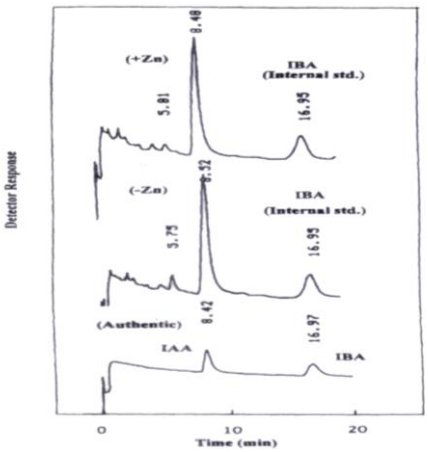


***Fig. 3: Gas** chromatogram of putative methylated IAA (peptidic + ester + free) from control (+Zn) and Zn-deficient (-Zn) radish shoots and authentic IAA and IBA.*

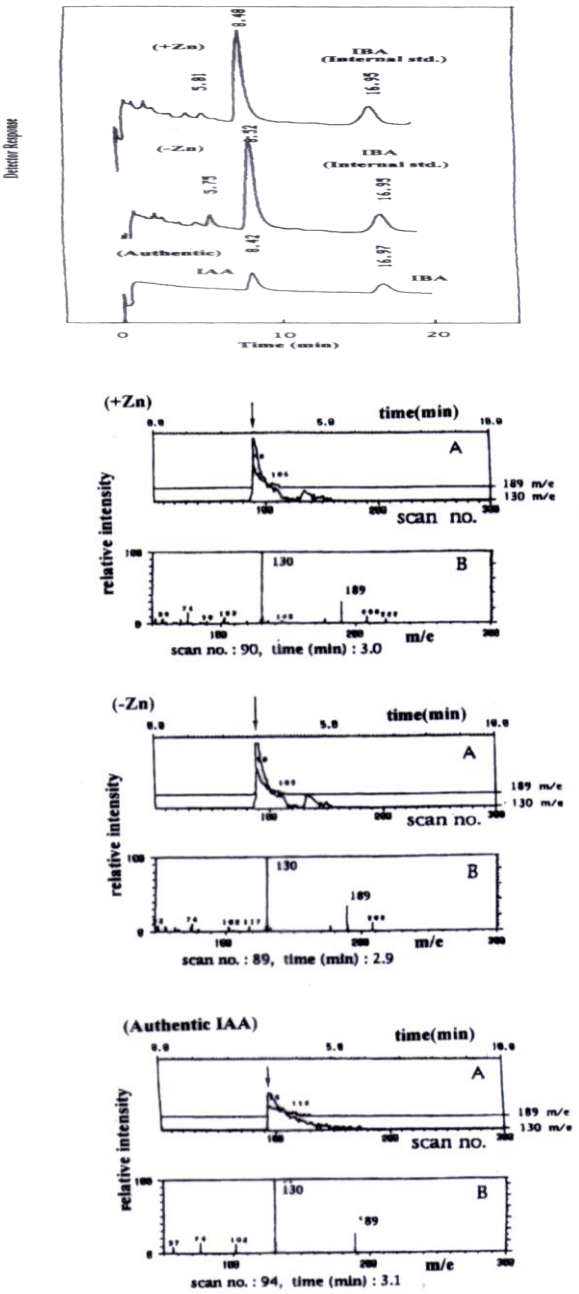


***Fig. 4 :*** *Electron-impact mass chomatogram and mass spectra of putative methylated IAA (peptidic + ester + free) from control (+Zn) and Zn-deficient (-Zn) radish shoots and authentic IAA (A: mass chromatogram, B: mass spectra).*

Results are shown in Table 1, 2 and 3. 50μL nonradioactive IBA was used to the plant extract as internal standard in the estimation of IAA. The content of IAA (peptidic + ester + free) and IAA (ester + free) (Table 1) in different three experiments detected in Zn deficient and control radish shoots were found to be comparable [4]. By the same procedure, the content of IAA (peptidic + ester +free) in three experiments detected (Table 2) in boron deficient radish shoots were found higher than that of control radish shoots [5]. The content of IAA (peptidic + ester +free) in Zn deficient maize, wheat and rice were also investigated (Table 3).

***Table 1:*** *The 7M NaOH-labile IAA (peptidic + ester + free) and 1N NaOH-labile IAA (ester + free) contents in control (+Zn) and Zn-deficient (-Zn) following purification prior to analysis by GC-MS in radish shoots.*

| | | IAA μg/100g fresh weight | |
|---|---|---|---|
| Expt. No | DAT | +Zn | -Zn |
| | | 7 N NaOH -Labile IAA | |
| 1 | 24 | 190 | 220 |
| 2 | 26 | 219 | 236 |
| 3 | 30 | 221 | 238 |
| | | 1 N NaOH - Labile IAA | |
| 1 | 24 | 10.3 | 10.1 |
| 2 | 25 | 11.5 | 10.8 |
| 3 | 31 | 17.9 | 14.9 |

DAT = days after transplanting

***Table 2:*** *The 7 M NaOH-labile IAA (peptidic + ester + free) content in control (+B boron) and boron deficient (-B) radish shoots.*

| | | IAA μg/100g fresh weight | |
|---|---|---|---|
| Expt. No | DAT | +B | -B |
| 1 | 18 | 178 | 332 |
| 2 | 21 | 214 | 331 |
| 3 | 22 | 212 | 333 |

DAT = Days after transplanting

***Table 3:*** *The 7 M NaOH-labile IAA (peptidic + ester + free) contents in control (+Zn) and Zn-deficient (-Zn) following purification prior to analysis by GC-MS in rice, wheat and maize plants.*

| | | IAA μg/100g fresh | |
|---|---|---|---|
| Plants Name | DAT | +Zn | -Zn |
| Rice | 26 | 4.5 | 5.9 |
| Wheat | 23 | 8.0 | 10 |
| Maize | 22 | 3.0 | 4.0 |

DAT = Days after transplanting

Based on these results obtained from GC of IAA determination it seems that the purification techniques employed were sufficient as evidenced from the results. Identification of the suspected IAA from the plant sample were made easier with the use of GC-MS. However, further refinement of the technique is suggestive to warrant conclusive results.

## Summary

Efforts to simplify the method of IAA determination particularly the purification techniques of the acid fraction. Insoluble polyvinylpyrrolidone in the column was used in the purification of IAA. Nonradioactive IBA was used as internal standard in the estimation of IAA. Identification was done by the method of GC-MS. The content of IAA(Table 1, 2, and 3) detected in Zn-deficient and control radish shoots, rice, wheat and maize were found to be comparable, with values of 231 (average), 5.9, 10, 4.0 and 210 (average), 4.5, 8.0, 3.0 μg $100^{-1}$ fresh weight, respectively. These results suggest that zinc nutrition does not affect the level of IAA in radish shoots, rice, wheat and maize.

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