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# IMPROVED PROCEDURE TO DETERMINE INDOLE-3-ACETIC ACID BY FLUORESCENCE DERIVATIVE

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**Key Word Index**—Indole-3-acetic acid; indole-α-pyrone; phytohormone analysis.

**Abstract**—An improved procedure is described to determine endogenous levels of indole-3-acetic acid in peach seed tissues. Unstability of indole-α-pyrone derivative is overcome by sodium hydroxide addition and further extraction in ethyl acetate. The limit of detection was under 0.9 ng/ml and the relative error lower than seven per cent, 95% confidence level. For peach seed samples relative standard deviations were close to 13 per cent.

## INTRODUCTION

Indole-3-acetic acid (IAA) has been thoroughly studied because it is a plant hormone. One of the first steps is to determine the harmone content in the plant tissues. The ideal analytical procedure must be capable of coping with the high sample diversity and very low hormone contents. Consequently, the literature contains many reports of attempts to find a sensitive and selective method to determine IAA in partially purified plant extracts.

The commonest methods of IAA assay are bioassays, immunological or physico-chemical procedures which include the fluorometric techniques [1,2]. The latter are the most sensitive but if the intrinsic fluorescence of this hormone is used they are only moderately specific. However, using the 2-methylindole-α-pyrone derivative, which can be obtained with a high quantum yield, improves selectivity and allows nanogram content determinations to be made [3]. This method has been extensively explored with samples from many diverse plants [4–10].

The measurement of the fluorescence intensities of indole-α-pyrone derivatives has two main shortcoming. Firstly, the high unstability of the derivative in aqueous acid solution causes poor reproducibility of the measurements. Secondly, the light scattering which occurs because of the high turbidity of most crude extracts of plants may cause overestimations of hormone content [4,6]. This problem can be satisfactorily resolved by synchronous derivative spectrofluorometry as described elsewhere [11].

The present paper describes the results of experiments designed to improve the indole-α-pyrone procedure; the capacity of the method to cope with problems of instability and light scattering was also investigated.

## RESULTS AND DISCUSSION

### *Absorption and fluorescence phenomena*

Any attempt to improve the Stoessl and Venis method [3] requires the determination of the optimum conditions required for indole-α-pyrone derivative formation. The compound appears to be very unstable if the original procedure described in ref. [3] is used. The rapid decomposition of indole-α-pyrone is the result of the energy input provided by the excitation light [3, 6, 7]. However, it seems reasonable to assume that an acid hydrolysis occurs. Band absorption at 450 nm, specific for IAA derivative [3], steadily decreases following first order kinetics. The hydrolysis was avoidable if the pH of the derivative solution could be of high basic value. The addition of 3 N sodium hydroxide to the derivative solution proved to be more than sufficient to stabilize the indole pyrone derivative. Under these conditions the maximum absorption wavelength occurs at 452 nm.

Despite the evident advantage of improved stability of the compound obtained by adding 3 N sodium hydroxide to the IAA derivative solution, IAA determination is still difficult when the fluorometric method is used. The relative fluorescence intensity of the derivative is very weak because of the high quenching effect of the NaOH. The alternative was a rapid extraction of the derivative using an organic solvent. For this purpose, several solvents were tested: diethyl ether, chloroform and ethyl acetate all successfully extracted the derivative. However, the high volatility of diethyl ether and the great capacity of chloroform [12] to extinguish fluorescence made them inappropriate for derivative extraction. Consequently, ethyl acetate was the solvent of choice to extract the indole-α-pyrone derivative as it was formed. This method gave efficient extraction of the derivative. The extraction yield was almost 100% as the fluorecent activity in the aqueous fraction following extraction was identical to that of a control solution containing acetic anhydride and trifluoracetic acid in 3 N sodium hydroxide.

The emission fluorescence spectrum in ethyl acetate is shown in Fig. 1A with a $\lambda_{max}$ for emission at 480 nm. In this solvent, however, there is a shift to 458 nm for maximum light absorption probably because of a weak solute–solvent interaction in the excited state which could cause the light scattering, as observed in the emission spectrum of the Fig. 1a at wavelength values below

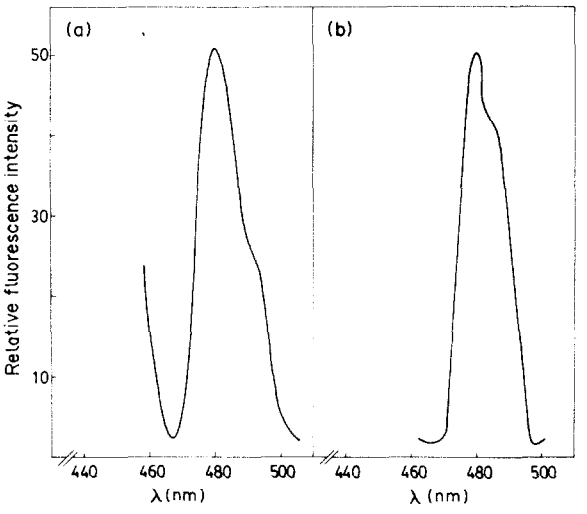


Fig. 1. (a) Fluorescence emission spectrum of the 2-methylindole-α-pyrone derivative in ethyl acetate (90 ng of IAA derivatized). (b) Synchronous fluorescence spectrum of the same solution ($\Delta\lambda = 40$ nm).

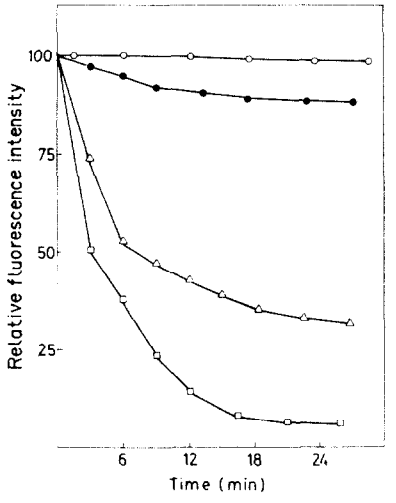


Fig. 2. Breakdown of 2-methylindole-α-pyrone in: (○) ethyl acetate; (●) 3N NaOH; (△) 1N NaOH; (□) water.

470 nm. This undesirable side effect can be avoided by using synchronous spectrofluorometry [11, 13]. This type of study was intended and the best results were for $\Delta\lambda$ of 40 nm (Fig. 1b). The maximum wavelength for emission remains at 480 nm, but emission fluorescence disappears at low values.

Derivative extraction with ethyl acetate not only improved the above fluorescence measurements, it also avoided derivative unstability. Figure 2 shows the relative fluorescence intensities of indole-α-pyrone derivatives in different solutions. The results lead to the conclusion that derivative stability is even higher in ethyl acetate than in basic solutions. The relative fluorescence intensity in this organic solvent was over 97% of the original value after 25 min.

*Quantitative analysis*

Standard curves were constructed after analysis of a series of samples with known IAA concentrations. Table 1 gives the results of the two different methods, normal and synchronous spectrofluorometry. Linear least squares regression analysis gave lines with intercepts that were not statistically different from zero. All correlation coefficients were better than 0.99. The detector response was linear over the IAA amount range of 10–80 ng.

The limit of detection ($C_L$), as defined by IUPAC [14], describes the lowest concentration level of the substance that can be determined to be statistically different from an analytical blank; it is given by $k$ x $S_b/S_c$, where $S_b$ is the standard deviation of the blank signal, $S_c$ is the slope of the calibration graph, and $k$ is the numerical factor corresponding to the selected confidence level ($k = 3$). To compare the sensitivity of the method, in a coherent unit system, analytical sensitivity was used, $S_a = S_s/S_c$, where $S_s$ is the standard deviation of the analytical signal at a particular concentration. All these values indicate the ability to distinguish concentration differences with determined confidence levels.

The reproducibility of the method was determined for a series of 10 measurements on 43.8 ng/ml of IAA, Table 2 summarizes this analytical parameter. Relative error was under seven per cent in both procedures for fluorescence measurements, normal and synchronous.

*IAA content in peach seeds*

IAA determination in crude extracts from plant tissues needs care after taking into account the specific measurement difficulties of the previous extraction. Consequently, the presence of impurities in the acid ether fraction, during the IAA extraction, causes high background fluorescence. However, since the sensitivity of the method is unchanged, the standard addition procedure was used to determine the IAA content of the extracts. This possibility has been studied by Reeve and Crozier [15]. Similarly, the standard addition method can minimize the proportional non-random error produced by impurities.

A problem would occur if there was a change from the standard straight line slope to that of standard addition.

Table 1. Characteristics of the analytical methods

| Method | Sb ($I_F$) | Ss ($I_F$) | Sc ($I_F$ ng$^{-1}$ ml) | Sa (ng ml$^{-1}$) | $C_L$ (ng ml$^{-1}$) |
|---|---|---|---|---|---|
| A | 0.32 | 4.61 | 1.10 | 4.11 | 0.87 |
| B | 0.24 | 4.62 | 1.11 | 4.16 | 0.65 |

A: Normal spectrofluorometry; B: synchronous spectrofluorometry; $I_F$: relative fluorescence intensity.

Table 2. Reproducibility of the analytical methods

| Method | Taken (ng ml$^{-1}$) | $n$ | Found (ng ml$^{-1}$) | Error*(%) |
|---|---|---|---|---|
| A | 43.80 | 10 | 44.70 | 6.67 |
| B | 43.80 | 10 | 44.79 | 6.51 |

*Relative error ($100\ ts/n\frac{1}{2}\bar{x}$, confidence level 95%).

Some authors consider quantitative interference when the change in the slope is more than 15% [16, 17]. The results obtained for peach seed extracts when the standard addition procedure was used gives straight line representations with similar slopes to a standard straight line, within a 2.5% difference range. This indicates a low level of impurities in the crude extracts. Some purification occurred during the extraction of IAA from the seed tissue, as indicated in the experimental section. The ethyl acetate extraction step for the indole-α-pyrone derivative, as described in the present paper, may also eliminate impurities.

The endogenous IAA levels found in peach seeds in two different extractions are summarized in Table 3. Relative standard deviations were 12.9 and 12.5% for both normal and synchronous fluorescence methods, respectively. The values for free IAA in this type of tissue is slightly higher than those previously reported for different seed samples [18]. Since great differences are to be expected at different stages of seed development [19] the free IAA changes should be followed throughout the seed growth period, to obtain any physiologically meaningful data.

In conclusion it appears that the proposed modifications for IAA determination of the Stoessl and Venis method [3], by the use of derivative extraction with ethyl acetate together with the synchronous fluorescence procedure, gave improved hormone determinations in crude extracts from different plant samples.

## EXPERIMENTAL

*Plant material.* Seeds of peach fruits (*Prunus persica*, cv. Merry) were harvested at mid stage II of development, immediately frozen and stored at −20° until used.

*IAA extraction.* IAA extraction and purification was carried out by slight modification of the Knegt and Bruinsma procedure [4]. Seed samples were homogenized in a Sorvall homogenizer at full speed for 1 min at 4°. The extraction by MeOH lasted 30 min at 20° in darkness. To this MeOH soln 10 μl of methanolic [1-$^{14}$C] IAA soln was added to correct for IAA loss during purification. The extracts were filtered through glass filters and evaporated to dryness under red. pres. at 35°. The dry residue was dissolved in 0.5 M $K_2HPO_4$ and fractionated according to ref. [6]. The final $Et_2O$ phase was evapd to dryness under red. pres. and stored at −20°.

*IAA derivatization.* Stock solns of indole-3-acetic acid were prepared in MeOH and stored in amber bottles at 4°. Aliquots of this stock soln were placed in 10 ml test tubes and the solvent was evapd by a stream of $N_2$ gas. The derivatization was started by the addition of 0.1 ml $Ac_2O$ and 0.05 ml trifluoroacetic acid. The reaction mixture was kept for 6 min in an ice bath before adding 1 ml of 3N NaOH immediately followed by 2 ml of EtOAc. The mixture was vigorously shaken for 1min and allowed to stand for 5 min. The fluorescence intensity of the organic phase was measured at $\lambda_{ex}$ 440 nm and $\lambda_{em}$ 480 nm.

*Determination of* [1−$^{14}$C] *IAA.* An aliquot of the final methanolic acid ether fraction was added to a vial containing 10 ml of scintillation liquid consisting of 5 g PPO (2,5-diphenyloxazol) and 300 mg dimethyl POPOP [1,4-bis-(4-methyl-5-phenyl-2-oxazolyl)benzene] in toluene. The radioactivity of [1-$^{14}$C] IAA was measured with a liquid scintallation counter. Quenching correction was made with the aid of an external standard. The recovery obtained was 61.6%. The recoveries are usually between 40 and 60% [9].

Table 3. Estimation of the endogenous IAA content in peach seeds (*Prunus persica*,) (values in ng per g fresh weight ± standard deviation)

| Method | Extraction I | Extraction II |
|---|---|---|
| A | 398±60 | 405±44 |
| B | 399±55 | 399±45 |

A: Normal spectrofluorometry; B: synchronous spectroflurometry.

*Analysis of seed samples.* The dry acid ether fraction, obtained as described above, was dissolved in 0.5 ml MeOH, and aliquots of this soln were added to test tubes which contained different amounts of the IAA stock soln. After the MeOH was evaporated by a flow of $N_2$ the IAA was derivatized following the above procedure.

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