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A RAPID, SENSITIVE AND ACCURATE DETERMINATION OF INDOLYL-3-ACETIC ACID

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Dedicated to Professor Dr. H. VELDSTRA on the occasion of his retirement from the chair of biochemistry of the University of Leiden

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Abstract—A method is described for the routine determination of absolute amounts of free indolyl-3-acetic acid (IAA) in plant material. The time required for a determination is in the order of 2 hr. Several samples can be handled at the same time. Depending on the contents of IAA, the amount of fresh plant material varies from 0·05 to 5 g. Losses are corrected for by applying ^{14}C-labeled IAA.

INTRODUCTION

THE DETERMINATION of hormonal amounts of indolyl-3-acetic acid (IAA) in plant tissues is generally a time-consuming and laborious procedure. Bioassays take time for the growth reaction to occur, are inaccurate and liable to interference by other substances affecting the growth process. Physicochemical methods of sensitivity equal to the bioassays and of good accuracy have become available, but the extraction and purification procedures are still lengthy and cause appreciable losses that are frequently not taken into account.[1]

The present procedure has been developed in order to reduce the extraction and purification from days to hours and, thereby, to minimize losses. The specific conversion of IAA into indolo-α-pyrone, as described by Stoessl and Venis,[2] permits the spectrofluorometric determination of very small amounts of IAA in relatively impure samples. Moreover, by the addition of a known amount of radioactive IAA to the extract, losses can be measured in the very samples that are treated, enabling the calculation of absolute amounts of IAA in the original extracts.

RESULTS AND DISCUSSION

To correct for losses in the procedure (see Experimental), the radioactive IAA should be added directly to the powdered plant material as it is weighed on the glass filter. However, because percentage recoveries were the same irrespective of whether IAA was added before or after the extracting filtration, addition to the extract is more convenient.

The ^{14}C-IAA used in the experiments was labeled in the carboxyl group. Repetitions with 2-^{14}C-IAA in a number of experiments under highly standardized manipulations gave recovery percentages matching exactly those with 1-^{14}C-IAA. The use of carboxyl-labeled IAA can therefore be considered to be safe.

[1] MANN, J. D. and JAWORSKI, E. G. (1970) *Planta* **92**, 285.
[2] STOESSL, A. and VENIS, M. A. (1970) *Anal. Biochem.* **34**, 344.

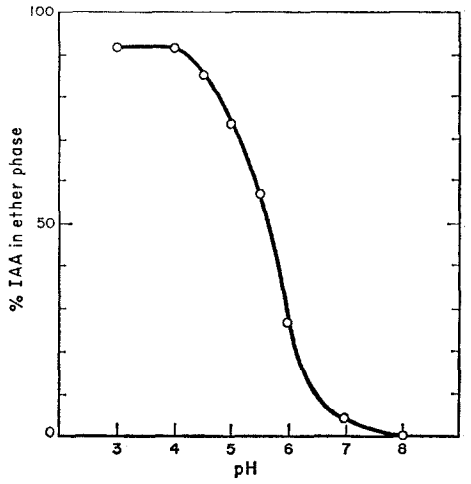

FIG. 1. PARTITION OF IAA BETWEEN EQUAL AMOUNTS OF Et_2O AND 0·05 M K_2HPO_4.

TABLE 1. IAA CONTENT (ng/g) OF YOUNG TOBACCO LEAVES

After spraying (hr)	Leaves sprayed with H_2O	Leaves sprayed with 10^{-4} M IAA
0	4·0 ± 0·9	
18	3·6 ± 1·0	11·0 ± 0·8
42	6·4 ± 0·7	7·7 ± 1·4
66	5·1 ± 1·1	8·9 ± 0·0

Determinations with 5 g leaves in duplicate.

TABLE 2. IAA CONTENT OF *Begonia* FLOWERS

Age class	Cultivar 1		Cultivar 2	
	ng/g	ng/bud or flower	ng/g	ng/bud or flower
Small buds	4·9 ± 0·8	0·08 ± 0·01	73·3 ± 3·0	1·25 ± 0·05
Half-grown buds	9·3 ± 0·2	0·65 ± 0·01	66·2 ± 1·2	4·64 ± 0·08
Large buds	6·7 ± 0·7	1·18 ± 0·12	26·1 ± 1·0	4·60 ± 0·18
Half-open flowers	3·1 ± 0·2	0·87 ± 0·06	10·5 ± 1·4	3·02 ± 0·40
Open flowers	13·2 ± 4·0	5·26 ± 1·60	27·8 ± 0·5	11·10 ± 0·20

Determinations with 5 g flowers in duplicate.

The partition of ^{14}C-IAA between H_3PO_4(pH 3) or 0·05 M K_2HPO_4 (pH 8·5) and Et_2O (Fig. 1) demonstrates that the pH during separation are safe: below pH 4, 92% of the IAA accumulates in the ether layer, above pH 8, all the AA is in the aqueous layer. Subsequent extractions as commonly practised, are not of much advantage. Careful rewashing of

fractions allowed recoveries up to 96% of the radioactive IAA. However, it suffices to collect only clear layers, and not to include such materials as chlorophylls and carotenoids from the interface which may cause quenching in the fluorescence measurement. With difficult separations, losses up to 50% can be tolerated because the recovery percentage is known.

The method gave very reproducible results. A plot of the amount of IAA against the relative fluorescence at 490 nm (excitation at 440 nm) gave a straight line (regression $x = 26{\cdot}3\ y + 247$ mg where y = relative fluorescence) and this could be corrected for recovery by measurement of the radioactivity.

TABLE 3. IAA CONTENT OF *Cleome* FRUIT PARTS

Days after pollination	Empty pods		Seeds	
	ng/g	ng/pod	μg/g	μg/pod
10	58 ± 16	3·6 ± 1·0	7·08 ± 0·94	0·23 ± 0·03
17	114 ± 8	26·6 ± 1·4	9·86 ± 0·09	1·60 ± 0·16
24	163 ± 52	44·5 ± 14·3	3·00 ± 0·29	0·81 ± 0·08
31	53 ± 7	18·4 ± 2·3	1·01 ± 0·05	0·31 ± 0·02

Determinations with 4 g pods and 40 mg seeds in duplicate.

In Tables 1–3, some results are collected from the various materials.

EXPERIMENTAL

Materials. The plant tissues used for testing the procedure were leaves of tobacco, flowers of *Begonia*, and pods and seeds of *Cleome spinosa*. The plants were grown under glasshouse conditions. The quantity of plant material was chosen to contain 25–125 ng IAA. With the leaves, the flowers and the pods, 5 g fresh material per sample was sufficient; with the seeds, 50 mg.

Methods. Extraction. The material is frozen in liquid N_2 and powdered within 5–10 sec in an IKA analysis mill A10 (Janke & Kunkel KG), precooled with liquid N_2. A weighed amount is extracted on a G-4 glass-filter in 5–10 min with 10 ml (×3) and once with 5 ml of cold MeOH. The MeOH was redistilled to reduce fluorescent impurities. During the extraction the suspension on the filter is stirred sideways with a magnetic stirrer and the extract is sucked directly into a 100 ml flask. To this extract 1 ml of a methanolic solution of 1-^{14}C-IAA is added, equivalent to 2 ng IAA, with an activity of about 1500 cpm. The extract is evaporated to an aqueous residue under reduced pressure in 5–10 min at 30°.

Purification. All the chemicals used are Merck p.a. To the aqueous residue, 10 ml of cold 0·5 M K_2HPO_4 solution is added, which brings the pH to about 8·5. In a 50-ml separating funnel, provided with a teflon tap (to avoid grease), the suspension is shaken with 10 ml light petrol. (×2) and then with 10 ml Et_2O. The lipid fractions are discarded. The H_2O layer is brought to pH 3 with 2 ml 2·8 M H_3PO_4 solution, the strength of which has been adjusted to the K_2HPO_4 solution. The 10 ml Et_2O into which the IAA passes subsequently, is extracted with 10 ml cold 0·05 M K_2HPO_4 solution. The lower concentration avoids salt accumulation in the final evaporation step. With 2 ml of about 0·28 M H_3PO_4 (adjusted to the 0·05 M K_2HPO_4 solution) the pH is brought back to 3 and the IAA is passed into a final 10 ml Et_2O. The Et_2O is evaporated in a few min under reduced pressure to 0·20 ml.

Measurement. To the purified extract 5·00 ml of cold redistilled MeOH is added. From this solution 1 ml is brought into 10 ml of a scintillation fluid, consisting of 4 g PPO, 0·2 g POPOP, 667 ml toluene and 333 ml Triton X100. The remainder of the solution is used to obtain a reference line for the fluorometric measurement. In each of four 10-ml flasks 1 ml of the solution is pipetted, and each of the flasks is provided with 1 ml MeOH containing 0, 10, 20 or 30 ng of IAA respectively. The 4 aliquots are dried completely under reduced pressure and cooled to 0°. At this stage the procedure may be interrupted for several days. To each flask 0·20 ml of an ice-cold reaction mixture is added, freshly prepared from equal parts of CF_3CO_2H and Ac_2O. The components must be precooled separately to prevent irregular results. The reaction in which IAA is converted

into indolo-α-pyrone is allowed to continue for exactly 15 min with the flasks put on ice; it is stopped with 3 ml H_2O.[2] The reading is taken after 60 sec on an Aminco-Bowman spectrophotofluorometer, provided with the off-axis ellipsoidal mirror accessory for improved sensitivity. The indolo-α-pyrone has an excitation maximum at 450 nm and an emission maximum at 480 nm. However, to avoid interference with the scatter-peaks, at the low concentrations used it is preferred to work at 440 and 490 nm respectively. A blank is occasionally prepared by adding first 3 ml H_2O to 1 of the 4 aliquots and the 0·20 ml reaction mixture after 15 min. The value of the blank, that is substracted from the readings, turns out to be constant, irrespective of the source of plant material. By running the whole procedure without plant material at the start, a low background is found (consisting partly of the radioactive IAA), which is corrected for in the calculations. From the regression line the unknown amount per flask is calculated. This figure is multiplied by the dilution factor, 5·2, corrected for the background and for the percentage recovery as determined by the radioactivity measurement.

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