Phytochemistry, 1975, Vol. 14, pp. 2455–2457. Pergamon Press. Printed in England.

A GLC PROCEDURE FOR DETERMINING SUB-NANOGRAM LEVELS OF INDOL-3-YL ACETIC ACID

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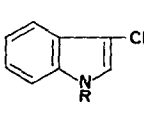
(*Received* 8 *January* 1975)

Key Word Index—Indol-3yl acetic acid; quantitative determination; chloro esters of IAA; GLC electron capture detector.

Abstract—Mono-, di- and tri-chloroethyl esters of indol-3-yl-acetic acid were synthesized and analysed by a gas–liquid chromatography using an electron capture detector. The response was found to be proportional to the number of chlorine atoms in the molecule. The trichloroethyl ester proved the most effective in the detection, permitting measurement of amounts of IAA as low as 0·02 ng. Good linear relationship was found between the amount of ester injected and the peak area. All the chloro derivatives of IAA showed auxin activity in the coleoptile elongation bioassay.

The development of a quick, simple and specific method for the quantitative estimation of endogeneous indol-3-yl-acetic acid (IAA, **1a**) is of crucial importance to plant physiologists working with plant hormones. At present, chemical, biological and GLC methods are used. The chemical methods call for rather large amounts of plant material and high purity of the hormone. The bioassays are more sensitive [1–2 ng IAA is the limit in the *Avena* section curvature test] but are of limited reliability both qualitatively and quantitatively, due to interfering substances which may either promote or inhibit the bioresponse. The most specific method, which is also the fastest, is GLC which was first applied to the analysis of plant hormones about 12 yr ago.



(**1a**) R = H; R' = H
(**1b**) R = H; R' = Me
(**1c**) R = $Si(Me)_3$; R' = Me
(**1d**) R = $Si(Me)_3$; R' = $Si(Me)_3$
(**1e**) R = F_3CCO; R' = Me
(**1f**) R = H, R' = CH_2Me, CH_2CH_2Cl, CH_2CHCl_2 or CH_2CCl_3

Prior to GLC analysis, the IAA must be converted to a derivative sufficiently volatile at reasonable temperature. Esterification is the most frequently-used method of increasing volatility, and most investigators [1–6] have used diazomethane for the preparation of the methyl ester (**1b**); in other cases a mixture of MeOH + BF_3 [7,8] was employed. In all cases, the threshold of sensitivity, when flame ionization detectors were used, ranged from 1–10 μg IAA.

Other workers [9,10] prepared the *N*-trimethylsilyl derivative of the methyl ester of IAA (**1c**) or the ditrimethylsilyl derivative (**1d**) (11); in both cases the threshold of sensitivity was around 1 μg of IAA. Diazomethylation followed by *N*-trifluoro acetylation [12] resulted in a product (**1e**) to which the electron capture detector was very sensitive due to the presence of 3 fluoro atoms in the molecule. When this compound was used, concentrations as low as 100 pg of IAA could be detected.

None of these methods were entirely satisfactory for detection of the minute amounts of IAA present in single plants, due to sensitivity limitations and purification problems. We have attempted to improve the methods currently available and to develop a simple, specific and highly sensitive method for GLC assay of IAA.

Theoretically, 10^{-15} mol levels of sensitivity can be reached by using gas chromatography equipped with an electron capture detector (i.e. 1·8 picograms of IAA). An approximate level of sensitivity can be achieved by use of halogenated derivatives of the analysed substance. Consequently,

Table 1. Retention times and threshold of sensitivity of various IAA esters on electron capture instrument

IAA ester	Retention time (min) 1·5% QF-1	1·5% SE-30	Threshold of sensitivity (ng)
Ethyl	8·6	8·4	10·0
Monochloroethyl	21·8	15·6	10·0
Dichloroethyl	6·7	4·9	0·50
Trichloroethyl	10·3	6·4	0·02

a series of chlorinated derivatives of IAA, namely, the mono-, di- and tri-chloro ethyl esters (1f) were synthesized. Esterification was carried out in benzene under azeotropic conditions using excess of the chloro alcohol and *p*-toluene sulfonic acid as the catalyst. Yields were above 85%, and the products were characterized by IR, NMR and elementary analysis. Esterification by this method, with synthetic IAA, proved to be simple and reproducible and could be completed within 60 min.

In preliminary GLC studies, retention values of the chloro esters, on 2 column packings (1·5% QF-1 and 1·5% SE-30) were compared (Table 1). Sharp symmetrical peaks with almost no tailing were obtained on both columns, and for routine work we chose to use 1·5% QF-1. The chloro derivatives were stable over a wide temperature range and showed no sign of decompositon or rearrangement.

Next we determined the lowest level of concentration of each ester, that could be detected by an electron capture detector. The limit of detection (Table 1) was taken to be the lowest concentration of ester that provided a response reproducible with 10% accuracy (12). The results obtained show that the sensitivity increases in a direct ratio to the number of chloro atoms in the molecule. With IAA ethyl ester and even with the monochloro derivative, no less than 0·01 μg could be detected. However, when the dichloro and trichloro esters were used, the sensitivity increased by factors of 20 and 1000 respectively. Use of the trichloro ester, and electron capture analysis enabled us to accurately detect 10 pg of IAA.

A good linear relationship was found between the amount of IAA esters injected and the peak area, over a wide range of concentration; for the

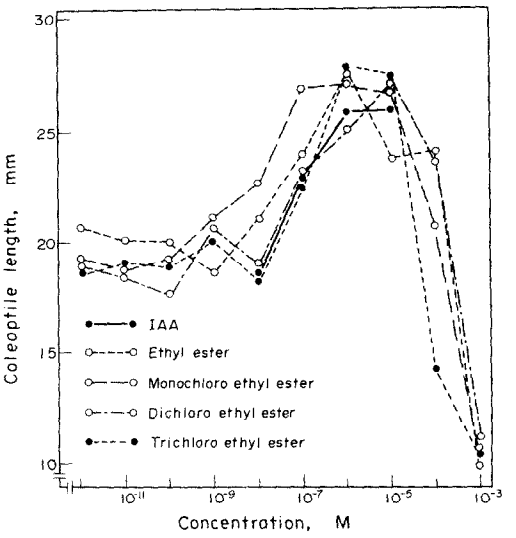


Fig. 1. Response of wheat coleoptiles to various concentrations of chloroethyl IAA and IAA esters.

trichloroethyl ester, the range was from 10–300 pg.

It was interesting to examine the hormonal activity of the hitherto-unknown chloro esters. In the wheat coleoptile growth bioassay the results accorded with what could have been anticipated of the original hormone (Fig. 1), namely, retention of biological activity. It is hard to determine whether the chloro derivatives themselves are active or whether they are hydrolysed on the test medium or *in vivo*, to give the free hormone.

So far, only the determination of synthetic IAA has been discussed. Attempts to apply the method to plant material met with several difficulties: The esterification method needs to be modified, since it proved successful at the milligram level but gave equivocal results at the sub-microgram level. The high sensitivity of our GLC method also calls for changes in the usual methods of extraction of endogenous IAA. The acidic IAA fraction contains several organic acids which do not interfere with existing GLC methods. Under the present method, however, transfer to trichloro-esters results in products to which the EC detector is highly sensitive, so that the IAA peak is appreciably masked.

We are now attempting to overcome these obstacles and to arrive at effective application of the method to plant material.

EXPERIMENTAL

All mp's were determined on a Thomas–Hoover capillary apparatus and are uncorrected. Indole acetic acid was purchased from Sigma and the chloroethanols from Aldrich.

Chloroethyl ester of IAA. IAA (0·5 g; 0·003 mol) was dissolved in dry C_6H_6 (40 ml) and 2-chloroethanol (5 ml) and *p*-toluene sulfonic acid (60 mg) were added. The reaction mixture was refluxed in azeotropic apparatus for 60 min, extracted with 4% $NaHCO_3$ (3 × 100 ml) [to eliminate any nonesterified substance], dried over Na_2SO_4 and solvent was evaporated *in vacuo*. The oily *ester* (0·68 g; 90%) was crystallized from isobutanol and gave mp 65–66°: (found: C, 60·50; H, 5·00; N, 5·80; Cl, 15·0. $C_{12}H_{12}NClO_2$ requires: C, 60·63; H, 5·05; N, 5·89; Cl, 14·94%).

Dichloroethyl ester of IAA. This ester was prepared by the same procedure as before using dichloroethanol (5 ml). The oily product was purified by use of Si gel column, for which 100–200 mesh Si gel (Merck), slurried in CCl_4 was used. The elution proceeded by a step-wise gradient of $CHCl_3$ in CCl_4; 50 ml of the following dilution were taken: 1, 2, 4, 8, 32 and 64% chloroform in CCl_4. Dichloro ester was obtained from the last fractions (64%). Solvents were evaporated *in vacuo* leaving a bright yellow oil (0·73 g; 86%). (Found: C, 52·95; H, 4·00; N, 5·25; Cl, 26·00. $C_{12}H_{11}NCl_2O_2$ requires: C, 53·13; H, 4·05; N, 5·16; Cl, 25·83%).

Trichloroethyl ester of IAA. It was prepared by the same procedure using trichloroethanol (5 ml). Purification of trichloro ester was performed with a Si gel column as described above and the compound eluted in the 32% $CHCl_3$ in CCl_4. Solvents were evaporated *in vacuo*, and the resulting oil crystallized from Et_2O–hexane. The white crystals of *the ester* (0·78 g; 88%) melted at 64–65°. (Found: C, 47·10; H, 3·20; N, 4·50; Cl, 34·65. $C_{12}H_{10}NCl_3O_2$ requires: C, 46·98; H, 3·26; N, 4·56; Cl, 34·74%).

GC. The analytical work was accomplished on a model 7400 Packard gas chromatograph, and a spiral glass column 1·8 m long × 3 mm internal diam was used. Column packing consisted of 1·5% QF-1 and 1·5% SE-30 coated on 60–80 mesh Gas-chrom Q. Column temp. 190°C with QF-1) or 180°C (with SE-30), with injection and detector temp. of 250°C. Columns were preconditioned at 240°C overnight. An electron capture detector was used with radioactive ^{63}Ni foil. N_2 carrier gas was used at a flow rate of 55 ml/min. The electrometer range was 3×10^{-10} A. A pulsating voltage of 50V amplitude, lasting 0·10 msec (Textronic pulse generator) was applied at 0·1 msec intervals, to the detector. One μl of each dilution was injected.

Bioassay procedure. The wheat coleoptile section elongation bioassay was used for detection of auxine activity of the chloro ester derivatives. The procedure used was similar to that of Nitsch and Nitsch (13) and of Hancock *et al.* (14). Various amounts of chloro ester were put into small vials. Three 10 mm coleoptile sections were placed in each vial with 0·75 ml Pi–citrate buffer pH 5·7 + 2% sucrose. Vials were rotated in a horizontal rotator (1 rpm). After incubation for 22 hr at 23°, Sections were measured to the nearest 0·5 mm. (see Fig. 1).

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