Journal of Chromatography, 205 (1981) 125–137
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,334

IDENTIFICATION OF 3-INDOLEACETIC ACID IN *PINUS SYLVESTRIS* L. BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY, AND QUANTITATIVE ANALYSIS BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH SPECTROFLUORIMETRIC DETECTION

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(Received September 9th, 1980)

SUMMARY

The phytohormone 3-indoleacetic acid (IAA) was conclusively identified by gas chromatography–mass spectrometry of its methyl ester in purified extracts of Scots pine, *Pinus sylvestris* L. Preceding purification included reversed-phase ion-pair chromatography on a Nucleosil C_{18} column with tetrabutylammonium ion as counter ion. A number of indole acids were separated in this high-performance liquid chromatographic (HPLC) system. In particular, IAA was separated from the reported plant constituents 4-chloro-IAA, 5-hydroxy-IAA, 3-indoleacrylic acid, 3-indolelactic acid, 3-indolepropionic acid, 3-indolebutyric acid and tryptophan. In connection with a spectrofluorimetric detector, the HPLC system was also used for the quantitative analysis of IAA in pine samples. Systematic errors caused a constant under-estimation of the IAA content by 12% and could easily be corrected for. The random error was 14%. Eight-week-old pine seedlings contained 46 ng g^{-1} of IAA. The sensitivity of the method, applied to the analysis of pine extracts, was 50 pg of IAA. Evidence is presented that the method, when applied to the analysis of pine extracts, is specific for IAA.

INTRODUCTION

Auxins are a group of plant hormones, among which 3-indoleacetic acid (IAA) is considered to be the major but perhaps not the sole active substance[1]. This compound has been identified in different plant materials in concentrations of 20–250 ng g^{-1} fresh weight. So far, only a few identifications have been made from conifers[2–4]

0021-9673/81/0000–0000/$02.50

Numerous chemical methods of analysis have been developed to replace the biological assays originally used. Different chromatographic methods have been tried. Gas chromatography (GC) with various detectors has been applied, but shows questionable specificity when used for the analysis of complex plant extracts. A recently described method using a nitrogen-sensitive alkali flame-ionization detector[5] may be superior to previously described GC systems in this respect. Methods based on mass spectrometry (MS) or combined GC–MS have been used primarily to identify IAA[2,4,6]. Single-ion monitoring has also been used for quantitative analysis[3,7,8]. Methods based on MS, if properly and cautiously used, are reliable but expensive. Thus, in spite of their advantages, they are seldom used for routine analyses.

A popular method for such purposes is the condensation of IAA with acetic anhydride to form 2-methylindole-α-pyrone, a compound with very specific fluorescent properties[9]. This method is specific for indoleacetic acids. It has recently been improved by the recording of entire emission spectra rather than monitoring the fluorescence at a fixed wavelength[8]. However, the pyrone method has been reported to be sensitive to interfering compounds frequently present in plant extracts[10,11]. We have found that the method cannot be used for the analysis of IAA in pine extracts because of a large random error and a sometimes irregular distribution function for the systematic error. It has also been shown[8,9,12] that the method cannot distinguish between IAA and the compounds 5-hydroxy-IAA (5-OH-IAA) and 4-chloro-IAA (4-Cl-IAA), which occur naturally in plants[13,14]. The last-mentioned substance shows strong auxin activity in many bioassays[15]. A radioimmunological assay for IAA has been described[16] but has so far not been widely used.

High-performance liquid chromatography (HPLC) has recently been used for the analysis of IAA. A number of systems have been described, such as anion-exchange[17], silica gel adsorption[17], straight-phase partition[18] and reversed-phase[17,19,20] HPLC with various detectors[17]. HPLC is particularly suitable for combination with additional analytical procedures, as no derivatization is needed and fractions can easily be collected. Detectors are available that show a considerable specificity for IAA[17].

We needed a system for quantitative analysis that combined resolution and specificity with the simplicity required for routine work. These factors have been difficult to combine for the HPLC techniques previously described, as the complexity of plant extracts normally necessitates the use of gradient elution in order to obtain acceptable resolution of IAA from other extract components. It has been shown[21–23], however, that ion-pair chromatography (IPC) is superior to the ordinary ion-suppression chromatography (ISC) for the separation of carboxylic acids on reversed-phase columns. The principle behind IPC of acidic components is that a quaternary ammonium compound containing four lipophilic groups is added to the eluent as a counter ion. The water content of the eluent can generally be lowered in comparison with ISP, and this leads to a higher column efficiency owing to lower viscosity. For IAA analysis in general, it is necessary to separate this compound from interfering substances. If a relatively selective spectrofluorimetric detector is used, interference will be restricted to compounds that absorb or emit light at the two wavelengths employed. In particular, indolic compounds interfere strongly. Non-acidic indolic compounds will be eliminated by the purification procedures that we use before the final IAA analysis[24]. Acidic indoles remain in the sample and have to be separated by

the HPLC system. Particular attention has to be paid to 4-Cl-IAA because of its high auxin activity[15] and demonstrated presence in plant extracts[14]. Other acidic indoles reported to occur in plants are 5-OH-IAA[13], 3-indoleacrylic acid[25], 3-indolelactic acid[26], 3-indolepropionic acid[27] and 3-indolebutyric acid[27]. All of these should be separated from IAA.

Before a method for IAA analysis can be adopted for the analysis of plant extracts, the identity of this compound in the extract has to be proved conclusively. At present there is no means of doing this except by MS. Also, before quantitative analysis can be attempted, the errors of measurement have to be evaluated. Such errors can be systematic or purely random. The two types of error have different causes, are different in character and have to be determined separately.

The purposes of the present investigation were as follows:

(1) To develop an ion-pair reversed-phase HPLC system with spectrofluorimetric detection having sufficient resolving power to separate IAA from other acidic indoles, particularly 4-Cl-IAA.

(2) To identify IAA as a constituent of purified pine extracts by MS.

(3) To evaluate the systematic and random error components of the entire analytical procedure, including extract purification preceding the HPLC analysis.

EXPERIMENTAL

Plant material

Eight-week-old pine seedlings (*Pinus sylvestris* L.) were used. These were grown in peat medium in a climatic chamber under the following conditions: 17-h day, photon flux density *ca.* 470 μE m^{-2} sec^{-1}, 25°C; and 7-h night, 15°C; relative humidity 75% throughout. The seedlings were watered daily and were given Wallco nutrient solution twice a week. Immediately upon harvesting the seedlings were stored at −80°C until analysed.

Reagents

The following were used: methanol, ethyl acetate and light petroleum ether (boiling range 60–71°C) (all redistilled in glass prior to use), diethyl ether (Mallinckrodt, St. Louis, MO, U.S.A.; analytical grade), buffer chemicals (Merck, Darmstadt, G.F.R.; analytical grade), poly-N-vinylpyrrolidone (PVP, purchased as Polyclar AT Powder; GAF Corp., New York, NY, U.S.A.), Celite (30–80 mesh) for GC (BDH, Poole, Great Britain), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), Insta-Gel scintillation cocktail (Packard, Downers Grove, IL, U.S.A.), [2-^{14}C]IAA (specific activity 2.10 TBq mol^{-1}; New England Nuclear, Boston, MA, U.S.A.), tetrabutylammonium hydrogen sulphate, IAA, 5-OH-IAA, 3-indolelactic acid, 3-indoleacrylic acid, 3-indolepyruvic acid, 3-indoleglyoxylic acid, 3-indolepropionic acid, 3-indolebutyric acid, tryptophan, 5-indolecarboxylic acid, 2-indolecarboxylic acid (all from Sigma, St. Louis, MO, U.S.A.) and 4-Cl-IAA (Kjeld Engvild, Risø, Denmark).

Extraction procedure for large samples

Ten-gram samples (fresh weight) of plant material were homogenized in 200 ml of refrigerated methanol. A carefully pipetted amount (122.6 kBq) of [2-^{14}C]IAA was added as internal standard, and the samples were extracted at 4°C for 24 h. The

methanol extract was filtered off and evaporated to dryness at reduced pressure and 40°C. The extract was then dissolved in 0.5 *M* phosphate buffer (pH 8.0) and washed three times with equal volumes of light petroleum. If emulsions formed, the sample was placed in a refrigerator or in a cooled ultrasonic bath until the phases separated. The buffer phase was acidified to pH 2.7 and extracted five times with half its volume of ethyl acetate. The ethyl acetate phases were combined, water was removed by freezing and filtering, and the ethyl acetate was evaporated to dryness at 40°C and reduced pressure. The residue was dissolved in 2 × 5 ml of 0.1 *M* phosphate buffer (pH 7.5) and applied to a column (10 mm I.D.) consisting of, from top to bottom, 24.5 cm of PVP, 19.5 cm of Sephadex LH-20 and 1.0 cm of Celite[28]. The column was eluted with 0.1 *M* phosphate–citrate buffer (pH 4.5) and the 150–450 ml fraction was collected, acidified to pH 2.7 and extracted five times with half its volume of ethyl acetate. The ethyl acetate phases were combined and evaporated to dryness as before. The final residue was dissolved in 2.5 ml of diethyl ether, transferred into a conical test-tube and evaporated to dryness with a stream of nitrogen.

Samples intended for identification of IAA were treated similarly, but no internal standard was added.

Extraction procedure for small samples

A single pine seedling (fresh weight 400 mg) was homogenized in 100 ml of refrigerated methanol and extracted for 2 h at room temperature. The methanol extract was filtered off and [2-^{14}C]IAA was added as internal standard. The extract was diluted with 20 ml of 0.5 *M* phosphate buffer (pH 8.0) and evaporated at reduced pressure and 40°C to a residual volume of 15 ml. This residue was adjusted to 100 ml with 0.1 *M* phosphate buffer (pH 8.0) then acidified to pH 2.7 and extracted with three 50-ml volumes of diethyl ether. The ether phases were combined and evaporated to dryness. The residue was dissolved in 2.0 ml of 0.1 *M* phosphate buffer (pH 8.0) plus 2.0 ml of methanol, and applied to a column (10 mm I.D.) consisting of, from top to bottom, 1.0 cm of Celite, 2.5 cm of PVP and 24.0 cm of Sephadex LH-20[28]. The column was eluted with 0.1 *M* phosphate buffer (pH 3.5) and the 175–280 ml fraction was collected, acidified to pH 2.7 and extracted three times with half its volume of diethyl ether. The ether phases were combined and evaporated to dryness. The residue was dissolved in 150 μl of ethyl acetate and applied to a thin-layer plate (Merck DC-Fertigplatte, Kieselgel F-254) and chromatographed in parallel with pure IAA in ethyl acetate–chloroform–formic acid (50:45:5). The part of the chromatogram corresponding to standard IAA was scraped off and eluted with water–diethyl ether (2:8). The ether was evaporated with a stream of nitrogen and the sample was finally evaporated to dryness in a freeze-drier. During the entire procedure the sample was protected from light to prevent photo-oxidation of IAA.

Liquid scintillation counting

Corrections for losses of IAA during the analytical procedures were made by liquid scintillation counting of sample aliquots containing [2-^{14}C]IAA as internal standard. This was done with an LKB-Wallac Ultrobeta 1210 instrument with Insta-Gel as the scintillation cocktail. All data were corrected for background and quenching.

HPLC equipment and analysis

The equipment consisted of a Milton Roy Minipump connected via a Valco 50-μl loop injector to a 40 × 4 mm I.D. pre-column packed with silica gel (120–160 mesh), followed by a 250 × 4 mm I.D. analytical column of 10-μm Nucleosil C_{18} (packed in our laboratory), and a Spectra-Physics SD-970 spectrofluorimetric detector with a 5-μl cuvette volume. The detector was adjusted to an excitation wavelength of 285 ± 5 nm. The emitted light was passed through an interference filter with the wavelength 360 ± 10 nm. The eluent was methanol in 0.01 *M* phosphate buffer plus 0.01 *M* tetrabutylammonium hydrogen sulphate and the flow-rate was 1.5 ml min^{-1}. Unless otherwise stated, the methanol concentration was 25% and the pH was 6.5. Samples were dissolved in 250 μl of 0.01 *M* phosphate buffer (pH 6.5). Injections were always carried out in the sequence IAA standard, sample, sample spiked with 10 ng of IAA. The fraction corresponding to IAA was collected for recovery measurements.

Equipment for GC–MS and identification of IAA

The gas chromatograph was equipped with a 30 m × 0.25 mm I.D. capillary column of SP 2401-1. The Grob-type injector was kept at 200°C. The column temperature was initially 90°C for 1.5 min; it was then increased at 30°C min^{-1} to a final value of 220°C, which was held for 15 min. The gas chromatograph was connected to a Finnigan Model 4023 mass spectrometer equipped with an INCOS computer system. The temperature of the interface was 200°C and that of the ion source 250°C. Spectra were recorded at 70 eV.

The identification of IAA was performed with extracts to which no internal standard had been added. To prevent photo-oxidation of IAA, the HPLC separation was performed with the detector light switched off.

Fractions of 2 ml corresponding to the putative IAA peak from five repeated injections of the same sample were combined to give a total volume of 10 ml. This combined sample was extracted with diethyl ether. The ether phase was methylated with diazomethane and finally analysed by GC–MS as the methyl ester of IAA (IAA-Me) in dichloromethane. The retention time for IAA-Me was 8 min. Blank samples from the HPLC step were treated identically.

Quantitative analysis

The random error of the analytical procedure as a whole was evaluated by dividing a large methanol extract into many identical parts, ten of which were analysed (extract purification and HPLC) separately. All values were corrected for losses independently by means of the internal standard. The corrected values were used for evaluation of the random error.

The systematic error was evaluated by addition of different carefully measured amounts of pure IAA to methanolic extracts identical with those used to study the random error. In addition, a number of samples consisting of pure IAA were extracted and analysed.

RESULTS AND DISCUSSION

The trend in HPLC work seems to be towards an increased use of reversed-phase systems instead of ion-exchange or straight-phase adsorption systems. Reversed-

phase systems primarily separate compounds according to differences in lipophilic properties. For carboxylic acids this means that separations have to be made at a low pH, so that the acids are undissociated and thus in their most lipophilic state. This is called ion-suppression chromatography (ISC). Generally, a large proportion of water has to be employed in the eluent. This increases the viscosity, thus decreasing the column efficiency, and causes impracticably high pressures or slow flow-rates. An alternative to ISC is ion-pair chromatography (IPC). To the eluent is added a lipophilic counter ion, commonly tetrabutylammonium ion (TBA), which increases the selectivity for negatively charged compounds. The system can work at higher pH values, which is of great importance if plant extracts are being analysed, as these are frequently difficult to dissolve at low pH values. Also, the proportion of water in the eluent can be lowered.

We compared ISC and IPC in the separation of IAA, 2-indolecarboxylic acid and 5-indolecarboxylic acid. In both systems the methanol concentration and the pH were varied, and a retention factor (k') of 8.0 for IAA was considered desirable. This k' value was selected as a compromise between speed of analysis and resolution power, based on our previous experience with HPLC analysis of pine extracts.

The results are shown in Fig. 1. Both systems easily separated IAA and 2-indolecarboxylic acid. The separation of IAA from 5-indolecarboxylic acid is more difficult, as the lipophilic properties of the two compounds are similar. With ISC we could achieve only partial separation, and this required very low methanol concentrations. As the two compounds have similar pK_a values, a decrease in the pH of the eluent gave no improvement in the resolution. With the use of IPC, the two compounds can be separated even at a relatively high concentration of methanol and at high pH. The value of $k' = 8.0$ for IAA was achieved at methanol concentrations of 22.0% for ISC and 27.5% for IPC.

Non-acidic indoles are efficiently removed from the samples by the purification steps preceding HPLC[24]. A number of acidic indoles are, however, reported to occur in plants, *e.g.*, 3-indoleacrylic acid[25], 3-indolelactic acid[26], 3-indolepropionic acid[27], 3-indolebutyric acid[27], 5-OH-IAA[13] and 4-Cl-IAA[14]. The last-mentioned compound, in particular, is of interest as a possible endogenous auxin[1]. The chromatography of twelve indole acids in the two systems is shown in Figs. 2 and 3. The superiority of the IPC system is obvious. In particular, the ISC system fails to separate IAA from 4-Cl-IAA. In our opinion the ISC system therefore cannot be used to analyse IAA in plant extracts. In addition to its separation efficiency, the IPC system is simple, works at reasonable pressures in the isocratic mode and thus does not require sophisticated equipment to generate and pump the eluent.

The concentration of the counter ion strongly affects the retention. For Nucleosil C_{18}, the optimal concentration of TBA is 0.01 *M*. As shown in Fig. 4, both lower and higher concentrations decrease the k' value for IAA. We noticed that the k' value is not the same for IAA chromatographed alone and with plant extract. The difference is pronounced at sub-optimal concentrations of TBA, and decreases if the extracts are additionally purified by thin-layer chromatography (TLC). The most likely explanation is that the extracts contain compounds that serve as counter ions. This is supported by the minimal extract effect at optimal or supra-optimal TBA concentrations. Extensive purification of the extracts prior to HPLC analysis is thus recommended. This will also be necessary in order to remove interfering compounds

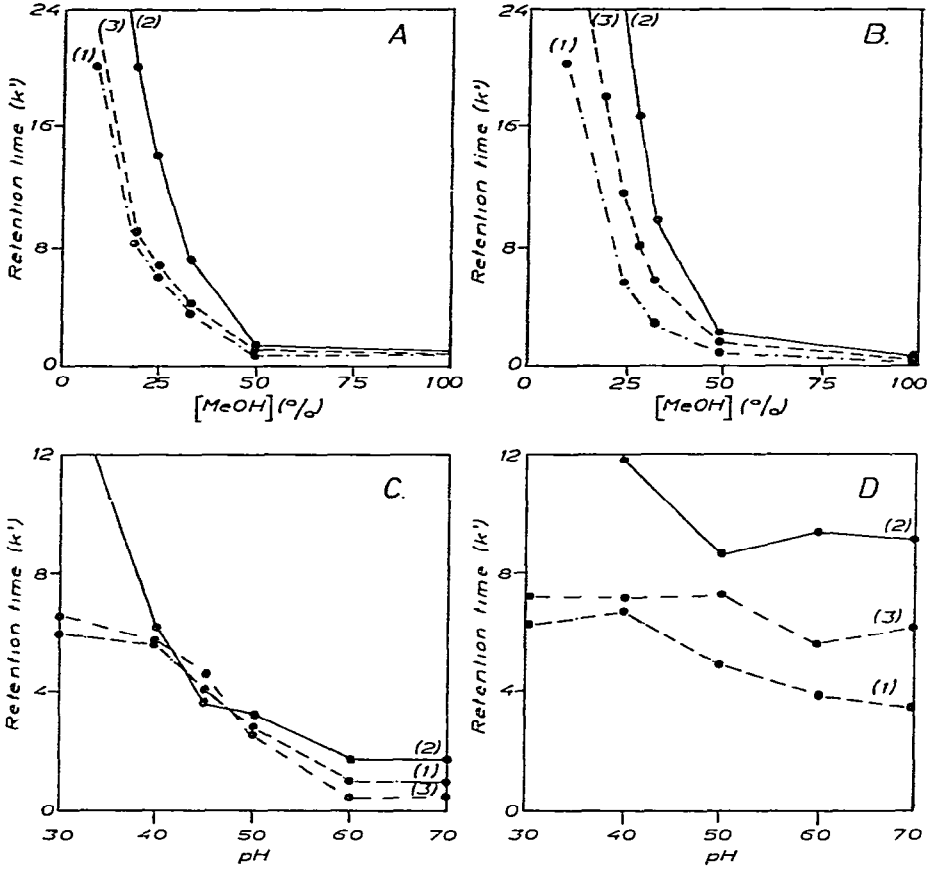


Fig. 1. HPLC separation of 5-indolecarboxylic (1), 2-indolecarboxylic (2) and 3-indoleacetic (3) acids on a Nucleosil C_{18} column. Influence of methanol concentration and pH of the eluent on retention. Eluent: methanol in 0.01 *M* phosphate buffer, without (A and C) or with (B and D) 0.01 *M* TBA. In (A) the pH is constant at 3.0 and in (B) at 6.5. In (C) and (D) the methanol concentration is 25%.

other than acidic indoles. Fluorescing compounds will give rise to peaks on the chromatogram and are thus simple to detect. Fig. 5 shows the final result of the analysis of a sample aliquot corresponding to 2.0 g fresh weight of pine tissue. The putative IAA peak is well separated from neighbouring peaks and shows no signs of asymmetry or shoulders. Slight changes in the composition or pH of the eluent did not affect the peak shape.

The sensitivity of the analytical method is a factor that seems to attract more interest than is warranted. It is often evaluated by analysis of pure standards devoid of all complicating extract components, so that the "sensitivity" claimed may have no practical significance. In most instances specificity and accuracy are of greater importance, and are also more difficult to evaluate. With the method described here it is possible to analyse the IAA content of single pine seedlings weighing 400 mg or less (see Fig. 6). For this to be achieved, however, the sensitivity had to be increased by raising the methanol concentration to decrease the k' value as low as 3.0 for IAA,

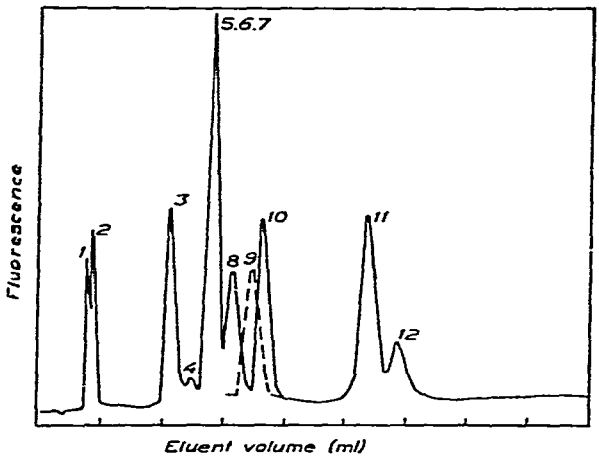


Fig. 2. HPLC separation of indolecarboxylic acids with ion-suppression chromatography on a Nucleosil C_{18} column. Eluent: 22.0% methanol in 0.01 *M* phosphate buffer (pH 3.0). Compounds: tryptophan (1), 5-OH-IAA (2), 3-indolelactic acid (3), 3-indolepyruvic acid (4), 3-indoleacrylic acid (5), 3-indoleglyoxylic acid (6), 5-indolecarboxylic acid (7), IAA (8), 4-Cl-IAA (9), 3-indolepropionic acid (10), 2-indolecarboxylic acid (11) and 3-indolebutyric acid (12). Peak 4 is uncertain because of the rapid degradation of 3-indolepyruvic acid in aqueous solution. Because of the small amounts available, 4-Cl-IAA (peak 9) was chromatographed separately.

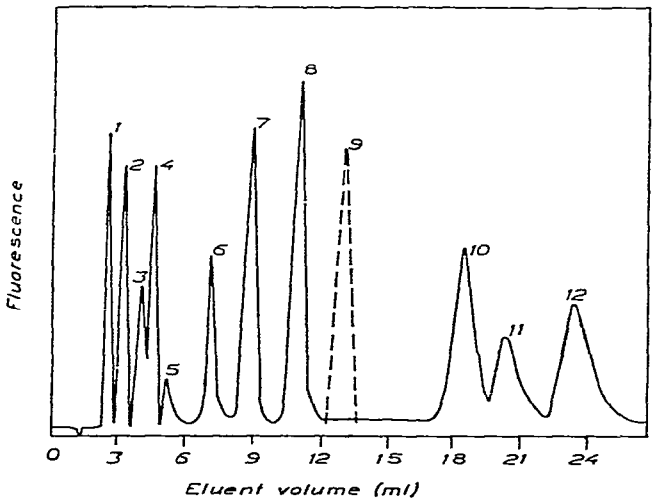


Fig. 3. HPLC separation of indolecarboxylic acids with ion-pair chromatography on a Nucleosil C_{18} column. Eluent: 27.5% methanol in 0.01 *M* phosphate buffer (pH 6.5) with 0.01 *M* TBA. Compounds: tryptophan (1), 5-OH-IAA (2), 3-indolelactic acid (3), 3-indoleacrylic acid (4), 3-indolepyruvic acid (5), 3-indoleglyoxylic acid (6), 5-indolecarboxylic acid (7), IAA (8), 4-Cl-IAA (9), 3-indolepropionic acid (10), 2-indolecarboxylic acid (11) and 3-indolebutyric acid (12). Peak 5 is uncertain because of the rapid degradation of 3-indolepyruvic acid in aqueous solution. Because of the small amounts available, 4-Cl-IAA (peak 9) was chromatographed separately.

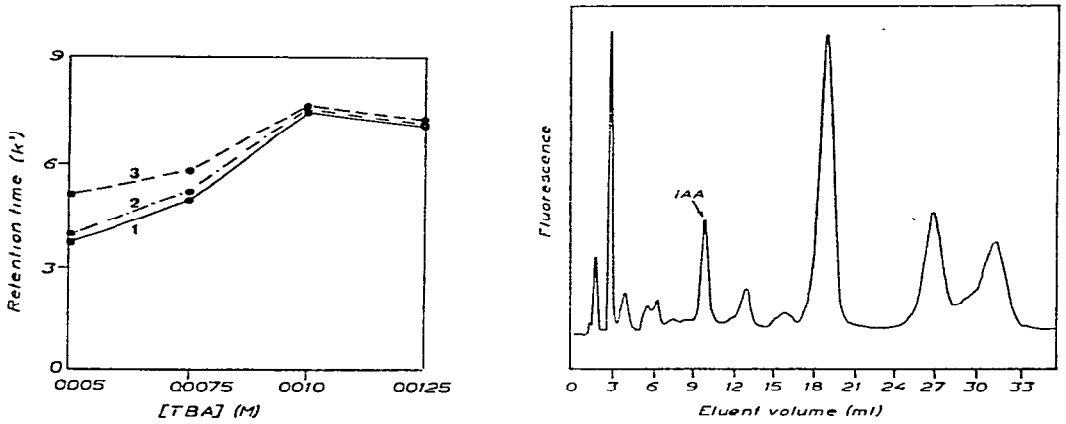


Fig. 4. Influence of the concentration of TBA in the eluent on the retention of IAA on a Nucleosil C_{18} column. Eluent: 27.5% methanol in 0.01 *M* phosphate buffer (pH 6.5). Compounds: standard IAA (1), IAA in pine extract purified by TLC (2) and IAA in pine extract that was not finally purified by TLC (3).

Fig. 5. HPLC of a pine extract. Column: 250 × 4 mm I.D., 10-μm Nucleosil C_{18}. Pre-column: 40 × 4 mm I.D., silica gel (120–160 mesh). Eluent: 27.5% methanol in 0.01 *M* phosphate buffer (pH 6.5) with 0.01 *M* TBA. Flow-rate: 1.5 ml min^{-1}. Detector: Spectra-Physics SD 970 spectrofluorimetric detector operating at 285 ± 5 nm (excitation) and 360 ± 10 nm (fluorescence). Extract equivalent to 2.0 g fresh weight of pine tissue was injected. The IAA peak corresponds to 26 ng. The recovery of IAA was 36%, giving an original concentration of IAA in the pine tissue of 36 ng g^{-1}.

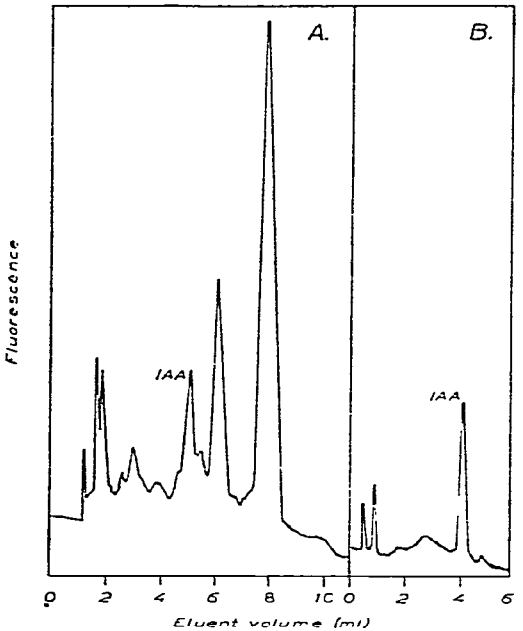


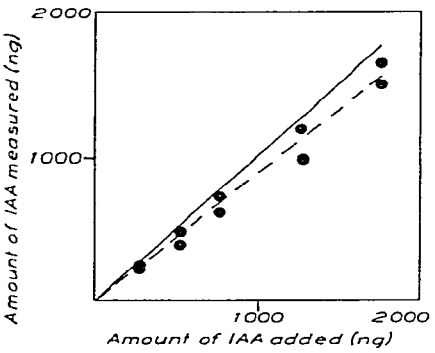
Fig. 6. HPLC of extracts from single pine seedlings (fresh weight 400 mg, one third of which was injected). Chromatographic conditions as in Fig. 5 except for the methanol concentration. Extract without final purification by TLC (A), methanol concentration 40.0%, $k' = 4.1$ for IAA, the IAA peak corresponds to 1.4 ng. Extract finally purified by TLC (B), methanol concentration 44.0%, $k' = 3.0$ for IAA, the IAA peak corresponds to 800 pg.

but the decreased separating power then necessitated additional sample purification by TLC. This, in turn, increased the losses of IAA. In extracts purified by TLC, amounts of IAA down to 50 pg can be analysed, however, and for our purposes this sensitivity by far exceeds our requirements.

The most important quality of an analytical method is its specificity. This can only be relative, *i.e.*, when applied in a particular context, the method measures what it is presumed to measure and nothing else. Considerable specificity is provided by the spectrofluorimetric detector. Indoles that could interfere in the detection of IAA are separated by the extract purification steps and the final HPLC analysis. The resulting chromatograms seem to indicate that the detector responds to a single fluorescing compound with an elution volume identical with that of IAA. The identity of this putative IAA peak was verified by GC–MS of its methyl ester. Although it cannot be definitely proved, the combined HPLC and GC–MS data lend strong support to the tentative conclusion that IAA is the single fluorescing compound recorded by the spectrofluorimeter detector in the IAA peak of the chromatogram. The additional compounds obviously present may either not interfere in the detection of IAA and thus be harmless, or by light absorption decrease the fluorescence signal from IAA and thus cause systematic errors. In either instance, the method would be specific for IAA when applied to pine extracts, although with possible systematic errors.

All analytical methods are inaccurate to some extent, and the value obtained in a single analysis deviates from the true value. The error of measurement has two components: random error and systematic error. The random error is by definition equal to the standard deviation of a population of measurements of identical samples, and shows a normal distribution. Ten independent analyses including corrections for losses gave the following result: mean value, 46 ng g^{-1} of IAA; standard deviation, 6.5 ng; random error, 14%. This magnitude of the random error is acceptable and probably far less than the variation between individual seedlings. The systematic error is often more critical and also more difficult to evaluate. For absolute quantifications, the systematic error must be accurately determined over the range of measurements. For relative quantifications, however, it is sufficient if the error can be shown to be proportional to the true value. It can be determined by analyses of known amounts of standard compounds, but may be different for analyses of plant extracts. For the latter, one method is the addition of known amounts of the standard compound, but this only shows the systematic error for extracts containing higher than normal concentrations of the compound, and extrapolation must be performed to cover the lower concentration range. The reliability of such procedures depends on the shape of the systematic error. Figs. 7 and 8 show that the systematic errors are identical for standard IAA and for IAA-spiked extracts, follows the linear function $y = k\,x$ and constantly under-estimates the true IAA content by about 12%. Because of the consistency and simplicity of the systematic error, we consider it safe to extrapolate to normal concentrations in extracts.

One obvious source of error is, as previously mentioned, the occurrence in extracts of light-absorbing components. As the systematic error is the same for extracts and for standard IAA, this cannot be the case with our extracts. If it were, the error would be larger for extracts. Other possible sources of systematic errors are the injection of sample on to the HPLC column and the collection of sample fractions



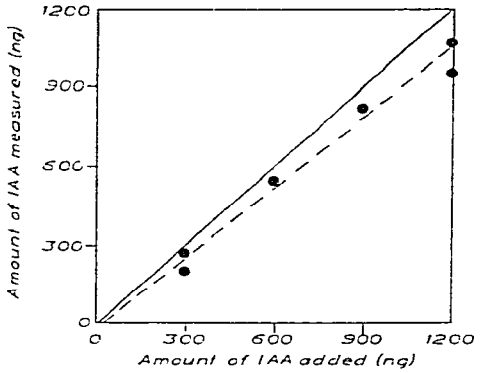


Fig. 7. Systematic quantification errors for standard IAA. The solid line represents the ideal situation with no error. The broken line depicts the actual result.

Fig. 8. Systematic quantification errors for pine extracts with added IAA. The solid line represents the ideal situation with no error. The broken line depicts the actual result.

for recovery measurements. As the total systematic error is small, follows a simple linear function and seems not to vary between the different extracts of various plant materials that we are analysing, we consider it unnecessary to trace its causes. Instead, we can easily correct the data.

CONCLUSIONS

For the following reasons we consider the method described here to be specific for IAA when pine samples are analysed:

(1) The putative IAA peak recorded by the detector contains IAA, as shown by GC–MS of its methyl ester. IAA-Me could not have been present in the extract during the HPLC analysis, as neutral compounds were previously removed, but must have been formed after HPLC upon treatment of IAA with diazomethane.

(2) The spectrofluorimetric detector was set for narrow wavelength bands that are relatively specific for indoles.

(3) Non-acidic indoles are efficiently removed by the sample purification procedures.

(4) Acidic indoles known to occur in plants, including the potent auxin 4-Cl-IAA, and a number of other acidic indoles are efficiently separated from IAA by the HPLC analysis.

(5) The resulting putative IAA peak has the same elution volume as standard IAA, and this is the case also if the methanol concentration or pH of the eluent is changed.

(6) The putative IAA peak is symmetrical without shoulders, and changes to the eluent did not alter its shape.

(7) The systematic error is small, follows a simple mathematical function and is identical for standard IAA and for IAA in extracts.

It should be noted, however, that the specificity of the method is only relative,

and that other types of plant extracts may contain interfering substances. The purification procedures we employ are efficient for pine extracts but may be of limited value for other types of material.

The method is sufficiently sensitive for our purposes. We can analyse as little as 50 pg of IAA in any purified pine sample. If recoveries of 20% can be obtained, which we have found to be no problem even if TLC is included, this would correspond to about 5 mg of normally IAA-rich pine tissue. A pair of needles, for comparison, may weigh about 60 mg.

The method is accurate and precise. Systematic errors cause a consistent underestimation by 12%, which can easily be corrected for. The random error is 14%. This is considerably less than the 20% we found for the indolo-α-pyrone method applied to the analysis of samples similar to those used for this investigation. It should be noted that we have determined the errors of the entire analytical procedure and not merely of the HPLC step. The systematic error in particular may be very different for other types of plant extracts, particularly if these have not been rigorously purified, and may even have characteristics that make quantitative analysis impossible. Before the method is used for purposes other than IAA analysis of pine tissue, an evaluation of the accuracy and precision for that particular application must be made.

ACKNOWLEDGEMENTS

We thank Dr. Kjeld Engvild, Risø, Denmark, for a gift of 4-Cl-IAA, Ms. Barbro Aldén and Mr. Kåre Eriksson for technical assistance and Dr. Kurt Andersson for advice and supervision. The staff at the National Board of Occupational Safety and Health, Umeå, allowed us to use their GC–MS equipment. The work was financially supported by the Swedish Council for Forestry and Agricultural Research (grants P316 and P331), the Swedish Natural Science Research Council (grant E 3769-100) and the J. C. Kempe and Seth M. Kempe Memorial Foundations.

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