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SIMULTANEOUS ANALYSIS OF INDOLE-3-ACETIC ACID AND DETECTION OF 4-CHLOROINDOLE-3-ACETIC ACID AND 5-HYDROXYINDOLE-3-ACETIC ACID IN PLANT TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THEIR 2-METHYLINDOLO-α-PYRONE DERIVATIVES

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SUMMARY

The use of high-performance liquid chromatography in the reversed-phase mode coupled to a spectrophotofluorimetric detector allowed the selective detection of acidic indoles possessing a C-3 methylene side chain, following the reaction of a purified plant extract with acetic anhydride. Of these acidic indoles, only 4-chloroindole-3-acetic acid and 5-hydroxyindole-3-acetic acid were found to have fluorescence activity greater than 1 % of that of indole-3-acetic acid, and good separation of these derivatives was obtained by high-performance liquid chromatography. In purified tissue of both *Pinus sylvestris* and *Chamaecyparis lawsoniana*, two fluorescing peaks were separated following derivatisation. The first had an identical fluorescence emission spectrum and retention time in high-performance liquid chromatography to the 2-methylindolo-α-pyrone derivative of indole-3-acetic acid. The second is so far unidentified. The presence of such unidentified compounds in extracts analysed by the standard spectrophotofluorimetric method would lead to an overestimate of the levels of indole-3-acetic acid. The use of high-performance liquid chromatography in the analysis of derivatised acidic indoles is discussed in relation to other published modifications of the method.

INTRODUCTION

The accurate quantification of endogenous auxins is essential if they are to be related to parameters of plant cell and tissue growth and changes in developmental patterns[1]. The introduction of a relatively simple and rapid technique which measures the fluorescence of 2-methylindolo-2,3:3′,4′-pyr-6-one (2-MIP), the derivative formed from the reaction of indole-3-acetic acid (IAA) with acetic anhydride (AA) in the presence of trifluoroacetic acid (TFA)[2,3], apparently provided such a physicochemical assay and has been extensively used (*e.g.*)[4]. This reaction detects both acidic and neutral indole compounds with a C-3 methylene side chain[5,6]. Whilst acidic indoles will remain in the purified extract, neutral indoles are removed during purification.

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IAA is likely to be the most important acidic indole present but attention should also be given to 4-chloroindole-3-acetic acid (4-Cl-IAA), which has been identified in plant tissues[7,8] and shown to have strong auxin activity in the *Avena* coleoptile straight growth test[9]. Of the other acidic indoles found in plant tissues, *viz.* 5-hydroxyindole-3-acetic acid (5-OH-IAA)[10], indole-3-proprionic acid and indole-3-butyric acid[11], indole-3-lactic acid[12] and indole-3-acrylic acid[13], only 5-OH-IAA can be detected by the spectrophotofluorimetric method described. Thus to date, IAA, 4-Cl-IAA and 5-OH-IAA can be detected by the spectrophotofluorimetric method but although it has been refined since its original publication[6,14–16] as presently used it does not distinguish between these three acidic indoles. However, the inclusion of a high-performance liquid chromatography (HPLC) step prior to derivatisation has been reported to allow the separation of IAA from these other acidic indoles[17,18]. Further there are two other possibilities to be considered. The first is the natural occurrence of so-far unidentified acidic indoles and the second the presence of other contaminating fluorescent compounds, which may even be present in an HPLC purified extract.

[^{14}C]IAA is routinely added at the beginning of purification procedures and assayed at the end to provide a measure of loss of IAA by isotope dilution. Breakdown products of IAA are removed in the various partition steps. The final stages in the preparation of the sample for the spectrophotofluorimetric method involve the removal of water from the solvent fraction by freezing and filtering, evaporation to dryness, solution in methanol and division into fractions. Known aliquots of authentic IAA standard are then added and these are reduced to dryness and derivatised. The use of IAA standards takes into account any losses incurred after their addition, but losses incurred during the evaporation step immediately prior to the addition of standards are not accounted for by the method as presently used. Significant IAA breakdown is considered to occur at each evaporation step[19] and, unless removed by partition, breakdown products may persist and falsify the final result for IAA content. This problem cannot be solved by partitioning, however, for each partition step must necessarily be followed by an evaporation step which causes further IAA breakdown.

This paper describes a modification of the standard spectrophotofluorimetric method, involving HPLC separation of the derivatised extract, which takes into account the last mentioned loss, allows the simultaneous analysis of IAA and detection of 4-Cl-IAA and 5-OH-IAA and provides an additional purification step for the separation of contaminating fluorescent compounds. In recent years various forms of HPLC separation, such as anion-exchange[20], reversed-phase ion-supression[21,22] and reversed-phase ion-pair chromatography[18,23,24] have been used for the separation and analysis of IAA, although they have been applied exclusively to "underivatised" plant extracts.

EXPERIMENTAL

Vegetative tissues from shrubs of *Cotinus coggygria* cv "Royal Purple" and hedge-grown *Chamaecyparis lawsoniana* cv "Fraseri" were harvested in autumn 1981, frozen immediately in liquid nitrogen, freeze-dried and stored at −20°C. Seedlings of *Pinus sylvestris* and *Pinus contorta* were grown in vermiculite on a mist bench under

natural daylight conditions in a polythene tunnel at a minimum temperature of 20 ± 1°C. Six-week-old seedlings were harvested in April 1982 and stored at −20°C until use.

Extraction

All solvents used were of analytical grade (BDH, Poole, Great Britain) and had previously been redistilled. Samples of plant material (0.5–2.0 g dry weight) were homogenised with 50 cm^3 methanol in an MSE homogeniser at 4°C for 10 min. The homogenate was filtered and the solid material rehomogenised. The first filtrate was then added to this homogenate and stirred for 10 h at 4°C in the dark. An internal standard of 5000 dpm of 305 μCi mg^{-1} [2-^{14}C]IAA (Radiochemical Centre, Amersham, Great Britain), was added and the homogenate filtered. The filtrate was evaporated to near dryness under vacuum at 30°C. The residue was dissolved in 0.5 *M* phosphate buffer (pH 8.0) and centrifuged for 1 h at 23,000 *g*. The supernatant was washed twice with light petroleum (b.p. 60–80°C) and once with diethyl ether, adjusted to pH 3.0 and extracted three times with half its volume of diethyl ether. The diethyl ether phases were combined, 0.5 cm^3 of 0.05 *M* phosphate buffer (pH 8.0) added and evaporated to near dryness at 30°C under vacuum. The residue was dissolved in 0.5 cm^3 of 0.05 *M* phosphate buffer (pH 8.0) and subsequently washed five times with the same volume of buffer and applied to a 150 × 10 mm column of insoluble poly-N-vinylpyrrolidone (Polyclar AT, GAF, Great Britain). The column was eluted by gravity flow with 0.05 *M* phosphate buffer (pH 8.0) and the 20–120-cm^3 fraction collected, adjusted to pH 3.0 and extracted three times with diethyl ether. The diethyl ether phases were then combined, water was removed by freezing and filtering and the diethyl ether evaporated to dryness under vacuum at 30°C. The residue was taken up in methanol and stored at −20°C prior to analysis.

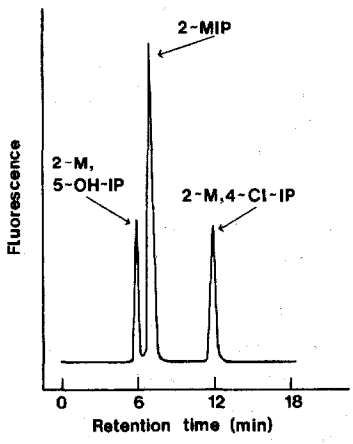
Derivatisation

Each sample for analysis was evaporated to dryness under a stream of nitrogen gas. The derivatisation was initiated by the addition of 25 mm^3 of a 1:1 mixture of AA–TFA (BDH). After 5 min the mixture was separated by HPLC (Advanced Chromatography Systems), as follows; the sample was injected via a Rheodyne valve and 200-mm^3 injection loop onto a semi-preparative 5 μm Hypersil ODS column (250 × 10 mm I.D.) or an analytical 5 μm Hypersil ODS column (250 × 5 mm I.D.), each fitted with a 55 × 5 mm pre-column packed with LiChroprep RP-18 (Merck). The high-performance liquid chromatograph, fitted with a digital programmer was connected to a detector system consisting of a UV absorbance monitor (Perkin-Elmer LC 75) set at 280 nm and a spectrophotofluorimeter (Perkin-Elmer MPF 43A) adjusted to an excitation wavelength of 445 ± 5 nm and an emission wavelength of 480 ± 10 nm. The mobile phase used for both columns was a gradient of methanol and water (adjusted to pH 3.5 with glacial acetic acid), both of which had been redistilled, filtered through a millipore filter of pore size 2.0 μm (Millipore) and degassed by stirring under vacuum. The fraction corresponding to authentic 2-MIP was collected, diluted with Lumagel scintillation fluid (Lumac Systems) and the percentage recovery of [^{14}C]IAA determined using a Hewlett-Packard Tricarb scintillation counter.

RESULTS

HPLC separation

2-MIP was eluted by a gradient of 50–100% methanol in pH 3.5 water at a flow-rate of 2 cm^3 per min, rising at 1% per min for 5 min, 2% per min for 10 min, and 5% per min for 5 min. The retention time of 2-MIP on a semi-preparative Hypersil ODS (5 μm) column was 13.5 min and 7 min on an analytical column. Of the other acidic indoles reported to be present in plant tissue, only 5-OH-IAA and 4-Cl-IAA were found to fluoresce following derivatisation. Using the above programme these three compounds could be well separated (Fig. 1). The fluorescence emission spectra of these compounds (Fig. 2) show that whilst 2-MIP, and 2-methyl-4-chloro-



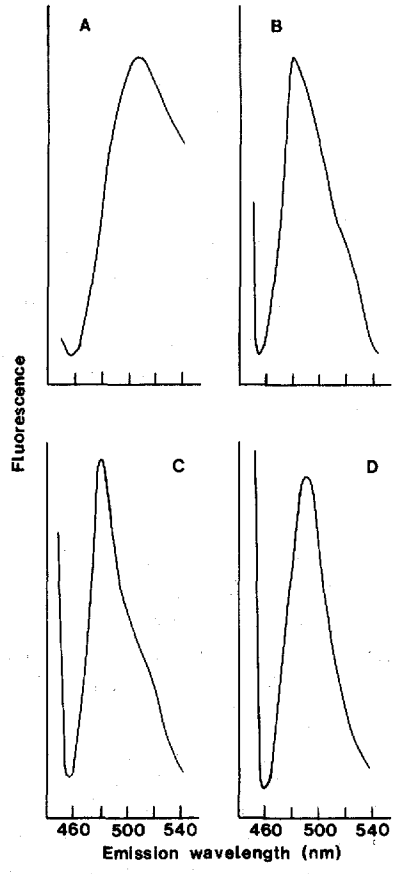


Fig. 1. HPLC separation of a 1:1:1 mixture of authentic 5-OH-IAA, IAA and 4-Cl-IAA standards following derivatisation with AA–TFA. Column, 5 μm Hypersil ODS (250 × 5 mm I.D.). Flow-rate: 2 cm^3/min. Mobile phase: gradient of 50–100% methanol in pH 3.5 water, at a rate of 1%/min for 5 min, 2%/min for 10 min, and 5%/min for 5 min. Spectrophotofluorimetric detection at 480 nm following excitation at 445 nm.

Fig. 2. The fluorescence emission spectrum of (A) AA, (B) 2-MIP, (C) 2-M,4-Cl-IP and (D) 2-M,5-OH-IP, formed by derivatisation with AA–TFA. Excitation wavelength 445 nm. The fluorescence sensitivity was adjusted to give peaks of similar intensity.

indolo-2,3:3′,4′-pyr-6-one (2-M,4-Cl-IP), the derivative of 4-Cl-IAA, peak at 480 nm, 2-methyl-5-hydroxyindolo-2,3:3′4′-pyr-6-one (2-M,5-OH-IP), the derivative of 5-OH-IAA, peaks at 489 nm and AA at 514 nm following excitation at 445 nm. Thus, the emission spectrum of a mixture of these compounds will not distinguish between 2-MIP and 2-M,4-Cl-IP and will distinguish 2-M,5-OH-IP only if large quantities are present.

Derivatisation

Following reduction to dryness of standards in a gas chromatography 'v'-vial under a stream of nitrogen gas, 25 mm^3 of AA–TFA was found to saturate the reaction. The examination of samples after 0.25, 0.5, 1.0, 2.0 and 5.0 min showed that derivatisation of IAA and 5-OH-IAA takes place almost instantaneously and was complete within 1 min. However, 4-Cl-IAA required 4 min. Conditions must be

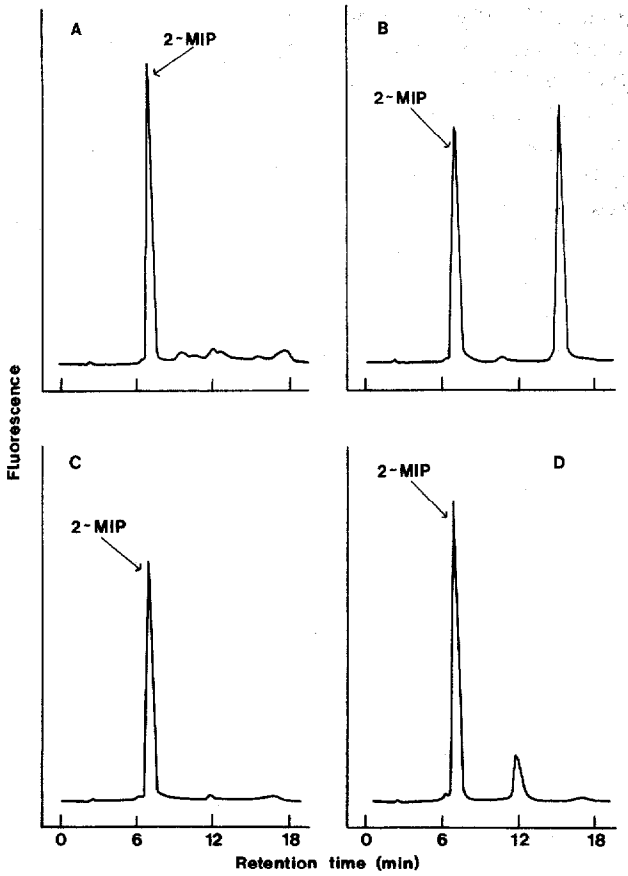


Fig. 3. HPLC separation of purified extracts following derivatisation with AA–TFA. Column: 5 μm Hypersil ODS (250 × 5 mm I.D.). Flow-rate: 2 cm^3/min. Mobile phase: gradient of 50–100% methanol in pH 3.5 water, at a rate of 1%/min for 5 min, 2%/min for 10 min, and 5%/min for 5 min. (A) *Cotinus* leaf tissue, (B) *Chamaecyparis* leaf tissue, (C) *Pinus contorta* seedlings and (D) *Pinus sylvestris* seedlings. Spectrophotofluorimetric detection at 480 nm following excitation at 445 nm.

established for maximum derivatisation of plant material; with an extract from 600 mg of freeze-dried *Cotinus* leaf material the reaction required 5 min.

It is known that partial inhibition of the reaction occurs in many species[6]. This was shown to be approximately 15% in an extract of 600 mg of *Cotinus* leaf tissue by the reduction in peak height following the addition of such extracts to known amounts of IAA prior to derivatisation. This does not present a problem because any [^{14}C]IAA internal standard not derivatised will be separated from 2-MIP during the HPLC separation and therefore possible errors from this source will be avoided.

Decay of 2-MIP

Both the long-term and short-term decay of 2-MIP need to be considered: (a) Long-term decay: 2-MIP is unstable in methanol–water mixtures and slowly breaks down with the loss of fluorescence activity. The magnitude of fluorescence after total decay is referred to by Mousdale[16] as the decay blank. As he has pointed out this value is often larger than that of the reagent blank suggesting the presence of stable fluorescing compounds. The presence of unstable fluorescing compounds must also be considered. If these decay at rates which are significantly different from that of 2-MIP their presence can be detected using Mousdale's method of decay kinetics. If an unstable interfering compound decays at the same rate as 2-MIP it will not be detected by the study of decay kinetics[23]. This problem can be resolved using HPLC as is shown by the use of derivatised extracts of *Chamaecyparis* and *Pinus silvestris* (Fig. 3). In *Chamaecyparis* good separation of two fluorescing compounds is obtained, one of which has an identical emission spectrum and retention time to 2-MIP, the other an unknown interfering compound. HPLC of derivatised extracts of *Pinus sylvestris* also separates two compounds. The first has an identical emission spectrum and retention time to 2-MIP, the second has an emission spectrum and retention time almost identical to that of 2-M, 4-Cl-IP (Figs. 1 and 3). No such interfering com-

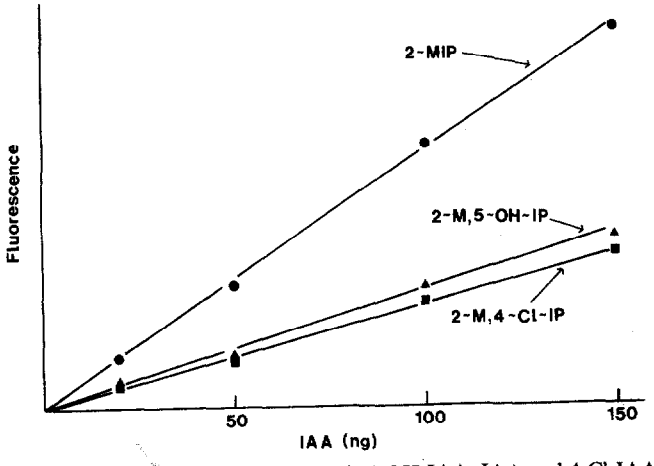


Fig. 4. Calibration curves of authentic 5-OH-IAA, IAA and 4-Cl-IAA standards following derivatisation with AA–TFA and HPLC separation. Column: 5 μm Hypersil ODS (250 × 5 mm I.D.). Flow-rate: 2 cm^3/min. Mobile phase: gradient of 50–100% methanol in pH 3.5 water at a rate of 1%/min for 5 min, 2%/min for 10 min, and 5%/min for 5 min. Spectrophotofluorimetric detection at 480 nm following excitation at 445 nm. Standard errors are contained within the points.

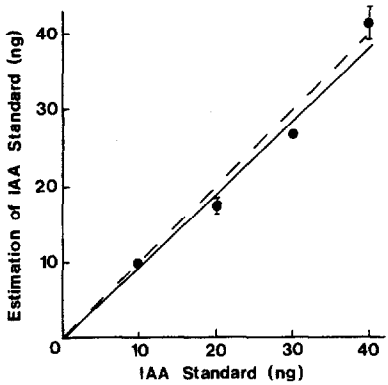


Fig. 5. Estimations of authentic IAA standards taken through the purification procedures, and analysed by HPLC coupled to spectrophotofluorimetry of the 2-MIP derivative. Estimates of IAA are indicated by the solid line; broken line represents the ideal situation. Standard errors are contained within the points except where shown.

pounds were found in extracts of *Cotinus* or *Pinus contorta* (Fig. 3). In all four species, the 2-MIP peak decayed to zero indicating the absence of stable fluorescing contaminants. (b) Short-term decay: as described above, the internal [^{14}C]IAA standard allows correction for losses incurred at all points in the procedures. It is inevitable that some breakdown of 2-MIP will occur between its formation in the derivatisation step and its assay after HPLC separation. Breakdown in a 1:1 mixture of methanol–water (pH 3.5), expressed as a percentage, was 5.8 $\pm$ 0.6 (standard error) and 10.1 $\pm$ 1.6 after 7 and 13.5 min, the approximate retention times of 2-MIP on an analytical and semi-preparative column respectively. This loss in the plant extract is corrected for by the addition of [^{14}C]IAA as an internal standard. Addition of [^{14}C]IAA to the external standard permits the production of a corrected calibration curve.

The calibration curves for 2-MIP, 2-M,5-OH-IP and 2-M,4-Cl-IP were found to be linear over the range 0 to 150 ng (Fig. 4) on both the semi-preparative and analytical columns. The use of the semi-preparative column allows the routine estimation of these compounds down to 250 pg.

TABLE I

ESTIMATIONS OF THE ENDOGENOUS IAA CONTENT OF PLANT TISSUES ANALYSED BY THE INTENSITY OF FLUORESCENCE OF THE 2-MIP DERIVATIVE FOLLOWING HPLC SEPARATION OF THE DERIVATISED EXTRACT

The means and standard errors of extractions of three replicate batches of tissue are shown. The presence of interfering fluorescing compounds, whose intensity of fluorescence is >5% of that of 2-MIP, is indicated.

Plant material	*IAA Estimation (ng per g dry weight)*	*Presence of interfering fluorescing compounds*
Cotinus leaf tissue	16.7 $\pm$ 1.3	–
Chamaecyparis leaf tissue	110.0 $\pm$ 7.4	+
Pinus contorta seedling	52.4 $\pm$ 2.1	–
Pinus sylvestris seedling	67.5 $\pm$ 5.3	+

To check the accuracy of the HPLC method a series of standard amounts of IAA were put through the purification and analysis procedures. The results (Fig. 5) demonstrated very close agreement. Typical values for IAA content are shown in Table I and the presence or absence of interfering fluorescing compounds indicated.

DISCUSSION

Several reports of improvements to the spectrophotofluorimetric method have recently been made. Knegt *et al.*[25] described fine-tuning adjustments to the standard method[3] but did not address the problems raised in this paper. Removal of 4-Cl-IAA and 5-OH-IAA prior to derivatisation has been achieved by HPLC separation[17,18] and by a thin-layer chromatographic separation[19,26], both of which are also designed to remove other fluorescing compounds if present. In these modified forms the spectrophotofluorimetric method would not allow for the detection of these compounds at

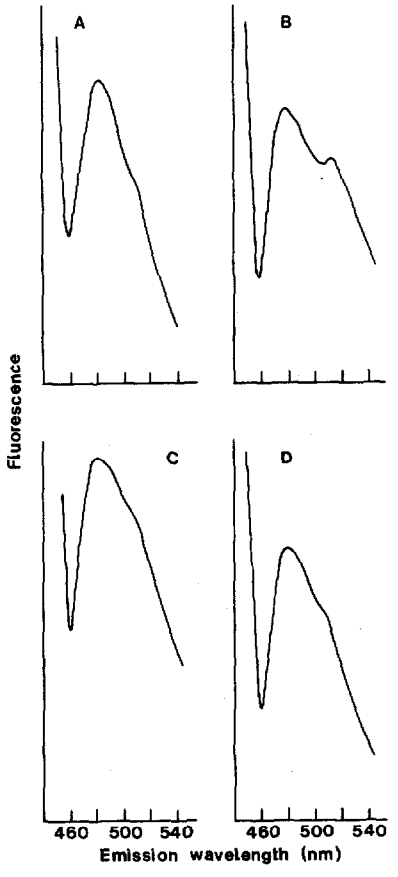


Fig. 6. The fluorescence emission spectrum of purified extracts of (A) *Cotinus* leaf tissue, (B) *Chamaecyparis* leaf tissue, (C) *Pinus contorta* seedlings and (D) *Pinus sylvestris* seedlings, following derivatisation with AA–TFA. Excitation wavelength 445 nm. The fluorescence sensitivity was adjusted to give peaks of similar intensity.

the same time as that of IAA. Whilst very close agreement has been demonstrated by Iino and Carr[26] between the spectrophotofluorimetric method and gas chromatography–mass spectrometry, the method has apparently so far been tested only on young etiolated maize shoots. Sandberg and Dunberg[23] compared natural fluorescence measurement following HPLC separation with the spectrophotofluorimetric method. Good agreement was demonstrated only after the inclusion of at least two HPLC purification steps prior to derivatisation and routine under-estimation of standards by 24% was obtained.

The HPLC separation of partially purified plant extracts *after* derivatisation by reaction with AA–TFA allows the separation and quantification of 4-Cl-IAA, IAA and 5-OH-IAA simultaneously (Fig. 1). It also enables the demonstration of the presence and the separation of interfering fluorescent compounds (Fig. 3). Sandberg and Dunberg[23] point out that the presence of compounds other than IAA will be indicated if the fluorescence maxima of their derivatives differ from that of 2-MIP. An example of this is shown by extracts of *Chamaecyparis*. The fluorescence emission spectrum indicated the presence of a contaminating compound (Fig. 6) and this was resolved by HPLC (Fig. 3). However, the fluorescence emission spectrum of derivatised extracts from *Pinus silvestris* did not indicate the presence of a contaminant (Fig. 6), yet two compounds were separated by HPLC. Thus the fluorescence emission spectrum will not always indicate the presence of an interfering compound. The contaminant in *Pinus silvestris* has the same retention time on HPLC as 2-M,4-Cl-IP (Figs. 1 and 3) and confirmation of its identity by gas chromatography–mass spectrometry is in progress.

After the final evaporation step of the purification procedure it is inevitable that some radioactive breakdown products of [^{14}C]IAA will remain in the extract. Unless the IAA is quantified by a technique which combines separation and analysis these breakdown products will persist in the fraction to be counted and thus lead to the underestimation of the levels of endogenous IAA. HPLC separation of the 2-methylindolo-α-pyrone derivative of IAA constitutes such a method.

Until now, HPLC has been applied exclusively to the separation of underivatised plant extracts, either as a purification step for spectrophotofluorimetry[17,23] or for the analysis of IAA by its natural fluorescence[18,20–22]. The use of the HPLC separation of derivatised extracts removes the need for both lengthy purification procedures[18,20,23] and several semi-preparative HPLC steps[20–22].

It is thus considered that the modifications to the standard spectrophotofluorimetry procedure as described above provide a significant improvement to its accuracy, specificity and sensitivity. Less tissue is required as the extract need no longer be divided and there is the added advantage of the ability to detect 4-Cl-IAA and 5-OH-IAA simultaneously with IAA thus enabling a more complete picture of the auxin profile of a tissue to be obtained. The accurate determination of the levels of 4-Cl-IAA or 5-OH-IAA can be achieved if specific radiolabelled standards are available for the estimation of losses during purification.

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