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PRECISION AND ACCURACY OF RADIOIMMUNOASSAY IN THE ANALYSIS OF ENDOGENOUS 3-INDOLEACETIC ACID FROM NEEDLES OF SCOTS PINE

GORAN SANDBERG, KARIN LJUNG and PER ALM*

Department of Forest Genetics and Plant Physiology, The Swedish University of Agricultural Sciences, S-901 83 Umeå, Sweden; *Division of Experimental Medicine, National Defence Research Institute, Department 4, S-901 82 Umeå, Sweden

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Key Word Index—*Pinus sylvestris*; Pinaceae; Scots pine; quantitative analysis; radioimmunoassay; high performance liquid chromatography; 3-indoleacetic acid; successive approximation.

Abstract—The precision and accuracy of a radioimmunoassay (RIA) for 3-indoleacetic acid (IAA) were assessed when applied to the analysis of extracts from *Pinus sylvestris* seedlings. It was found that extracts contained contaminants that adversely affected both the precision and accuracy of quantitative estimates of IAA. When samples were subjected to purification procedures prior to RIA, accurate and more precise estimates were obtained. Parallel analysis with high performance liquid chromatography using a fluorescence detector (HPLC-FL) indicated that HPLC-FL provided accurate estimates of the IAA content of *Pinus* extracts at the same stage of purification as RIA. The precision of HPLC-FL estimates was similar to that obtained with RIA.

INTRODUCTION

Quantitative analysis of 3-indoleacetic acid (IAA) has been the object of numerous investigations since the early days of auxin research in the 1920's and 1930's. Several sensitive analytical procedures for IAA are currently available. They include combined gas chromatography–mass-spectrometry (GC/MS) in the multiple and single ion mode [1, 2], high performance liquid chromatography with fluorescence (HPLC-FL) and/or polarographic detection [3, 4] and gas chromatography (GC) with electron capture [5] or alkali-flame detection [6]. Quantitative analysis of IAA by fluorescence detection of the condensation product of IAA with acetic anhydride, 2-methylindole-α-pyrone (2-MIP), is also used widely [7]. The accuracy of this technique when applied to extracts from conifer tissues has, however, been questioned [8].

The analysis of IAA in plant extracts is not a straightforward task because trace quantities of IAA have to be measured against a background of large amounts of contaminants. The application of published standardized purification procedures to samples from diverse sources, without prior investigation, is an approach that almost always leads to problems. A procedure optimized for a specific tissue, with its own characteristic assortment of impurities, is not necessarily suitable for other samples containing a different array of contaminants.

Recently, a new approach to IAA analysis has involved the use of radio (RIA) and enzyme linked immunoassays (ELISA) [9–12]. Immunological techniques for phytohormone analysis have attracted much attention as very little sophisticated equipment is required apart from either a liquid scintillation counter or a spectrophotometer. The methods are also suitable for processing large numbers of samples especially when automated equipment is available. Immunoassays undoubtedly have the potential for large scale collection of data and this could open a new dimension to whole plant hormone physiology. However, the reliability of these techniques remains to be proved, especially when applied to the analysis of impure samples.

The production of antisera against phytohormones was first reported in 1971 by Fuchs *et al.* [13]. Immunological procedures for IAA were further developed in 1977 by Pengelly and Meins [9], who linked the IAA molecule to a protein via the nitrogen in the indole ring. Subsequently, Weiler [11] devised an alternative to this procedure by linking the protein to the carboxyl group and using [^{3}H]- or [^{125}I]IAA as tracers. The simplicity of the RIA is obvious. The reliability of immunological assays, however, was not discussed until Pengelly *et al.* [10] published a paper on a validition of the RIA technique by a comparison with selected ion monitoring GC/MS. Unfortunately, this excellent study does not provide a general view of the relationship between sample purity and the accuracy of the RIA. This can, however, be achieved through the application of a 'successive approximation', as described by Reeve and Crozier [14]. A successive approximation works in the following manner. When given a sample portion shown to contain a given quantity of IAA (E_1) the test for accuracy consists of purifying the sample and re-estimating the IAA content (E_2). An internal standard is used so sample losses encountered during purification can be assessed. If E_1 is accurate, E_2, taking into account the precision of the method, should not be significantly different. If a difference is found, E_1 must be rejected as inaccurate and E_2 tested by further purification and analysis. This process is continued until an estimate is obtained that does not change on purification. At this point it becomes possible to conclude that there are no grounds for believing that the estimate is inaccurate [14].

RESULTS AND DISCUSSION

An investigation of the reliability of the RIA technique for IAA analysis must be based on sera with the same specificity and sensitivity as used in other investigations. The diluted serum used in the present investigation had a linear detection range of 0.6–12 ng. The specificity of the IAA serum compared with other indoles, such as tryptophan, 3-indoleacetaldehyde, 5-hydroxy-3-indoleacetic acid, 3-indolealdehyde and 3-indoleacetyl aspartic acid, was equal or higher than in earlier reports [11]. The cross reactivity with 3-indoleethanol, 3-indolemethanol and 3-indolecarboxylic acid was less than 5% for each compound The characteristics of the RIA are, thus, similar to those of immunoassays utilized by other investigators [9–12].

An outline of the successive approximation procedure employed is presented in Fig. 1. Tissue was extracted with methanol containing [^{14}C]IAA as internal standard, phosphate buffer was added, the methanol extract reduced to the aqueous phase *in vacuo* and purified on a polyvinylpyrrolidone (PVP) column, an XAD-7 concentrator column and a silica gel Sep-Pak cartridge prior to sequential analysis in three HPLC systems each utilizing different separatory mechanisms. Seven replicate extracts were analysed. Quantitative estimates of endogenous IAA by RIA were based on: (i) triplicate analysis of methylated sample aliquots; and (ii) triplicate analysis of methylated sample aliquots mixed with known amount of IAA methyl ester. The apparent endogenous IAA content obtained by comparing values of per cent immunotracer bound with a standard curve was plotted against the amount of exogenous IAA added. This procedure makes it possible to detect inactivation of the antibody or binding of compounds other than IAA in the samples that would give a spurious estimate of IAA content. In principle, the exogenous IAA curve should be a straight line with unit slope and a *y*-interscept equal to the amount of IAA originally present in the methylated sample. The presence of interfering compounds that adversely affect accuracy will be indicated by a change in the slope of the curve. This procedure was described by Pengelly and Meins [9] and elaborated by Reeve and Crozier [14] in a discussion of potential sources of error in analyses with the 2-MIP assay.

An outline of the successive approximation procedure used is presented in Fig. 1 and the data obtained are summarized in Table 1. RIA of samples to which known amounts of exogenous IAA were added provide the more precise estimates of endogenous IAA (Table 1). They also

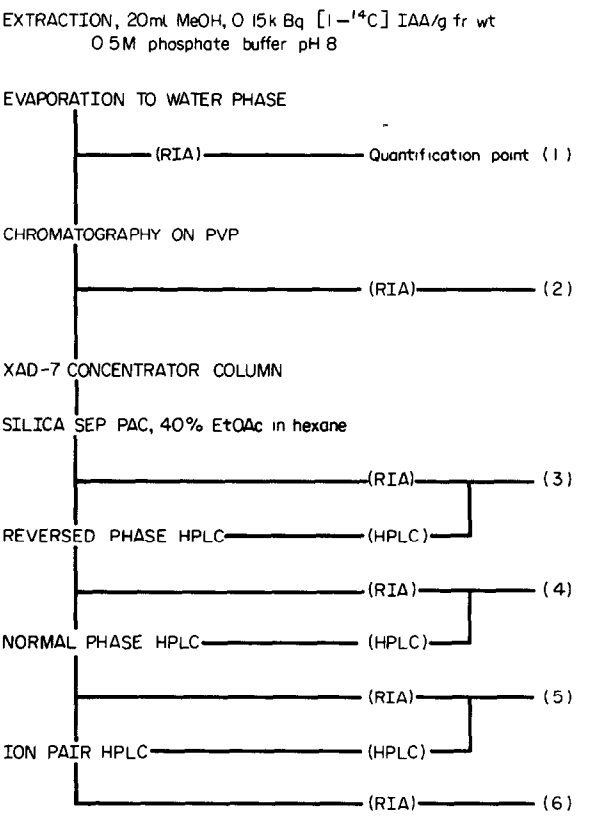

Fig. 1. The successive sample purification.

detect the presence of interfering compounds which adversely affected accuracy at quantification points 1 and 2. Interference was not apparent at quantification point 3 (see Fig. 2) and beyond. The precision of estimates obtained with both RIA procedures improved with sample purification up to point 3. It is, thus, apparent that unless samples are purified adequately, contaminants have detrimental effects on both the precision and accuracy of RIA estimates.

For comparative purposes parallel analyses with HPLC-FL were performed. The HPLC-FL estimates provide an example of the purity required for analysis of endogenous IAA by a physicochemical method. The data obtained are presented in Table 1. HPLC-FL estimates

Table 1. Successive approximation of estimates of the endogenous IAA content of *Pinus sylvestris* extracts obtained with RIA and HPLC-FL

Quantification point	RIA	RIA with added exogenous IAA	HPLC-FL exogenous IAA
1	85.4 ± 40.4 (47.3%)	87.6 ± 36.1 (41.2%)	—
2	68.6 ± 18.4 (41.4%)	62.3 ± 21 5 (34.6%)	—
3	60.3 ± 17.6 (29.2%)	51.5 ± 11 0 (21.4%)	43 5 ± 7.6 (17.4%)
4	54.5 ± 12.8 (23.4%)	54 0 ± 8.8 (16.3%)	40 6 ± 7 0 (17 0%)
5	51.2 ± 13.1 (25.6%)	50.2 ± 8 7 (17 4%)	44 5 ± 6.4 (14.0%)
6	56.4 ± 13.1 (22.1%)	53.4 ± 10.3 (19 2%)	—

Data expressed as ng IAA/g fr. wt. ± s.d. (per cent precision).

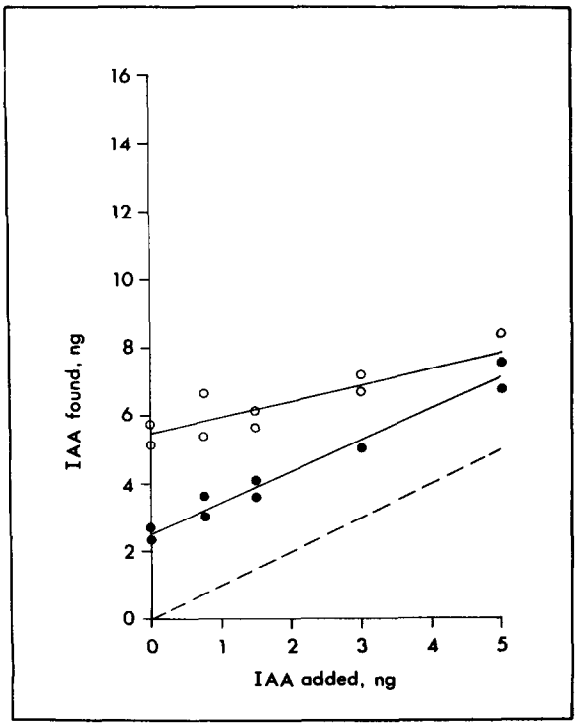

Fig 2. Internal standard curves for authentic IAA (---) and extracts at on purification levels 1 (○) and 3 (●). Aliquots of methylated extracts were analysed in triplicate after the addition of 0, 0.75, 1.5, 3.0 and 5.0 ng standard IAA methyl ester.

were obtained at quantification level 3 and did not change significantly with further purification. It was not possible to make realistic measurements prior to quantification point 3 because samples contained large amounts of fluorescent contaminants. The data, thus, indicate that HPLC-FL provides an accurate estimate of the IAA content of *Pinus sylvestris* extracts at the same stage of purification as RIA. The precision of HPLC-FL compares favourably with that of RIA. Although the HPLC-FL estimates were consistently lower than those obtained by RIA, the figures were not significantly different.

At least five procedures for quantitative analysis of IAA are now available; GC with selective detection, GC/MS in the single and multiple ion mode, HPLC-FL, 2-MIP fluorescence assay and immunological methods such as RIA and ELISA. The reliability of three of these assays, 2-MIP, HPLC-FL and RIA, when applied to conifer tissue, can be compared when data obtained in the present study are related to an earlier investigation with the 2-MIP and HPLC-FL assays [8]. The same experimental design and the use of identical plant material simplifies this comparison. The data presented in this report enable one to conclude that accurate estimates of IAA in Scots pine extracts can be obtained at the same purification level with RIA and HPLC-FL. The findings of Sandberg and Dunberg [8] suggest that the 2-MIP assay requires more extensive sample purification if reliable estimates are to be obtained. The three techniques all give a precision of *ca* 14% after adequate sample purification. Weiler [15] claims that: "The most prominent advantage of immunoassay over other techniques is its potential to assay rather impure samples". This is, in fact, not the case, at least as far as the analysis of the IAA content of extracts from Scots pine is concerned. The real value of immunological assays lies in the rapidity with which samples can be analysed. A further advantage is that there is less demand on equipment and, as a consequence, investment in instrumentation and maintenance are reduced. Finally, it should be noted that this study, as well as the work of Pengelly *et al.* [10], clearly shows that the RIA technique is sensitive to interfering substances in plant extracts. It is, therefore, important that the accuracy of the assay, for the samples under investigation, is thoroughly checked before routine quantification of endogenous hormones are undertaken.

EXPERIMENTAL

Extraction and purification. Scots pine seedlings (5-week-old) grown as described in ref. [16] were homogenized in MeOH at −20° (20 ml/g fr. wt). Aliquots of this extract were 'spiked' with [1-^{14}C]IAA as an int. standard (0.15 kBq/g fr wt, sp act. 1.8 GBq/mmol, Amersham), and 0.02 mol/l of the antioxidant sodium diethyldithiocarbamate. NaPi buffer (10 ml, 0.5 M pH 8) was added and the homogenate extracted for 2 hr at room temp. filtered and reduced to the aq. phase under red. pres at 40°

The aq phase was diluted to 10 ml with H_2O and run through a 10 × 1 cm i.d. PVP column. The eluant from the PVP column was acidified with HOAc to pH 2.7 and applied to a 150 × 4 mm i.d. column of Amberlite XAD-7 (0.3–1 mm) [17] A silica Sep-Pak cartridge (Waters Assoc.), pre-washed with 5 ml 1 M HOAc and 20 mM HOAc in hexane, was coupled to the XAD-7 column. The sample was eluted from the XAD-7 concentrator column and run through the silica gel Sep-Pak cartridge in a solvent comprising 20 mM HOAc in EtOAc–hexane (2:3) The organic phase was reduced to dryness under red. pres. at 40°.

High performance liquid chromatography. The sample was further purified on reversed-phase HPLC using a Gilson gradient system based on two Gilson Model 302 pumps with an Apple II microcomputer as gradient manager. Gradient elution from 20% to 70% of MeOH in 1% HOAc was carried out over 35 min Samples were introduced off column via Valco loop injector on a 250 × 4 6 mm i.d. 5 μm LiChrosorb RP-18 column (Merck, Darmstadt) eluted at 1 ml/min Column effluent was monitored with a Spectra Physics SF 970 fluorimetric detector (excitation 285 ± 5 nm, emission 360 ± 10 nm) with 5 μl flow cell and a radioactivity monitor (Reeve Analytical) in the heterogeneous mode

The IAA fraction was collected, evaporated to dryness, redissolved in 100 μl EtOAc–hexane (1:1) and introduced onto a 250 × 4.6 i.d. 5 μm Nucleosil CN column (Skandinaviska Genetec) eluted at 1 ml/min with EtOAc–hexane–HOAc (49:49:2). The IAA fraction was collected and reduced to dryness and introduced onto the RP-18 column described above and eluted with 35% MeOH in 0.01 M pH 6.5 NaPi buffer, containing 0.01 M tetrabutylammonium hydrogen sulphate as ion pair agent. The system was eluted at 0.7 ml/min. For more detailed information on the HPLC procedures used see refs [5, 8].

Immunization and antiserum production. Rabbits (12–15-week-old) were immunized with an emulsion of an IAA–bovine serum albumin conjugate coupled to COOH-11 in buffered physiological saline soln and Freunds complete adjuvant Blood was collected 2 weeks after the first injection and thereafter each month. The blood serum was stored at −18°. Proteases and free albumin was removed from the sera on a CA-G(K)B column (Bio Rad). The collected fraction was made up to 45% satd soln with

$(NH_4)_2SO_4$, stirred for 2 hr and centrifuged at 1000 *g* for 20 min. The pellet was resuspended in 45% satd $(NH_4)_2SO_4$ soln and again centrifuged at 1000 *g* for 20 min. The pellet was resuspended in PBS buffer (0.1 M K_2HPO_4, 0.14 M NaCl, pH 8) and desalted on a Bio Gel P-6 DG column (Bio Rad). The sera was thereafter stored at −18° until further use.

Radioimmunoassay. Samples were methylated with ethereal CH_2N_2 before the RIA assay. A standard assay mixture consisting of 80 μl sample, 30 μl tracer ([^{3}H]IAA Me ester, 0.33 kBq, sp. act. 1070 GBq/mmol) and 20 μl serum, all in PBS buffer were added to 1.5 ml disposable Eppendorf centrifuge tubes. After vortex mixing for 15 sec the tubes were incubated overnight at 4°. Non-specific binding was determined through a parallel incubation in PBS buffer without sample. Saturated $(NH_4)_2SO_4$ soln (150 μl) was added and, after 10 min pptn at room temp., the tube was centrifuged at 12 000 *g* for 15 min at 4°. After separation of the pellet and the supernatant, the pellet was dissolved for 30 min at 4° with 100 μl NCS protein solubilizer (Amersham International) and mixed with 500 μl Riafluor scintillation cocktail (New England Nuclear) before radioactivity was measured in a Model 1210 Wallac liquid scintillation counter. Methylated extracts were analysed in triplicate and assays of 0, 0.3, 0.6, 1.5, 3.0, 6.0, 12.0, 30.0 and 60.0 ng IAA Me ester were used to construct a standard curve. In certain instances 0, 0.75, 1.5, 3.0 and 5 0 ng IAA Me ester were added to the methylated samples prior to assay as described in ref [9].

Calculation. After correction for non-specific binding and the contribution of cold and labelled IAA from the labelled IAA added as int. standard during the extraction and purification, the per cent of ^{3}H bound was calculated on the basis of the amount of radioactivity present in the pellet.

Determination of errors and analysis. The accuracy of the HPLC-FL method and the RIA procedures was determined as described in ref. [14]. A large homogenous MeOH extract was divided into identical aliquots. Each aliquot was 'spiked' with [1-^{14}C]IAA and, thereafter, purified as described earlier. At the quantification points defined in Fig. 1, aliquots of the extracts were analysed for endogenous IAA. The precision of the methods at each quantification level was determined by calculation of the s.d. of the IAA content in each aliquot. Differences between estimates at the different purification levels were tested with the Student's *t*-test ($P = 0.05$).

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