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IMPROVEMENTS IN METHODS FOR DETERMINATION OF ABSCISIC ACID AND INDOLE-3-ACETIC ACID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Improvements and simplifications in the early steps of the procedure for analysis of indole-3-acetic acid and abscisic acid by high-performance liquid chromatography (HPLC) are described. These include sample preparation, extraction and purification of the hormones. The method was applied to the leaves of *Fagus silvatica* and pines of *Picea abies*. With acetonitrile as extraction solvent, the hormones were recovered with high efficiency and the amounts of other substances were reduced. Partitioning of the extract between chloroform and water at different pH values obviates the need for laborious methods for prepurification of the material, simplifies the procedure and permits the use of an analytical column for further purification of the hormones by HPLC. Some terms used for characterization of the analysis are discussed.

INTRODUCTION

The growth and development of plants depends upon the balance between stimulatory and inhibitory hormones. This balance can change drastically under stress conditions, *e.g.*, water deficit, low temperature, air and soil pollution. The changes in the hormone level can also signify a disturbance in normal physiological events, and give information about these processes in the plant.

The content of hormones in plants is very low (in the range of nanograms to micrograms) and the amount of these substances is dependent not only upon environmental conditions, plant species and tissue type, but also upon the individual features of the material. Moreover, substances such as organic acids, phenolic and polyphenolic compounds present in the plant can interfere with the analysis of hormones; not only the hormone level, but also the amount of contaminants vary greatly from one species of plant or tissue type to another.

The large, and still increasing, interest in phytohormones has resulted in the elaboration of many methods for their determination. In the last 10 years, modern techniques using high-performance liquid chromatography (HPLC) have been increasingly applied to the purification and analyses of hormones in various types of

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plant material. Both abscisic acid (ABA) and indole-3-acetic acid (IAA) can be detected by UV absorption[1–5] or by electrochemical means[6]. For IAA, a more selective and sensitive means of detection is by fluorescence measurement[6–10]. Separation methods based on different adsorbents, *e.g.*, ion-exchange resin[6,9], reversed phase[10,11], ion pair–reversed phase[5,12] and silica, have also been used. Many methods elaborated by various authors are precise and selective, and have resulted in great progress in the analyses of the hormones. However, these procedures are mostly very material-specific, and not applicable to all plant materials. As mentioned earlier, the specificity of plant materials requires analyses of a very great number of samples in a short time. Most of the methods include very laborious sample preparation, *e.g.*, filtration, purification on polyvinylpyrrolidone (PVP), evaporation of large volumes of solvents, etc., making them very difficult for automatization and routine use[4,7,9,13].

This paper is concerned with some improvements in the HPLC method for analysis of the main representatives of the two various groups of hormones, ABA and IAA. Special attention was paid to the preparation of samples, extraction and purification of hormones which effectively determine the simplicity of the given method. Applications of the procedure to analyses of a large number of samples in a short time are presented. Some terms used for characterization of the analysis are discussed.

EXPERIMENTAL

The HPLC system used was a Waters 820 including two Model 510 pumps, an automatic sample injector WISP 710B, an UV absorbance detector (254 nm) Model 420 and a controlling–data processing computer Professional 350 (Digital). The UV detector was coupled in series with a fluorescence detector Model FS 970 (Kratos), excitation at 254 nm and emission at >370 nm (KV 370 filter).

Analytical grade hexane, chloroform, acetonitrile and methanol were obtained from Merck (Darmstadt, F.R.G.). Radioactive standards of *cis-trans*-abscisic acid and indole-3-acetic acid from Amersham (U.K.) were used. Water was double distilled from glass apparatus. Buffer solutions of pH = 7.0 were prepared from 5.7 g/l KH_2PO_4 and 0.2 *M* sodium hydroxide. Dissolved air was removed from already mixed solvents by direct sonification (1–2 min) with the Ultrasonifier B-12 (Branson).

Extraction of hormones from plant material

Leaves of *Fagus silvatica* L. and pines of *Picea abies* Karst. were used to test the method for hormone analyses. The plant material was harvested from trees about 20 years old, then immersed in liquid nitrogen, transported from the forest on solid carbon dioxide and stored in the laboratory at -20°C until analysis. Portions (up to 15 g) of the frozen material were placed into 250-ml centrifuge-tubes with 200 ml of liquid nitrogen and ground with Ultraturax for 3 min (20 000 rpm) with aggregate diameter 10 mm, followed by 10 min (27 000 rpm) with aggregate diameter 8 mm. After evaporation of nitrogen and thawing of the powdered plant material, samples of about 1 g were weighed in 10-ml centrifuge-tubes and lyophilized. To each dry sample, 5 ml of acetonitrile containing 200 ppm of 2,6-di-*tert.*-butyl-*p*-cresol (BHT) were added as an antioxidant. The tubes were tightly closed and shaken at 4°C to

extract the hormones. After 4 h, the samples were centrifuged at 2200 *g*, 3 ml of supernatant were pipetted from each sample into a 10-ml centrifuge-tube and evaporated to dryness under vacuum.

Purification of extracts by partitioning

To each tube containing the dry extract, 2 ml of phosphate buffer pH = 7.0 and 2 ml of chloroform were added, tightly sealed and placed horizontally on a shaker. Very gentle shaking for 1 h was required to prevent emulsion formation and to allow sufficient extraction of impurities into the chloroform. The samples were centrifuged for 10 min at 2200 *g* for better phase separation, 1.80 ml of buffer phase were transferred to a new tube and the extraction was repeated after addition of 2.00 ml of fresh chloroform. In this and the next steps of purification, the extraction process could be shortened to 5 min by vigorous shaking of the samples. A 1.60-ml volume of the buffer phase was transferred into new tubes and acidified to pH = 2.1 with 100 µl of formic acid. To each sample, 2.00 ml of chloroform were added and after shaking and separation of the phases, 1.80 ml of the organic phase containing the hormones was taken up. The procedure was twice repeated with portions of fresh chloroform.

Purification of hormone extracts by HPLC

After evaporation of chloroform, the samples were redissolved in 20.0% methanol, and 150 µl of the solution were injected onto a reversed-phase column containing LiChrospher 100 CH-18 Super (Merck, 4 µm, cartridge 125 × 4 mm) which had been equilibrated in the starting mobile phase: methanol–water–acetic acid (20:79.4:0.6). Analysis for 5 min in the starting solvent was followed by a linear gradient over 30 min to the final solvent ratio: methanol–water–acetic acid (70:29.4:0.6). A further period of 5 min in the end solvent removed the remaining impurities, and a reverse gradient over 5 min followed by 15 min of equilibration in the starting solvent prepared the column for the next injection. The flow-rate was 1 ml/min, and the pressure remained below 2000 p.s.i. Fractions corresponding to *trans-trans*-ABA, *cis-trans*-ABA and IAA were collected and evaporated to dryness under vacuum.

Analysis of hormones by HPLC

Samples were dissolved in 200 µl of 7.5% 2-propanol in hexane, and 150 µl of the solution were injected onto a silica column containing LiChrospher Si 60 Super (Merck, 4 µm, cartridge 125 × 4 mm) eluted isocratically. The mobile phase for analysis of *trans-trans*-ABA, *cis-trans*-ABA and indole-3-acetic acid was 2-propanol–hexane–formic acid (7.5:91.5:1)[7]. The flow-rate was 2 ml/min for ABA and 1 ml/min for IAA analysis. *trans-trans*-ABA and *cis-trans*-ABA were detected by their absorption at 254 nm; for detection of IAA a fluorescence detector was used (excitation at 220 nm, emission at >370 nm).

RESULTS AND DISCUSSION

The main steps of extraction and purification of hormones from plant material are presented in Fig. 1. Homogenization in methanol of materials such as pines was

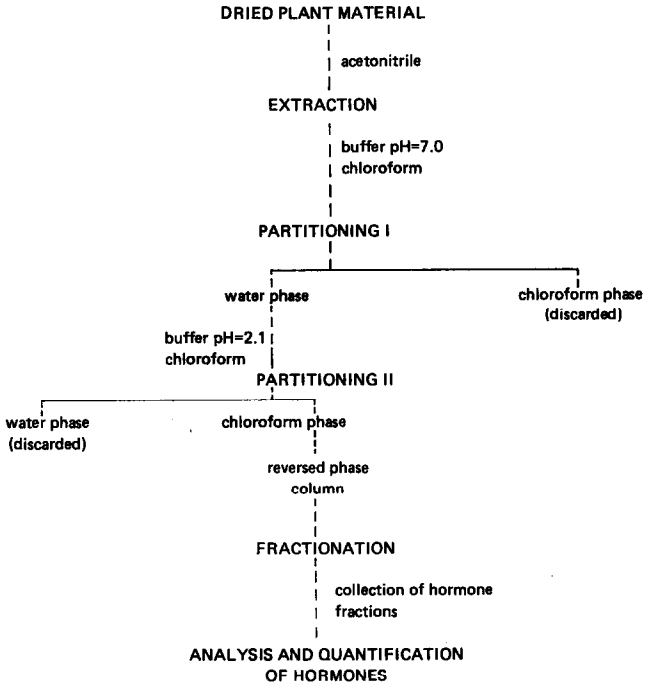

Fig. 1. Steps of the extraction and the purification of hormones.

not effective. Grinding the material in liquid nitrogen gave a fine homogenate powder, which allowed easy "penetration" of the solvent. A condensation of moisture on the frozen plant material, and loss of weight in the short time after thawing was not observed. The ground material was especially convenient for freeze drying, and for pines this was possible only after grinding. This method of grinding plant material has been used successfully for over a year on both the micro- and macroscale and the samples obtained have been divided and used for various other analyses.

In previously reported extraction procedures, methanol or methanol–water mixtures were used. The use of such polar solvents leads to extraction of many other compounds which interfere with hormone estimation, *e.g.*, phenolic acids are easily extracted with water[2]. In previous studies these polar compounds caused serious problems, and removing them required the use of laborious and often insufficient methods of purification, causing additional losses of hormones.

We found that the extraction efficiency, calculated as the recovery of radioactive standards, was increased by using less polar solvents and dry plant material (Table I). The amount of impurites extracted was proportional to the solvent polarity and amount of water. However, the use of solvents not miscible with water decreased the efficiency and reproducibility of extraction. This could be explained by less solvent "penetration" into the material and partition effects caused by traces of water. The use of acetonitrile and freeze-dried plant material gave the optimum extraction. In this step the recovery of added radioactive hormones was 97.0 ± 2% and 94.6 ± 2% for *cis-trans*-abscisic acid and indole-3-acetic acid, respectively. In comparison

TABLE I

RECOVERY OF RADIOACTIVE HORMONE STANDARDS FROM DRIED SAMPLES OF *PICEA ABIES* WITH VARIOUS EXTRACTION SOLVENTS

Solvent	*Recovery of ABA (%)*	*Recovery of IAA (%)*
Methanol	82.4	92.2
Acetonitrile	97.0	94.6
Ethyl acetate	85.5	76.1
Hexane–2-propanol (90:10)	72.6	58.1

with extraction using methanol or methanol–water, only traces of phenolic compounds were found in the extract. The time required for extraction was short, but to exclude any possible oxidation of indoles we used BHT (200 ppm) as an antioxidant. The high extraction efficiency permitted the use of only a part of the extract (3 ml from 5 ml) and eliminated the filtration step, making this procedure very simple.

The small amounts of phenols in the extracts do not require adsorption on PVP, and hormones can be purified from phenols and other impurities by a simple partition step using the large difference in partition coefficients for ABA and IAA when extracted from buffers into chloroform[11]. We found that the partitioning of hormone extracts was most effective when the one extraction solvent was applied, and the process was only pH dependent. Aqueous solutions at pH = 7.0 and pH = 2.1 with chloroform as the extraction solvent were optimal; chloroform was more effective than the dichloromethane used by Wurst *et al.*[14].

Eliminating the use of a separation funnel during partitioning by transferring a part of the sample using a precise, automatic pipette makes this normally inconvenient procedure very simple and routine. Most of the impurities are washed out from the buffer phase pH = 7.0 with chloroform, and after changing the pH to 2.1, ABA and IAA are easily extracted with chloroform leaving the more polar compounds in the water phase. The residue obtained after evaporation of the combined chloroform extracts is readily soluble in 30–40% methanol. Sandberg *et al.*[12] emphasized the great importance of the specificity of a method for successful hormone analysis. This was demonstrated by the fact that the best results were obtained by applying two different chromatographic systems, HPLC and gas–liquid chromatography (GLC)[4,11,15–17], and different chromatographic conditions for the sample preparation and analysis[1,3,6,7,9,10,18–22]. The combination of as varied methods of separation as possible alone increased the specificity of the separation method. Therefore, it is understandable that better results could be obtained when the chromatographic system used for the prepurification significantly differs from that applied to the analysis[23].

Taking into consideration the results reported in the literature and our experience, we chose as the optimum system purification on the reversed-phase C_{18} column and analysis on the silica column as described in Materials and Methods.

Chromatograms of samples from *Picea abies* and *Fagus silvatica* are shown in Fig. 2B and C.

In our procedure the impurities were present at much lower concentrations

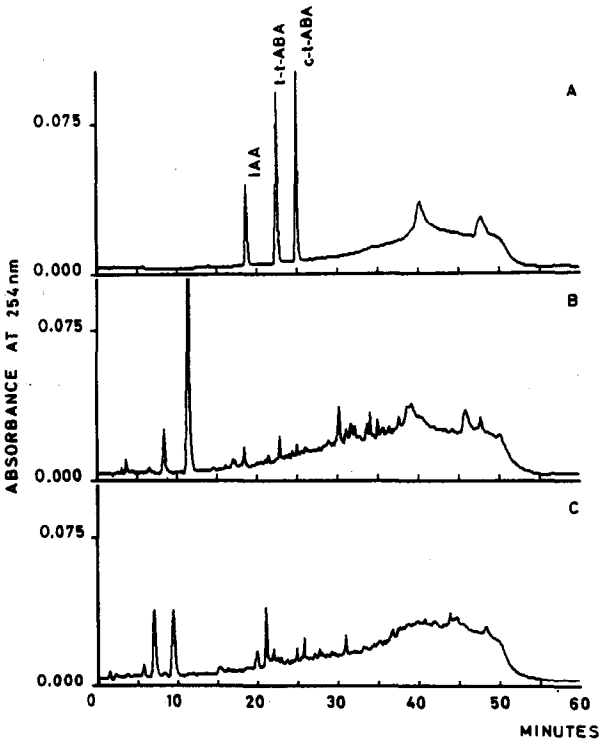

Fig. 2. Prepurification of a plant extract on a reversed-phase column. (A) Solution of standard substances; (B) sample from *Picea abies*; (C) sample from *Fagus silvatica*. Chromatographic conditions: column, LiChrospher 100 CH-18 Super (125 × 4 mm); mobile phase, gradient 20–70% methanol in 0.1 *M* acetic acid, flow-rate 1 ml/min.

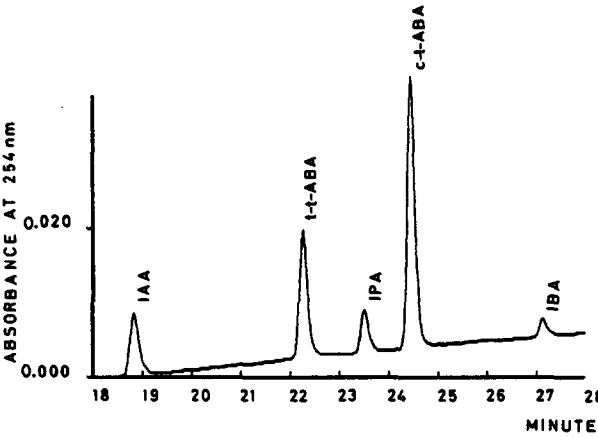

Fig. 3. Chromatogram of a standard solution of ABA, IAA, IPA and IBA. Chromatographic conditions as in Fig. 2.

than in earlier studies[13], therefore it was possible to use the analytical column instead of the preparative one for the purification step. Fractions corresponding to *trans-trans*-ABA, *cis-trans*-ABA and IAA are easy to collect (also using a programmable fraction collector), and the collection of broader fractions, if necessary, prevents losses of hormones in the event of changes in the retention time. The collection of fractions corresponding to indolepropionic acid (IPA) and indolebutyric acid (IBA) is possible as well (Fig. 3).

The same mobile phase and column for analysis of ABA and IAA allowed continuous work, without changes and reequilibration of the HPLC system (Fig. 4). Quantification by peak area or peak height measurement gave similar results.

Generally, in the analysis of compounds occurring in only very small amounts in the sample, not only the total amount of a substance, but also the correlation between this amount and the amount of impurities is important. Therefore, increasing the sample amount without increasing the efficiency of purification is not helpful. Using modern HPLC detectors it is at present possible to determine several ng of substances with the generally required accuracy.

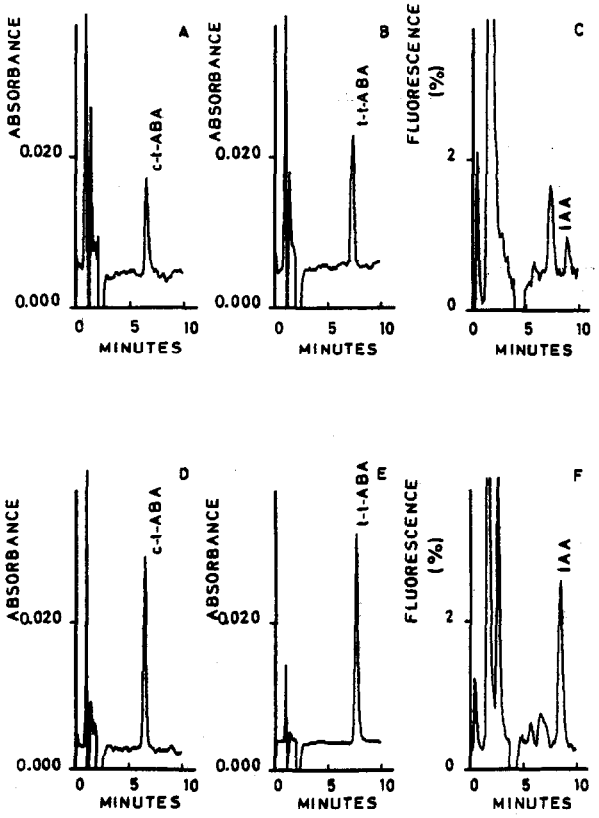

Fig. 4. Analysis of *cis-trans*-ABA, *trans-trans*-ABA and IAA by HPLC. (A) *cis-trans*-ABA from *Fagus silvatica*; (B) *trans-trans*-ABA from *Fagus silvatica*; (C) IAA from *Fagus silvatica*; (D) *cis-trans*-ABA from *Picea abies*; (E) *trans-trans*-ABA from *Picea abies*; (F) IAA from *Picea abies*. Column: 125 × 4 mm LiChrospher Si 60 Super. Mobile phase: 2-propanol–hexane–formic acid (7.5:91.5:1). Flow-rates: 2 ml/min (A, B, D, E); 1 ml/min (C, F).

The sample amount can therefore be chosen according to the concentration of substance to be analysed. It should be noted that a part of the sample is lost during processing, and the final amount of substances should be in the optimum range of determination. Loss of analysed compounds occurs in two ways: by an efficiency or yield of less than 100%, and by dilution or dividing of the sample. In the first case, the losses can be determined with radioactive or cold standards and described as 100% minus recovery (%). Some authors use this factor, without a clear explanation, to indicate the recovery of a certain step, *e.g.*, extraction, and not for the whole method. The term "recovery" in this paper means the amount of substance obtained in the last quantification step in relation to the amount of substance added to the plant material before extraction, and is expressed as per cent.

Losses by dilution or by dividing of the sample are easy to control and calculate throughout the whole method. The total dilution factor is calculated by multiplication of step dilution factors, as is normally done in the calculation of total recovery. An example of such a step dilution factor in our method is 0.6; for separating the extract from the plant material we used 3 ml from 5 ml. Losses of these types are very often characteristic of the method, and elimination of them can change a convenient routine procedure into a very laborious one (for example only part of the solution can be extracted and injected onto the column).

However, it should be noted that the recovery and the dilution factors do not describe the quality of the method, but together with the range of quantification they allow the calculation of the amount of material required for analysis, and this finally gives information about the usefulness of the method for certain problems. To determine the precision, 500 ng/g fresh weight radioactive hormone standards were added to the ground material. Analyses of six 1-g samples gave precision values of 7 and 12% for IAA and *trans-trans*-ABA respectively; the precision was equal for both plant materials. These values, sufficient for routine analyses, agreed well with previously presented data[12,14,20]. The calibration curve obtained from experiments with plant material of equal hormone concentrations to which known amounts of hormone standards were added corresponded well with the calibration curve from the pure hormone standards. The previously mentioned dependence of the systematic and random errors on the type of plant material[8] was probably the reason for the necessity of separate calibration for both plant materials. Quantification was based on peak area measurements, however, peak heights could also be used.

The limit of detection with our method was 0.1 ng, calculated for a peak signal-to-noise ratio of 3. Sandberg *et al.*[12] cautioned against the overestimation of this parameter, as it is less significant than often indicated in the literature. The definition of the detection limit which we used in accordance with other authors[10] should be understood as a characteristic parameter of the method with a strong dependence on the HPLC system used, and is useful only when calculated from the real sample. We propose to use this datum for calculation of the "determination limit", which describes the minimum amount of substance which can be quantified. The detection limit may be understood as the minimum detectable difference between two hormone concentrations and therefore corresponds to the precision of the determination. The determination limit is expressed by:

$$\text{Determination limit (Dt)} = \frac{100}{\text{precision}} \cdot \text{detection limit}$$

This means that, with 7% precision, it is possible to quantify an amount of substance 14.3 times higher than the detection limit. In this work the Dt value was 1.43 ng for IAA and 0.83 ng for ABA.

It is already possible to define the important parameter describing the method of determination, the minimum assayable amount of substance in the sample (Am):

$$\text{Am} = \frac{100}{\text{recovery (\%)} \cdot \text{dilution factor}} \cdot \text{Dt}$$

Together with the precision and accuracy as defined by Sandberg *et al.*[12], the minimum assayable amount describes the usefulness of any method for quantitation of a certain substance in a certain material. It enables the comparison of different methods and thus the possibility of finding the optimum analysis for any one problem. After taking into consideration the other important factors, *e.g.*, equipment required, time consumption or possibility of routine use, it should become easier to choose the optimum method.

The previously discussed recovery and dilution factors are presented in the Table II; the recovery achieved was better than or comparable to that in many earlier works[3,15,17,19,20]. In this study the minimum assayable amount (Am) of *cis-trans*-ABA (or *trans-trans*-ABA) in the sample was calculated as 3.63 ng; the minimum amount for analysis of IAA was 7.95 ng. This means that this amount of hormone could still be quantified with 10% precision. We used, in two examples, 1 g of material, but 200–300 mg of sample could also be used with the same precision of quantification. The Am value may also be decreased by changing the dilution factor, *i.e.*, by simply changing the volume of sample transferred during the purification procedure. We found, however, that the step dilution factors used in this work represented the optimum choice between the required Am, precision and simplicity for routine application.

The described procedure improves and simplifies the routine use of HPLC methods for plant hormone analysis. It is not specific for *Fagus silvatica* and *Picea*

TABLE II

DILUTION FACTORS CALCULATED FOR INDIVIDUAL STEPS OF THE PROCEDURE AND TOTAL RECOVERY OF THE HORMONES

Step of procedure	*Step dilution factors for ABA and IAA*	*Recovery of ABA (%)*	*Recovery of IAA (%)*
Extraction	0.60	–	–
Partitioning I (pH = 7.0)	0.80	–	–
Partitioning II (pH = 2.1)	1.00	–	–
Fractionation (HPLC)	0.75	–	–
Analysis (HPLC)	0.75	–	–
Total	0.27	84.7	66.6

abies, but could probably be applied to the determinations of ABA and IAA in any other plant material. The simplicity of operations and possibility of automatization allow the routine use of this method. With this method it is possible to extract and prepurify by partitioning at least 50 samples per day. About 20 samples can be prepurified by HPLC and analysed in 1 day. Moreover, the basic principles of the described procedure can be utilized in the determination of many other phytohormones.

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