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Note

Use of Amberlite XAD-7 as a concentrator column in the analysis of endogenous plant growth hormones

BARBRO ANDERSSON*
Department of Organic Chemistry, University of Umeå, S-901 87 Umeå (Sweden)
and
KURT ANDERSSON
National Board of Occupational Safety and Health, Department of Occupational Health in Umeå, Box 6104, S-900 06 Umeå (Sweden)
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Amberlite XAD resins are synthetic adsorbents displaying unique chemical and physical properties, and have been used successfully in the analysis of organic compounds[1,2]. Four kinds of Amberlite XAD are commercially available: XAD-2 and XAD-4 are both copolymers of styrene and divinylbenzene with non-polar and hydrophobic properties but with differences in active surface area and pore diameter. XAD-7 and XAD-8 are polymers of acrylic esters, of medium polarity and with different active surface area and average pore diameter. The ability of these adsorbents to retain large amounts of organic compounds is utilized in the analysis.

The first analytical use of Amberlite XAD resins was in the analysis of small amounts of organic compounds in water[3–6]. These compounds were adsorbed on XAD from large volumes of water and desorbed by buffer solutions at various pH values or by an organic solvent. A several hundred-fold concentration of organic compounds was effected. Thermal desorption has also been used in water analysis[7], but as the XAD resins are rather unstable at higher temperatures this method could give rise to a very high background in the analysis. Nowadays, there are control programmes for testing water quality all over the world[8] where the analysis is based on the use of XAD resins as concentrator columns. Another field of application of these adsorbents is in the analysis of organic compounds in air[9–13]. As in water analysis, small amounts of organic compounds are adsorbed on the resin and desorbed by a few millilitres of organic solvent. A further development is the use of XAD resins as supporting material for various reagents[14–16]. Specific compounds are derivatized directly and adsorbed from air on the pre-coated column (chemisorption) and are desorbed by an organic solvent. The reagents are selected so as to afford detectable derivatives suitable for direct analysis in very small amounts by gas chromatography (GC) or high-performance liquid chromatography (HPLC).

Amberlite XAD resins have also been used in the analysis of organic compounds in various biological materials. The first such use was in the analysis of flavouring compounds in meat tissue extract, where excess of picric acid used as deproteinator was removed by adsorption on XAD-2[17]. These resins have also been

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employed in the analysis of pharmaceuticals in blood, stomach contents and tissue extracts[18]. Nieman *et al.*[19] used XAD-2 in the analysis of nucleotides in plant extracts. Interfering phenolic compounds were removed by adsorption on the resin. In addition, it was shown that even nucleotides can be adsorbed on the same resin at appropriate pH values.

In all these analyses where organic compounds have been adsorbed on XAD resins, different kinds of organic solvents were used for desorption, for example, diethyl ether, dichloromethane, methanol and acetone. In previous papers we reported the analysis of the endogenous plant growth hormones abscisic acid[20], 3-indoleacetic acid[21] and the conjugate N-(3-indoleacetyl)aspartic acid[22] in pine or spruce shoots. Large volumes of organic solvents and buffer solutions were used in the clean-up preceding the final analysis of very small amounts of plant hormones. These routine analysis are outlined in Fig. 1.

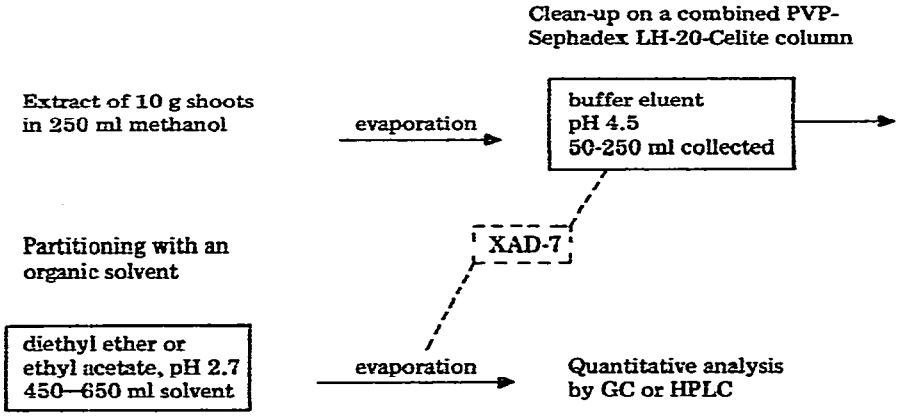


Fig. 1. Outline of methods employed to the analysis of plant hormones.

In the development of an analytical method for N-(3-indoleacetyl)aspartic acid, it was found that partitioning with an organic solvent was not very successful, and part of the analysis was therefore substituted by a concentrator column of Amberlite XAD-7. This meant a simple transfer of the extract from an acidic aqueous solution to a few millilitres of ethanol and a simultaneous purification. In the present work the XAD-7 concentrator column was tested with four standard hormones and one standard conjugate: 3-indoleacetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA_3), zeatin and N-(3-indoleacetyl)aspartic acid (IAAsp). Formulae of these compounds are presented in Table I.

EXPERIMENTAL

Reagents

The following reagents were used: [1-^{14}C]3-indoleacetic acid (specific activity 2.18 TBq/mol), (±)-*cis–trans*-[2-^{14}C]abscisic acid (specific activity 947 GBq/mol), [1,7,12,18-^{14}C]gibberellic acid (specific activity 370 GBq/mol), all from New England Nuclear; 3-indoleacetic acid, (±)-*cis–trans*-abscisic acid, gibberellic acid, *cis–trans*-zeatin, all from Sigma; N-(3-indoleacetyl)aspartic acid (synthesized according to refs.

22, 23), citric acid and disodium hydrogen phosphate ($C_6H_8O_7 \cdot H_2O$ and $Na_2HPO_4 \cdot 2\,H_2O$, Merck, analytical grade), diethyl ether, ethanol (abs.) and methanol of p.a. grade, Instagel scintillation cocktail (Packard) and Amberlite® XAD-7 (purchased as Servachrom® XAD-7, pract., 300–1000 μm; Serva, G.F.R.).

Purification and sieving of XAD-7

The synthetic resin was washed seven times with water in a beaker and the fines were discarded by decanting. Further washings were performed with methanol (seven times) and the resin was dried by filtration and sieved (U.S. Bureau of Standards). Two fractions were collected: 30–45 and 45–60 mesh, respectively. Each fraction was finally washed with diethyl ether in a Soxhlet apparatus for 2 × 12 h and then dried in air.

XAD-7 column

This column, 15 × 0.5 cm I.D. (*e.g.*, a 2-ml measuring pipette with glass-wool), was packed with about 0.3 g XAD-7, 30–45 mesh, in 20 ml water and pre-eluted with 30 ml of 0.1 *M* citrate–phosphate buffer at an appropriate pH value and with a flow-rate of 1.9 ml/min. A 5-μg amount of standard compound in 50 ml buffer at the same pH value was applied to the column and also 50 μl of a standard with activity 20,000 dpm were added for scintillation when using radioactive standard for yield determination. The column was washed with a few millilitres of buffer solution before the eluent was switched to ethanol. The fractions were collected from the time when the ethanol reached the top of the column (a meniscus between the buffer solution and the ethanol could be seen in this narrow column) and the first fractions of 0.8 ml were discarded. Table I shows the different volumes of the collected second fractions. These were evaporated to dryness in a stream of nitrogen ($<40°C$) and redissolved in 1.0 ml ethanol or in the appropriate HPLC eluent. Columns of XAD-7, pore diameter 45–60 mesh, were packed and used in the same way.

Determination of yields by liquid scintillation

The yields of IAA, ABA and GA_3 were determined by liquid scintillation counting of samples containing the radioactive standard. This was done with an LKB-Wallac Ultrobeta 1210 instrument with Instagel as the scintillation cocktail. All data were corrected for background and quenching.

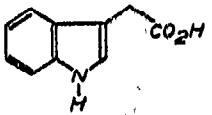
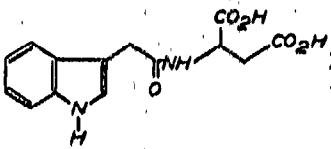
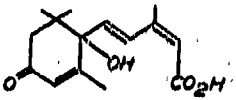
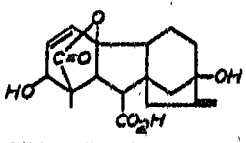
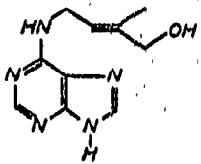
Determination of yields by HPLC

The yields of IAAsp and zeatin were determined by HPLC. For IAAsp, details of the HPLC equipment and the parameters were as described previously[22]. The analysis of zeatin was performed on a 15 × 0.46 cm I.D. analytical column of 5 μm Nucleosil C-18 (Skandinaviska GeneTec AB, Sweden), with methanol–0.01 *M* phosphate buffer at pH 3.0 (30:70 v/v) as the eluent and a flow-rate of 0.9 ml/min. A UV-detector (Pye-Unicam) with a wavelength of 298 nm was used, and the retention time of zeatin in this system was 9 min. The quantification was made by using an external standard.

TABLE I

RECOVERIES OF SOME ENDOGENOUS PLANT GROWTH HORMONES FROM A XAD-7 CONCENTRATOR COLUMN

The total adsorption–desorption yield also includes the evaporation of ethanol.

Standard	*pH 2.7*			*pH 4.5*			*pH 7.0*		
	*ml**	*Yield***	*RSD****	*ml**	*Yield***	*RSD****	*ml**	*Yield***	*RSD****
 3-Indoleacetic acid (IAA)	4.0	95	3	4.0	87	5		!	
 N-(3-Indoleacetyl)aspartic acid (IAAsp)	2.7	96	3	2.7	92	4		!	
 Abscisic acid (ABA)	3.5	92	3		!			!	
 Gibberellic acid (GA_3)	4.0	93	3	4.0	83	3		!	
 Zeatin		!			!		2.0	93	3

* Volume of the collected ethanol fraction.

** Each yield is calculated in per cent as an average of six replicates.

*** Relative standard deviation (%, coefficient of variation).

! Not retained, elutes with buffer.

RESULTS AND DISCUSSION

Five groups of endogenous plant growth hormones have been reported in plants: ethylene, IAA, ABA, the gibberellins and the cytokinins. Ethylene as a gas cannot be analysed in our routine analysis and is not included in this work. Up to now 60 gibberellins have been identified in plant tissue; gibberellic acid is chosen as a representative of this group. The last group, the cytokinins, are all adenine derivatives and are represented here by zeatin. In addition, an endogenous conjugate of a growth hormone and an amino acid, IAAsp, is included in this work. In pine and spruce shoots the amounts of our reference compounds are estimated at about 5 μg per 10 g fresh weight, and that is the amount of the added standards in our work.

Since the reference compounds used are all polar compounds, the choice of a suitable Amberlite XAD resin fell on one of the polar adsorbents, namely, XAD-7. These resins are not synthesized for analytical purposes and must be rigorously purified before use. The resin was sieved in two fractions with different particle sizes, 30–45 and 45–60 mesh, respectively. However, the latter fraction with smaller particles was difficult to use since the particles sedimented very slowly in the column. This fraction was accordingly not used further in this work.

The adsorption of the reference compounds was noted at three different pH values in order to ascertain whether the pH value of the buffer, and thereby the charge on the molecules, had any influence on adsorption efficiency. The compounds were adsorbed in both charged and uncharged forms. At pH 7.0 the carboxylic acids are in the anionic form while zeatin as an adenine derivative can be regarded as uncharged (adenine is an uncharged molecule at this value). But at pH 2.7 the acids are uncharged and zeatin is in the cationic form. As is seen in Fig. 1, this value was used in our routine analysis. The third pH value, 4.5, was chosen since the buffer eluent of the clean-up column has this value and it would be very convenient if the reference compounds could be adsorbed on XAD-7 directly after the clean-up column without changing the pH value of the collected fraction. However, as can be seen in Table I, excellent adsorption–desorption yields are obtained when the reference compounds are adsorbed in uncharged form —the acids at pH 2.7 and zeatin at pH 7.0.

This is in agreement with a report of Burnham *et al.*[3] who found that non-ionic compounds were adsorbed in a quantitative yield while ionic solutes pass through the column without retention. Moreover, the adsorption of such compounds as carboxylic acids and amines depends on the pH of the solution. Nieman *et al.*[19] found the same dependence for adenine derivatives and phenolic compounds on XAD-2. At pH 7.0 the adsorption of the adenine derivatives was found to occur quantitatively. Among the organic solvents used for desorption, ethanol was preferred since it gave dry extracts after evaporation (azeotrope with water), which is desirable for further analysis.

The aim of this work was to substitute the partioning with an organic solvent in our routine analysis of plant growth hormones (Fig. 1). Large volumes of diethyl ether or ethyl acetate are used in the clean-up procedure after column purification. This partitioning is tedious and laborious and constitutes a health risk that must be taken into account. Large volumes of solvents must be evaporated in an evaporator and not in a stream of nitrogen, and the risk of contamination is evident. Furthermore, if the separator funnel is not shaken carefully enough, it is hard to obtain a

sufficient separation due to emulsion problems. We can now substitute this partitioning with an organic solvent by means of the small XAD-7 column, and large volumes of diethyl ether or ethyl acetate are substituted by a few millilitres of ethanol. In addition, the change to the XAD-7 column results in a higher yield from the concentration step.

The excellent yield of the amino acid conjugate IAAsp indicates the possibility of using Amberlite XAD-7 as a concentrator column in the analysis of amino acids in biological samples. The adsorption should preferably be performed near the isoelectric point of the amino acid. XAD-7 could thus replace ion-exchange resins in this kind of analysis. The use of Amberlite XAD-7 results in both a concentration and a purification of the plant extract. The purification properties will be investigated further in our studies of plant hormones in pine and spruce shoots.

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