*Journal of Chromatography*, 294 (1984) 485–488
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,687

## Note

# Isolation and purification of plant hormones by XAD-2 column chromatography

ALLAN E. STAFFORD*, JUDITH A. KUNHLE*, JOSEPH CORSE and EARL HAUTALA
*Plant Physiology and Chemistry Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710 (U.S.A.)*
(First received December 13th, 1983; revised manuscript received February 27th, 1984)

The use of selective detectors such as the nitrogen–phosphorus detector[1–3], electron-capture detector[4] and selected-ion monitoring (SIM)[5] with a mass spectrometer makes the development of simplified and rapid extraction and purification procedures of plant hormones from natural sources possible. This is important in the quantitative analyses of plant hormones since extensive extraction and purification procedures invariably result in their loss[6,7]. In qualitative analyses these losses can usually be countered by the use of larger samples. This is not always possible and hormones present in very low concentrations could be missed even though they may be important physiologically to the system under study. Even if the hormones are detected, large quantitative errors will obscure their physiological role[8].

The non-polar cross-linked divinylbenzene resins, Amberlite XAD-2 and -4 and the medium polar acrylic ester copolymers, XAD-7 and -8 have been used to isolate many undissociated organic compounds from a wide variety of aqueous systems with good efficiency[9–11]. Plant hormones are present in aqueous systems such as exudate[12], extrudate[13], culture media[14] and sea water[15]. In many analytical procedures plant extracts are taken to dryness and redissolved in water prior to further separation and purification procedures[7].

The XAD-7 resin has been tested as a concentrator column for a small number of plant hormones in model systems[9]. In our study we tested XAD-2 resin to adsorb from aqueous buffers a wide variety of cytokinins, 3-indolacetic acid (IAA) and abscisic acid (ABA) in model systems and report their recoveries. To increase the resin's usefulness a simple step gradient was used not only to isolate, but also to separate and purify several of the plant hormones. The step gradient elution was used with the non-polar XAD-2 resin for the analysis of cytokinins in coconut milk and our results compared with those using strong cation-exchange resins for extraction[2,16].

## EXPERIMENTAL**

### *Reference compounds*

All cytokinins used in this study were synthesized at this laboratory by reported

* Present address: Hawaiian Sugar Planters' Association, 99-193 Aiea Heights Drive, Aiea, HI 96701, U.S.A.

** Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

0021-9673/84/$03.00 

procedures[18]. Commercially available IAA and ABA were from Calbiochem-Behring (La Jolla, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. Standard solutions were made up in methanol.

*Materials*

Amberlite XAD-2 (20–50 mesh beads) was obtained from Mallinckrodt (Paris, KY, U.S.A.). Chemicals used in preparing buffers were reagent grade. Solvents were HPLC grade or glass distilled. All water used was passed through deionizing and organic removal cartridges before being glass distilled. Two Waters HPLC columns were used in this study, a μBondapak $C_{18}$ and μBondapak phenyl (30 cm × 3.9 mm I.D., 10 μm particles).

*Column preparation*

The resin columns used in these experiments measured 25 × 1.2 cm and contained resin which had a bed volume of 11 ml. The resin was extracted with acetone before use and stored in methanol in columns when not in use. The columns were equilibrated with buffer or water before sample application.

*Recovery experiments*

Three standard solutions were prepared, one contained 13 cytokinins, the second IAA, and the third ABA. Individual hormone concentration was 10 ng/μl in the three solutions. To 100 ml of 0.1 *M* phosphate buffer (pH 3, 5, 7 or 9) 30 μl of a standard solution were added and the mixture was applied to the column at a flow-rate of 3 ml/min. The column was eluted with three bed volumes of water and three of methanol at a rate of 3 ml/min. The three bed volumes of methanol containing the hormones were taken to dryness in a vacuum rotary evaporator at 30°C, transferred in methanol to a conical vial, dried with a stream of nitrogen and made up to 60 μl with methanol. Recovery was determined by comparing sample (25 μl) HPLC chromatograms with those of a reference chromatogram of equal concentration and content of standard solution chromatographed the same day. The Waters Assoc. chromatograph was equipped with two M6000A pumps, a 660 solvent-flow programmer, a U6K injector and a Tracor 970 variable-wavelength detector, operated at 265 and 280 nm. A μBondapak $C_{18}$ column was used with a 1-h concave gradient (No. 7) from 6% to 30% acetonitrile in water at a flow-rate of 1.4 ml/min. Both solvents contained 1% acetic acid. Integration was accomplished using the integrator on a Hewlett Packard 5840 gas chromatograph equipped with an external input board and remote start.

*Coconut milk analysis*

Coconut milk (10 ml) was adjusted to approximately pH 9 with 1 *M* sodium hydroxide and centrifuged. The supernatant was applied to the resin column at the rate of 3 ml/min. The column was then washed with 6 bed volumes of water and the cytokinins eluted with a stepwise gradient of 6 bed volumes of 15% methanol, 6 bed volumes of 50% methanol and 6 bed volumes of 100% methanol. The water and 15% methanol fractions were discarded. Remaining bed volumes were combined in groups of three. These four combined bed volume fractions (33 ml each) were taken to dryness and transferred in methanol to conical reaction vials for HPLC separation

using the μphenyl column. The elution program was a 1-h concave gradient (No. 7) from 10% to 60% methanol in water at a flow-rate of 1.4 ml/min. Only the water contained 1% acetic acid. The cytokinin fractions were dried and permethylated for gas chromatographic analysis with a nitrogen–phosphorus detector[1].

## RESULTS AND DISCUSSION

The recoveries of plant hormones used in this study are summarized in Table I. The analyses of the hormones were monitored at 265 and 280 nm since the wide variety of hormones showed two general absorption maxima at these wavelengths but since the data were very similar they were combined for Table I. These data in general show the most consistent high recoveries for the undissociated cytokinins, ABA and IAA in agreement with earlier work with XAD-2 resin which found the greatest retention of organic bases[19] and acids[20] occurs with neutral, undissociated molecules. In our study this was illustrated with ABA which showed an approximate 100% recovery at pH 3 and 86% at pH 9. The unaccounted for ABA at pH 9 could be recovered from aqueous eluate after acidification to pH 3 and re-running it through the column. The methanol eluent contained the balance of ABA. Our results with the non-polar XAD-2 resin shown in Table I and discussed above contrast markedly with those of Andersson and Andersson[9] who used the moderately polar XAD-7 resin. The XAD-7 resin showed complete reversal, compared with XAD-2, in retention of hormones with pH changes in the buffer solution used to load the hormones onto the resin. For example ABA was unretained by the resin with buffers of pH 4.5 and higher, IAA was unretained with pH 7.0 buffer and zeatin was unretained with buffer solutions of pH 4.5 and lower.

TABLE I

RECOVERY VALUES (%) OF PLANT HORMONES FOR XAD-2 RESIN COLUMN

Recovery values are given as mean ± S.D. of 2 runs at 265 nm and 280 nm. An amount of 300 ng per component was applied to the resin column.

| *Hormones* | *pH* | | | |
|---|---|---|---|---|
| | *3* | *5* | *7* | *9* |
| *trans*-Zeatin | 74 ± 4 | 90 ± 9 | 76 ± 4 | 94 ± 9 |
| Dihydrozeatin | 72 ± 3 | 82 ± 12 | 80 ± 5 | 96 ± 6 |
| *cis*-Zeatin | 74 ± 5 | 96 ± 10 | 80 ± 3 | 96 ± 6 |
| Ribosyl-*trans*-Zeatin | 84 ± 9 | 97 ± 13 | 92 ± 3 | 104 ± 3 |
| Ribosyl-*cis*-Zeatin | 83 ± 10 | 84 ± 13 | 95 ± 3 | 102 ± 4 |
| Isopentenyladenine | 72 ± 9 | 89 ± 7 | 82 ± 4 | 104 ± 9 |
| Isopentenyladenosine | 74 ± 6 | 93 ± 7 | 91 ± 8 | 99 ± 9 |
| O-Hydroxylbenzyladenosine | 88 ± 6 | 92 ± 6 | 96 ± 5 | 96 ± 8 |
| 2-Methylthiodihydrozeatin | 102 ± 8 | 91 ± 5 | 70 ± 4 | 87 ± 5 |
| 2-Methylthioribosyl-*cis*-zeatin | 87 ± 9 | 100 ± 17 | 98 ± 14 | 100 ± 6 |
| 2-Methylthioribosyldihydrozeatin | 81 ± 6 | 87 ± 10 | 92 ± 5 | 102 ± 4 |
| 2-Methylthioisopentenyladenine | 99 ± 13 | 90 ± 4 | 98 ± 3 | 89 ± 5 |
| 2-Methylthioisopentenyladenosine | 74 ± 19 | 105 ± 10 | 101 ± 11 | 90 ± 6 |
| 3-Indolacetic acid | 99 ± 6 | 97 ± 6 | 103 ± 18 | 67 ± 9 |
| Abscisic acid | 108 ± 7 | 104 ± 8 | 97 ± 9 | 86 ± 5 |

A step gradient was used to separate and purify the cytokinins in coconut milk prior to HPLC. Using polar and non-polar cytokinin standards, the resin was found to act as an absorbant via a reversed-phase mode[21] and the elution order of some of the cytokinins of present interest was determined. For example, *trans*-zeatin and *trans*-zeatin riboside eluted with the first three bed volumes of 50% methanol and isopentenyladenine and isopentenyladenosine eluted with the first three bed volumes of 100% methanol. Using this information, a small sample of coconut milk (10 ml), was run through the resin column. Permethylation of the HPLC fractions from the first three 50% methanol bed volumes showed the presence of two cytokinins, *trans*-zeatin riboside and a trace of *trans*-zeatin. This agrees in general with previous work of Letham[17]. None of the other three bed volume fractions showed cytokinins for which we have standards.

Whenham[2] and Van Staden and Drewes[16] report these two cytokinins to be present in roughly equal concentration. This difference could be caused by sampling, but the latter results rely on the use of a strong cation-exchange resin (Dowex 50W-X8) to separate and purify the cytokinins. It has been reported that zeatin riboside was hydrolyzed on Dowex $H^+$ cation-exchange resin[21,22]. Experiments at this laboratory also show hydrolysis of *trans*-zeatin riboside by Dowex 50W-X8 resin.

## REFERENCES


1 A. E. Stafford and J. Corse, *J. Chromatogr.*, 247 (1982) 176.
2 R. J. Whenham, *Planta*, 157 (1983) 554.
3 C. A. Burgett, D. H. Smith and H. B. Bente, *J. Chromatogr.*, 134 (1977) 57.
4 M. Ludewig, K. Dörffling and W. A. König, *J. Chromatogr.*, 243 (1982) 93.
5 M. Claeys, E. Messens, M. van Montagu and J. Schell, in A. P. Deleenheer, R. R. Roncucci and C. van Peteghem (Editors), *Quantitative Mass Spectrometry in Life Sciences II*, Elsevier, Amsterdam, 1978, p. 409.
6 M. L. Brenner, *Annu. Rev. Plant Physiol.*, 32 (1981) 511.
7 H. M. Dekhuijzen and E. C. T. Gevers, *Physiol. Plant.*, 35 (1975) 297.
8 J. Van Staden, *J. S. Afr. Bot.*, 39 (1973) 261.
9 B. Andersson and K. Andersson, *J. Chromatogr.*, 242 (1982) 353.
10 A. K. Burnham, G. V. Calder, J. S. Fritz, G. A. Junk, H. J. Svec and R. Willis, *Anal. Chem.*, 44 (1972) 139.
11 G. Ibrahim, S. Andryauskas and M. L. Bastos, *J. Chromatogr.*, 108 (1975) 107.
12 J. E. Davey and J. Van Staden, *Planta*, 130 (1976) 69.
13 R. O. Morris, J. B. Zaerr and R. W. Chapman, *Planta*, 131 (1976) 271.
14 L. M. S. Palni and R. Horgan, *Plant Sci. Let.*, 24 (1982) 327.
15 M. Pederson, *Physiol. Plant.*, 28 (1973) 101.
16 J. Van Staden and S. E. Drewes, *Physiol. Plant.*, 34 (1975) 106.
17 D. S. Letham, *Physiol. Plant.*, 32 (1974) 66.
18 J. A. Kuhnle, G. Fuller, J. Corse and B. E. Mackey, *Physiol. Plant.*, 41 (1977) 14.
19 C. Chu and D. J. Pietrzyk, *Anal. Chem.*, 46 (1974) 330.
20 D. J. Pietrzyk and C. Chu, *Anal. Chem.*, 49 (1977) 860.
21 J. R. Tegley, F. H. Witham and M. Krasnuk, *Plant Physiol.*, 47 (1971) 581.
22 J. A. Zwar and M. I. Bruce, *Aust. J. Biol. Sci.*, 23 (1970) 289.