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Purification of Cytokinins on a Polyvinylpyrrolidone Column Followed by Analysis on a Reversed-Phase C_{18}ODS HPLC System

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Abstract. It has been found that cytokinins, as a class, can be separated from co-occurring phenolics by column chromatography using polyvinylpyrrolidone (PVP) with methanol as eluant. Subsequent fractionation of the cytokinins can then be achieved by HPLC on a C_{18}ODS reversed phase system using methanol : water (60 : 40) as the mobile phase. The system shows considerable promise as an extremely mild separation process and has been used to separate two unknown cytokinins from seedlings of *Hordeum vulgare* cv. Steptoe.

There are two main disadvantages of many clean-up procedures of leaf extracts, prior to analysis for cytokinins by HPLC. Firstly, they generally employ treatment with cation exchange resins; a relatively harsh procedure which usually results in partial breakdown of the cytokinins. Secondly, the use of cation exchange resins does not separate cytokinins from the simpler low molecular mass phenolics such as *p*-coumaric and caffeic acids, *p*-hydroxybenzyl alcohol *etc.* (CHALLICE 1982). Since many phenolics are known to inhibit response to cytokinins in the *Amaranthus* bioassay (CHALLICE 1977a) and in the soya bean callus bioassay (CHALLICE 1977b) there is a strong possibility of false negative results here.

The use of PVP columns with aqueous buffers — a much used procedure for removing phenolics (*e.g.* BIDDINGTON and THOMAS 1973a) — failed in this laboratory to remove the simpler low molecular mass phenolics entirely from the common cytokinins. Here, it was only the more complex flavonoids which were removed. Likewise, a system devised by HAHN (1975) using silica gel columns failed in this respect. Even Sephadex LH-20/aq. ethanol columns (much used as a final purification stage) proved incapable of separating cytokinins from a range of simpler phenolics (CHALLICE 1976). Many phenolics run coincidentally with cytokinins in the HPLC separation systems which are available, so there is also the possibility of false positive results because both groups of compounds respond to the UV-detection procedures

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used. The latter can, of course, be obviated by an *Amaranthus* bioassay of the fractions in question.

However, a PVP-methanol system recently devised by MOUSDALE and KNEE (1979) — primarily developed in order to separate IAA from other classes of plant growth substances — has shown considerable promise in separating cytokinins, as a class, from all contaminating phenolics.

In HPLC separation systems, the use of pellicular column packings such as Zipax SCX (strong cation exchange resin) and Pellamidon (pellicular polyamide) *e.g.* — CHALLICE 1975 — has largely been superseded by reverse-phase microparticulate systems such as C_{18}ODS 10 μm/aqueous methanol (*e.g.* HAHN 1976); these latter systems display considerably improved separatory power.

MATERIAL AND METHODS

Amaranthus Bioassay

— by the modified method of BIDDINGTON and THOMAS 1973b as used previously in CHALLICE (1977a, b).

High Pressure Liquid Chromatography

A Whatman Partisil PXS 10/25 C_{18}ODS (10 μm) pre-packed column was used. The dimensions were 25 cm × 4.6 mm with methanol : water (60 : 40) as eluant at ambient temperature. Isocratic elution proved to be quite satisfactory in the present investigations. The eluant was Helium — degassed and run at a constant 1.5 ml per min which generated a back pressure of 1050 psi. The HPLC system consisted of a Pye-Unicam LC3XP constant flow rate pump and a LC3 UV detector set at 268 nm. A simple septum injection system was used.

Polyvinylpyrrolidone (PVP)

— obtained from BDH Chemicals Ltd as Polyclar AT. 100 g of PVP was slurried with methanol and packed into a column to give a bed volume occupying 24 cm × 5.5 cm diam. Methanol was employed as eluant.

Processing of Plant Material

— 50 g fresh matter of 40 day old seedlings of barley (*Hordeum vulgare*) was homogenized with methanol in a Wareing Blender and filtered on a Büchner funnel. The solid residue was re-extracted with methanol and the combined filtrates evaporated to near-dryness in a rotary evaporator at 50 °C. 150 ml of water + 50 ml 80/100 °C petroleum ether was added and the stoppered flask + contents vigorously shaken. The petroleum ether layer was evaporated away in the rotary evaporator and the coagulated chlorophyll + protein, lipids *etc.* was filtered off using a fluted filter paper. The clear light-brown filtrate was then evaporated to a small volume and then taken up in ~ 10 ml methanol. The whole of this concentrate was placed on the top of the PVP column and eluted with methanol, 20 ml fractions were collected on a fraction collector. Each fraction, up to 1400 ml, was evaporated down to 2.0 ml for bioassay and HPLC analysis.

TABLE 1
HPLC analysis of cytokinins

Peak		Retention time [min]
Solvent peak (MeOH)		2.2
Adenosine*	50 ng	2.7
Zeatin riboside	50 ng	3.1
Adenine*	50 ng	3.4
Zeatin	50 ng	3.8
N^6-benzyladenosine	100 ng	4.0
Dihydrozeatin	25 ng	4.1
N^6-isopentenyladenosine	100 ng	4.4
N^6-benzyladenine	100 ng	5.6
N^6-isopentenyladenosine	100 ng	6.4
N^6-benzyladenine N^9-tetrahydropyran	100 ng	9.5
PVP fraction 20–100 ml		5.1
PVP fraction 740–800 ml		2.7

* Adenine and adenosine do not display cytokinin activity at the concentrations employed with the other compounds.
For experimental details, see 'Material and Methods' section.

RESULTS

Using the *Amaranthus* bioassay, sharp peaks of activity were found in the fractions corresponding to 20—100 ml and 740—800 ml. These two peaks, when analysed by HPLC (4—10 µl injections) corresponded to sharp HPLC peaks at 5.1 min and 2.7 min, respectively. As shown in Table 1, these peaks did not correspond to any known, commercially available cytokinin. Paper chromatographic analysis of all the fractions indicated that phenolics did not appear in the eluate until ~ 800—900 ml methanol has passed through the column. Thus the cytokinin fractions are phenolic-free.

DISCUSSION

The two sharp peaks of cytokinin activity are probably N^7or N^9-glucosides or ribosides of the commonly encountered zeatin or isopentenyladenine, perhaps with phosphorylated glycoside moieties. Further work is in progress to provide precise identities of these unknowns.

The PVP-methanol procedure for cytokinin analysis shows considerable promise as a general and extremely mild clean-up procedure for plant extracts; it will probably supersede the cation-exchange procedure which has never been entirely suitable, partly because of the relatively harsh conditions which have led to partial hydrolyses of cytokinin ribosides and glucosides, but more seriously because of the failure to remove all phenolic contaminants.

The purity of the two cytokinin fractions means that further purification by means of solvent partitioning (LETHAM 1974) and column chromatography on Sephadex LH-20/aqueous ethanol (*e.g.* LEONARD 1974) can now be used in a more rational manner.

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BOOK REVIEW

ELLENBERG, H., ESSER, K., KUBITZKI, K., SCHNEPF, E., ZIEGLER, H. (ed.): FORTSCHRITTE DER BOTANIK. MORPHOLOGIE. PHYSIOLOGIE. GENETIK. SYSTEMATIK. GEOBOTANIK. VOL. 44. — Springer-Verlag, Berlin—Heidelberg—New York 1982. XVI + 449 S. Geb. DM 168,—. US $ 67.20.

28 Literaturübersichten bilden den Inhalt des 44. Bandes der Fortschritte der Botanik, die alljährlich in oben genannten Teilgebieten der Botanik die neueste Literatur (meist aus den letzten drei bis fünf Jahren) zusammenfassen und die daraus resultierenden neuen allgemeinen Erkenntnisse herausarbeiten. Besonders zahlreich ist in diesem Band der Teil Physiologie mit zehn Übersichten vertreten. Die Referierenden dieses Bandes kommen aus sechs Ländern, es überwiegen Beiträge von Autoren aus der Bundesrepublik Deutschland. Einige der Autoren gehören zu den regelmässigen, alljährlichen Mitarbeitern des Autorenkollektivs, einige sind neu dazugekommen. Ähnlich wie in Band 43 sind nur zwei der Beiträge in Deutsch, alle anderen in Englisch verfasst worden. Die einzelnen Literaturübersichten sind wie üblich gegliedert, enthalten nur die allernötigsten Illustrationen, weisen ebenfalls auf neue Bücher und Review hin und sind ausser als reiche Literaturquellen hauptsächlich dadurch wertvoll, dass die Fülle der neuen Daten zusammengefasst und verallgemeinert wird. Ein Sachregister mit eingearbeiteten Hinweisen auf die im Band hervorgehobenen Pflanzen ermöglicht die schnelle Orientierung im Band. Auch dieser Band, wie schon alle vorhergehenden, ermöglicht dem Leser eine schnelle und fundierte Übersicht über den Fortschritt der Disziplin zu gewinnen.

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