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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR SEPARATION AND QUANTIFICATION OF ZEATIN AND ZEATIN RIBOSIDE FROM PEARS, PEACHES AND APPLES

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SUMMARY

    A method for purification of zeatin and zeatin riboside from plant extracts for quantification by high-performance liquid chromatography (HPLC) is described. Initial separation is by chromatography on insoluble polyvinylpyrrolidone (PVP) and C_{18} Porasil B columns, followed by thin-layer chromatography on silica gel H. The final separations and quantification are done with methanol–water (pH 7) and then acetonitrile–water (pH 3) on μBondapak C_{18} analytical HPLC columns.

INTRODUCTION

    To measure the zeatin content of developing fruit of pear, peach, and apple, we have developed a purification procedure adaptable to final quantification of zeatin (Z) and zeatin riboside (ZR) by high-performance liquid chromatography (HPLC). HPLC has been frequently used in the separation of cytokinins from plant extracts[1–11], but only Arteca et al.[1] and Dekhuijzen[4] used HPLC for final quantification. Hahn[5], Kannangara et al.[8] and Morris et al.[10] showed chromatograms of cytokinin separation, but the peaks were difficult to quantify, and bioassay was generally relied upon. Buban et al.[2] and Monselise et al.[9] used silica columns, but peaks were not satisfactory for quantification.

    The disadvantage of bioassays and some of the problems associated with derivatization of cytokinins for determination by gas chromatography (GC) have been pointed out by others[5,11,12]. There are a number of advantages associated with the use of HPLC for quantifying Z and ZR: relatively large sample volumes can be injected; derivatization is not necessary; peaks with elution times similar to known cytokinins can be recovered and bioassayed; and HPLC is faster and more precise than bioassay.

    The non-specificity of the HPLC UV-detector is a disadvantage. While as little as 5 ng Z can be easily detected, numerous UV absorbing coextractives of Z generally occur in plant extracts and mask the presence of Z. The problem is to remove those impurities that strongly absorb in the UV and interfere with the detection of Z and ZR.

    A major class of UV interferants, phenols, can be at least partially excluded

from plant extracts by chromatography on insoluble polyvinylpyrrolidone (PVP)[1,2,3,9,12-14] and the dry weight of the extract reduced as well[8,14]. PVP chromatography is an attractive first step[8], since the extract is generally aqueous. The buffered aqueous fraction from PVP containing cytokinins can be passed over a column of C_{18} Porasil B*,[10] and the cytokinins concentrated on the column while other impurities such as sugars are not retained. This step is more rapid and more nearly quantitative than partitioning with n-butanol and does not require handling large volumes of organic solvents.

This report describes the use of columns of PVP and C_{18} Porasil B to initially separate and concentrate the cytokinins Z and ZR from plant extracts and the subsequent steps necessary to allow final quantification by HPLC.

MATERIALS AND METHODS

Initial purification
Five to 10 ml of the filtered aqueous fraction from an alcoholic extract of small pear fruit, equivalent to 33 g fresh weight (f.w.) were adjusted to pH 3.5 and loaded on top of a 1.9 × 20 cm column of PVP (Polyclar AT, GAF Corp.). PVP was prepared by suspending it in distilled water several times and decanting the fines. After the column was prepared, it was washed with several column volumes of the developing buffer, 0.013 M KH$_2$PO$_4$, pH 3.5[15], prior to use. The column had a flow-rate of about 3 ml min^{-1}. The extract was washed slowly onto the column with the developing solvent. The first 40 ml of eluent, including the extract volume, were discarded and the next 80 ml, which contained Z and ZR, collected and the pH adjusted to 7.

The entire 80 ml were then passed through a 0.8 × 4.0 cm column of C_{18} Porasil B (Waters Assoc.) which retained and concentrated the Z and ZR[10]. This column was prepared by slurrying 1 g of C_{18} Porasil B in acetonitrile, pouring the slurry into the column, and allowing it to settle by gravity. Several more column volumes of acetonitrile were pumped through the column with sufficient pressure to generate a flow-rate of about 3 ml min^{-1}, before changing over to distilled water prior to the introduction of the eluent from the PVP column. After the eluent had passed through the column, the column was washed with about 5 ml of distilled water to remove the initial buffer; 10 ml of 5% acetonitrile buffered at pH 7 with 0.01 M (NH$_4$)$_2$HPO$_4$ to remove the most polar compounds; and finally with 5 ml distilled water to remove the acetonitrile and buffer. Zeatin and ZR were eluted with 4 ml of ethanol and the ethanol removed under reduced pressure. The residue was dissolved in 3 × 100 μl methanol and stripped on a 500-μm silica gel H (silica gel 60H, EM Reagents) thin-layer plate that was previously washed in methanol–acetone (1:1) and then activated at 110°C for 30 min. The plate was developed in water saturated n-butanol in an ammonia atmosphere with appropriate standards. After development the standards were located with a 254-nm UV lamp while the plate was still moist. Zones comparable to the Z and ZR standards were scraped from the plate after it was dried. The silica gel was eluted four times with 2 ml of 90% methanol and the

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methanol taken to dryness under reduced pressure. The residue was redissolved in 200 μl of the mobile phase for the first HPLC separation.

HPLC analysis

We used a Waters Associates Model 244 liquid chromatograph equipped with a 6000 p.s.i. pump and Model 440 ultraviolet fixed wavelength detector (254 or 280 nm) and a Valco injector with a 100-μl loop. The columns used were Waters Associates μBondapak C_{18}, 300 × 3.9 mm I.D. All separations were done isocratically. Peak areas were measured with a single-channel computing integrator (Spectra-Physics Minigrator).

The mobile phase for the initial separation was methanol–water (20:80) buffered at pH 7 with triethylammonium bicarbonate (TEAB)[7] with a flow-rate of 1.0 ml min^{-1}.

Zones with the same elution time as Z and ZR were collected and taken to dryness under reduced pressure. Even though the peaks were not readily apparent during separation on the first HPLC column, zones corresponding to those of Z and ZR standards could be collected and Z and ZR recovered reproducibly. The residue was dissolved in 200 μl of the second mobile phase and 100 μl chromatographed on the second column with a mobile phase of acetonitrile–water (8.5:91.5) buffered at pH 3 with 0.02 M ammonium acetate, with a flow-rate of 1.0 ml min^{-1}. The alcoholic extract from small pear fruits (33 g f.w.) treated as described yielded a chromatogram with easily quantifiable peaks of Z and ZR (Fig. 2). When the procedure described was found to yield a relatively clear chromatogram with quantifiable peaks of Z and ZR, each step was examined for recovery and reproducibility using Z and ZR standards of 50, 100, 200, 400, and 800 ng. Finally, Z and ZR standards were taken through the entire procedure and the results examined by linear regression.

RESULTS AND DISCUSSION

PVP

The advantages and usefulness of PVP for purifying and initially separating plant extracts are well documented[1,2,8,9,13,14]. We agree with Kannangara et al.[8] that PVP is ideal for the initial purification of plant extracts. However, the columns must be carefully prepared and the pH carefully controlled to keep Z and ZR in the expected elution volume. Properly used, little loss of Z or ZR is anticipated for this step[9].

C_{18} *Porasil B*

There are a number of advantages in using a small open column of C_{18} Porasil B. The column used did not conform to the general rule that relates column height to column width because the shorter column allowed a flow-rate of about 3 ml min^{-1} without the necessity of high pressure. Concentration and recovery of Z and ZR from this column is preferable to exhaustive solvent extraction, which, by its nature, leads to some losses and requires handling and removal of large volumes of solvent. Thus, the C_{18} Porasil B column saves time, energy, and solvent, and elution of Z and ZR from it is essentially quantitative. The column can be manipulated to be more selective than solvent extraction since many substances which interfere with Z determi-

nation are coextracted with Z into n-butanol. Sugars and very polar compounds are not retained by the C_{18} Porasil B column. By using a column with the same functionality as the analytical columns, those compounds irreversibly bound in the C_{18} phase can be removed and prolong the analytical column life.

Recoveries with the standard deviations of Z and ZR standards passed through PVP and C_{18} Porasil B were 90.3 ± 5.1 % and 96.7 ± 4.7 %, respectively.

Thin-layer chromatography

Adsorption chromatography on silica gel was a technique with another mechanism to separate Z and ZR from interfering coextractives. The first problem we encountered was finding a solvent that would completely remove Z and ZR from the origin and then separate them as much as possible from other UV absorbing compounds. Chromatography on silica gel H with water saturated n-butanol in an ammonia atmosphere was effective for this purpose. Z and ZR were compact spots or zones at R_F 0.70 and 0.45, respectively, with these conditions. A more difficult problem was recovery of Z and ZR from silica gel. Best results were achieved by preparing our own plates from silica gel H as described and eluting with 90 % methanol. Recoveries and standard deviations for Z and ZR under these conditions were 80.4 ± 4.4 % and 78.5 ± 6.1 %, respectively.

This step was slow, but effective for separating Z and ZR from other materials that interfered with final separation and quantification by HPLC. We found no substitute for it.

HPLC

The isocratic mobile phase compositions chosen for the two separations provided different, but easily reproducible conditions to isolate finally and quantify Z and ZR[8]. The dedicated columns can be easily and quickly cleaned by increasing the solvent strength prior to equilibration for another sample. The use of TEAB[7] to adjust the pH of the first mobile phase was important because no solvent residue was left after evaporation. The sample residue was easily dissolved in the second mobile phase for injection on the second column. The usefulness of two columns with the same functionality but with different mobile phases[8] provided the selectivity necessary for separating Z and ZR from interfering substances on the first column and then quantifying them in a relatively clear area of the chromatogram from the second column. This procedure is effective as well as quicker and simpler than the commonly used Sephadex separations[2-4,6-9]. Recoveries and standard deviations of Z and ZR standards through these two columns were 82.2 ± 8.9 % for Z and 88.6 ± 10.2 % for ZR.

A series of standards of Z and ZR were analyzed by the procedure described and the recovery data related to the initial amounts by linear regression. Ninety-five percent confidence bands were then calculated for each line (Fig. 1a and 1b). This information suggests that the amount of plant material chosen for this type of analysis should contain at least 100 ng of Z or ZR. More accurate determinations of Z or ZR could be made near the midpoint of their recovery curves, which in this case was 400 ng. The regression lines for both Z and ZR standards accounted for 98 % of the variability in recovery of Z and ZR attributable to a change in the initial amount of each.
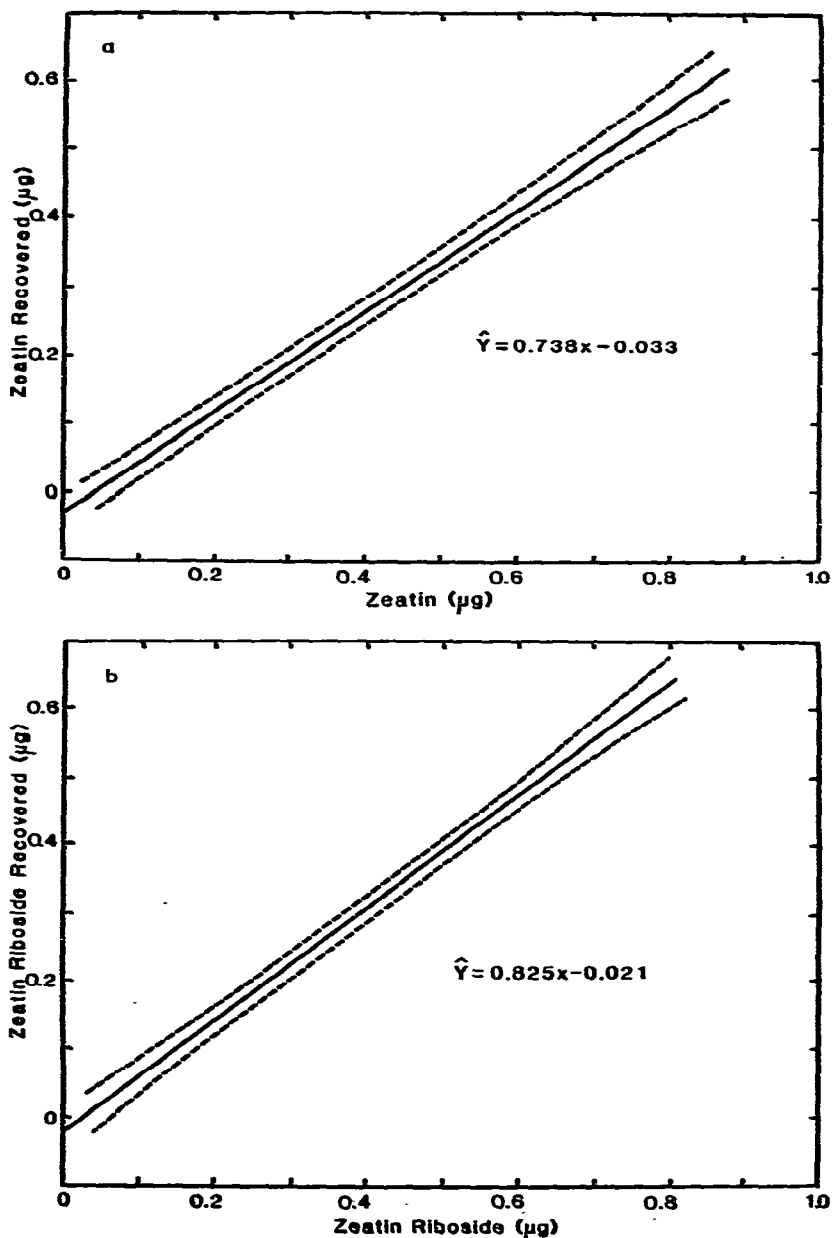
Fig. 1. Regression lines for recovery with 95% confidence bands for zeatin (a) and zeatin riboside (b) standards through purification procedure described in text.

The regression lines with their 95% confidence bands, once constructed, can be used to calculate sample unknowns. Preferably a minimum of two or three determinations can be done for each experimental sample. Then the amount of Z or ZR with its confidence interval can be taken from the appropriate regression line.

A pear fruit extract carried through the procedure described yielded a chroma-

togram with two peaks with retention times corresponding to authentic *trans*-zeatin and *trans*-zeatin riboside (Fig. 2b). When a similar extract was "spiked" with Z and ZR, and carried through the same procedure, peaks in the Z and ZR zone were enhanced (Fig. 2c). Quantification of the two peaks at 254 nm and 280 nm gave the same values for zeatin, indicating the presence of only one UV absorbing compound in that peak. There was a slight discrepancy between values for ZR suggesting there may have been another peak associated with ZR in the pear extract. The peaks were active in an *Amaranthus caudatus* bioassay[16].
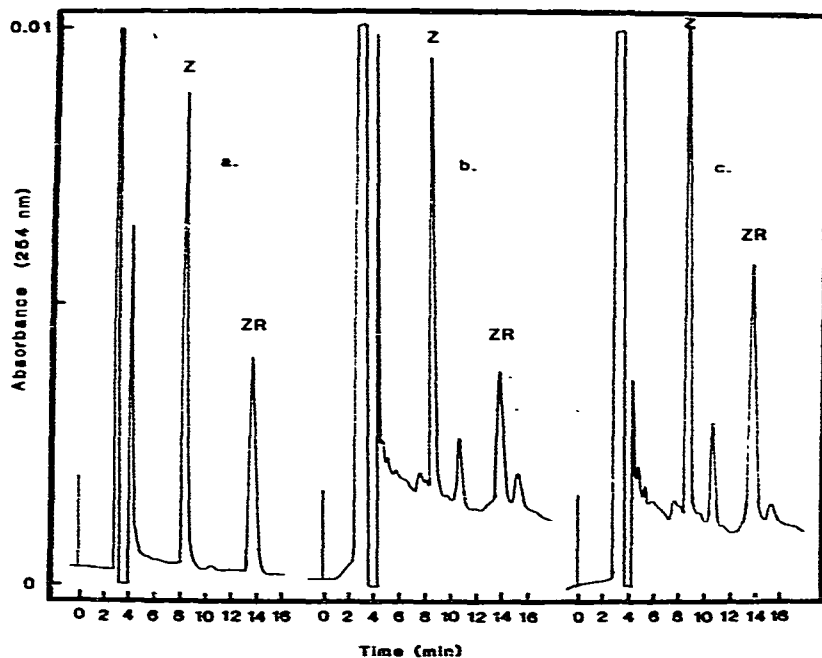

Fig. 2. Separation of zeatin and zeatin riboside standards (a), from pear fruit extract (b), and pear fruit extract spiked with zeatin and zeatin riboside (c) by reversed-phase HPLC. Column: μBondapak C_{18} (300 $\times$ 3.9 mm I.D.). Flow-rate: 1 ml min^{-1}. Mobile phase: acetonitrile–water (8.5:91.5) buffered at pH 3 with 0.01 M ammonium acetate.

After the procedure was developed, it was further evaluated by making duplicate ethanolic extracts of 127-g f.w. samples of small apple fruits from about 50 days after bloom. One extract was separated into three equal subsamples and the other into two subsamples for analysis. Zeatin estimates for the triplicate samples were 3.3, 2.9, and 2.9 ng/g f.w. and for the duplicate samples 2.8 and 2.7 ng/g f.w. The ZR peak was not adequately separated from a strongly absorbing impurity for the integrator to make an accurate estimate. However, peak heights from the duplicate samples were measured to be about one and one-half times those from the triplicate samples.

This procedure was useful for separating Z and ZR in extracts of small pear and apple fruits. Interfering constituents of extracts change with plant tissue chosen as well as the stage of development or physiological state of the plant material, so

adjustments may be necessary to maintain Z and ZR in relatively clear areas of the final chromatogram. The changes necessary can usually be made in the TLC step by changes in the developing solvent. Some manipulation of the composition of the mobile phases for the HPLC columns can also be made to shift Z and ZR into clear areas of the final chromatogram.

The procedure described avoids extremes of pH and has a minimum number of steps. Recovery through the entire system was reproducible. Any step could be omitted if the extract warranted. Each step has sufficient latitude for modification, so the method could be adapted to other plant extracts. By adjusting conditions, starting with PVP separation, abscisic acid (ABA), indoleacetic acid (IAA), and gibberellins may also be isolated. For instance, we found ABA had an elution volume of 100 ml from PVP as we used it, and could easily be included with the cytokinins fraction.

The number of steps could probably be reduced further through the use of a preparative HPLC column[11] in place of TLC and the first HPLC analytical column. Another option that may be promising for reducing the number of steps is the use of an analytical column with some residual hydroxyl sites, perhaps 10–20%, in place of TLC and the first HPLC column.

ACKNOWLEDGEMENTS

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* *Editor's note:* See also E. M. S. MacDonald, D. E. Akiyoshi and R. O. Morris, *J. Chromatogr.*, 214 (1981) 101, and G. C. Martin, R. Morgan and I. M. Scott, *J. Chromatogr.*, 219 (1981) 167.