IDENTIFICATION OF SOYBEAN, *GLYCINE MAX* (L.) MERR., CYTOKININS

by

Kevin Edward Crosby

Dissertation submitted to the Graduate Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Agronomy

APPROVED:

Dr. L.H. Aung, Co-Chairman

Dr. G.R. Buss, Co-Chairman

Dr. J.L. Hess

Dr. D.G.I. Kingston

Dr. E.R. Stout

May 1982

Blacksburg, Virginia

DEDICATION

To my wife Lois, whose unflagging support made this work possible

Acknowledgements

The author thanks Dr. Louis Aung for his patience, guidance and friendship over the last five years. The help and advice of Dr. Glenn Buss were immeasurable and is deeply appreciated. I also thank my committee members Dr. John Hess for the use of HPLC and many helpful ideas, Dr. David Kingston for his suggestions and criticisms and Dr. Ernie Stout for his concern and service. The technical assistance of Mr. Allen Shaver and Dr. Margie Pyle in the planting and harvesting of the soybean crop is gratefully acknowledged. I greatly appreciated the assistance of Mr. Kim Harich with GC-MS analyses and Mr. Ted Ellmore with the electronic balance. Last but not least, I thank Mrs. Carolyn Rahnema for typing the manuscript.

TABLE OF CONTENTS

page

DEDICATION ii

ACKNOWLEDGEMENTS iii

LIST OF TABLES vi

LIST OF FIGURES vii

INTRODUCTION 1

REVIEW OF LITERATURE

Characterization of Cytokinins from Plant Sources 3
Identification of Cytokinins using Bioassays 3
Identification of Cytokinins using Physico-Chemical Methods 4

MATERIALS AND METHODS

Plant Growing and Harvesting 11
Extraction 11
Chromatography 12
Bioassay 14
Gas chromatography-Mass spectrometry 16

RESULTS

Paper Chromatography 18
PVP Chromatography 18
Sephadex LH-20 Chromatography 18
High Performance Liquid Chromatography 20

EXPERIMENT I
Chromatographic Behavior of Cytokinin-like Substances in Soybean fruits 20
Mass Spectra of Cytokinin Standards 36
Quantitative Estimate of Cytokinin-like Substances 39

EXPERIMENT II
GC-MS Characterization of Bioactive Fractions 46

EXPERIMENT III
Further Purification and Characterization of Bioactive Fractions 51

DISCUSSION 70

SUMMARY AND CONCLUSIONS 81

TABLE OF CONTENTS (continued)

	page
LITERATURE CITED	83
APPENDIX I Calculation of Endogenous Cytokinin content	87
APPENDIX II Calculation of Amount of BAP applied to Soybean Fruits	88
APPENDIX III Thin layer Chromatography	89
VITA	91

LIST OF TABLES

		page
Table 1.	Biological Activities of Cytokinin-like substances in Partially purified Extracts of Legumes ..	5
Table 2.	Influence of flow rate on elution time of zeatin and zeatin riboside from Partisil ODS 2	21
Table 3.	Effects of aqueous phase pH on elution times of adenosine, zeatin riboside, zeatin and dihydrozeatin from Partisil ODS 2	22
Table 4.	Peak intensities for permethylated zeatin	42
Table 5.	Peal intensities for permethylated zeatin riboside .	42
Table 6.	Estimation of the total cytokinin content in 15 grams dry weight of 10-25 mm Essex fruits	45
Table 7.	Estimated cytokinin-like substances in Essex and Shore pericarps	47
Table 8.	Bioassay responses of HPLC purified fractions of Essex fruits	56

LIST OF FIGURES

page

Figure 1. Chemical structure of selected naturally occurring cytokinins 2

Figure 2. Schematic diagram of Sephadex LH-20 chromatography apparatus 19

Figure 3. *Amaranthus* bioassay of PVP eluate for 15 grams dry weight of immature Essex fruits 24

Figure 4. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from PVP 0-100 ml fraction, paper R_f 0.2-0.4 Cultivar ESSEX 26

Figure 5. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from PVP 0-100 ml fraction, paper R_f 0.5-0.8 Cultivar ESSEX 27

Figure 6. Bioassay of paper chromatograms of (A) Shore and (B) Essex extracts, PVP 100-250 ml fraction 29

Figure 7. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from PVP 100-250 ml fraction, paper R_f 0.5-0.8 Cultivar ESSEX 30

Figure 8. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from PVP 100-250 ml fraction, paper R_f 0.5-0.8 Cultivar SHORE 31

Figure 9. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from PVP 100-250 ml fraction, paper R_f 0.2-0.4 Cultivar ESSEX 32

Figure 10. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from PVP 250-650 ml fraction, paper R_f 0.5-0.8 Cultivar ESSEX 34

Figure 11. Absorbance at 268 nm and bioassay activity of Sephadex LH-20 fractions from phosphocellulose ammonia eluate of alkaline phosphatase digested ("bound") aqueous phase after n-butanol extraction... 37

Figure 12. Flow chart of purification of cytokinin-like substances in Essex soybean fruits (10-25 mm) 38

LIST OF FIGURES (continued)

page

Figure 13. Mass spectra from m/e 50 to 300 for permethylated zeatin standard 40

Figure 14. Mass spectra from m/e 100 to 500 for permethylated zeatin riboside standard 41

Figure 15. Multiple ion monitoring detection of 50 ng of permethylated zeatin standard 43

Figure 16. Multiple ion monitoring scan of 50 ng of permethylated zeatin riboside standard 44

Figure 17. Cytokinin-like activity of developing ovules at four stages of development 48

Figure 18. Multiple ion monitoring spectrum of Z and ZR standards in Experiment II 49

Figure 19. Multiple ion monitoring detection of ZR in Sephadex LH-20 fractions (Experiment II) 50

Figure 20. Multiple ion monitoring scan for Z in Sephadex LH-20 fractions (Experiment II) 52

Figure 21. UV absorbance of Shore Z and ZR fraction from Sephadex LH-20 chromatographed on Partisil ODS 2 ... 53

Figure 22. UV absorbance of Essex Z and ZR fraction from Sephadex LH-20 chromatographed on Partisil ODS 2 ... 54

Figure 23. Total ion current recording for Shore ZR fraction from Partisil ODS 2 57

Figure 24. Partial spectrum of peak 1 in figure 23, identified as dioctylphthlate 58

Figure 25. Spectrum of peak 2 in figure 23, identity unknown .. 59

Figure 26. UV detection of HPLC eluate of ZR fraction from Sephadex LH-20 after phosphocellulose 61

Figure 27. UV detection of HPLC eluate of ZR fraction from Sephadex LH-20 after phosphocellulose, cultivar ESSEX 62

Figure 28. UV detection of HPLC eluate of Z fraction from Sephadex LH-20 after phosphocellulose, cultivar SHORE 63

LIST OF FIGURES (continued)

page

Figure 29. UV detection of HPLC eluate of Z fraction from Sephadex LH-20 after phosphocellulose, cultivar ESSEX 64

Figure 30. UV detection of HPLC eluate of polar fraction from Sephadex LH-20 after PVP and paper chromatography . 65

Figure 31. Multiple ion monitoring scan for permethylated unknowns from HPLC fractions of figure 30 66

Figure 32. Multiple ion scan of chemical inoization mass spectrum of polar fraction (figure 30)............. 67

Figure 33. Multiple ion monitoring recording of permethylated unknowns from polar fraction (figure 30) 69

INTRODUCTION

Cytokinins are N^6-substituted adenine derivatives (figure 1) with growth regulating properties which influence many aspects of plant development (Skoog and Schmitz 1979). They are involved in the processes of cell division, cell enlargement, assimilates transport and senescence. Cytokinins have been detected in young fruits and ovules and are important for successful fruit and seed development.

A previous study (Crosby 1979) demonstrated the presence of cytokinin-like substances in developing seeds of soybeans. Exogenous cytokinin applications to young fruits have also been shown to increase fruit-set and seed yield of soybeans (Crosby et al.,1981). Therefore, information on the identity of endogenous cytokinins of soybeans will be of great interest for understanding the role(s) in regulating soybean growth and fruitng.

A systematic review of soybean literature shows there is little or no information on the chemical identities of cytokinins in soybeans. This investigation was undertaken to study the extraction, purification and identification of the cytokinin-like substances of soybean fruits.

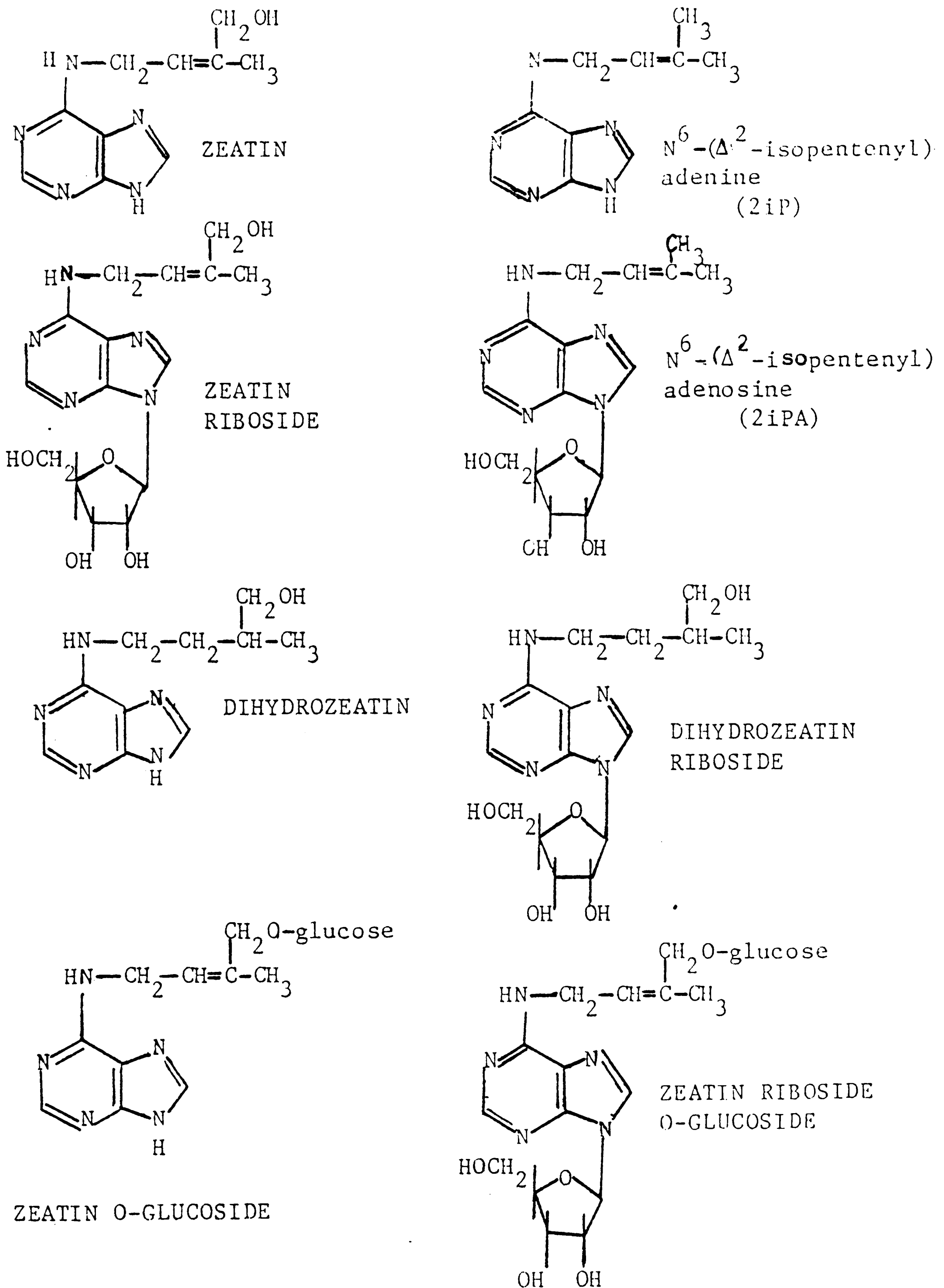


Figure 1. Chemical structures of selected naturally occurring cytokinins.

REVIEW OF LITERATURE

Characterization of Cytokinins from Plant Sources

The isolation and identification of cytokinins from plants are problematical because of the minute quantities usually found in plants (Horgan, 1980). Ideally, therefore for such studies, tissues or organs relatively "rich" in cytokinins and readily available for bulk extraction are desireable. However, tissues and organs from crop plants of interest may not often meet these considerations. In these cases, sensitive and selective methods for cytokinins identification are required.

Characterization of cytokinins in legumes has been limited to relatively few organs which are rich in cytokinins such as root nodules (Syono and Torrey, 1976), immature fruits (Summons *et al.*, 1979; Yokota *et al.*, 1980) and primary leaves (Wang *et al.*, 1977). Two general approaches are involved in these investigations, namely bioassays and certain physico-chemical methods. For cytokinin identification it is important to select and use appropriate techniques since the reliability of the results depends upon the specificity and sensitivity of the method(s) chosen.

Identification of Cytokinins using Bioassays

Bioassays are commonly and routinely used for plant growth substances studies because of their relative sensitivity, convenience and low cost. However, bioassays lack specificity and cannot be relied upon to quantify the amount of plant growth substances such as

cytokinins in plant tissues. In addition, variation between bioassays done at different times can prevent direct comparison of results.

Since bioassays lack specificity, a characterization of a substance eliciting a response is limited to descriptions such as 'cytokinin-like'. However, bioassays are useful for screening unknown plant extracts before further characterization by physico-chemical methods.

The occurrence of cytokinin-like substances in legumes is summarized in table 1. A common feature of these studies is the relatively low degree of purification using paper and column chromatography. Immature seeds and fruits of four species contain cytokinin-like substances. While inconclusive, these studies suggest that these organs contain cytokinins which can be extracted and partially purified in a biologically active form.

Identification of Cytokinins by Physico-chemical Methods

Compared to the biological detection of cytokinin-like activity in partially purified plant extracts, identification by physio-chemical methods is more difficult. Cytokinin concentrations in plant tissues are generally in the nanogram per gram concentration range (Horgan, 1980). Extracts of plant tissues may contain many different compounds at much higher concentrations than cytokinins. The substances of interest may constitute only a very small percentage of a complex mixture and a sufficiently large quantity of plant tissues must therefore be extracted and separated from the bulk of the other constituents. In general several chromatography steps are obligatory before a sample

Table 1. Biological Activities of Cytokinin-like Substances in Partially Purified Extracts of Legumes

SPECIES	SEPARATION TECHNIQUES	BIOASSAY	REFERENCE
Pisum sativum, nodules	Thin layer chromatography	Soybean callus	Syono and Torrey,1976
Pisum arvense, young seeds	no purification	Soybean callus	Burrows and Carr,1970
Phaseolus vulgaris, young seeds	Paper chromatography	Soybean callus	Nesling and Morris,1979
Lupinus albus, young seeds	Ion exchange and LH-20 chromatography	Soybean callus	Davey and Van Staden,1979
Glycine max, lateral buds	Ion exchange and LH-20 chromatography	Soybean callus	Dua *et al*., 1978
Glycine max, young fruits	PVP chromatography	*Amaranthus*	Lindoo and Nooden, 1978
Glycine max, young seeds	PVP chromatography	*Amaranthus*	Crosby,1979

is enriched enough in cytokinins before an analytical technique can be applied for chemical determination.

A powerful technique for structure determination of cytokinins is combined gas chromatography-mass spectrometry (GC-MS). The great advantage of this method is that absolutely pure compounds do not have to be isolated because the gas chromatograph can separate the components of mixtures. The mass spectrometer can then give unique structural information about the individual components of the mixture. When the GC-MS is coupled to a computer system it can quickly generate large amounts of structural data about unknown compounds. This capability has made combined GC-MS the method of choice for identification of cytokinins and other naturally occurring compounds. There are two major problems to solve before GC-MS can be used for cytokinin identification. First, the impure extracts must be partially purified and the cytokinins concentrated. Second, the polar nature of cytokinins makes them non-volatile and prevents the direct use of gas chromatography which requires a volatile sample. Therefore the compounds must be derivatized to a less polar form before GC-MS analysis. There are two methods of derivatization commonly used for cytokinins, each with advantages and disadvantages.

The trimethylsilyl (TMS) derivatives are sensitive to traces of moisture and samples must be kept dry before, during and after derivatization. TMS derivatives do not give a very diagnostic fragmentation pattern or molecular ion when an electron impact (EI) source is used, but this can be overcome by using a chemical ionization (CI) technique (Summons _et al_., 1980). They do have the advantages of giving sharp

GC peaks, widely available reference spectra in the literature and are a proven method with high purity reagents commercially available.

The permethylated derivatives have the advantage of being stable to water and giving diagnostic fragmentation patterns and observable molecular ions (Young, 1977). Permethylation reagents are not available commercially and must be prepared by the user. Also, GC peaks of permethylated cytokinins tend to be less sharp than TMS derivatives. Finally, reference spectra have not been published for all the cytokinins.

An example of isolation and identification of an unknown cytokinin without using mass spectrometry is the identification of dihydrozeatin in immature lupin fruits by Koshimizu _et al_. (1967). A methanolic extract of 206 kg of immature fruits (stage of development not given) was purified by chromatography on charcoal followed by ion-exchange chromatography. The ammonia eluent was treated with silver nitrate to selectively precipitate cytokinins. The precipitate was further purified by ion-exchange and paper chromatography. Finally the bioactive fraction was treated with picric acid and the picrate salt recrystallized. The UV spectra and melting point indicated a saturated side chain. The saturated zeatin analogue, dihydrozeatin was synthesized and found to be identical to the isolated compound. From all this evidence it was concluded that dihydrozeatin was present in immature lupin seeds.

The first report of combined GC-MS to identify a cytokinin from a plant was by Horgan _et al_. (1973) who found zeatin riboside in xylem sap of sycamore (_Acer pseudoplatanus_) trees. The same method was used

by Wang et al. (1977) to identify dihydrozeatin glucoside in leaves of decapitated bean plants, and confirmed the earlier report of Englebrecht (1972) that leaves of decapitated beans accumulated high levels of cytokinin-like substances. Wang et al. (1977) used ion-exchange, Sephadex LH-20 and paper chromatography to purify methanolic leaf extracts. Cytokinin-like substances were detected with the soybean callus bioassay. Bioactive fractions were treated with B-glucosidase, extracted into butanol and derivatized for GC-MS. The TMS derivative of dihydrozeatin was identified by its mass spectrum, while glucose was detected in the glucosidase incubation mixture. An unknown compound identified as dihydrozeatin-O-B-D-glucoside (DHOG) was found.

Palmer et al. (1981) further investigated the metabolism of DHOG in bean leaves using a stable isotope method developed by Summons and co-workers (1979). Decapitated and disbudded bean plants accumulated high levels of DHOG in leaves. When a shoot was allowed to develop, DHOG levels fell over a 10 day period to levels comparable to untreated controls.

The studies cited demonstrate the utility of GC-MS for identification of cytokinins in vegetative tissues of legumes.

The cytokinin complex in developing lupin fruits has been identified and quantified using a stable isotope method (Summons et al., 1979). The glucosyl derivatives of zeatin and dihydrozeatin were initially identified as metabolites of exogenously applied radiolabeled zeatin (Letham et al., 1975; Parker and Letham, 1973; Duke et al., 1979). Bioassays of chromatograms suggests the glucoside-like compounds occur

naturally in diverse species (Davey and Van Staden, 1979; Henson, 1978).

Seeds and pericarps of developing lupin fruits harvested 14 days after petal abscission were extracted with methanol and fractionated with cellulose phosphate columns. The ammonia eluates were subjected to preparative HPLC and the cytokinin-like eluents were further purified by HPLC on microparticulate columns with C_{18} and phenyl bonded phases. After this purification procedure individual cytokinin peaks were evident. The collected fractions were dried and TMS derivatives prepared. Direct insertion mass spectrometry was used to identify the derivatives. The addition of deuterated internal standards at the start of the procedure allowed for correction of losses and accurate estimation of endogenous cytokinins by comparisons of selected peak heights.

The total cytokinin content of the pericarp was greater than the seeds, with O-glucosyldihydrozeatinriboside (OGDZR) the major constituent of seeds and pericarps. Pericarps contained no zeatin riboside, which was a major component of the seeds, while dihydrozeatinriboside occurred at equivalent concentrations in seeds and pericarps. Amounts of the O-glucosides of zeatin, dihydrozeatin and zeatin riboside were relatively low in both tissues.

These results show the cytokinin complex in lupin fruits is not equally distributed between seeds and pericarps. The large amount of OGDZR in pericarps may represent a storage pool of inactive cytokinin which might be converted to more active forms required for seed growth. Interconversion of cytokinin forms is known in <u>in vitro</u> systems

(Van Staden and Davey, 1977; Chen and Petschow, 1978) and may be a regulatory mechanism controlling seed cytokinin content. Enzymes controlling the glucosylation of cytokinins have been isolated (Entsch and Letham, 1979) lending credence to this view.

Extracts of immature seeds of the hyacinth bean (Dolichos lablab L.) contain cytokinin-like components active in the Amaranthus bioassay (Yokota et al., 1981). Purification of the crude butanol extracts by cation-exchange, PVP and Sephadex LH-20 chromatography yielded four active fractions. These were further purified by paper and thin layer chromatography before final purification using reversed phase HPLC. The purified components were permethylated and analyzed by GC-MS. Zeatin, zeatin riboside (cis and trans isomers), O-glucosyl zeatin and O-glucosyl zeatin riboside were identified on the basis of their retention times and mass spectra. For this study 5.6 kg of seeds were extracted and purified. This large sample permitted the detection of the unknowns using the bioassay to screen fractions of interest.

The detection of cytokinins in developing fruits of other legume species and the known presence of cytokinin-like substances in soybean fruits strongly suggests the presence of cytokinins in soybeans.

MATERIALS AND METHODS

Plant Growing and Harvesting: Two determinate soybean cultivars (maturity group V), Essex and Shore, were grown at the Virginia Polytechnic Institute and State University Horticulture Research farm at Blacksburg, Va. during the growing seasons of 1980 and 1981. The soil is a Lodi series (typic hapludult) with pH 6.5-7.0. The soil was fertilized with 90 kg ha^{-1} P_2O_5 and 189 kg ha^{-1} K_2O equivalents before planting. No inoculant was used because the field had previously been cropped to soybeans. Weeds were controlled with a pre-plant incorporated treatment of 1.8 liters ha^{-1} trifluralin and timely cultivation.

Planting was done in early June at a seeding rate of approximately eight to ten seeds per 30 cm. of row with 0.9 m between rows.

Plants were harvested in mid to late August at stage R2 and R3 (Fehr and Caviness, 1977) by uprooting and transporting to the laboratory coldroom. Approximately 100 linear meters of row were harvested for each cultivar in each year. This corresponds to about 3000 plants for each cultivar. All fruits 10-25 mm length were removed and kept on ice until a quantity suitable for processing was collected. The bulked fruits were frozen and lyophilized at -40 C until dry and then stored at -20 C until extracted.

<u>Extraction</u>: Fruits were ground in a Virtis 45 homogenizer for 2 minutes at a setting of 50. Cold (-20 C) 80% ethanol was used at a rate of 15 ml for each gram of tissue. The homogenate was stirred overnight at 5 C, then decanted and extracted with another volume of cold 80% ethanol. After 24 hours the residue was filtered and washed

twice with 50 ml of cold ethanol. The combined extracts and washes were reduced to the aqueous phase _in vacuo_ at 40 C. The aqueous phase was refrigerated overnight and the precipitates collected by centrifugation at 35,000 g for 45 minutes.

The supernatant was decanted and adjusted to pH 8.4 with 1 N KOH, extracted twice with 25 ml of petroleum ether and then four times with 50 ml water saturated n-butanol. The butanol phase was reduced to dryness _in vacuo_ at 60 C. At the end of the distillation 10 ml of 95% ethanol were added to ensure the complete removal of butanol residues. The dried residue was then chromatographed.

CHROMATOGRAPHY

A) Column chromatography

i) Polyvinylpyrrolidone (PVP) - 2.5 x 15 cm columns were prepared using 15 grams of insoluble PVP (Sigma) slurried in 250 ml of water. The fines were discarded by pipetting the upper 50 ml of the water off and discarding. The column was packed under gravity flow and washed with 200 ml of distilled water followed by 200 ml of 16 mM KH_2PO_4 buffer, pH 6.4. A layer of clean, acid washed sand was placed over the top of the column to prevent disturbance when applying samples.

The dried butanol residue was taken up in four ml of buffer and run onto the column followed by two ml of buffer. The column was eluted at 35 ml hr^{-1} and three fractions were collected. The first fraction consisted of 0-100 ml which included the 40 ml void volume. The bulk of green pigments eluted in this fraction. The second fraction consisted of the 100-250 ml volume and the third fraction

included the 250-260 ml volume. Authentic zeatin and zeatin riboside elute in the second fraction.

ii) Phosphocellulose ion exchange - Phosphocellulose (Sigma) was swelled with consecutive rinses in water, 0.5 N HCl and 0.5 N NH_4OH, with fines decanted after every rinse. After swelling, 0.5 N NH_4OH is run through the column until the eluate is strongly basic. The column was then rinsed with water (pH 3.1 with acetic acid) until the pH was less than 7.0. The sample pH was adjusted to 3.0 with dilute HCl and the insoluble precipitates collected by centrifugation. The supernatant was applied to the column and five bed volumes (one bed volume = 50 ml) of water (pH 3.1 with acetic acid) were run through the column. The sample was then eluted with 5 bed volumes of 0.5 N NH_4OH. Standards of zeatin and zeatin riboside elute with the first 15 ml of basic eluent.

iii) Sephadex LH-20 - A 1.5 x 45 cm column was slurry packed with LH-20 swollen with 35% ethanol. Samples dissolved in 2 ml of 35% ethanol were applied by gravity flow. Elution was with 35% ethanol at 25 ml hr^{-1}. Flow rate was maintained with air pressure and a needle valve (Figure 2). Fractions were collected with a 5 ml siphon and absorbance determined at 268 nm for each fraction.

B) Paper chromatography

Whatman 3 MM paper was used for all chromatograms. Concentrated samples were applied to the paper with a syringe under a stream of warm air to remove the solvent. The solvent system used for cytokinins was isopropanol (iPrOH)/NH_4OH/H_2O 10:1:1 (v:v:v). Elution was for 40 cm in a descending manner and after development the

chromatogram was air dried and cut into appropriate R_f zones for extraction with 80% ethanol.

C) High Performance Liquid Chromatography (HPLC) - The instrument used was a Spectra Physics SP 3500B with dual pumps and gradient programmer module. The detector was a Schoeffel model 770 variable wavelength UV-VIS detector. A 4.6 mm by 25 cm column of Partisil 10 ODS 2 reversed phase material was used.

Cytokinins were chromatographed using an aqueous phase of high purity water adjusted to pH 7.0 with triethylammonium bicarbonate (TEAB). The organic phase was HPLC grade acetonitrile (Fisher). All solvents were degassed and filtered through a 2 micron filter before use. A linear gradient of 8 to 38% acetonitrile over 15 minutes was routinely used for elution of zeatin like cytokinins. Standards were included in each set of samples to correct for any variability from run to run. Unknown sample peaks were collected via a sampling port located immediately after the detector cell.

Bioassay: The *Amaranthus* bioassay of Biddington and Thomas (1973) was modified as described by Reda and Rasmussen (1975) and Eliot (1979).

Seeds of *Amaranthus caudatus* cv. Love Lies Bleeding (Stokes Seeds, Buffalo, NY) were germinated in a 5 x 13 x 22 cm plastic tray (Rubbermaid JB1-2916) lined with six sheets of Whatman No. 2 filter paper. The papers were moistened with 40 ml distilled water and seeds were uniformly distributed on the papers. The trays were placed in plastic bags, sealed, covered with aluminum foil and incubated at 37 C for 48 hours.

Assays were performed using 16 mM KH_2PO_4 buffer, pH 6.4 containing 1 mg ml^{-1} of L-tyrosine. The tyrosine was solubilized by grinding in a small mortar and pestle followed by autoclaving at 116 C for 30 minutes.

Chromatographic fractions for bioassay were reduced to dryness and dissolved in 7.5 ml of hot assay buffer with agitation. Equal parts were transferred to 5.5 cm petri plates lined with two 4.5 cm Whatman No. 1 filter discs.

After 48 hours incubation, the roots were removed from seedlings under a primary green safe lamp. Explants consisted of cotyledons and 5 to 10 mm of hypocotyl. Explants were incubated in distilled water for one hour before use. Twenty explants were used for each petri plate.

A randomized complete block design was used with three replications, each containing a reagent blank, zeatin standards and samples. After the explants were placed in the petri plates they were incubated in darkness for 24 hours at 25 C.

After incubation the explants were ground in two ml of 10% acetic acid and the tissue grinder rinsed with a further one ml. The combined acetic acid fractions were centrifuged for 10 minutes at 2500 rpm in an IEC Model K centrifuge. The supernatant was pipetted into glass cuvettes and absorbance measured at 542 and 620 nm. The difference in absorbance was recorded as the response. All data were analyzed by analysis of variance and fractions giving a significant response greater than the control were identified.

GAS CHROMATOGRAPHY-MASS SPECTOMETRY

Purified chromatographic fractions were reduced to dryness in 1 ml Reacti-vials (Pierce Chemical Co.) under a stream of N_2 and then dried for 24 hours *in vacuo* over P_2O_5.

Permethylation: The methylation procedure of Hakamori (1963) as modified by Young (1977) was used. Dimethyl sulfoxide anion is generated by reacting 200 mg ether washed sodium hydride (oil dispersion, Aldrich Chem. Co.) with four ml of dry dimethylsulfoxide at 65 C for two hours. This solution is stable for up to one month, but only two week old solutions were used. Derivatization was done in a plastic glove bag (I^2R Industries) inflated with dry N_2. Samples were reacted for one hour in 100 µl DMSO anion with occasional agitation. Then, 10 ul methyl iodide was added and reacted for one hour with occasional agitation. The reaction was quenched by adding 0.9 ml water and the methylated products extracted into three aliquots of chloroform (one ml each). The chloroform layers were cleaned with two water washes (one ml each) and reduced to dryness with a stream of N_2. The residue was then dissolved in 50 µl of chloroform.

Gas chromatography: A Varian 1400 GC equipped with an SP 2300 column was used for sample introduction. A linear temperature program of 10 C $minute^{-1}$ from 250 to 300 C was used. Carrier gas flow rate was 40 ml $minute^{-1}$.

Mass Spectrometry: A Varian MAT 112 mass spectrometer equipped with a Varian Data System and jet separator was used for sample analysis. The electon impact source was operated at 70 eV. Multiple ion monitoring was used for detection of cytokinin standards and unknowns.

In addition to the gas chromatograph, a direct insertion probe could be used for sample introduction. The probe was equipped with an electric heating accessory for sample volatilization.

RESULTS

Standards of zeatin (Z), dihydrozeatin (DHZ) and zeatin riboside (ZR) were purchased from Sigma Chemical Co., St. Louis, MO. Solutions of 1 mg ml^{-1} were made in 95% ethanol and stored at 5 C. These solutions were stable for at least six months.

Paper Chromatography: Aliquots of 50 ul of each standard were applied to Whatman 3MM paper and developed to 40 cm in a descending manner with a solvent mixture of isopropanol (iPrOH):NH_4OH:water (10:1:1 v:v:v). The chromatograms were air dried, cut into 20 R_f zones and eluted with 80% ethanol. The absorbance of each R_f zone was measured at 268 nm. Zeatin had an R_f of 0.7 $\pm$ .1, zeatin riboside 0.6 $\pm$ .1 and dihydrozeatin 0.75 $\pm$ .1.

PVP Chromatography: A 2 x 13 cm column of PVP was prepared by slurrying 15 grams of PVP (Sigma Chemical Co., St. Louis, MO.) in 250 ml of distilled water and pipetting off the fine particles that floated to the surface. The standard samples were applied dissolved in 16 mM KH_2PO_4 buffer, pH 6.4. Elution with this buffer at 35 ml hr^{-1} gave elution volumes of 100-150 ml for zeatin riboside and 150-200 ml for zeatin and dihydrozeatin.

Sephadex LH-20 Chromatography: Preliminary experiments with a 1.5 x 45 cm column packed and eluted by hydrostatic pressure gave variable results. This was caused by compression of the packing and a subsequent decrease in flow rate.

To overcome this problem, a system using air pressure to maintain solvent flow rate was constructed (Fig. 2). A needle valve gives

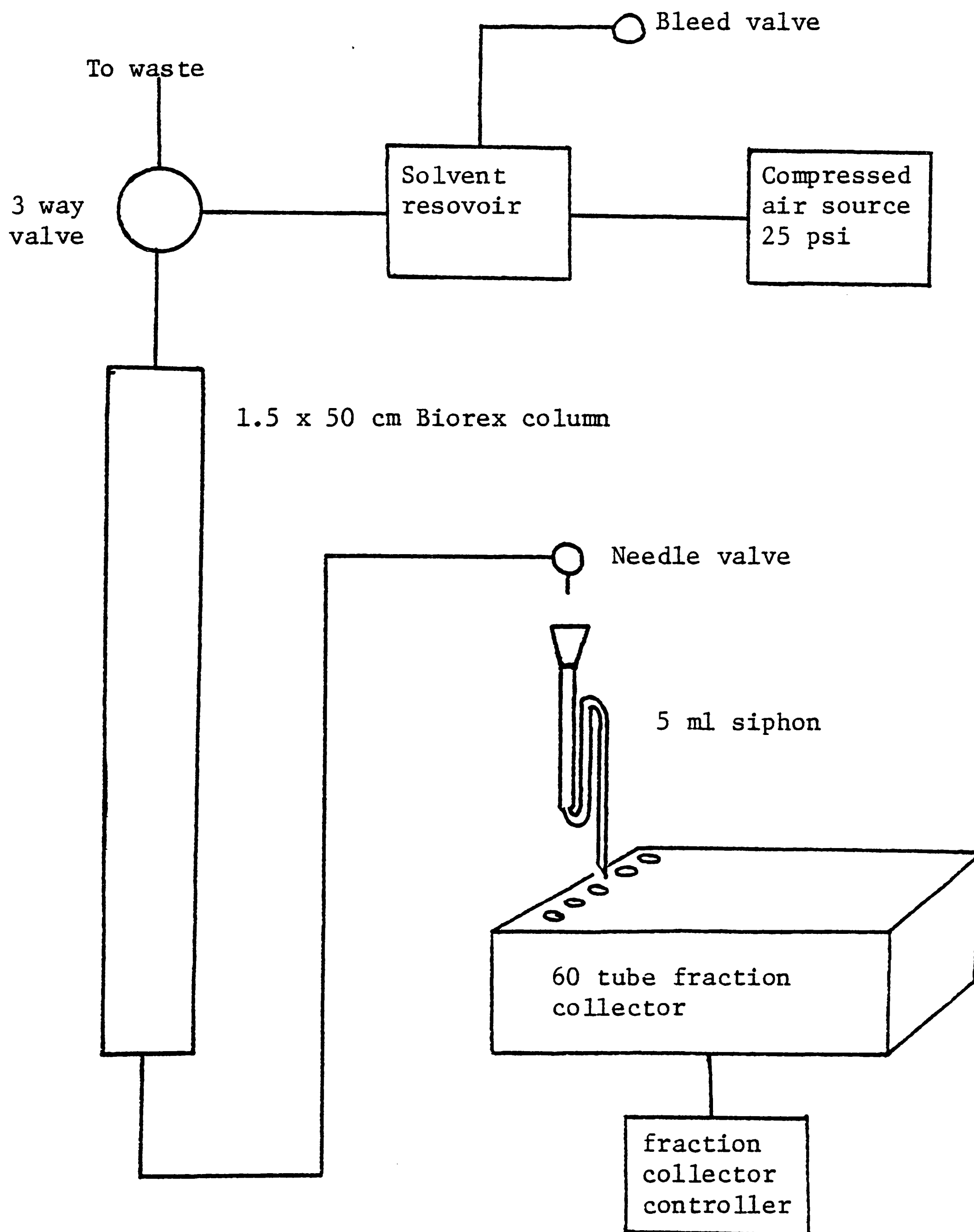


Figure 2. Schematic diagram of Sephadex LH-20 chromatography apparatus

accurate control of flow rate. Elution volumes of Z and ZR are reproducible to within five percent.

The packing was prepared by swelling in 35% ethanol prior to use followed by slurrying in 100 ml 35% ethanol. Just prior to packing the column the slurry was degassed by placing the flask containing the slurry in an ultrasonic bath for three minutes.

The column was packed by gravity flow, then compressed for approximately 2.5 cm. The sample was applied to the column in 2.0 ml of 35% ethanol and eluted with the same solvent at 25 ml hr^{-1} in five ml fractions.

Zeatin riboside eluted at 95-115 ml and zeatin eluted at 120-135 ml. Dihydrozeatin eluted at the same volume as zeatin, while the non-hydroxylated analogue of zeatin, N^6-Δ^2-isopentenyladenine (2iP) eluted at 145-165 ml. The riboside of 2iP elutes in the same volume as zeatin.

High Performance Liquid Chromatography: The solvent system of Horgan and Kramers (1980) will rapidly separate Z,DHZ and ZR on C_{18} reversed phases Partisil 10 ODS 2 columns (Whatman). The aqueous phase was buffered to pH 7.0 with triethylammonium bicarbonate (TEAB) and the organic phase was HPLC grade acetonitrile (Fisher). Elution of standards was influenced by flow rate (table 2) while changes in pH in the range of 5-7 had negligible effect on retention (table 3).

EXPERIMENT I

The objective of this experiment was to determine the chromatographic behavior of the cytokinin-like substances in soybean fruits.

PVP Chromatography: Fifteen grams of lyophilized Essex fruits (10-25 mm) were extracted and partitioned with n-butanol. The butanol

Table 2. Influence of flow rate on elution time of zeatin and zeatin riboside from a 4.5 x 250 mm Partisil 10 ODS-2 column. 8-38% acetonitrile gradient over 15 minutes. Aqueous phase; water adjusted to pH 7.0 with triethylammonium bicarbonate.

	Elution time	
Flow rate	zeatin	zeatin riboside
1 ml min^{-1}	16.0±0.22	14.7±0.16
2 ml min^{-1}	9.9±0.10	8.9±0.20

Table 3. Effects of aqueous phase pH on elution times of adenosine, zeatin riboside, zeatin and dihydrozeatin from a 4.5 x 250 mm Partisil ODS 2 column. 8-38% acetonitrile gradient over 15 minutes. Aqueous phase; water adjusted to pH 6.0 and 7.0 with triethylammonium bicarbonate, water adjusted to pH 5.0 with 0.05 KH_2PO_4. Flow rate = 1 ml min^{-1}

	pH		
Compound	5.0	6.0	7.0
adenosine	9.3±0.1	9.4±0.1	9.1±0.1
zeatin	13.7±0.1	13.8±0.2	13.8±0.2
zeatin riboside	15.1±0.1	15.2±0.1	15.1±0.1
dihydrozeatin	16.1±0.1	16.3±0.2	16.2±0.1

was reduced to dryness *in vacuo* at 50 C then flushed with 5.0 ml 95% ethanol to remove any traces of butanol.

The residue was dissolved in four ml 15 mM KH_2PO_4 buffer pH 6.4 and applied to a 2 x 13 cm column of PVP. Fractions of 25 ml each were collected, adjusted to pH 8.4 using 1 N KOH and extracted with n-butanol. The butanol was evaporated *in vacuo* and the residue taken up in assay buffer. The bioassay results obtained are shown in Fig. 3. Most of the cytokinin-like substances eluted in the region of Z and ZR. The early fractions (0-75 ml) contain most of the visible pigments and high levels of UV absorbing materials. After elution for 250 ml the column still contains at least nine distinct UV absorbing bands.

Based on these results, and the known elution of standards, three fractions were taken for further analysis, the 0-100, 100-250 and 250-650 ml fractions. Each of these were subjected to further purification by paper and LH-20 chromatography.

Analysis of PVP Eluate: 0-100 ml Fraction

Initially the 0-100 ml fraction was discarded because it showed no activity when bioassayed. This may have been caused by the large amounts of pigments and UV absorbing materials inhibiting the bioassay. Later, after further purification, bioassay activity was found in this fraction.

After adjusting the pH to 8.4, the 0-100 ml fraction was extracted three times with 50 ml of H_2O saturated n-butanol and reduced to dryness *in vacuo* at 60 C. The residue was taken up in two ml of 80% ethanol, centrifuged to remove particulates and applied to Whatman 3MM paper.

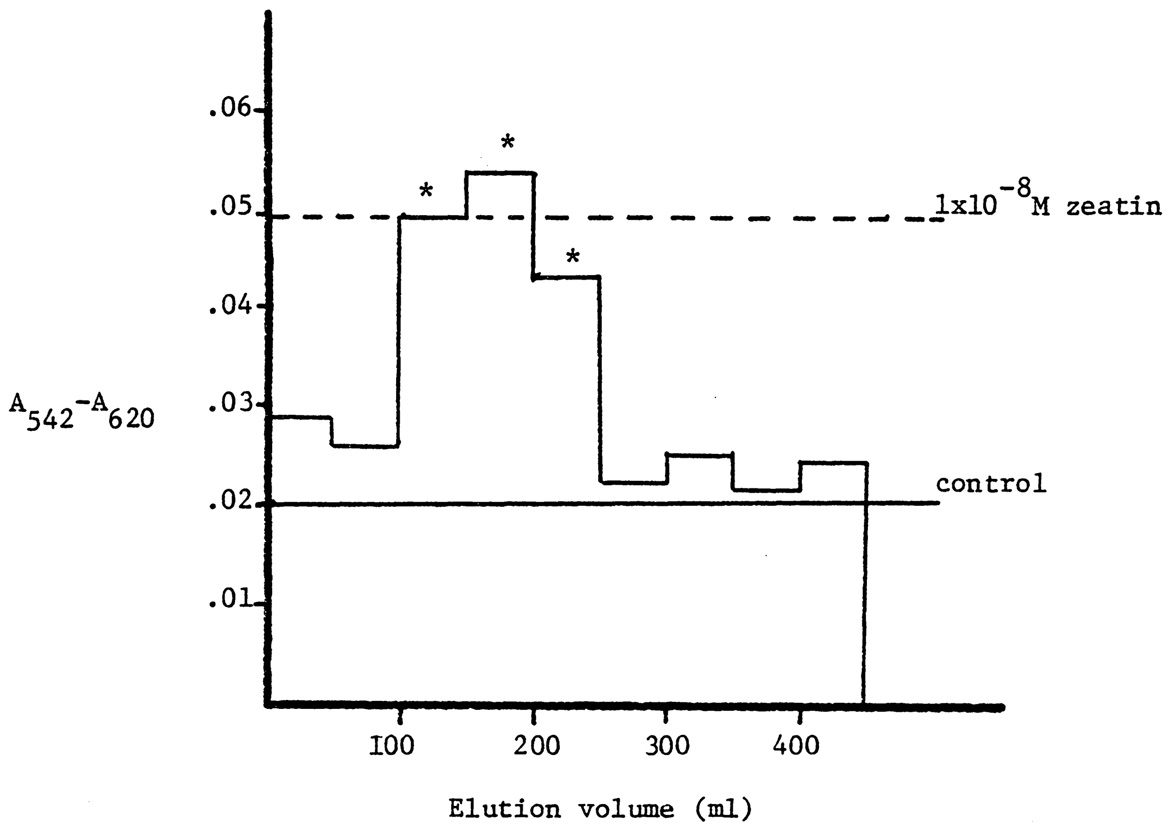


Figure 3. *Amaranthus* bioassay of PVP eluate for 15 grams dry weight of immature Essex fruits (10-25 mm).
* denotes significant difference from control at 5% level of probability.

The paper was developed to 40 cm in a descending manner using a solvent of iPrOH:NH_4OH:H_2O (10:1:1, v:v:v) and 4 R_f zones (0-0.2,0.2-0.5,0.5-0.8, 0.8-1.0) were extracted with 80% ethanol and reduced to dryness *in vacuo* at 40 C. The 0.2-0.4 and 0.5-0.8 R_f zones were dissolved in 35% ethanol and chromatographed on Sephadex LH-20. The elution pattern of the 0.2-0.4 zone measured at 268 nm and bioassay responses are shown in Fig. 4. Only the fourth fraction had cytokinin-like activity, equivalent to 10^{-9} M zeatin. The elution pattern of the 0.5-0.8 fraction is shown in Fig. 5. The second fraction, which rapidly eluted from this column had cytokinin-like activity greater than 10^{-8} M zeatin. The fourth fraction had activity equal to 2 x 10^{-9} M zeatin.

None of these fractions could be correlated with a distinct UV absorbing peak. This was not surprising since the calculated amounts of cytokinins would not be detectable with the cuvette volumes and spectrophotometer used.

The 0-100 fraction contained at least 3 chromatographically distinct zones of cytokinin-like activity that were not detectable when the PVP eluate was directly bioassayed. This is evidence for the presence of an unknown inhibitor(s) in this fraction. Further purification removed the inhibitors and allowed the detection of the cytokinin-like activity.

100-250 ml PVP Fraction

This fraction contained the bulk of cytokinin-like activity when directly assayed. This was also the fraction where authentic Z,DHZ, and ZR eluted. Thus, the cytokinin activity of this fraction resembled zeatin-like substances.

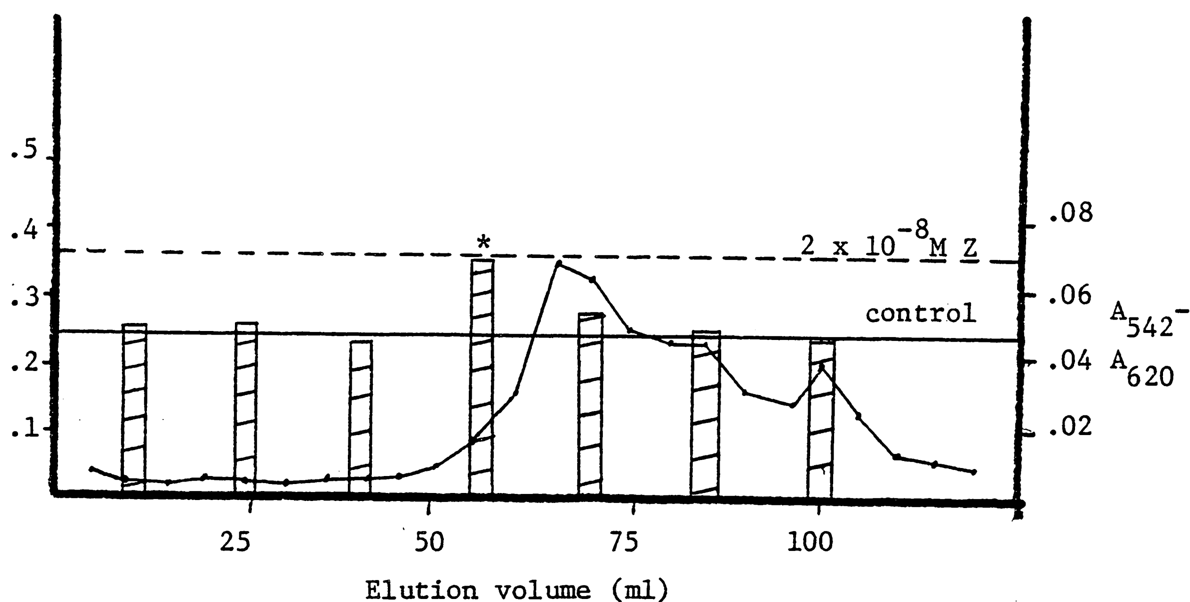


Figure 4. Absorbance at 268 nm (•—•) and bioassay activity (A_{542}-A_{620}) of Sephadex LH-20 fractions from PVP 0-100 ml fraction, paper R_f 0.2-0.4. Absorbance determined from 5 ml fractions, and 15 ml fractions used for bioassay. Bioassay response indicated by striped bars. * denotes significant difference from control at the 5% level of probability.

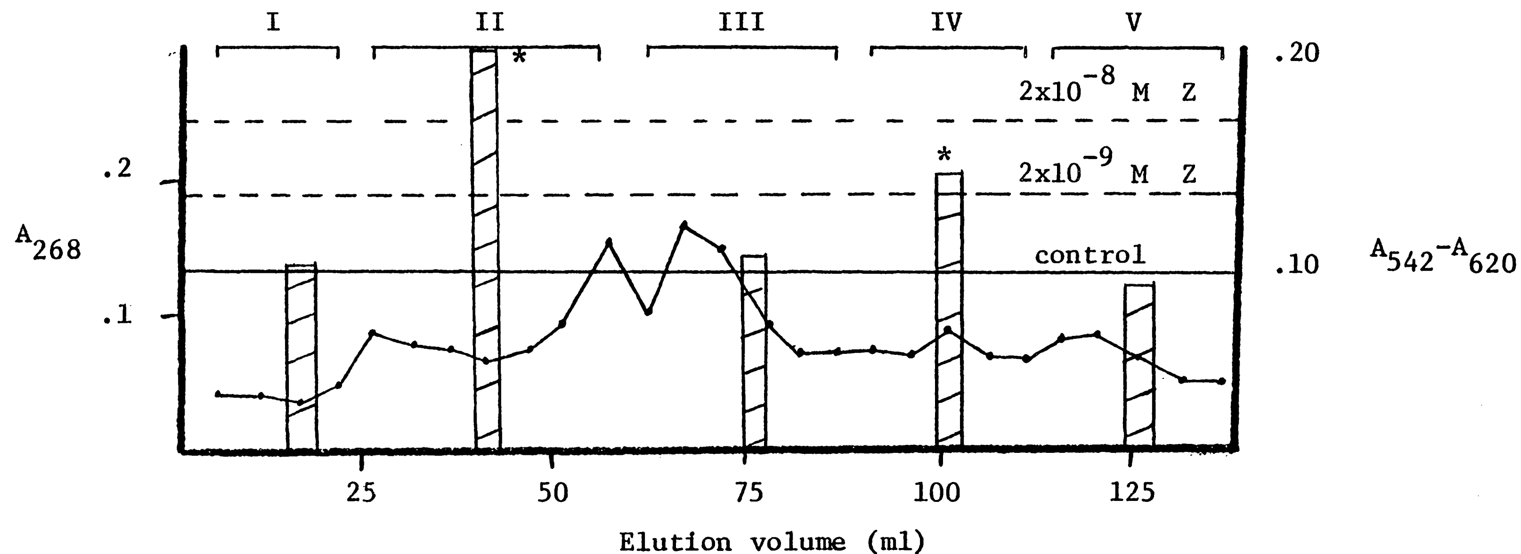


Figure 5. Absorbance at 268 nm (•——•) and bioassay activity (A_{542}-A_{620}) of Sephadex LH-20 fractions from PVP 0-100 ml fraction and paper R_f 0.5-0.8. Absorbance determined from 5 ml fractions, bioassay fractions indicated I-V. Bioassay response indicated by striped bars, * denotes significant difference from control at 5% level of probability.

The 100-250 ml fraction was adjusted to pH 8.4 and extracted with n-butanol. The n-butanol phase was concentrated and streaked on paper and developed to 40 cm. The dried chromatograms were cut into 10 R_f zones and eluted two times with 25 ml of 80% ethanol. The eluates were centrifuged to remove particulates, reduced to dryness in vacuo at 40 C and bioassayed. The bioassay results are shown in Fig. 6. Significant cytokinin-like activity was found at R_f 0.5-0.7, corresponding to where Z,DHZ and ZR migrated. The biological activity was equivalent to 4×10^{-8} M zeatin.

After detection of cytokinin-like activity on R_f 0.5-0.7, this zone was investigated further. Extracts of five g of Essex and Shore fruits (10-25 mm long) were purified by PVP and paper chromatography. The 0.5-0.8 R_f zones were extracted with 80% ethanol, reduced to dryness and redissolved in two ml 35% ethanol. This was chromatographed on LH-20 at a flow rate of 25 ml hr^{-1} with 35% ethanol. The bioassay results are shown in Fig. 7 and 8 for Essex and Shore respectively.

Fractions active in the bioassay included a zone where Z and ZR eluted and a zone just before ZR. The zone of activity eluting before ZR has been identified in other species as a zeatin glucoside (Wang and Horgan, 1977) and this is where authentic zeatin glucosides elute from LH-20 columns (Horgan, 1979). A rapdily eluting zone of activity was detected in both cultivars.

Further analysis of the 0.2-0.4 zone indicated the presence of a cytokinin-like substance in the 85-115 ml fraction when chromatographed on LH-20 (Fig. 9). This fraction had activity equivalent to 2×10^{-9} M zeatin.

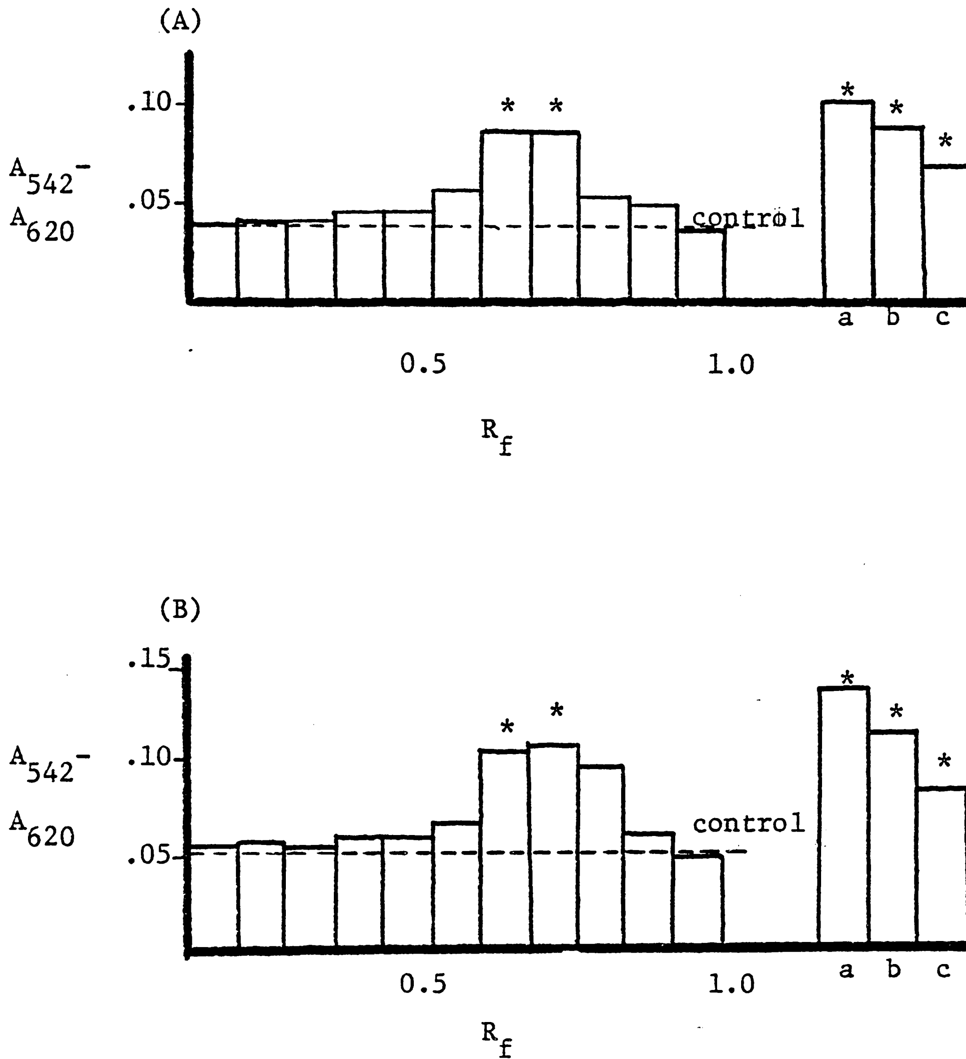


Figure 6. Bioassay of paper chromatograms of (A) Shore and (B) Essex extracts, PVP 100-250 fraction.

a= 2×10^{-7} zeatin, b= 2×10^{-8} zeatin, c= 2×10^{-9} zeatin

* denotes significant difference from control at 5% level of probability

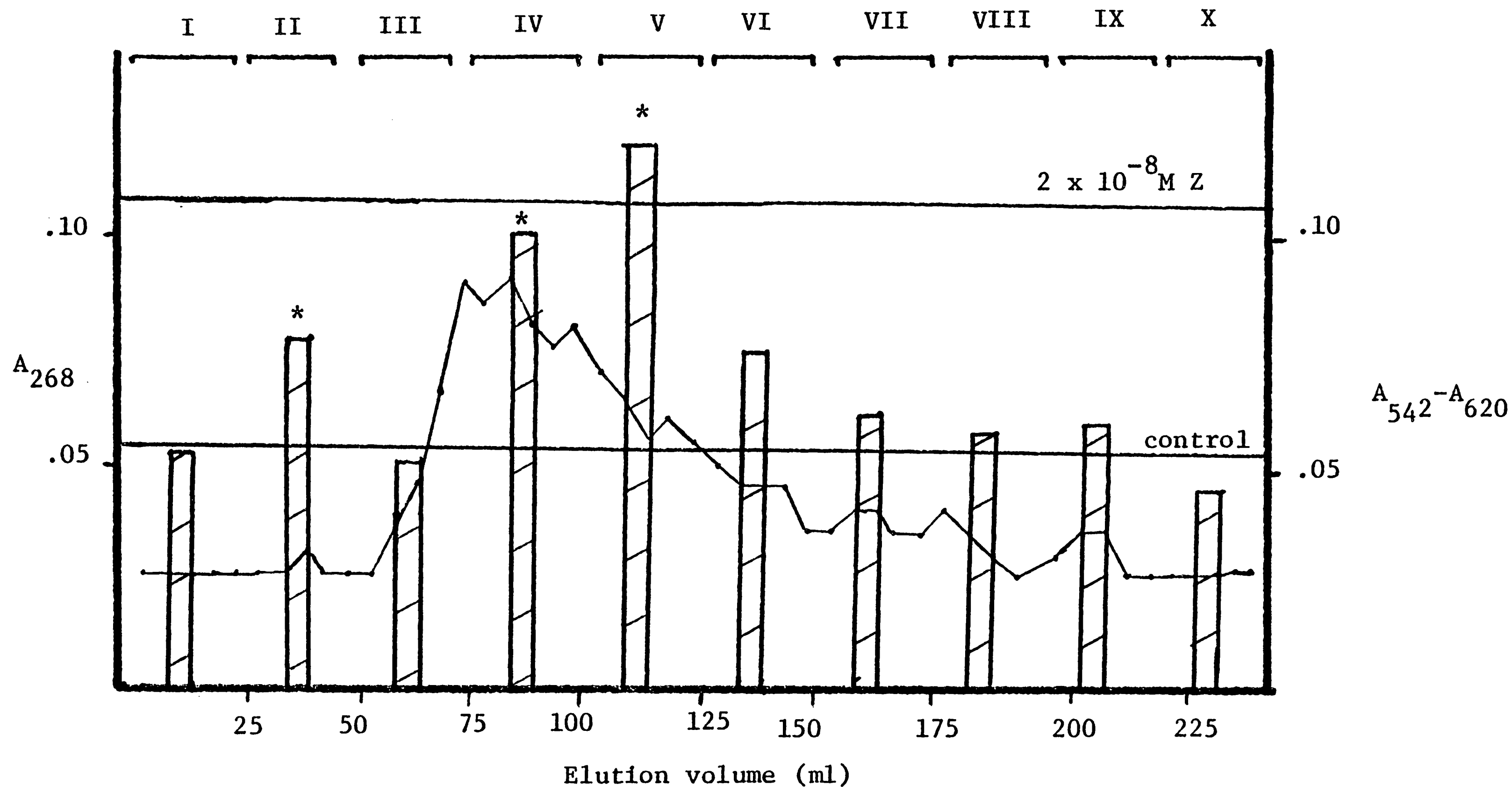


Figure 7. Absorbance at 268 nm and bioassay activity ($A_{542}-A_{620}$) of Sephadex LH-20 fractions from PVP 100 - 250 ml and paper R_f 0.5-0.8. Absorbance determined from 5 ml fractions, bioassay fraction indicated I-X. Bioassay response indicated by striped bars. * denotes significant difference from control at 5% level of probability. Cultivar ESSEX

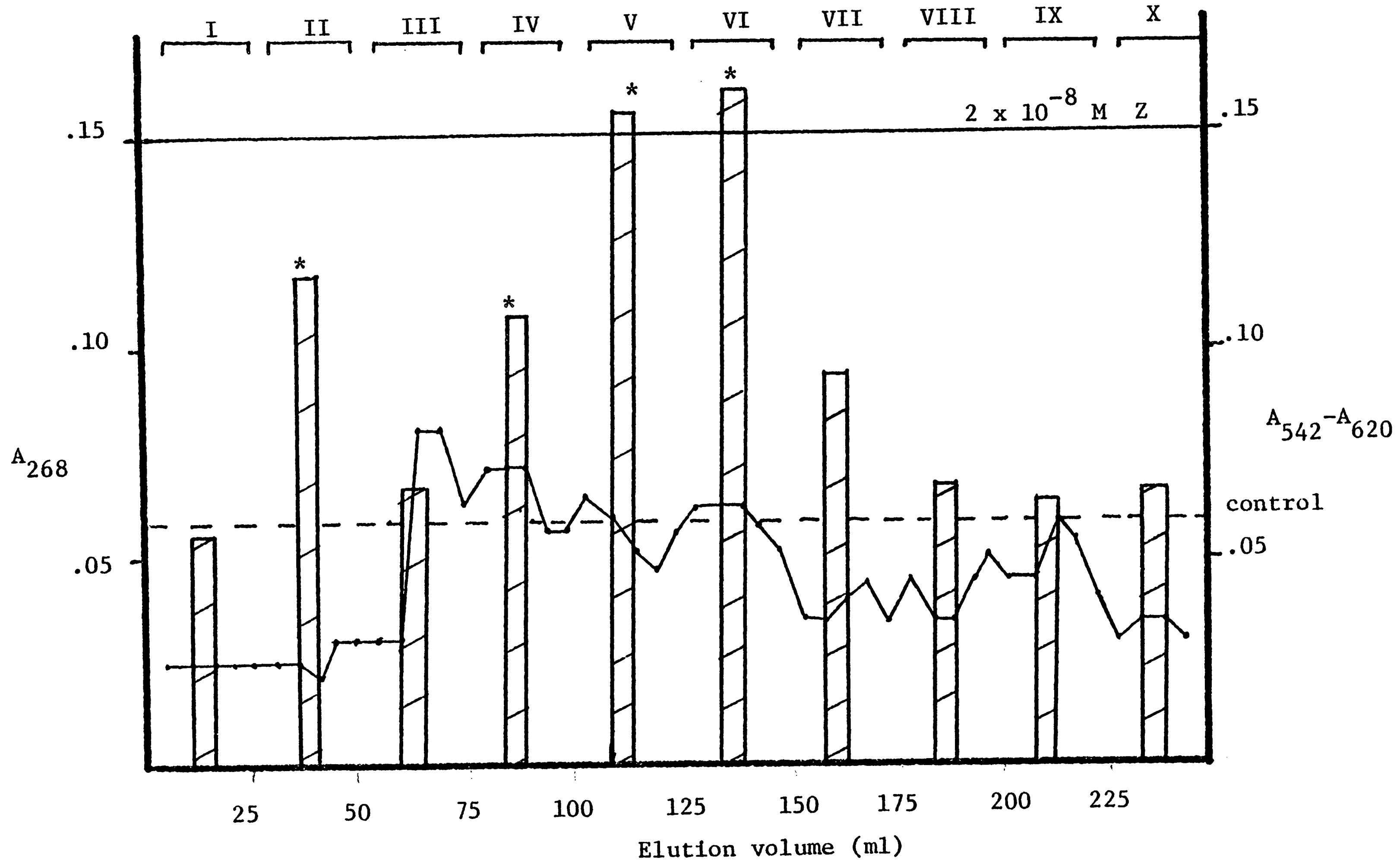


Figure 8. Absorbance at 268 nm (•—•) and bioassay activity (A_{542}-A_{620}) of Sephadex LH-20 fractions from PVP 100-250 ml and paper R_f 0.5-0.8. Absorbance determined from five ml fractions, and bioassay fractions indicated I-X. Bioassay response indicated by striped bars. * denotes significant difference from control at the 5% level of probability.

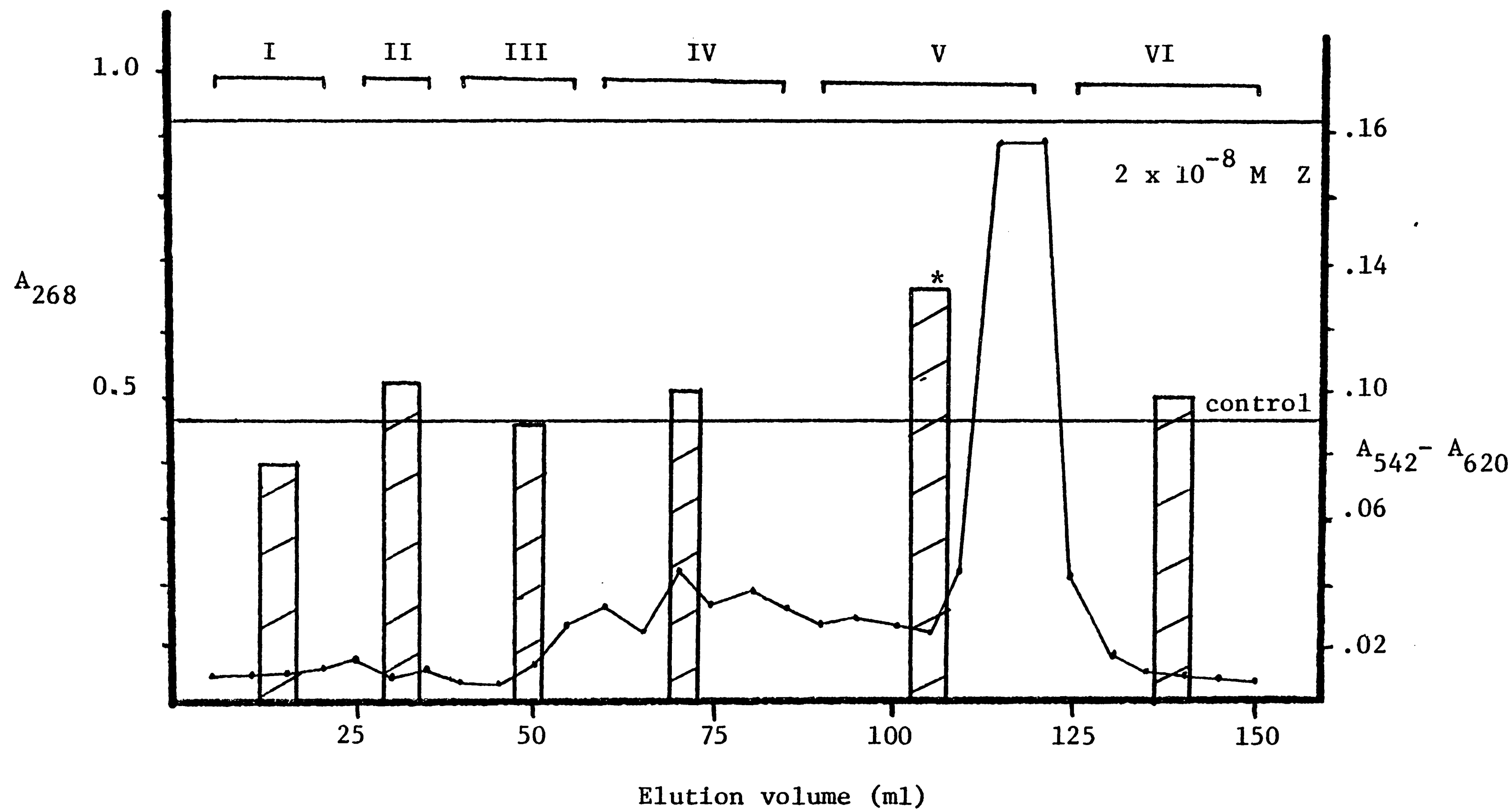


Figure 9. Absorbance at 268 nm (•—•) and bioassay activity (A_{542}-A_{620}) of Sephadex LH-20 fractions from PVP 100-250 ml and paper R_f 0.2-0.4. Absorbance determined from 5 ml fractions, bioassay fractions designated I-VI. Bioassay response indicated by sriped bars. * denotes significant difference from control at the 5% levle of probability. Cultivar ESSEX

The data indicate there were at least four separate zones of cytokinin-like substances in the 100-250 ml fraction derived from PVP chromatography.

Analysis of the 250-650 ml PVP Fraction

In initial experiments, only the first 250 ml of the PVP eluate was retained for analysis while the later eluting compounds were discarded. Under a UV lamp however, there were at least nine bands of material detectable in the PVP column. For convenience, the 250-650 ml fraction was collected at 70 ml hr^{-1}, double the flow rate for the first 250 ml.

The PVP eluate was adjusted to pH 8.4 and extracted five times with 50 ml n-butanol. The combined butanol extracts were reduced to dryness *in vacuo* at 60 C and the residue dissolved in 80% ethanol. The concentrated ethanol was streaked on Whatman 3 MM paper and developed to 40 cm with iPrOH:NH_4OH:H_2O (10:1:1, v:v:v). Three fractions (R_f 0.0-0.3, 0.3-0.6,0.0-0.9) were eluted with 80% ethanol dried *in vacuo* and the residue redissolved in buffer for bioassay. Only the R_f 0.6-0.9 zone contained cytokinin-like activity.

When the R_f 0.6-0.9 zone was further purified by Sephadex LH-20 with 35% ethanol 2 fractions were found to have cytokinin-like activity as shown in Fig. 10. The first peak of activity co-eluted with isopentenyladenosine (2iPA) while the second peak eluted much later, indicating it was less polar. Thus, there were at least two cytokinin-like substances in soybean fruits less polar than zeatin.

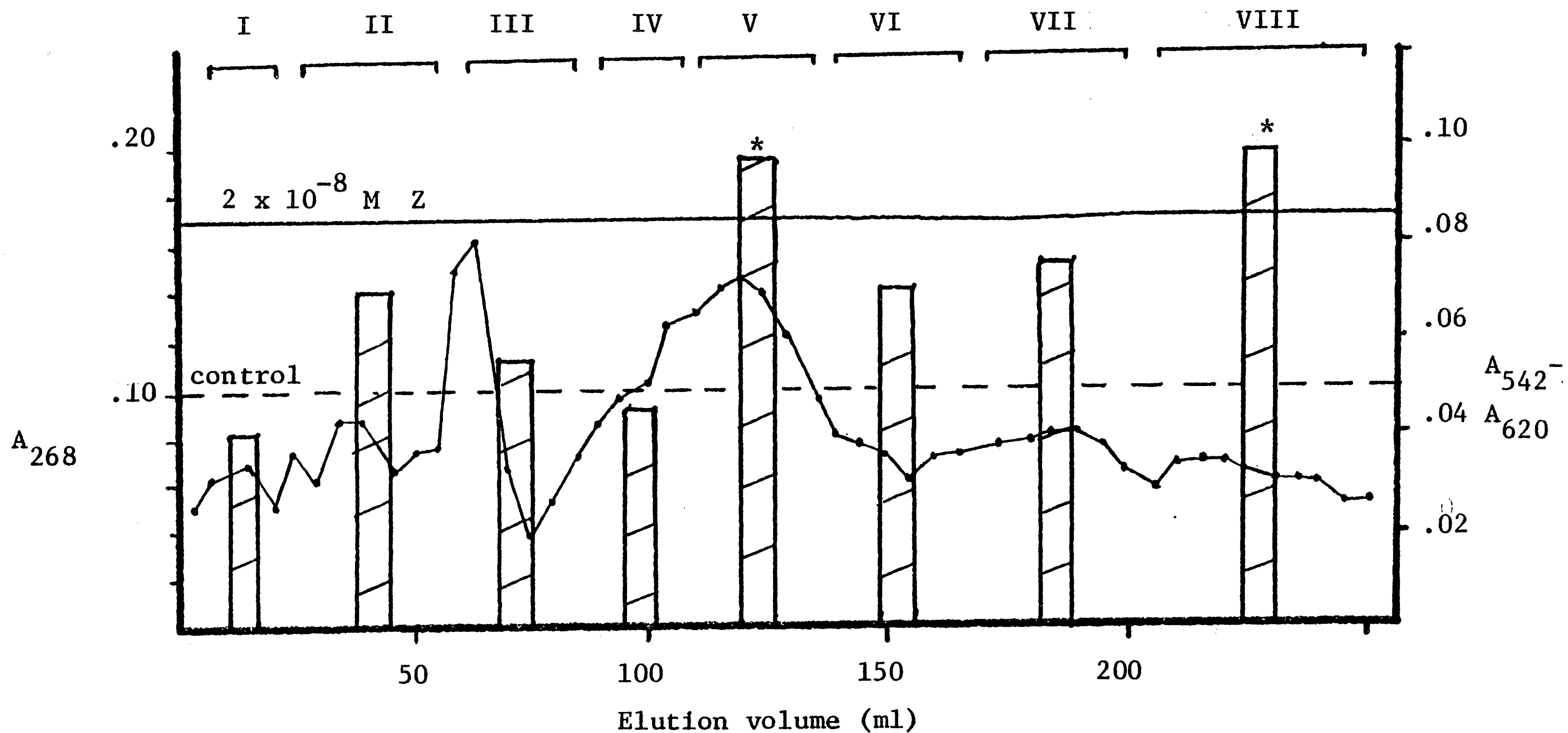


Figure 10. Absorbance at 268 nm and bioassay activity (A_{542}-A_{620}) of Sephadex LH-20 fractions from PVP 250-650 ml and paper R_f 0.5-0.8. Absorbance determined from 5 ml fractions, bioassay fractions indicated I-VIII. Bioassay response indicated by striped bars. * denotes significant difference from control at the 5% level of probability. Cultivar ESSEX.

Analysis of Alkaline Phosphatase Solubilized ("Bound") Cytokinin Activity

Cytokinin nucleotides possessing an acidic phosphoric acid moiety do not readily partition into n-butanol at alkaline pH. Therefore the aqueous phase remaining after n-butanol extraction of the crude ethanol extract should contain cytokinin nucleotides if they are present in the tissue. The study of this fraction may further be complicated by the presence of endogenous phosphatase activity, which has been shown to remain active in cold 80% ethanol (Bielski, 1964). Also, it is not known if lyphilization prior to extraction inactivates phosphatases in soybean fruits.

Bioassay of unpurified aqueous phase did not reveal any cytokinin-like activity. The residue, however, was very viscous and contained pigments which might have interfered with the bioassay.

To solubilize the nucleotide, the aqueous phase was buffered at pH 8.2 with 1.0 N TRIS buffer containing 0.1 N Mg^{++}. Calf Intestine Alkaline Phosphatase (E.C 3.1.3.1., type 1, 1-3 units mg $protein^{-1}$, #p-3877, Sigma Chem. Co.) was added at the rate of 1 mg per 10 ml solution. The mixture was incubated with shaking for 24 hours at 30 C in a water bath, and then extracted with three times 50 ml of n-butanol. The butanol was reduced to dryness *in vacuo* at 60 C and the residue redissolved in 4.0 ml of distilled water. The aqueous fraction was adjusted to pH 2.0 with 0.1 N HCl and centrifuged to remove precipitates. The supernatant was applied to a 2 x 10 cm column of cellulose phosphate (NH_4^+ form) equilibrated to pH 3.1 with water and acetic acid. The column was washed with five bed volumes (150 ml) of water, pH 3.1, and then eluted with five bed volumes of 1 N NH_4OH.

The ammoniacal eluate was reduced to dryness *in vacuo* at 40 C. The residue was dissolved in 2.0 ml of 35% ethanol and loaded on a 1.5 x 45 cm Sephadex LH-20 column. Fractions of five ml were collected and the absorbance read at 268 nm. Six bulk fractions were obtained and dried for bioassay. The results are shown in Fig. 11. Significant activity was found to co-elute with ZR while the third and fourth fractions were strongly inhibitory.

These results suggest that the phosphoric acid ester of ZR was present in the crude aqueous phase. Also, inhibitory compounds which interferred in the bioassay were present. The presence of inhibitors might explain why bioassay of the crude extract failed to exhibit any cytokinin activity.

The results of Experiment I are summarized as a flow chart in Fig. 12. There are 9 distinct fractions of cytokinin-like activity separated by a combination of PVP, paper, phosphocellulose and Sephadex LH-20 chromatography.

Mass Spectra of Cytokinin Standards

Cytokinin standards were permethylated using the procedure of Young (1977). One ug each of Z and ZR were dried *in vacuo* over P_2O_5 then reacted with 100 ul of DMSO anion solution followed by 10 ul of methyl iodide. The reaction products were extracted into chloroform which was washed twice with one ml distilled water to remove contaminants. The chloroform was reduced to dryness under a stream of N_2 and the residue was dissolved in 50 μl of chloroform.

Ten ul samples were introduced into the Varian MAT 112 Mass Spectrometer with a Varian 2400 gas chromatograph equipped with a

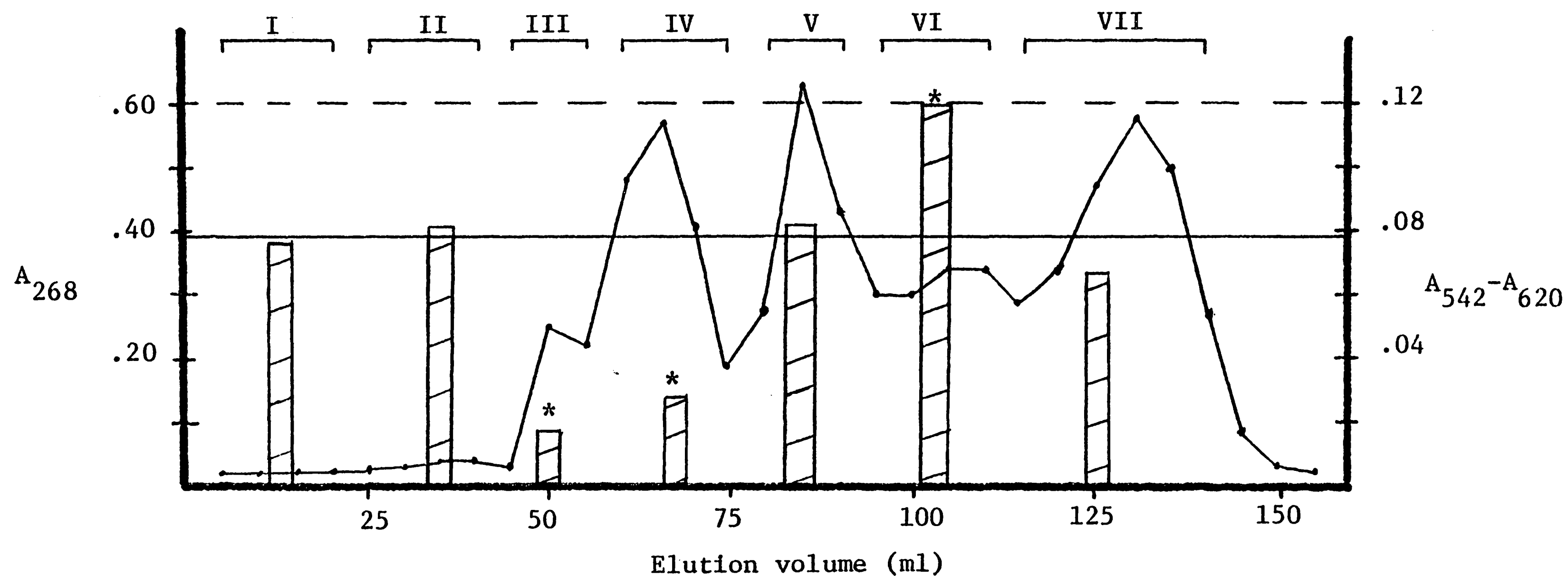


Figure 11. Absorbance at 268 nm (•—•) and bioassay activity (A_{542}-A_{620}) of Sephadex LH-20 fractions from phosphocellulose ammonia eluate of alkaline phosphatase digested ("bound") aqueous phase after n-butanol extraction. Absorbance dtermined from 5 ml fractions, bioassay fractions indicated I-VII. Bioassay response indicated by striped bars. * denotes significant difference from control at the 5% level of probability. Cultivar ESSEX.

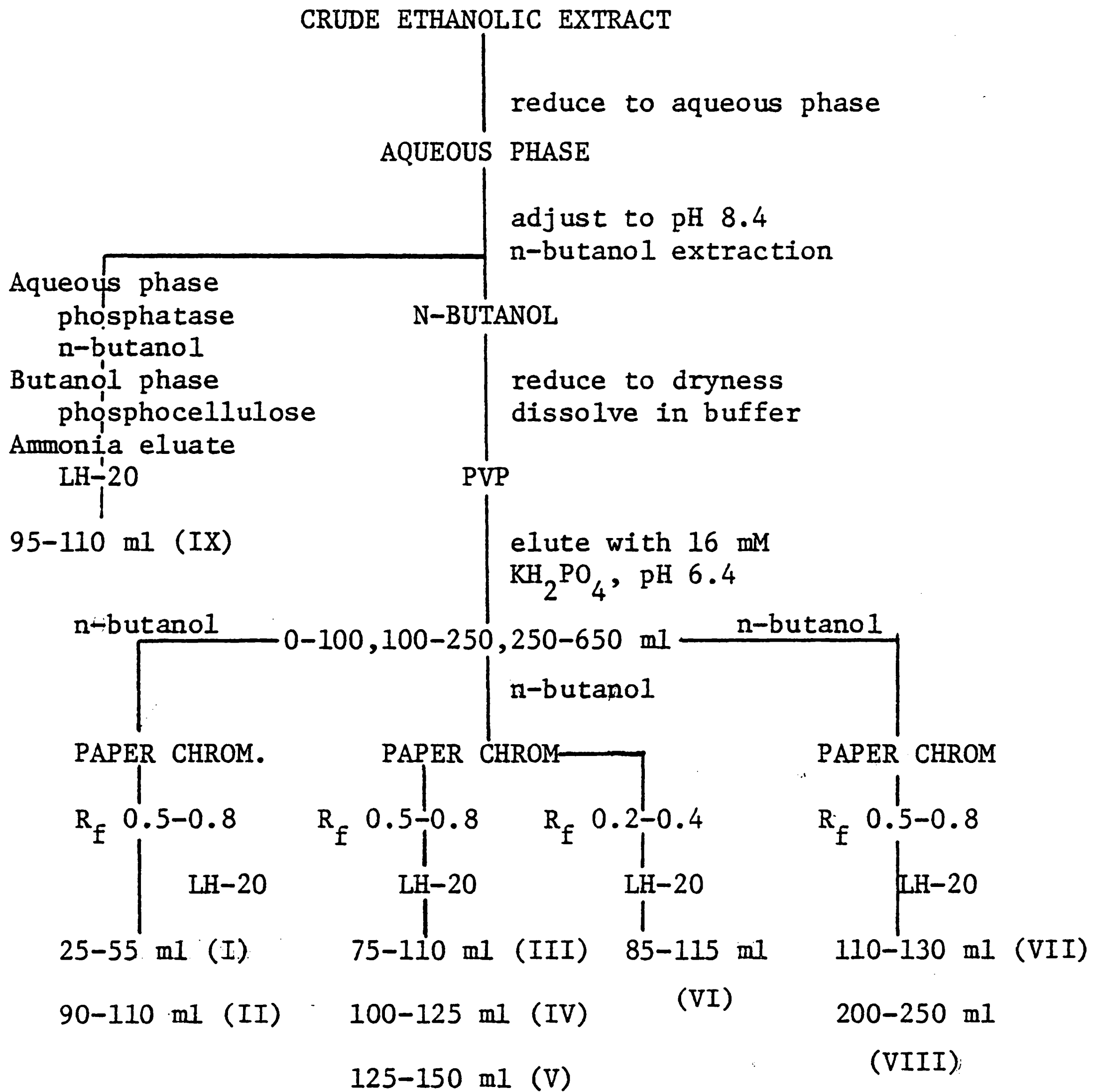


Figure 12. Flow chart of purification of cytokinin-like substances in Essex soybean fruits (10-25 mm)

three meter SP 2300 column and an SGE jet separator. Helium flow rate was 40 ml min^{-1} and the temperature was programmed from 250 C to 300 C over five minutes.

Complete spectra for Z and ZR are plotted in Fig. 13 and 14 and peak intensities are listed in tables 4 and 5.

Multiple ion monitoring scans for Z and ZR are plotted in Fig. 15 and 16. The peaks chosen for monitoring are based on intensity and diagnostic value, both of which are needed for sensitivity and selectivity.

Quantitative Estimate of Cytokinin-like Substances

Estimating the quantities of cytokinin-like substances is a difficult problem. In each bioassay, zeatin was used as the standard for comparison. Work by several authors (Letham, 1967; Van Staden and Papaphillipou, 1977;Biddington and Thomas,1973) demonstrated that different cytokinins gave different biological responses. Zeatin is one of the most bioactive cytokinins and it is likely that the quantities of less bioactive cytokinins will be underestimated.

Cytokinin-like substances were estimated by comparing the bioassay response of the unknown fractions with a series of standard zeatin concentrations. A linear regression program produced a regression equation for zeatin response and the unknowns were compared with this equation to compute the amount of zeatin equivalents in the unknown. The quantities estimated for each fraction are given in table 6.

The computed value of 27 ng per g of tissue of total cytokinins is in good agreement with the values obtained for Essex pericarps of

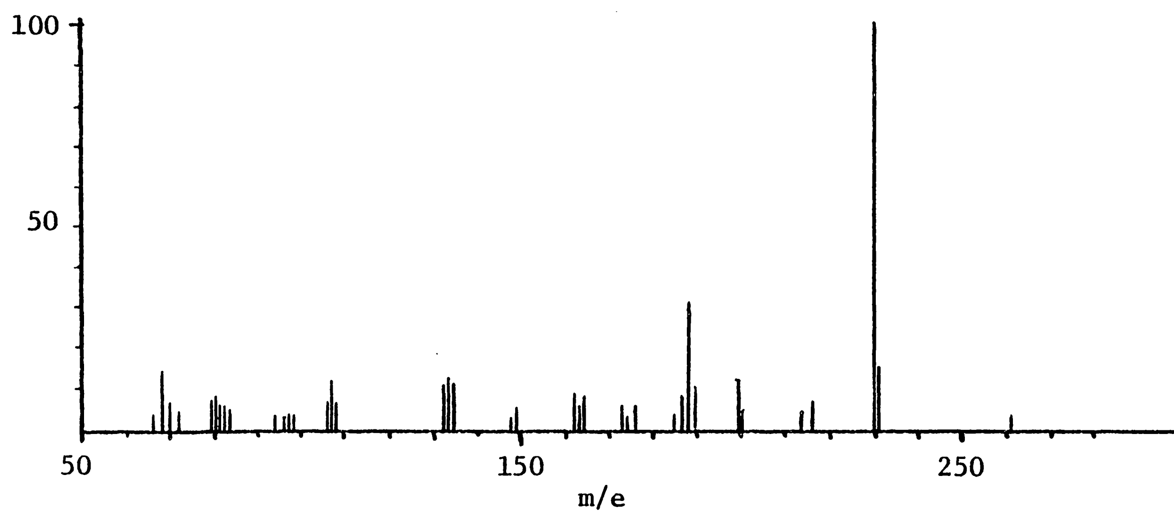


Figure 13. Mass spectra from m/e 50 to 300 for permethylated zeatin standard.

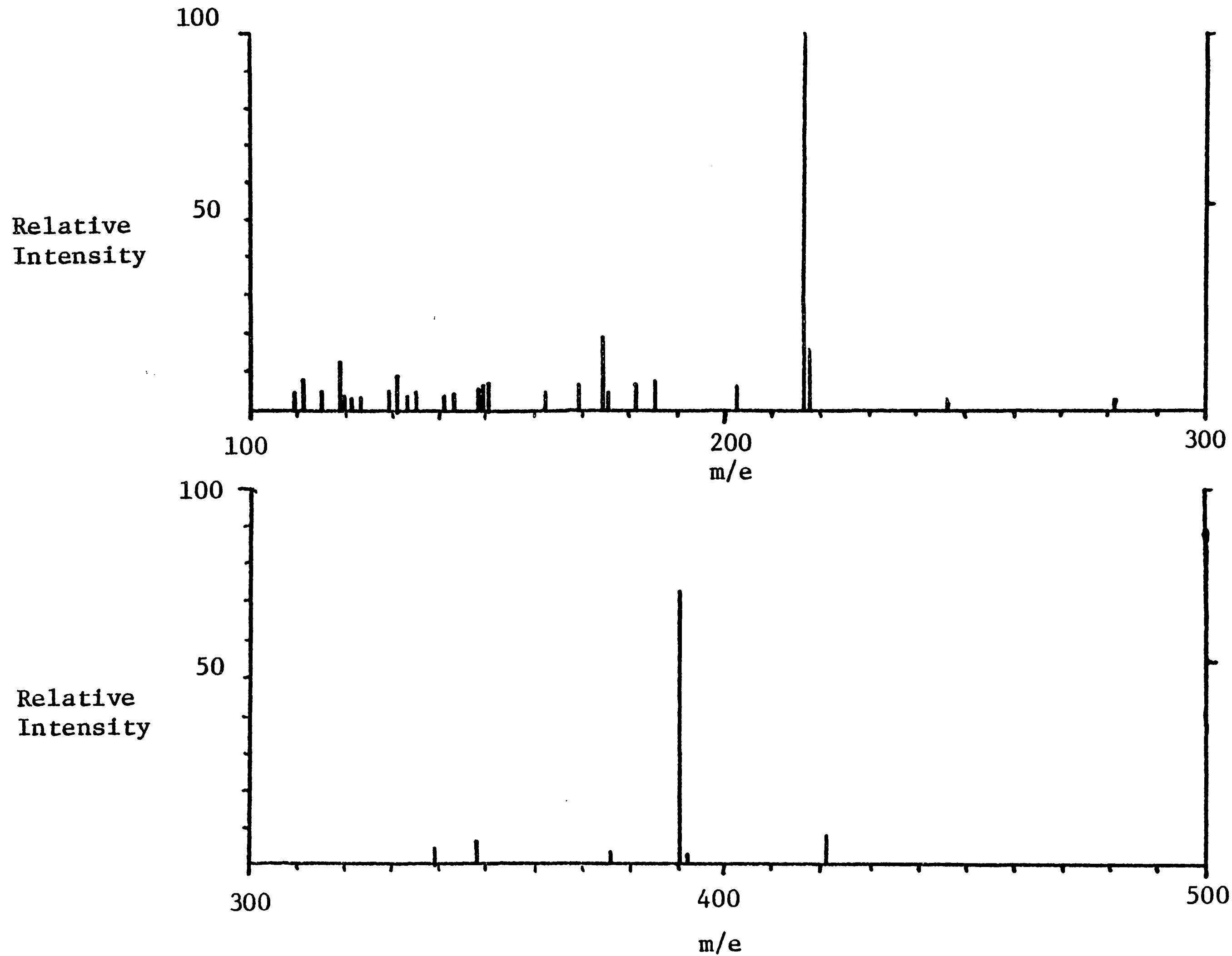


Figure 14. Mass spectra from m/e 100 to 500 for permethylated zeatin riboside standard.

Table 4. Peak Intensities for permethylated zeatin

mass	relative intensity
68	14.6
80	5.7
81	7.4
107	12.2
133	9.9
134	11.9
135	10.2
162	9.2
188	30.5
199	10.0
216	6.9
230	100.0
261	3.7

Table 5. Peak Intensities for permethylated zeatin riboside

mass	relative intensity
111	7.7
119	12.7
131	8.4
148	5.0
149	6.3
150	6.7
169	6.5
174	20.0
181	6.5
185	7.6
202	6.5
216	100.0
217	15.5
348	6.5
390	72.6
421	7.6

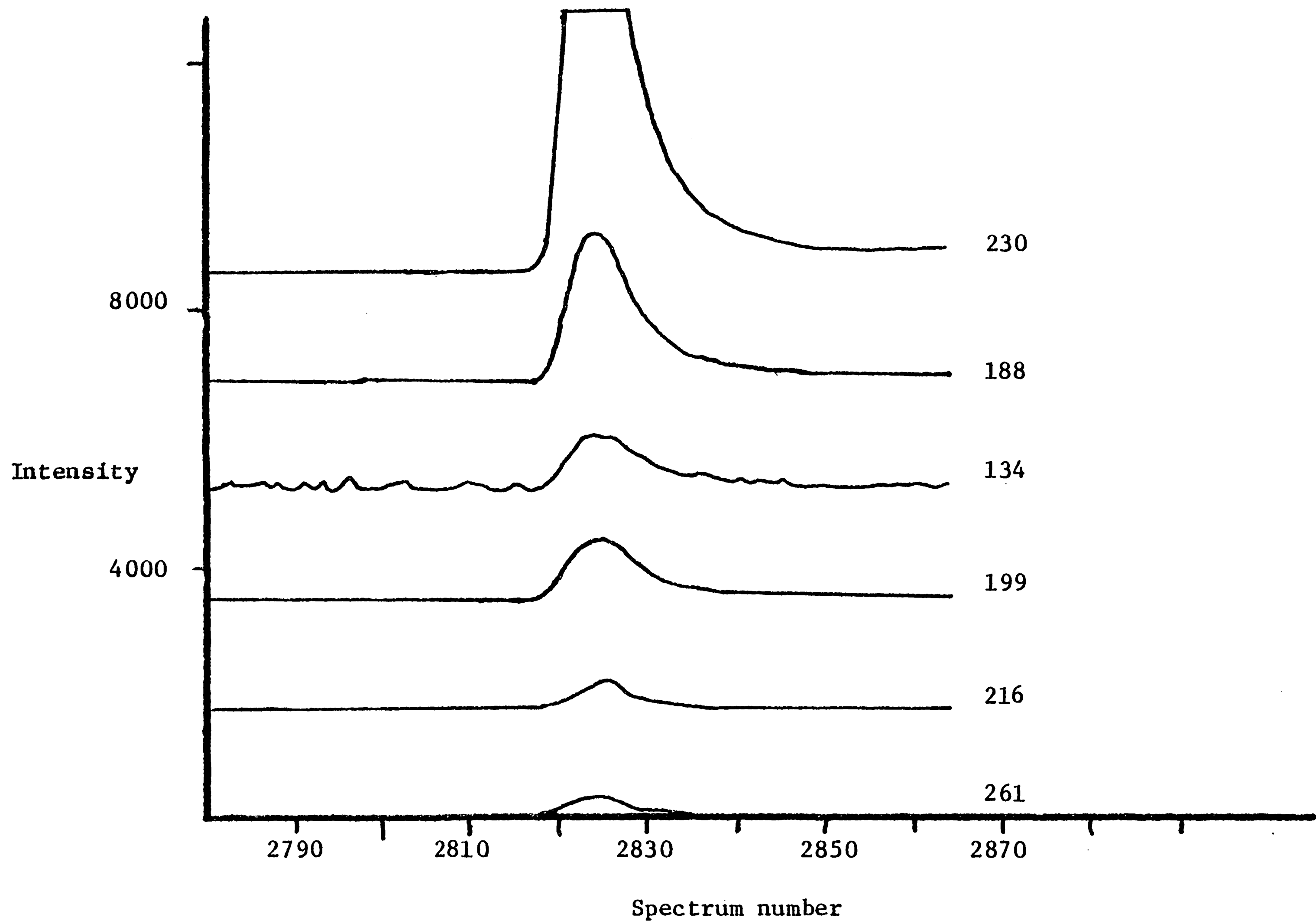


Figure 15. Multiple ion monitoring detection of 50 ng of permethylated Zeatin standard

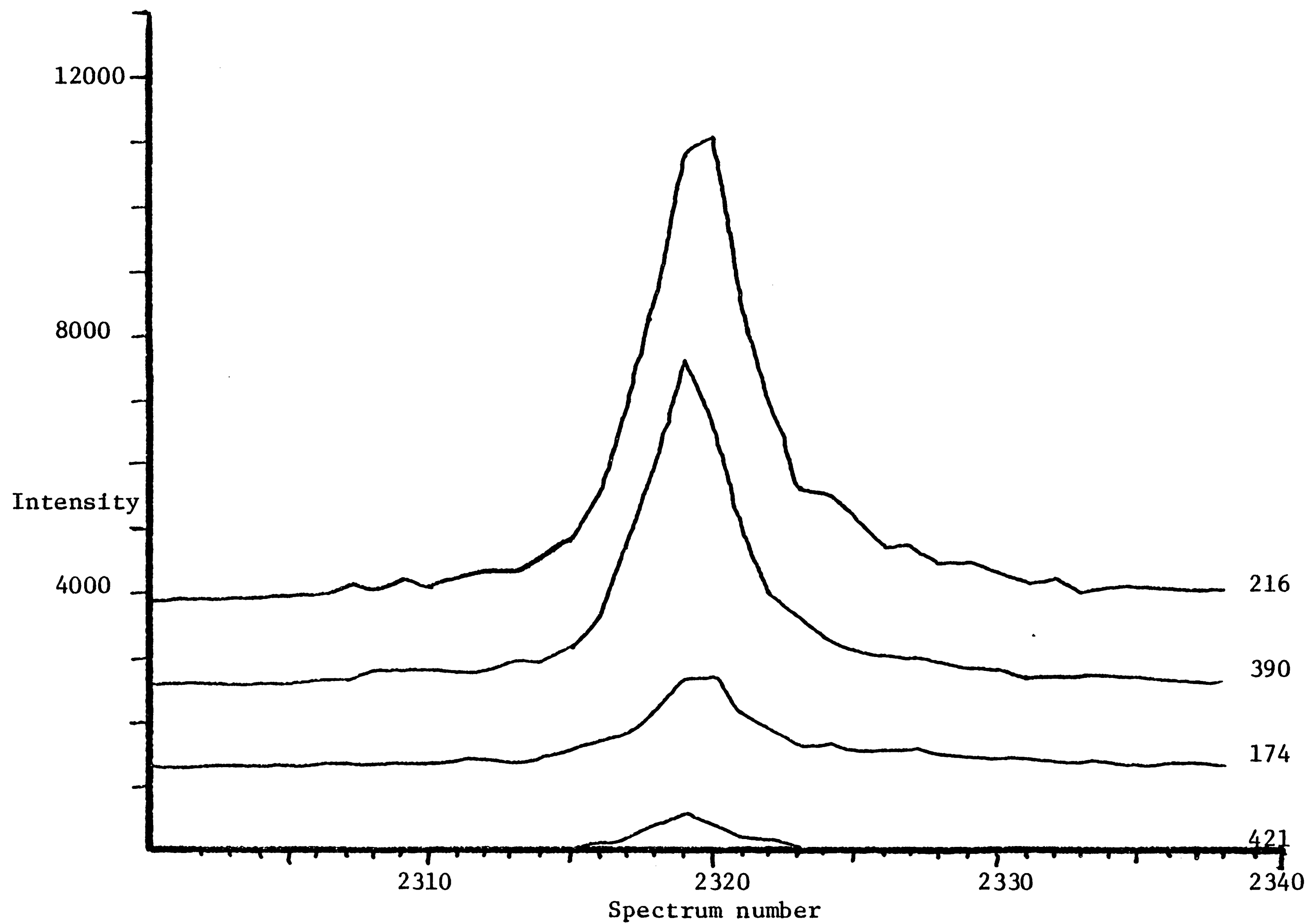


Figure 16. Selected ion monitoring scan of 50 ng of permethylated zeatin riboside using m/e 174,216,390 and 421.

Table 6. Estimation of the total cytokinin content in 15 grams dry weight of 10-25 mm Essex fruits (1980 field grown samples) based on Amaranthus bioassay.[a,b]

Fraction	Equivalent zeatin concentration (molar)	Total zeatin equivalents (ng)	zeatin equivalents (ng g^{-1})
I	$6.3 \pm 0.6 \times 10^{-8}$	105	6.8
II	$1.5 \pm 0.4 \times 10^{-9}$	3	0.2
III	$1.5 \pm 1.1 \times 10^{-8}$	18	1.2
IV	$6.0 \pm 0.8 \times 10^{-8}$	100	6.7
V	$6.0 \pm 0.7 \times 10^{-9}$	10	0.7
VI	$3.0 \pm 0.2 \times 10^{-9}$	5	0.4
VII	$4.5 \pm 1.1 \times 10^{-8}$	75	5.0
VIII	$4.5 \pm 0.8 \times 10^{-8}$	75	5.0
IX	$1.0 \pm 3.0 \times 10^{-8}$	16	1.1
		407	27.0

[a] Values for equivalent zeatin concentrations based on three replications, since each replication represented one third of actual material present in sample the values for total zeatin equivalents(ng) are computed as the total over three replictions and are not means.

[b] The data in this table is from five different bioassays; fractions I and II, III, IV and V, VI, VII and VIII and IX were assayed separately, regression coefficients for each assay given in appendix I

20-25 mm in 1978 and 1979. These values are given in table 7. Since fruits of 10-25 mm length consisted mainly of pericarp (98%), it might be presumed to be the major contributor of the cytokinin-like activity. However, the cytokinin-like activity had been estimated to be much greater in ovules (Aung *et al*., 1982), particularly at the early stages extracted here (Fig. 17). Therefore, even though the ovules formed only a small part of the total fruit weight, they contributed a substantial amount of the total cytokinin activity of the immature fruit.

EXPERIMENT II

Fifteen g of immature Shore fruits (10-25 mm) were extracted and chromatographed on PVP, paper and Sephadex LH-20. The fractions from the Sephadex LH-20 corresponding to Z and ZR-like substances were bulked. An aliquot representing 25% of the total volume was taken for bioassay and the remainder reduced to dryness *in vacuo*, dissolved in 80% ethanol and transferred to a 1.0 ml Reacti-vial (Pierce Chem. Co.). The ethanol was removed with a stream of N_2 and then the samples were dried *in vacuo* over P_2O_5. The sample was permethylated and reduced to a final volume of 50 μl.

The permethylated samples were analyzed by multiple ion monitoring using m/e 216 and 390 for ZR and 188 and 230 for Z. Standards of permethylated Z and ZR were run immediately prior to the samples to determine retention times accurately. The standard run is shown in Fig. 18 and the scan for ZR is shown in Fig. 19. The retention time and ratio of 216 to 390 in the sample is identical to the standard.

Table 7. Estimated cytokinin-like substances in Essex and Shore pericarps purified by PVP and paper chromatography. Values expressed as zeatin equivalents in ng per gram of tissue.[a]

	1978	1979	Mean[b]
Essex	19±8	10.5±5	14.8±6
Shore	57±22	23.2±12	40±24

[a] Values for three replications ±standard deviation

[b] Values for two years ±standard deviation over years

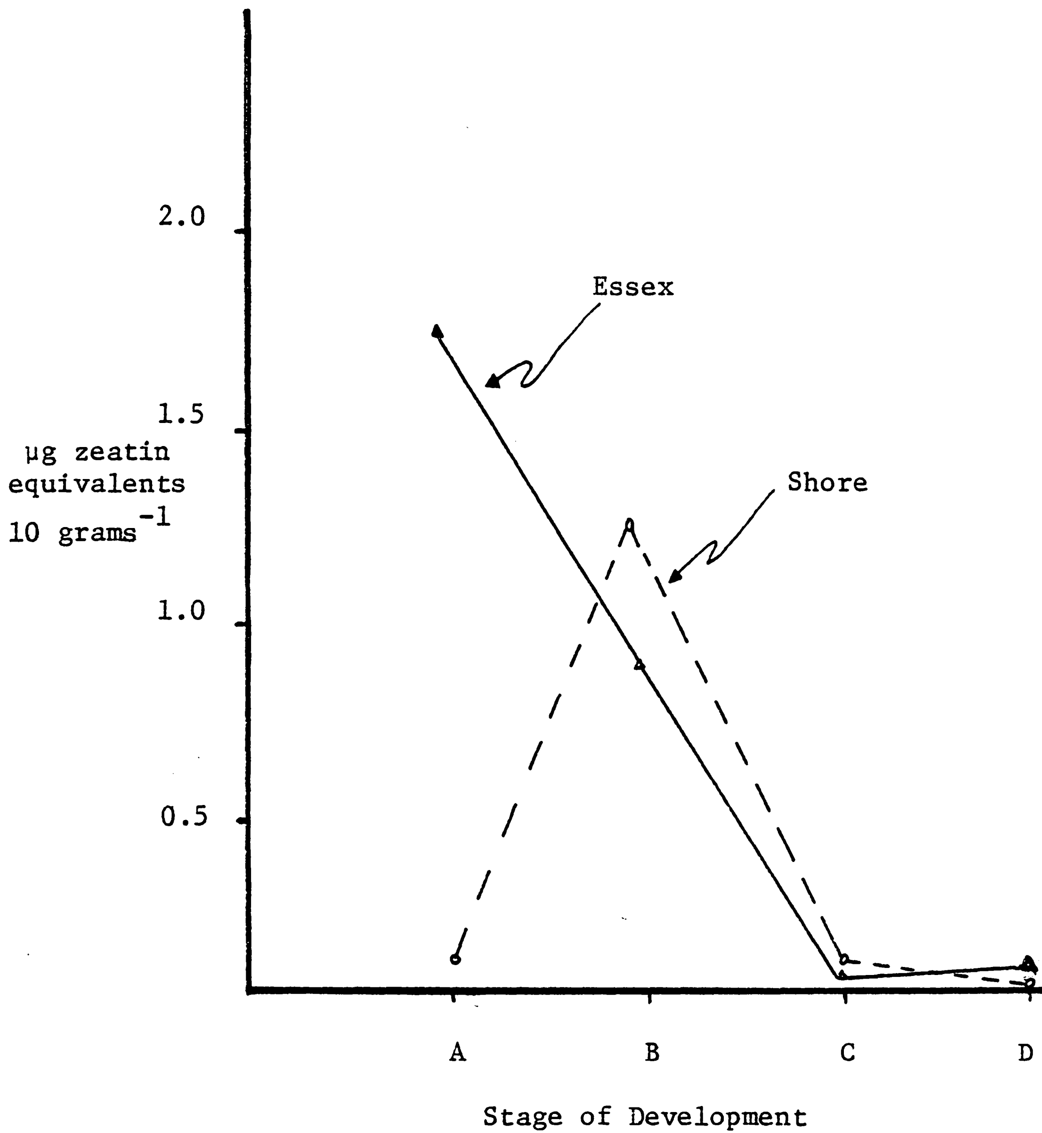


Figure 17. Cytokinin-like activity of developing ovules at four stages of development
A= whole ovaries less than 5 mm long
B= ovules from fruits 20-25 mm long
C= ovules from fruits 40-50 mm long
D= seeds 10-20 mm long

(Adapted from Aung *et al.*, 1982)

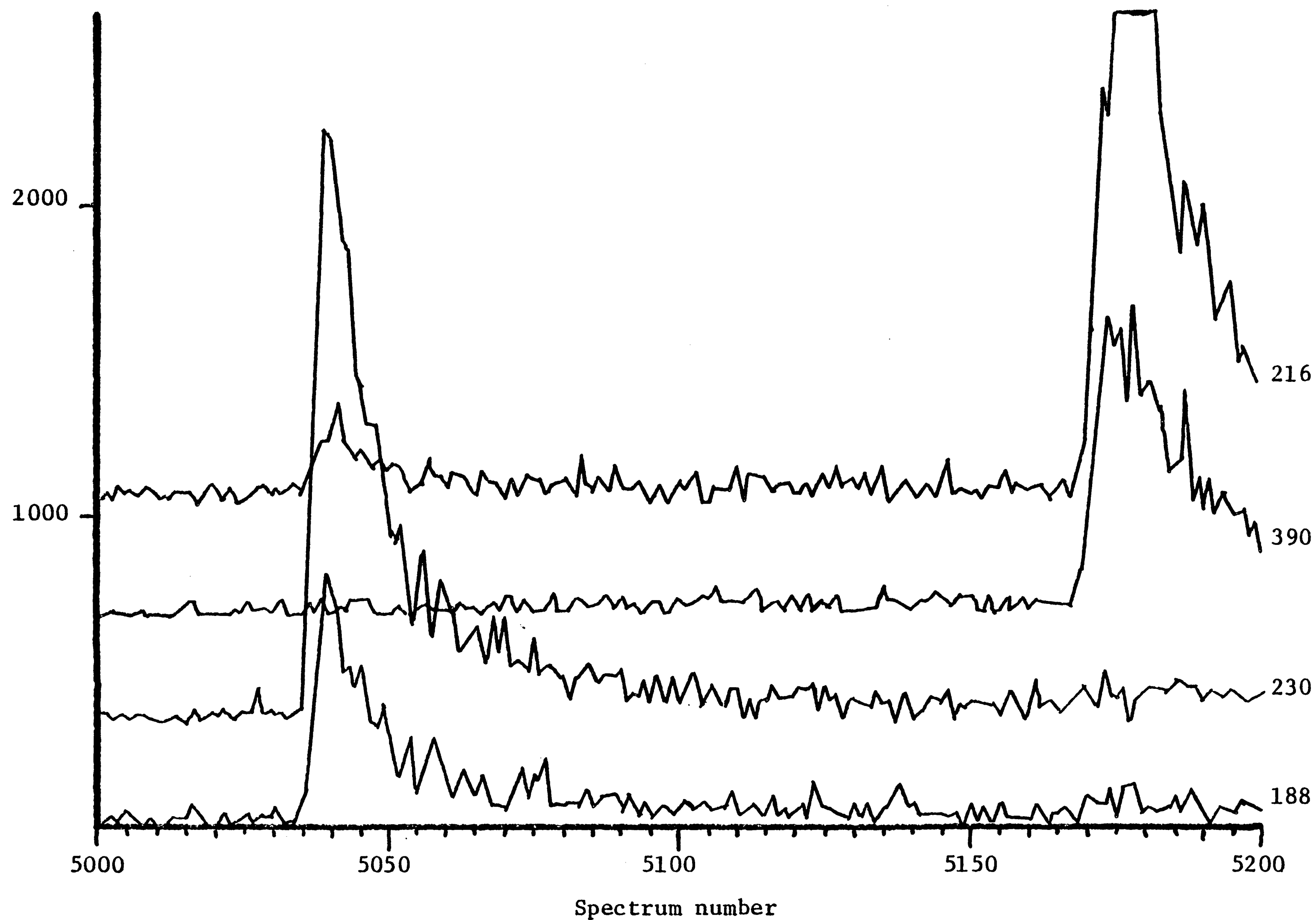


Figure 18. Multiple ion monitoring spectrum of Z and ZR standards in Experiment II.

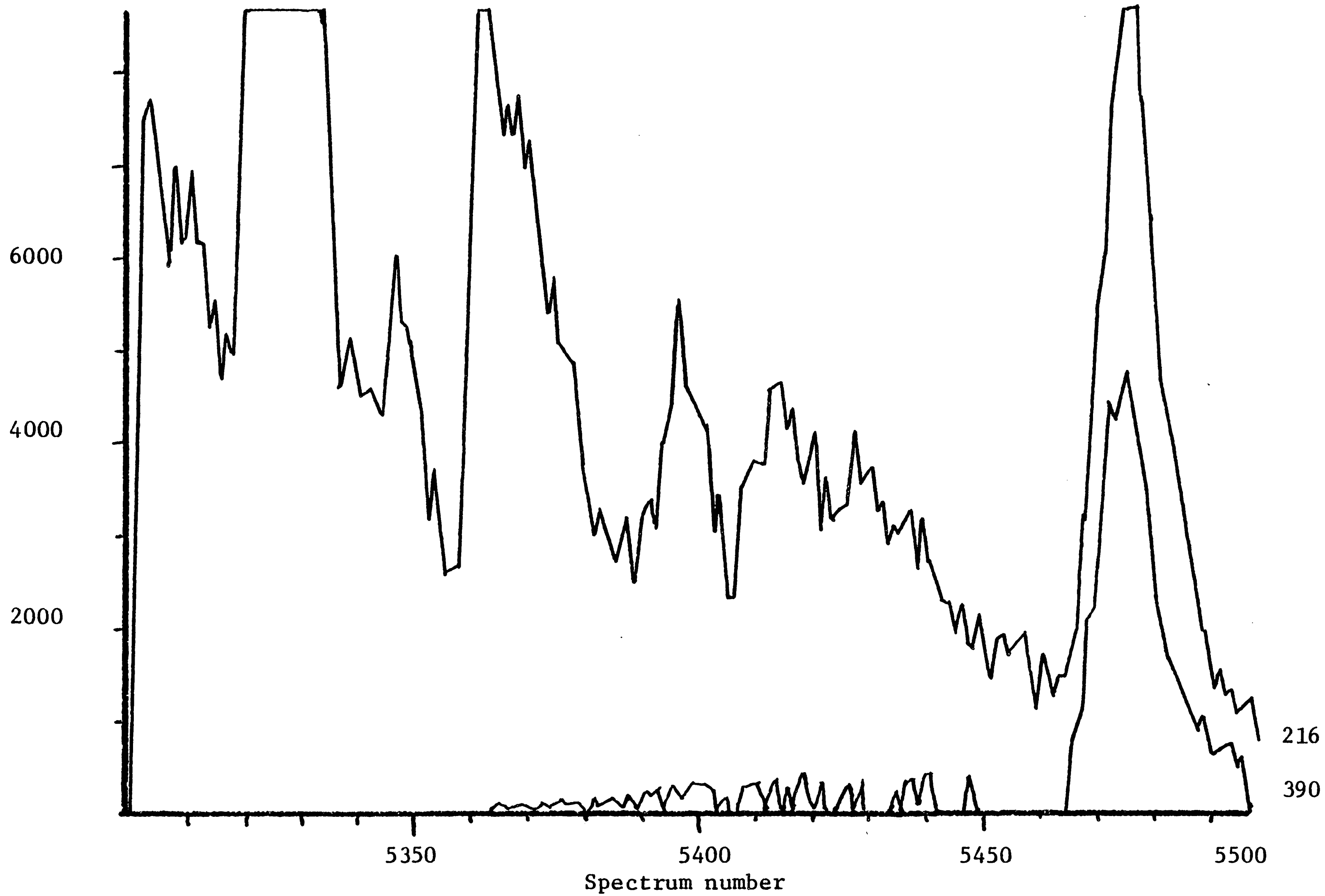


Figure 19. Multiple ino monitoring detection of ZR in Sephadex LH-20 fractions (Experiment II)

These results were considered conclusive evidence for the presence of ZR (Young, 1977).

The scan for Z is not clear (Fig. 20). The 188 peak co-elutes with the standard, but the 230 peak went off scale and was not accurately measured. Therefore the identity of Z was not firmly established from these data.

It was apparent from the high levels of background signal for both the 216 and 230 peaks that there were several cytokinin-like compounds present in the Sephadex LH-20 fractions. The selectivity of the multiple ion technique permitted the detection of ZR among these compounds. Further analysis for Z and other unknowns however, required greater purification.

EXPERIMENT III

The objective of this experiment was to further purify the Z and ZR fractions from Sephadex LH-20 obtained in experiment II by reversed phase HPLC.

Fifteen g of Essex and Shore fruits were extracted and purified by PVP, paper and Sephadex LH-20 chromatography. The fractions co-eluting with Z and ZR that were bioactive in earlier experiments were collected, and dried. The residue was dissolved in 200 µl of 95% ethanol. This solution was filtered to remove particulates and 100 µl aliquots were analyzed by HPLC. The UV absorbance at 268 nm of the Essex and Shore HPLC eluate is shown in Fig. 21 and 22. The region where Z and ZR elute are obscured by a large UV absorbing peak. Based on the UV absorbance profile there are several components in the LH-20 fractions at concentrations much greater than Z and ZR.

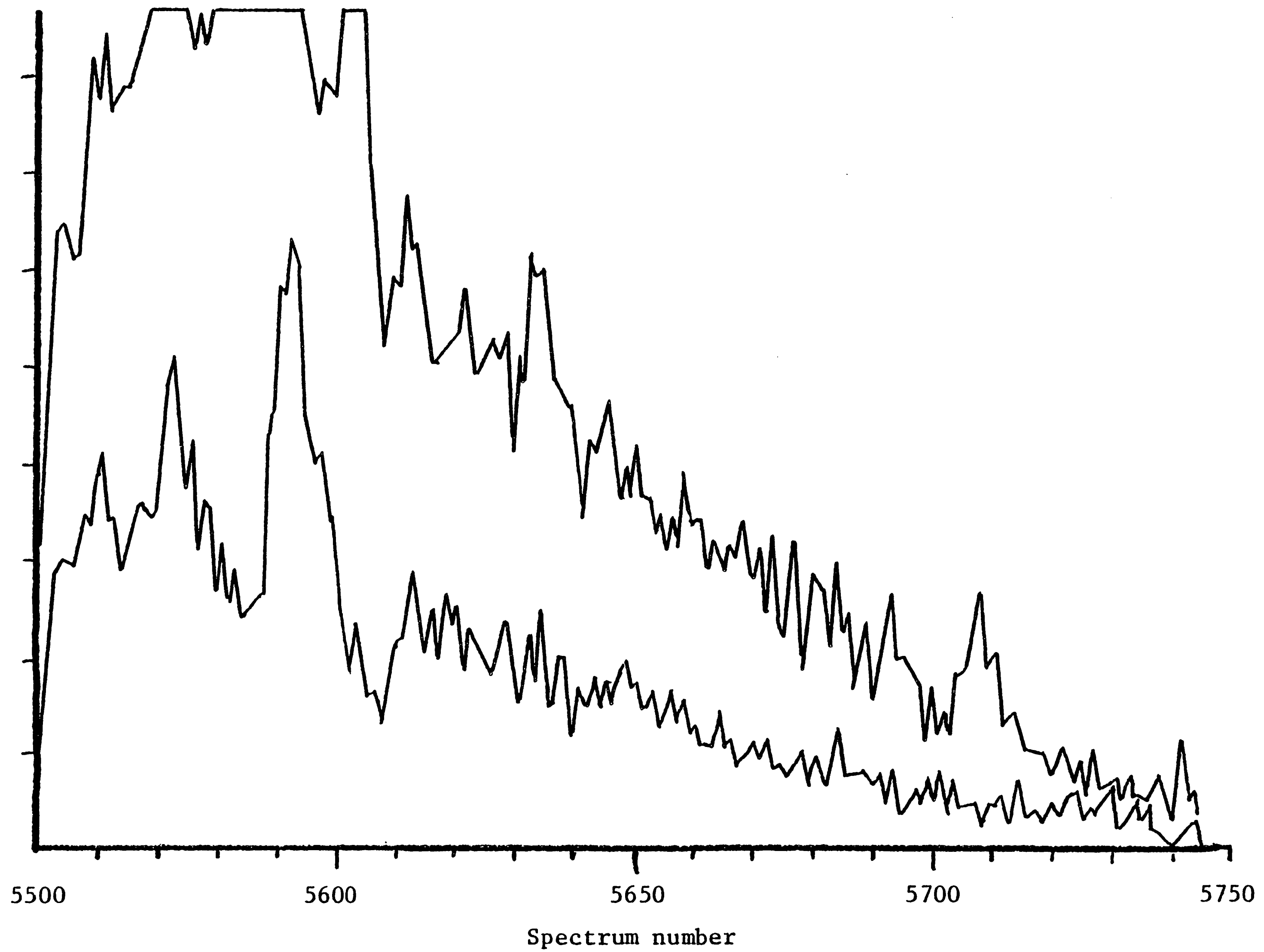


Figure 20. Multiple iom monitoring scan for Z in Sephadex LH-20 fractions (Experiment II).

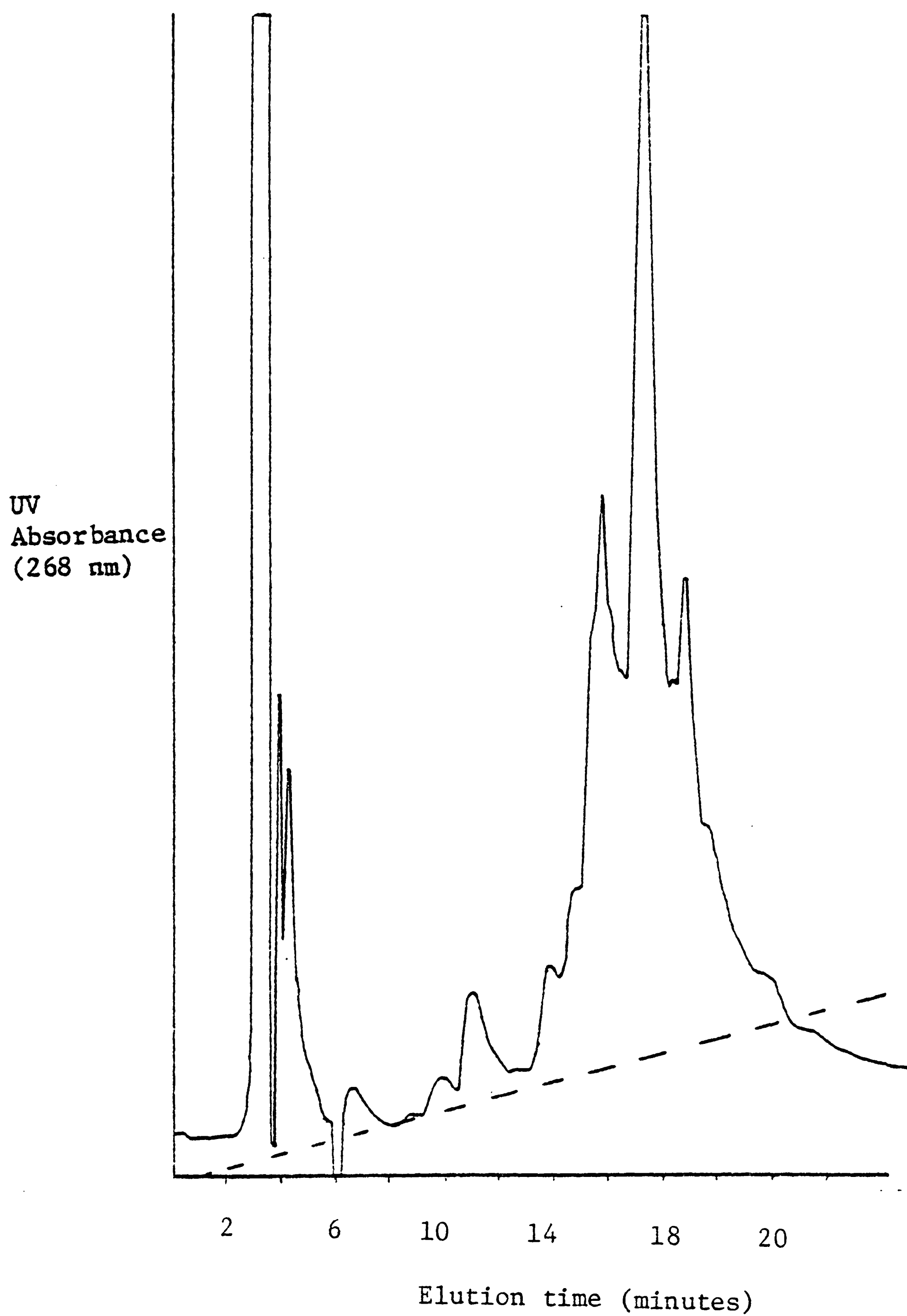


Figure 21. UV absorbance of Shore Z and ZR fraction from Sephadex LH-20 chromatographed on Partisil ODS 2. Acetonitrile gradient 8-38 % over 15 minutes (- - -). Flow rate = 1 ml min-1.

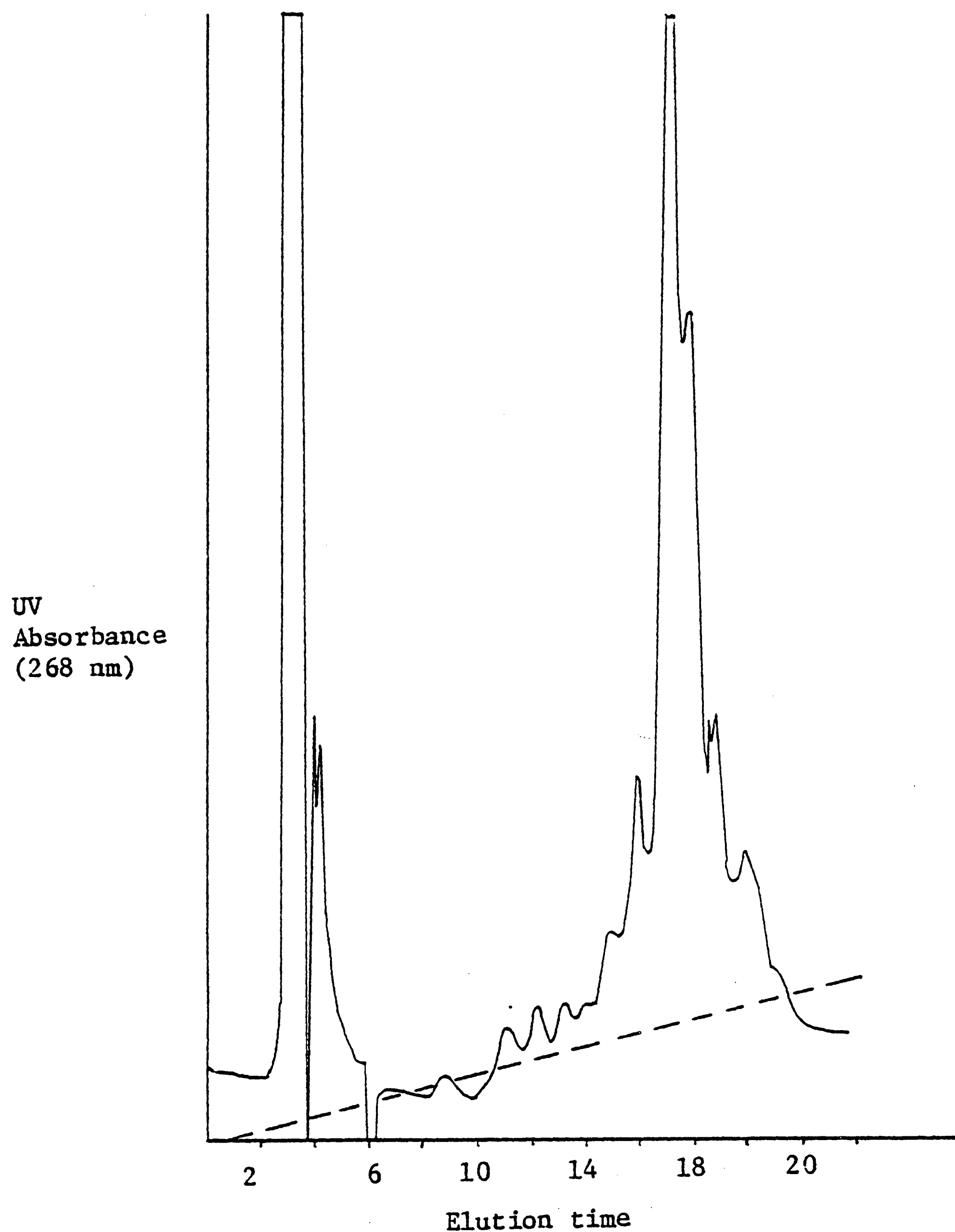


Figure 22. UV absorbance of Essex Z and ZR fraction from Sephadex LH-20 chromatographed on Partisil ODS-2. Acetonitrile gradient 8-38 % over 15 minutes(- - -) Flow rate = 1 ml min^{-1}.

An aliquot representing 30% of the total volume was taken for bioassay and the remainder derivatized for GC-MS. The bioassay results are shown in table 8. Cytokinin-like activity was detected in the fractions co-eluting with Z and ZR. Activity was also found eluting before the ZR fraction.

The GC-MS analysis of the derivatized Z and ZR fractions did not reveal any Z or ZR however, in either Essex or Shore samples using multiple ion monitoring. Three prominent peaks were evident in the total ion current for Shore (Fig. 23). The first peak was identified as dioctylphthlate (Fig. 24), a common plasticizer. This may have been introduced from the plastic components of the Sephadex LH-20 chromatography apparatus or as a contaminant in the chloroform used in the derivitization procedure.

The second peak was not identified. Its mass spectrum (Fig. 25) had prominent ions at m/e 410, 367, 341 and 206, which are not indicative of permethylated zeatin-like cytokinins. Also, the spectrum of this compound contains a series of prominent ions between m/e 150 to 200 that are separated by 14 mass units. This result indicates a hydrocarbon (loss of $-CH_2$) and suggests that a) the unknown is similar in structure to hydrocarbons or b) the sample is contaminated with a hydrocarbon. This contamination may have come from the N_2 used to remove the solvents during derivatization.

The third peak was not identified. Prominent ions included m/e 429, 415, 401, 355, 341, 281 and 206. Again these do not correlate with permethylated cytokinins and are indicative of a hydrocarbon.

Table 8. Bioassay responses of HPLC purified fractions of Essex fruits

Bioassay 1. Essex fruits, PVP 100-250 ml fraction, paper R_f 0.5-0.8, Sephadex LH-20 fraction 90-135 ml. HPLC conditions: Flow rate = 1 ml min^{-1}, 8-38% acetonitrile over 15 minutes.

Fraction (minutes)	Bioassay response ($A_{542} - A_{620}$)
Control	0.055
2 x 10^{-8} M zeatin	0.121 *
0-13.5 minutes	0.113 *
13.5-15	0.119 *
15-16.5	0.103 *
16.5-18.5	0.075
18.5-22.5	0.064

* Significantly different from control at 5% level of probability

Bioassay 2. Essex fruits, phosphocellulose ammonia eluate, Sephadex LH-20 fraction 90-135 ml. HPLC conditions: flow rate = 2 ml min^{-1}, 8-38% acetonitrile over 15 minutes.

Fraction (minutes)	Bioassay response ($A_{542} - A_{620}$)
Control	0.068
2 x 10^{-8} M zeatin	0.113 *
0-6 minutes	0.118 *
6-8	0.099 *
8-9	0.085
9-10.5	0.109 *
10.5-14	0.073

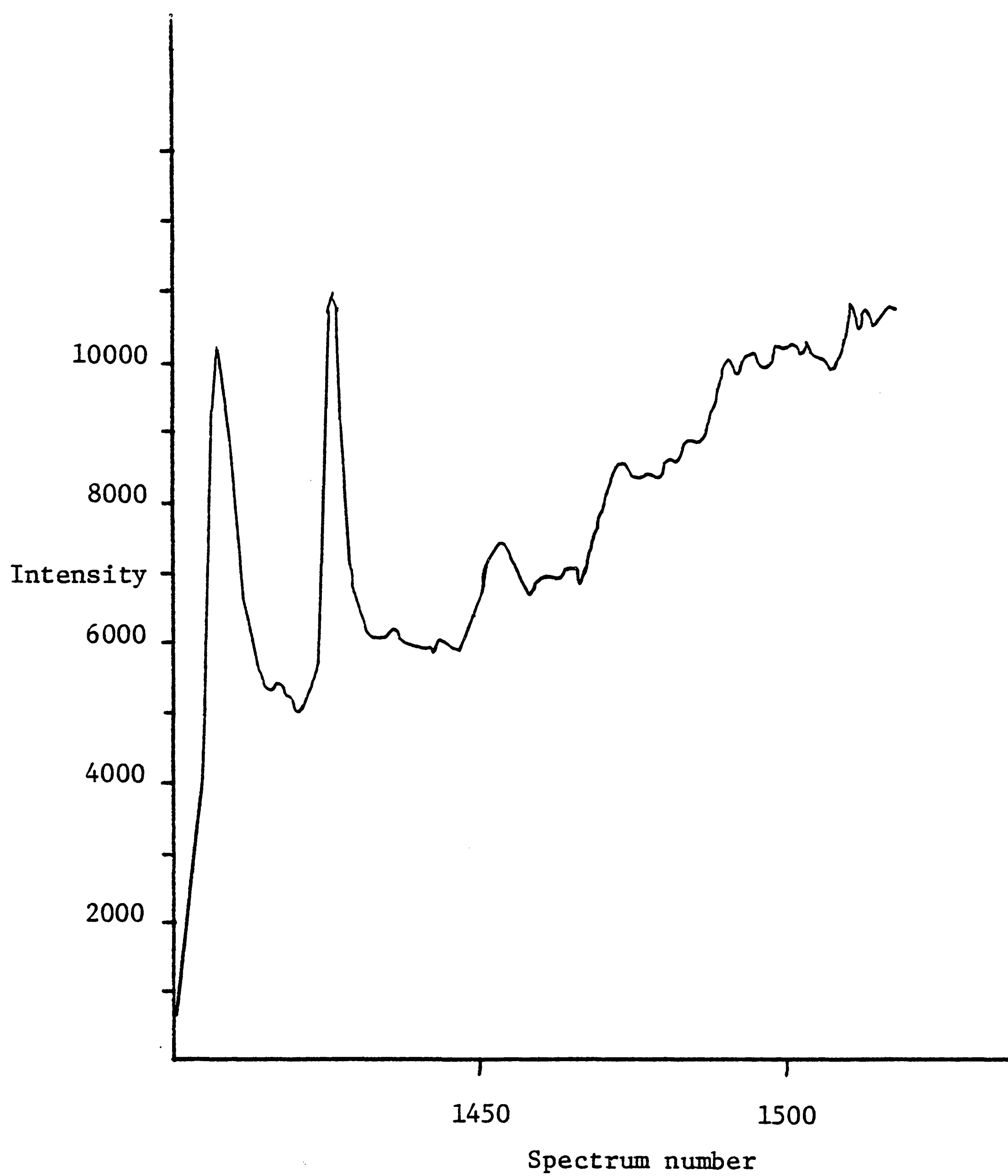


Figure 23. Total ion current recording for Shore ZR fraction from Partisil ODS 2.

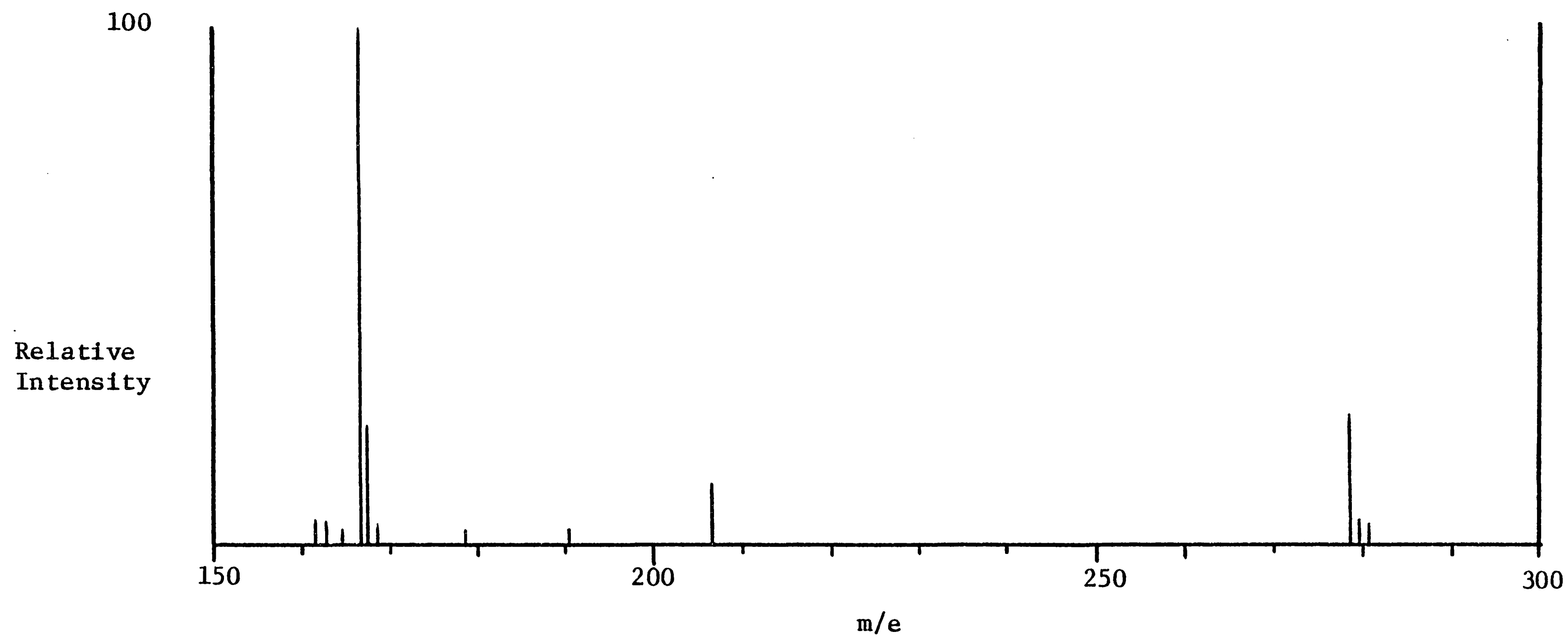
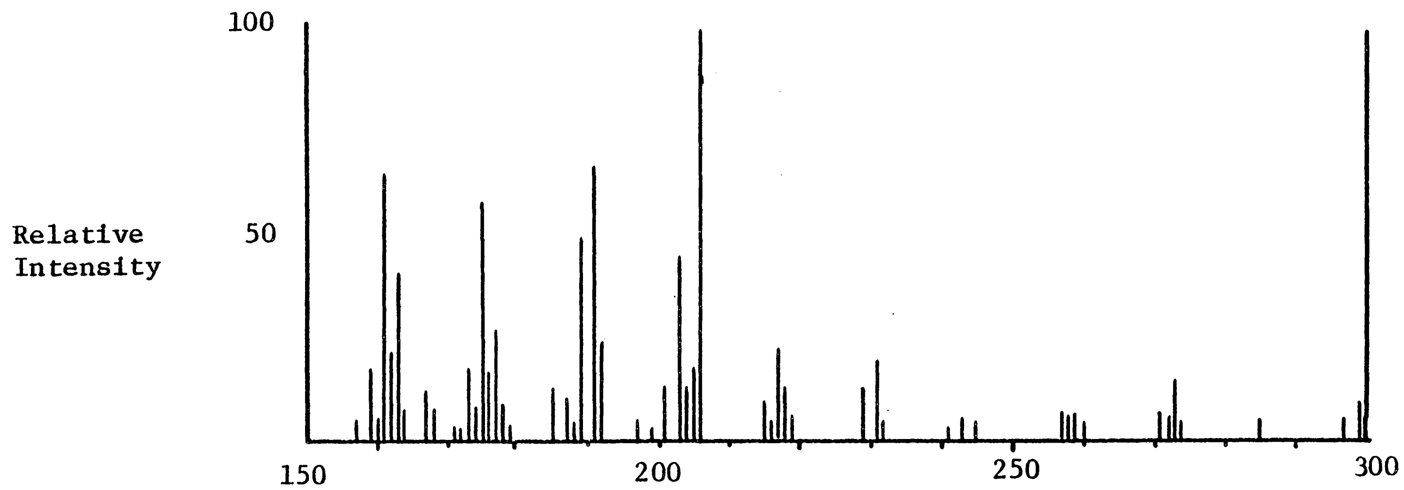


Figure 24. Partial spectrum of peak 1 in figure 23, identified as dioctylphthlate.



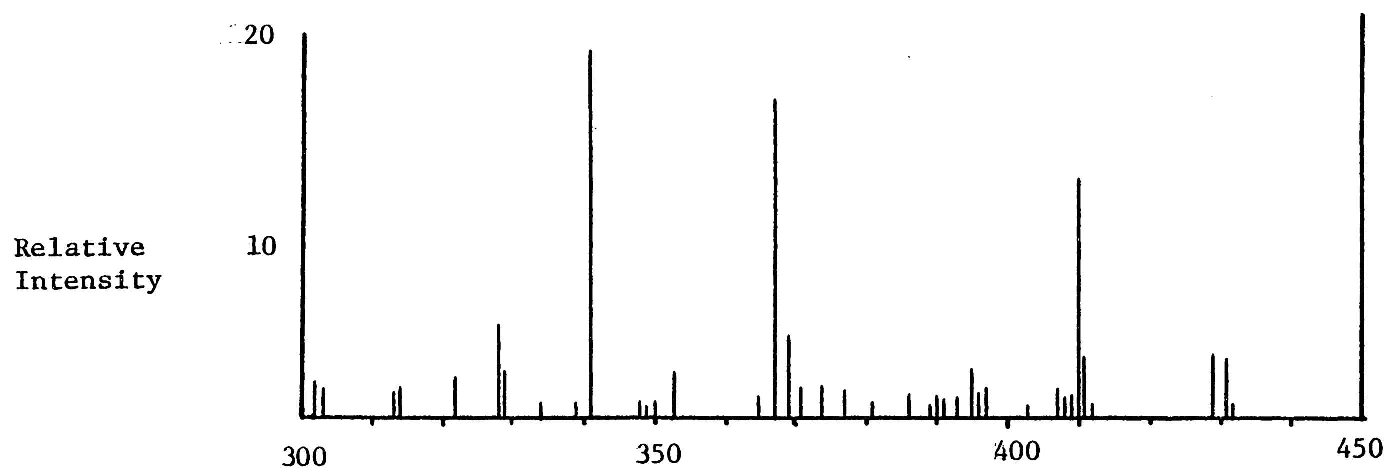


Figure 25. Spectrum of peak 2 in figure 23, identity unknown.

The total ion current of Essex had only one discernible peak which was identified as dioctylphthalate.

Because Z and ZR were not discernible in the chromatograms, a modified purification procedure was used. Ion-exchange chromatography on phosphocellulose (Horgan, 1979) was used instead of PVP and paper.

The Z and ZR fractions from Sephadex LH-20 gave distinct peaks in the region where Z and ZR elute in the HPLC chromatograms (Fig. 26-29). These peaks were collected separately, derivatized and analyzed by GC-MS. Again, no Z or ZR was detected with multiple ion monitoring. Aliquots taken for bioassay indicated significant activity in the Z fraction but not the ZR fraction. The more polar activity previously found to elute before ZR was also detected.

The polar fraction from Sephadex LH-20 (PVP 0-100, R_f 0.5-0.8 on paper) was chromatographed on Partisil ODS-2 and found to contain six components; all the components eluted within five minutes (Fig. 30).

Significant bioassay activity was found for the group, but the procedure did not sufficiently separate the components for an accurate assessment of individual peak activities. Isocratic elution with low percent acetonitrile did not improve the separation of the observed peaks.

When samples of the bulked HPLC effluent (0-4 minutes) were derivatized and analyzed by GC-MS the results were inconclusive. Fig. 31 shows a multiple ion scan of some expected peaks for zeatin glucoside but the results are not conclusive. Fig. 32 shows the detection of a m+1 peak using chemical ionization, and this provides supporting evidence for the identification of the glucoside although not

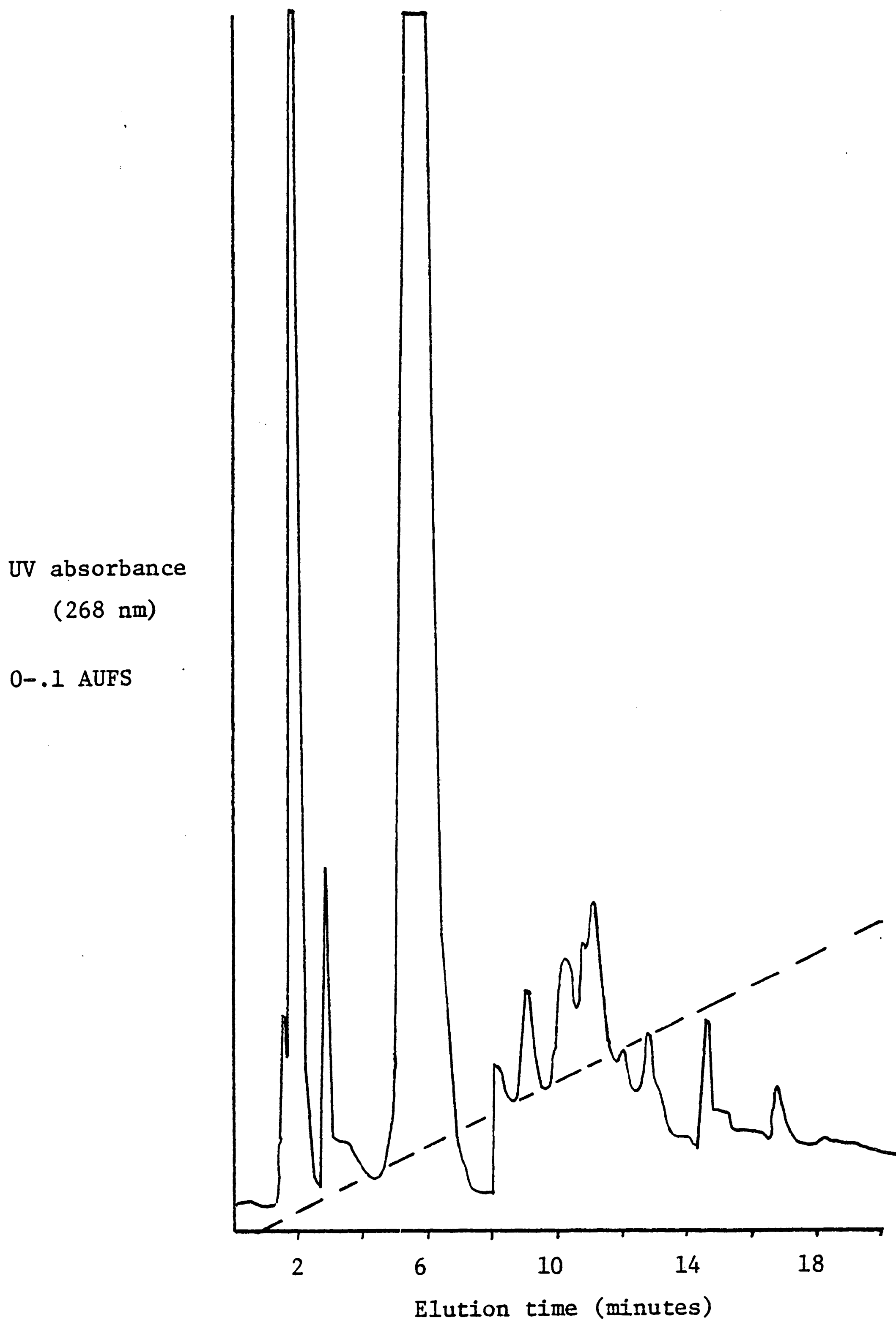


Figure 26. UV detection of HPLC eluate of ZR fraction from Sephadex LH-20 after phosphocellulose . Flow rate = 2 ml min^{-1}. Acetonitrile gradient 8-38% over 15 minutes.

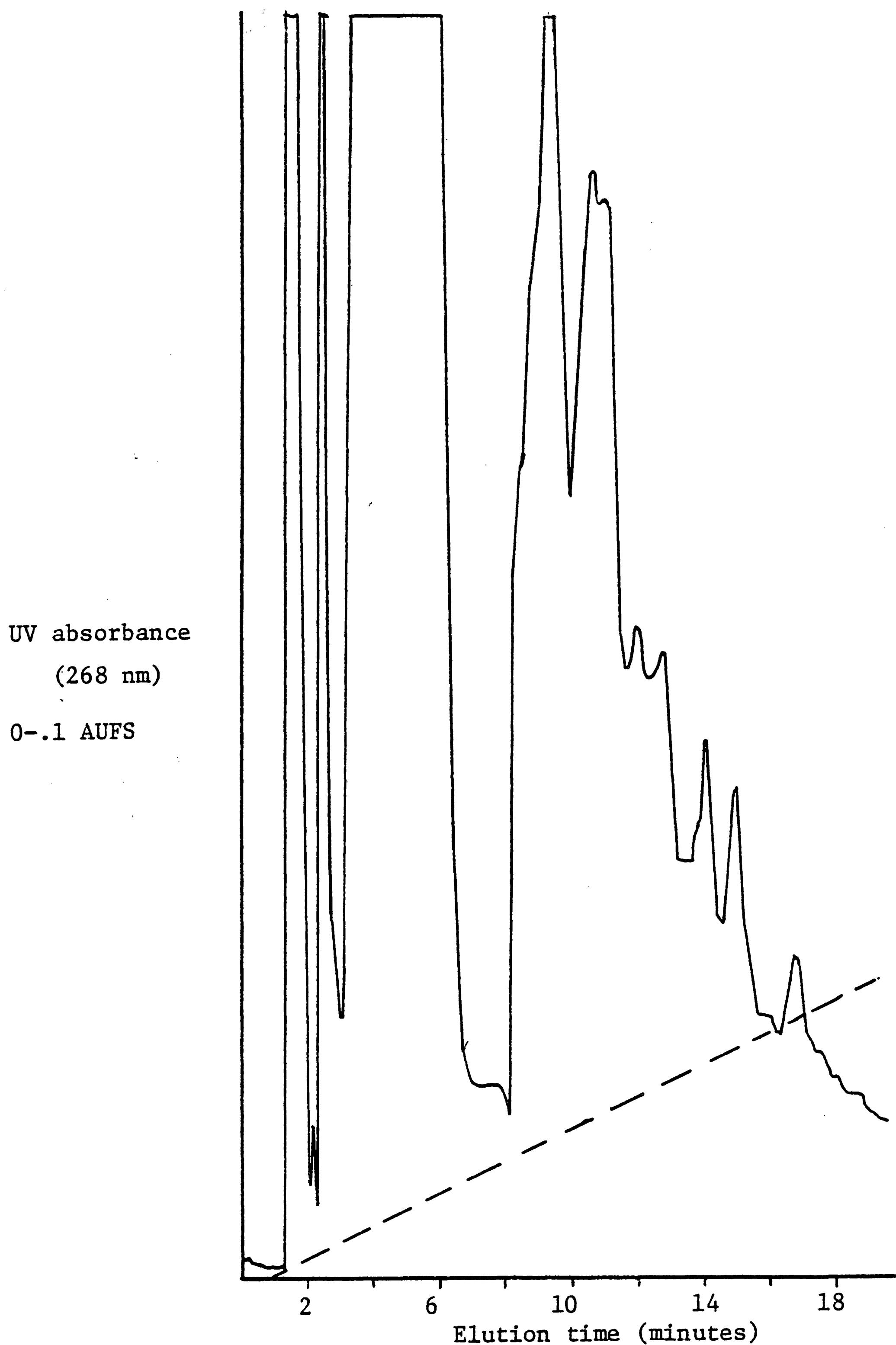


Figure 27. UV detection of HPLC eluate of ZR fraction from Sephadex LH-20 after phosphocellulose. Flow rate = 2 ml min^{-1}. Acetonitrile gradient 8-38% over 15 minutes. Cultivar ESSEX

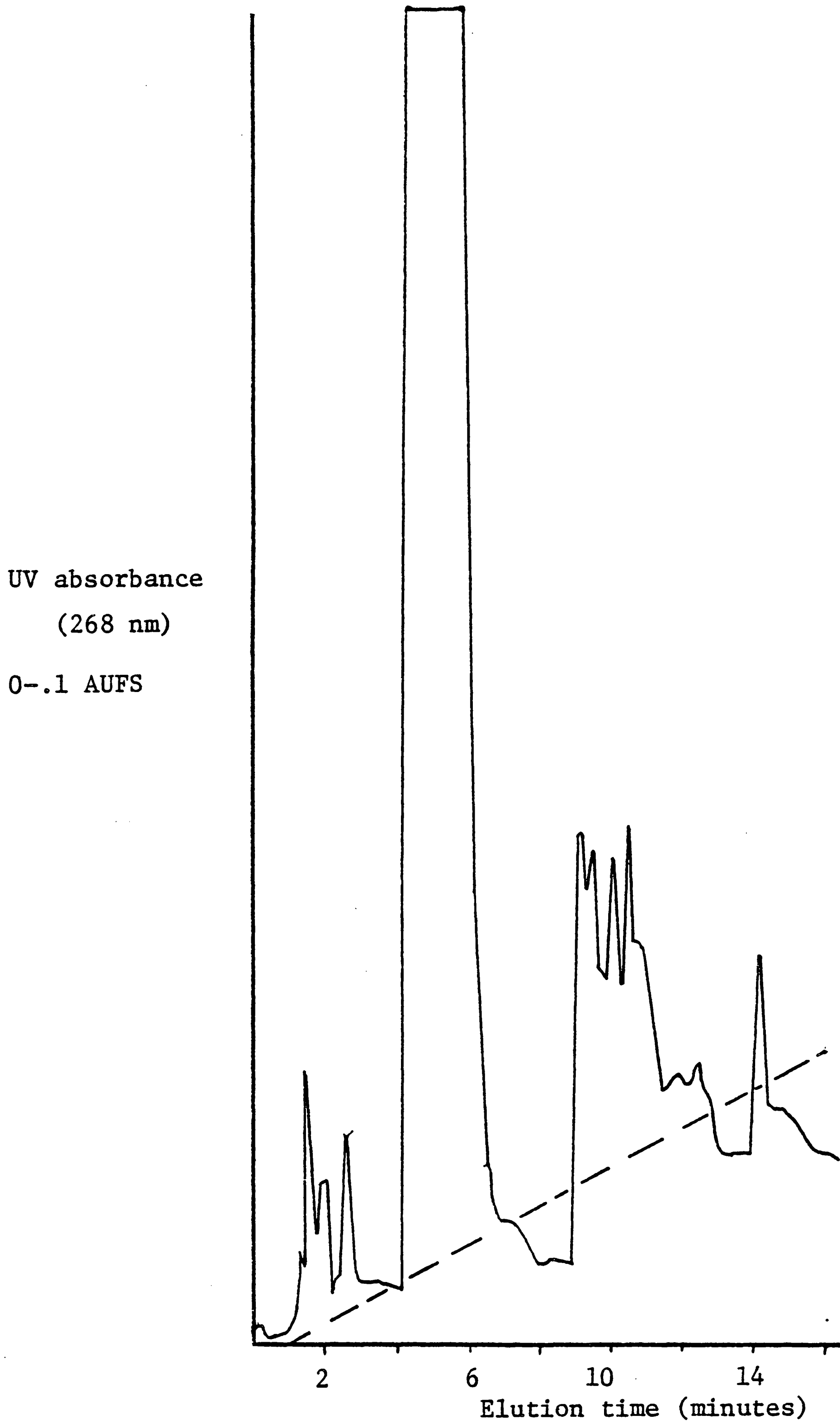


Figure 28. UV detection of HPLC eluate of Z fraction from LH-20 after phosphocellulose. Flow rate = 2 ml min^{-1}. Acetonitrile gradient 8-38% over 15 minutes. Cultivar SHORE

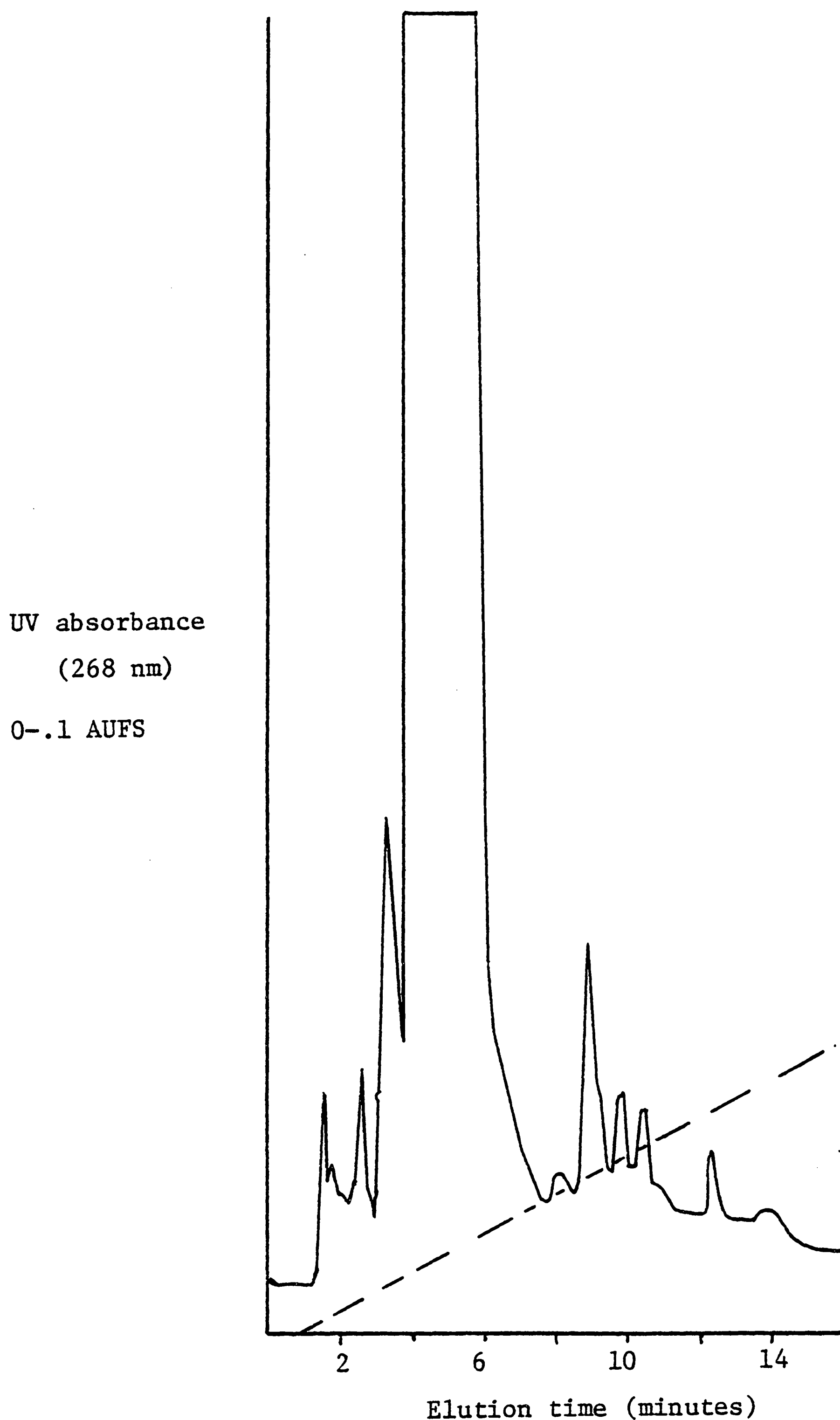


Figure 29. UV detection of HPLC eluate of Z fraction from Sephadex LH-20 after phosphocellulose. Flow rate = 2 ml min^{-1}. Acetonitrile gradient 8-38% over 15 minutes. Cultivar ESSEX

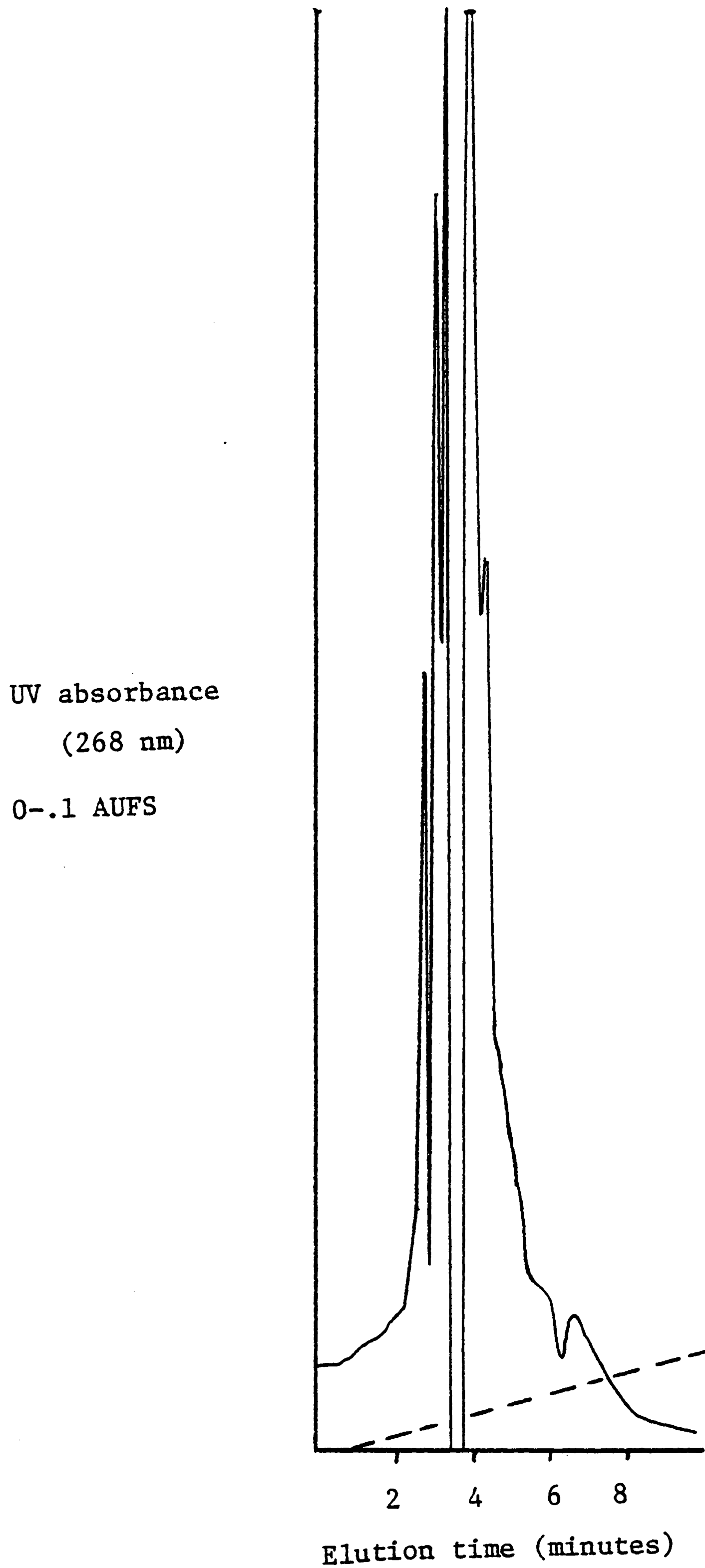


Figure 30. UV detection of HPLC eluate of polar fraction from Sephadex LH-20 after PVP and paper chromatography. Flow rate = 2 ml min^{-1}. Acetonitrile gradient 8-38 % over 15 minutes.

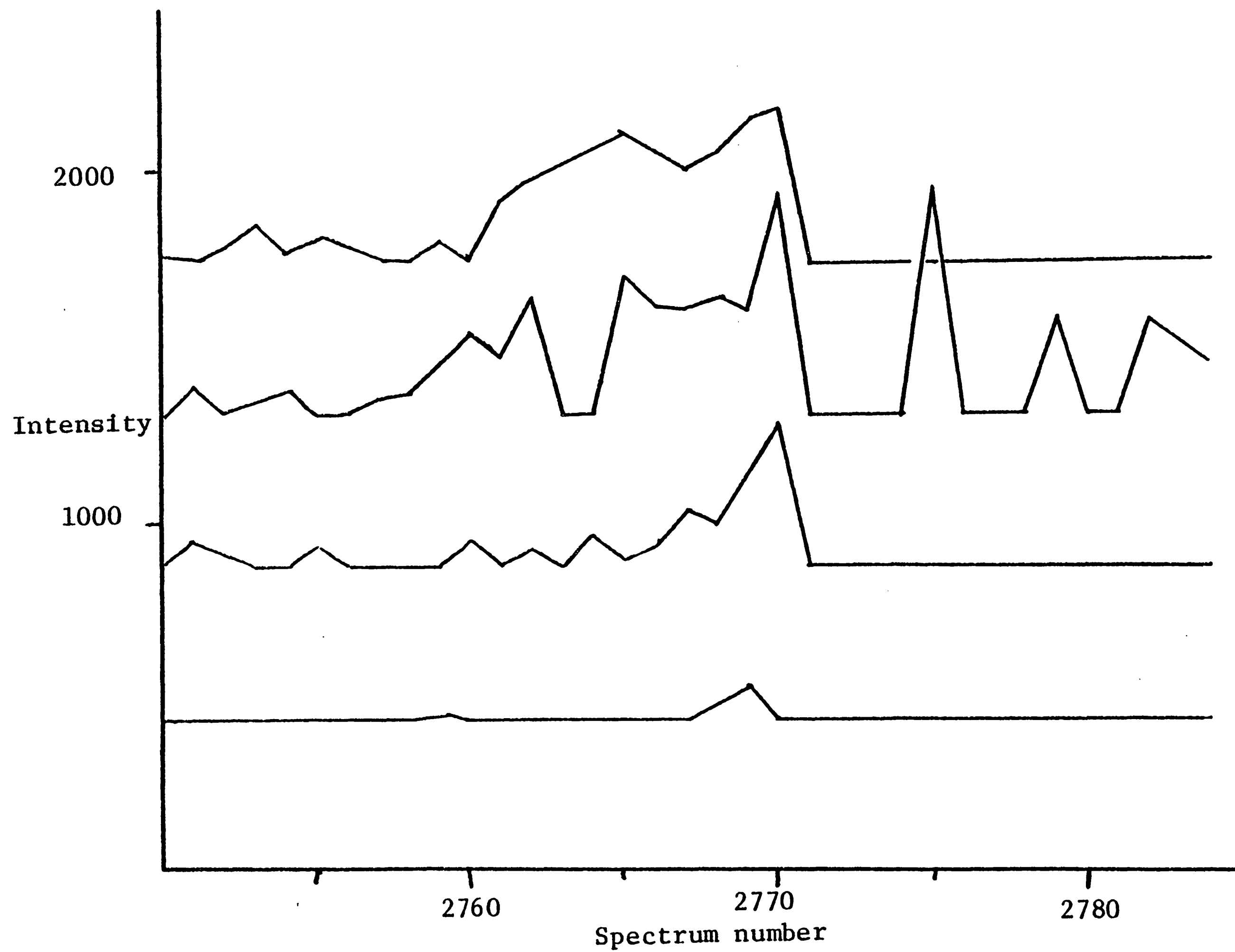


Figure 31. Multiple ion monitoring scan for permethylated unknowns from HPLC fractions of figure 30.

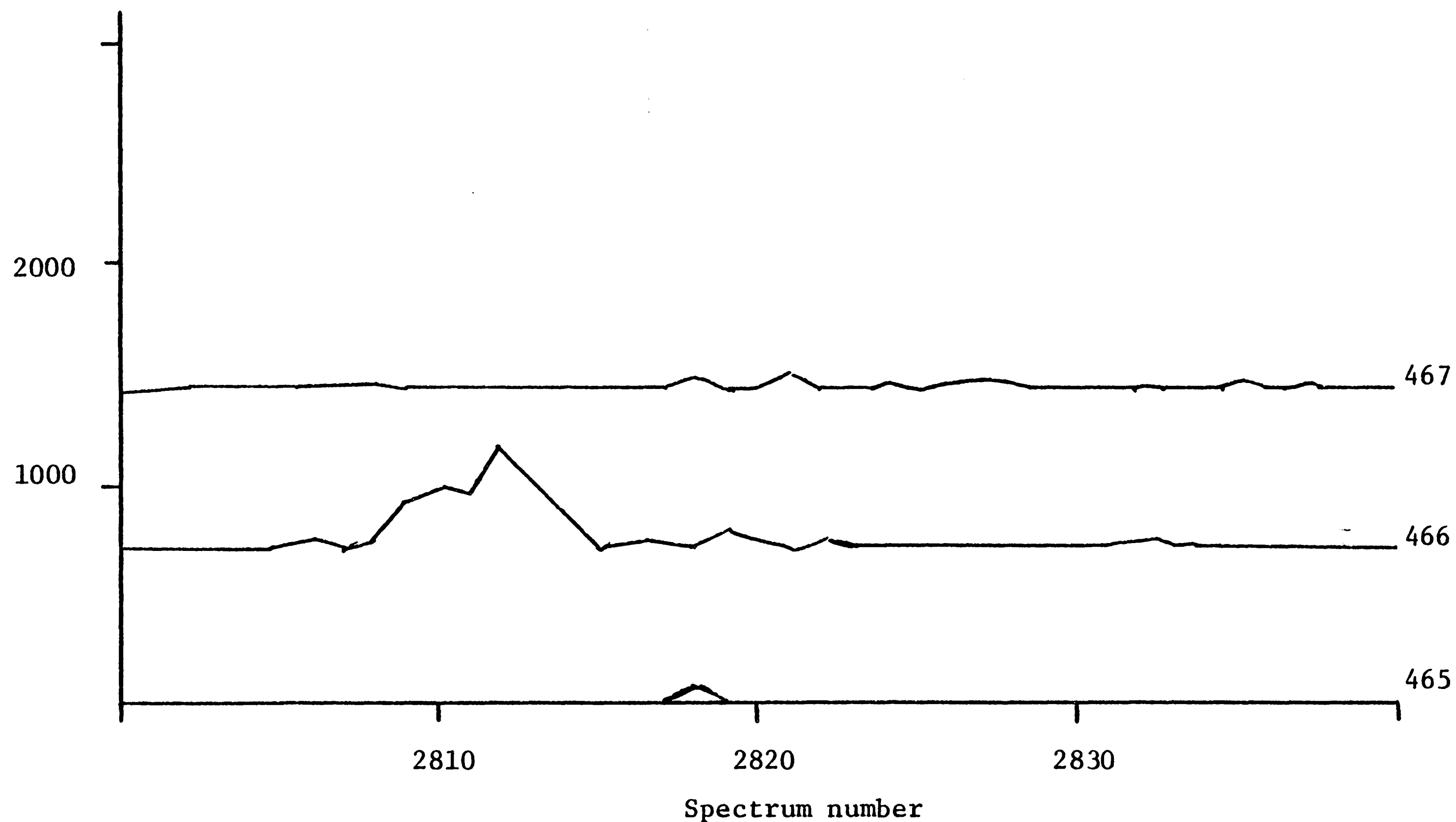


Figure 32. Multiple ion scan of chemical ionization mass spectrum of polar fraction (figure 30). m/e 466 is the postulated M+1 ion of zeatin glucoside.

conclusive. Fig. 33 shows a scan for diagnostic ions in the spectrum of ZR O-glucoside, which suggests the presence of this compound.

The lack of authentic standards prevents the comparison of retention times and ion ratios between unknowns and standards. This greatly impeded and prevented the identification of the unknowns.

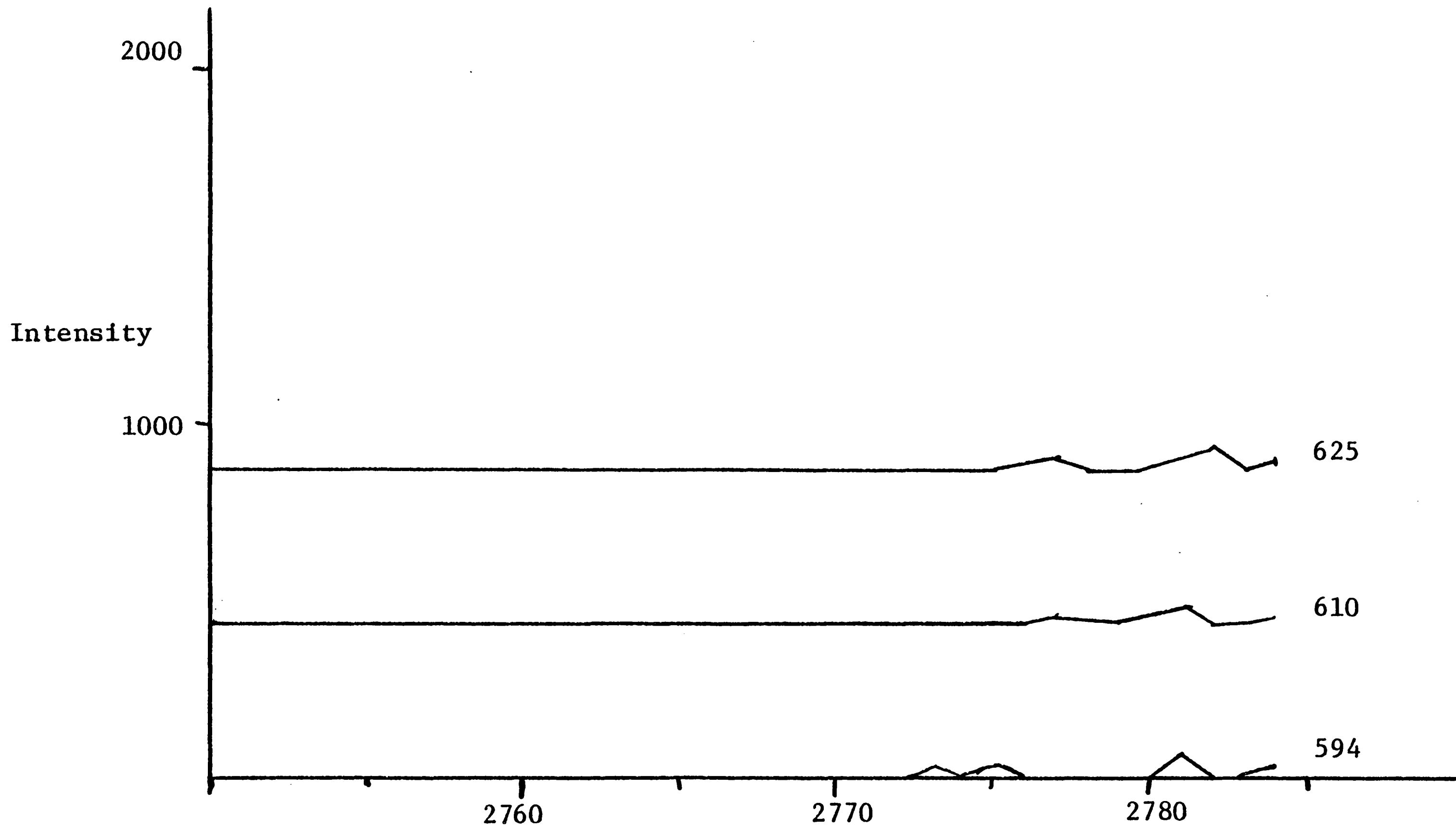


Figure 33. Multiple ion monitoring recording of permethylated unknowns from polar fraction (figure 30). Ions recorded are diagnostic high mass ions for ZROG.

DISCUSSION

The identification of endogenous cytokinins in soybean fruits is a complex problem. The concentration of cytokinins estimated by bio-assays is low and there are many other compounds present in the ethanolic fruit extracts. Thus, cytokinins of the soybean extracts must be concentrated several fold and separated from impurities for identification. The fractionation procedure used in this work separated nine fractions active in the <u>Amaranthus</u> bioassay. This activity could be followed through four different chromatographic procedures namely PVP, paper, Sephadex LH-20 and reversed phase HPLC. Finally, GC-MS was employed for identification. Although GC-MS gave additional information, the technique failed to provide conclusive evidence for the identity of the suspected cytokinins of soybean fruits.

The fractionation procedure used in this study separated three distinct groups of cytokinin-like substances based on their polarity. The cytokinin activity in the PVP 0-100 ml fraction when chromatographed on Sephadex LH-20 and Partisil ODS-2 exhibited characteristics consistent with polar compounds. The biological activity in this fraction eluted from Sephadex LH-20 immediately after the void volume, and on Partisil ODS-2 HPLC the activity eluted at low percent acetonitrile (10%) during gradient elution.

The 100-250 ml PVP fraction contained significant amounts of Z and ZR like activity. Chromatography on Sephadex LH-20 and Partisil ODS-2 indicated a compound very similar to ZR in the extract. Also, a Z-like compound was present in the Sephadex LH-20 and HPLC eluate.

These compounds were less polar than the compounds in the 0-100 ml fraction, and required 2-4 column volumes for elution from Sephadex LH-20 and 25-30% acetonitrile for elution from Partisil ODS-2.

The 250-650 ml fraction from PVP also contained significant amounts of biological activity and eluted from Sephadex LH-20 in 2 fractions. The first active fraction eluted at the same volume as ZR, while the second active fraction eluted at 10-15 column volumes (200-250 ml). Since ZR was not in the 250-650 ml PVP fraction, the first peak of activity was probably a compound similar to 2iPA, since 2iPA was not separated from ZR on Sephadex LH-20. The second peak of activity eluted much later than any of the standards and was less polar than Z or ZR. The chemical identity of this fraction was unknown.

The conjugated or "bound" fraction only exhibited one peak of activity, which eluted in the region of ZR. This suggests the presence of zeatin ribonucleotide in soybean fruits that was not hydrolyzed by endogenous phosphatases during extraction (Bielski, 1964).

Chromatography of cytokinins on PVP has been described for certain standards and plant extracts (Biddington and Thomas 1973). The elution of standards showed that the more polar cytokinins eluted before the less polar ones. Thus, the free base cytokinins eluted after their respective ribosides and 2iP eluted after zeatin. Based on these results, PVP separated cytokinins in an order similar to chromatography on Sephadex LH-20 (a C_3 reversed phase) or Partisil ODS-2 Columns. The elution order of standards was similar for all three materials.

The chromatographic behavior of cytokinins influenced the selection of solvents for HPLC analysis. On Partisil ODS-2 acetonitrile gave

better separations of a standard mixture than methanol. Conversely, methanol gave lower k' values. Therefore acetonitrile was chosen over methanol for chromatography on Partisil ODS-2. The choice of organic phase may vary with other column packings because different separations have been reported by several authors using other packings (Morris, 1981; Holland et al., 1980). The pH of the aqueous phase had negligible effect on elution in the range 5-7. These results were in good agreement with Haastert (1981) who found that for separation of nucleotides, nucleosides and free bases by reversed phase HPLC that methanol only changed k' values and not selectivity and that pH only affected k' when the pH was in the region of the pK_a of the compound, Since the pK_a's of cytokinins are generally 3-4 the pH change from 5-7 should have little effect on retention.

The indication of numerous cytokinin-like substances observed in soybean fruits was not unexpected in view of what was known of cytokinin metabolism. When ^{14}C-zeatin was fed to lupin seedlings at least 16 metabolites were formed (Parker, et al., 1978). Modifications included side chain cleavage to form adenine, adenosine, AMP and other purines, reduction of the side chain double bond to form DZ and a variety of ribosylations and glucosylations giving several Z and ZR ribosides and glucosides. Thus the metabolism of exogenous zeatin can include both a degradative pathway and several modification pathways.

Similar results were obtained with leaves of bean plants fed with ^{14}C labeled Z, DZ, Z O-glucoside (ZOG) and DZ O-glucoside (DZOG) (Palmer et al., 1981a). Zeatin was rapdily metabolized with less than 2% of the label found as zeatin after 72 hours. Urea and ureides

accounted for 55% of the total radioactivity recovered. Increasing amounts of label were recovered as intact molecules for DZ, ZOG, and DZOG (18, 38 and 53%, respectively). These results indicated that progressive modification of the zeatin molecule conferred stability _in vivo_, with DZOG being very stable. DZOG was therefore likely to accumulate _in vivo_ regardless of the source of cytokinin (endogenous or exogenous). This in fact had been found in primary leaves of decapitated bean plants (Wang and Horgan, 1977). The synthetic cytokinin 6-benzylaminopurine (BAP) also was glucosylated and ribosylated when applied to intact plant tissues (Fox _et al_., 1973; Ramina, 1979).

The polar glucosylated derivatives were therefore thought to be a deactivated storage form of cytokinins. This interpretation was supported by the observation of Palmer _et al_. (1981b) who found ZR O-glucoside (ZROG) levels to decrease rapidly in primary leaves of decapitated bean plants when a shoot was allowed to develop. The finding suggested the glucosides were mobilized from the leaf for use by the growing shoot.

The existence of polar glucosides _in vivo_ and the rapid conversion of applied cytokinins in both intact and detached systems necessitated enzymes for carrying out these reactions. Whitty and Hall (1971) demonstrated enzyme activity in developing corn kernels that was capable of cleaving the side chain from the zeatin nucleus. Interestingly, BAP was not a substrate for this enzyme, which readily degraded Z, ZR and 2iP.

Chen and Kristopeit (1981) isolated a 5'-nucleotidase which specifically catalyzed the formation of ribonucleosides from ribonucleotides. This enzyme had no detectable acid or alkaline phosphatase activity.

Several other enzumes capable of converting free bases to their corresponding nucleoside (Chen and Petschow, 1981) and other interconversions between nucleosides, nucleotides and free bases had been described (Chen and Eckert, 1977; Terrine and Laloue, 1980).

The observations of polar cytokinins in soybean fruits (Fig. 5 and 30) could have important implications for soybean development. Heindl et al. (1980) found increases in the cytokinins in xylem sap of soybeans during early fruiting. When seeds began to accumulate dry matter rapidly the content of cytokinin-like substances in the xylem sap fell rapidly. ZR and DZR comprised most of the cytokinins in the xylem sap. Therefore, it was conceivable that ZR and DZR imported into the fruits were converted to the polar glucosides and deactivated. Aung et al. (1982) also found that as soybean ovules enlarged past the 0.2 mg $ovule^{-1}$ stage cytokinin content rapidly declined. This observation might correspond to the decreased import observed by Heindl et al. (1980), and the probable metabolism of active cytokinins to less active and/or inactive metabolites.

The study of Summons et al. (1979) with lupin fruits might also be relevant for soybean. Although lupins and soybeans differ, the lupin study could serve as a good example for soybeans since a state-of-the-art instrumental method was used for quantification. Deuterium labeled internal standards were added to samples of seeds and pericarps prior

to extraction. Cytokinins were purified by phosphocellulose ion-exchange chromatography and preparative and analytical HPLC. The isolated compounds were derivatized (TMS) and analyzed by direct probe MS. Comparison of peak intensities of diagnositc ions derived from the deuterated and non-deuterated compounds allowed quantification of endogenous cytoinins by the ratio of peak heights. This technique also corrected for losses during extraction and purification.

Seeds and pericarps harvested 14 days after petal fall had different levels of cytokinins. No ZR was detected in the pericarps, while seeds contained 200 ng g^{-1}. DZR was found at 200 ng g^{-1} in seeds and 160 ng g^{-1} in pericarps. The glucosyl derivatives of Z, ZR, DZ and DZR were detected at 7, 17, 37 and 370 ng g^{-1}, respectively in seeds and 6, 81, 39 1100 ng g^{-1}, respectively in pericarps. Thus, in pericarps, the polar glucosyl derivatives comprised 88.5% of total cytokinins but only 49.5% in seeds. The result suggested the pericarp might store cytokinins for utilization by the seeds during fruit development.

In the soybean, distribution between seeds and pericarps was not directly determined, but was estimated. The polar fraction contained the largest fraction of total cytokinin-like activity. The result was similar to the pericarps of lupin where glucosyl ZR and glucosyl DZ constituted the bulk of pericarp cytokinins.

Since seeds constituted less than 2% of the dry weight of 10-25 mm soybean fruits, the bulk of cytokinins might be expected to reside in the pericarp. Developing ovules, however, were high in cytokinin activity (Aung et al., 1982) and contributed a significant part of the

total activity estimated in whole fruits. Comparison of quantitative estimates between lupin and soybean studies was made difficult by different methods used and species differences.

The reasons for not conclusively identifying the cytokinin-like substances active in the bioassay are not clear. It is apparent however, that the bioassay technique has sensitivity equal to or greater than the GC-MS instrument used in this study. The *Amaranthus* bioassay consistently detected 10^{-8} M zeatin, which is equal to 2.2 ng ml^{-1}. This level of detection is superior to the 25-50 g of permethylated cytokinins detectable by the GC-MS procedure. Therefore the quantities of cytokinins in the extracts may have been below the minimum detectable amount.

Yokota *et al.* (1981) used PVP and reversed phase HPLC for the purification of cytokinins from 5.6 kg of immature fruits of the hyacinth bean (*Dolichos lablab* L.). The *Amaranthus* bioassay was used to detect cytokinin active fractions before chemical analysis. GC-MS analysis of permethylated derivatives of the HPLC active fractions identified 5 cytokinins. The polar compounds ZOG and DHOG, as well as Z and ZR were identified. A major difference between the work of Yokota *et al.*(1981) and the soybean study was the relatively large fruit sample and the greater HPLC purification of the hyacinth bean extracts before GC-MS.

The bioassay results indicated several fractions with activity equal to 1 to 6 x 10^{-8} M zeatin. The estimate might be low or underestimated for the polar fractions, which are likely to be less active than Z or ZR in the bioassay (Van Staden and Phillipandou, 1977).

The estimates for the Z and ZR fractions were likely to be more accurate since Z was used as the standard for comparison in the bioassay. Therefore, the quantities of cytokinins might have been lower than the minimum required for GC-MS.

To overcome the problem of detecting minute quantities of cytokinins, a relatively large sample of fruits will be needed. Using 15 g dry weight fruit samples, cytokinin-like activity could be detected by bioassay. The estimated total cytokinin content of 27 ng zeatin equivalents g^{-1} dry weight was low and could not be detected by GC-MS. Therefore, an even larger fruit sample size of 10-100 times may be needed for identification. The collection of large fruit samples poses a problem because of the difficulty of collecting uniform young fruits 10-25 mm long. Also, Essex and Shore are determinate cultivars and they flower and produce fruits over a relatively short period of time. Once fruit development begins, it proceeds rapidly. Therefore, plants have to be sampled promptly with a relatively large labor force to provide the needed sample size for extraction.

The estimated cytokinin-like content of whole fruit approximates the estimated values for pericarps. The average 10-25 mm fruits are mainly pericarps, with less than 2% of dry weight being ovules. Based on bioassay estimate, the pericarps contributed only about 50% of the bioassay activity. Therefore, significant amounts of the total cytokinin-like activity resided in the ovules. This observation is similar to our earlier results (Aung _et al_, 1982) which showed cytokinin-like activity in ovules of 20-25 mm fruits equivalent to 1000 ng g^{-1}. Thus ovules are a relatively rich source of cytokinins for

extraction and identification. But at the same time, since several million ovules will be needed to provide a kilogram of tissue for extraction, the collection of this many ovules becomes an immense task.

An attendent problem with increased sample sizes is the need to alter chromatography procedures for handling the increased sample load. A desirable modification would involve preparative scale HPLC, which would increase speed and separating cytokinins from the crude extracts for GC-MS. Another modification to consider is altering the types of chromatography used. Since PVP, Sephadex LH-20 and Partisil ODS-2 all separate cytokinins in a similar way, a contrasting separatory procedure would be useful to separate out contaminating materials from the cytokinins. The use of phosphocellulose ion-exchange chromatography removed many of the UV absorbing materials that obscured the cytokinins when analyzed by HPLC after purification on PVP and paper. Another bonded phase for HPLC might be useful as demonstrated by Summons _et al_. (1979) and Yokota _et al_. (1981)

The identification of cytokinins of soybeans was further hampered by the lack of standards for DZR and the glucosyl derivatives of Z, DZ ZR and DZR. This prevented an accurate description of their chromatographic behavior in the systems used in this study and also precluded reference mass spectra for comparisons with unknowns.

The occurrence of cytokinins in soybean fruits might indicate an important role of these hormones in early seed and fruit development. Exogenous BAP application increased fruit-set in terminal inflorescences of determinate soybeans (Crosby _et al_., 1978, 1981a). Similar effects

of cytokinins had also been reported in mung beans (Clifford, 1981), broad beans (Chapman and Sadjadi, 1981) and soybeans (Peterson, 1979). These observations indicated an important function for cytokinins, but provided no direct evidence of endogenous cytokinins on fruit-set. Such a relationship needs further investigation.

The amount of BAP applied to soybeans was 37,500 times the estimated endogenous cytokinin content. Some of the applied BAP was undoubtedly lost or not absorbed, and some rapidly metabolized (Fox et al., 1973). Some however, penetrated to the site of action and prevented fruit abscission. It was also conceivable that the excess BAP could trigger responses not normally elicited by endogenous cytokinins, or it supplemented suboptimal endogenous cytokinin levels causing fruit set. The differences observed in the behavior of the two cultivars to applied cytokinin suggested genotypic differences in soybean cytokinin metabolism which could affect fruit set. It is tempting to speculate that if differences exist between genotypes for cytokinin metabolism, then it might be possible to select for genotypes with altered enzyme characteristics to regulate fruit-set and yield.

When Essex and Shore were compared, the active fractions were similar in types of cytokinin-like substances, but the amounts varied. While not all the fractions detected in Essex were assayed in the Shore samples, it appears the cultivars are similar qualitatively. This needs to be determined with greater precision.

The relationship between fruit-set and yield in soybeans is complex. Genotypes that differ widely in fruit number may have similar yields

while genotypes with similar fruit numbers may differ in yield (Crosby *et al.*, 1981b)

Therefore, the relationship of cytokinins, fruit-set and yield needs more investigation. A thorough quantitative and qualitative study on cytokinins of genotypes with contrasting yields is needed. If cytokinin metabolism and response to exogenous cytokinins is to be used as a selection criteria for plant breeders, these relationships must be better understood. Also, more powerful techniques are necessary for rapidly screening large numbers of samples that contain relatively low amounts of cytokinins.

Cytokinins have potential as physiological probes of soybean development, especially for yield components such as fruit-set and ovule abortion. Exogenous cytokinins might serve as growth regulating chemicals for increasing soybean yields. These potential uses of cytokinins depend on more refined identification and quanitification of soybean cytokinins.

SUMMARY AND CONCLUSIONS

Soybean fruits 10-25 mm long contained 9 fractions possessing cytokinin-like activity in the Amaranthus bioassay. Based on their polarity, these were placed in 3 goups: polar, moderately polar and relatively non-polar fractions.

The PVP 0-100 ml volume contained the polar fraction and substances inhibitory in the Amaranthus bioassay. Further purification (paper and Sephadex LH-20 chromatography) separated the inhibitor(s) and allowed detection of the cytokinin-like substances. The polar fraction was the single most active fraction detected by bioassay with activity equivalent to 6×10^{-8} M zeatin. The polar fraction contained 5 components when chromatographed on Partisil ODS-2 HPLC.

The moderately polar fraction (PVP 100-250 ml volume) contained activity similar to zeatin and zeatin riboside in 4 chromatographic procedures. The activity in this fraction was detected after PVP chromatography, and no increase in activity was seen upon further purification.

The non-polar fraction (PVP 250-650 ml volume) contained 2 active components: one which co-eluted with 2iPA on Sephadex LH-20, and the second component was much less polar than any reference cytokinins and required 10-15 column volumes for elution from Sephadex LH-20.

After initial fractionation, the aqueous phase had no cytokinin activity. Digestion with alkaline phosphatase released cytokinin-like substance(s) that co-eluted with zeatin riboside on Sephadex LH-20. This showed there were conjugated cytokinins that were not totally

degraded by endogenous phosphatases during extraction. Compounds that were inhibiting in *Amaranthus* bioassay were also extracted from the phosphatase-treated aqueous phase. These substances eluted before ZR.

Identification of individual cytokinins by GC-MS proved inconclusive. The reasons for this were not clear. It is likely the sample sizes were too small for accurate identification by GC-MS. Also, the chromatographic purification included 3 procedures that separated cytokinins in a similar order. Incorporation of a contrasting chromatographic step, such as a different bonded phase for HPLC, might remove interfering materials from the extracts before GC-MS. Care must be taken to avoid contamination of the extracts by chemicals such dioctylphthalate or hydrocarbons. The purest reagents available should be used at each step of the purification.

For improved conclusive detection of cytokinins larger samples and improved purification techniques are needed. Also more cytokinin standards are needed for accurate assessment of chromatographic fractions and GC-MS spectra.

LITERATURE CITED

Aung LH, GR Buss, KE Crosby,SS Brown 1982. Changes in hormonal levels of soybean fruit during ontogeny. Φhyton 42 (in press)

Burrows WJ and DJ Carr 1970. Cytokinin content of pea seeds during their growth and development. Physiol. Plant. 23:1064-1070

Bielski RL 1964. The problem of halting enzyme action when extracting plant tissue. Anal. Biochem. 9:431-442

Biddington NL and TH Thomas 1973. Chromatography of 5 cytokinins on an insoluble polyvinylpolypyrrolidone column. J. Chrom. 75:122-123

Chen C-M and RL Eckert 1977. Phosphorylation of cytokinin by adenosine kinase from wheat germ. Plant. Physiol. 59:443-447

Chen C-M and SM Kreistopeit 1981. Metabolism of cytokinin: Dephosphorylation of cytokinin nucleotide by 5' - nucleotidases from wheat germ cytosol. Plant Physiol.

Chen C-M and B Petschow 1978a. Cytokinin biosynthesis in cultured rootless tobacco plants. Plant Physiol. 62:861-865

Chen C-M and B Petschow 1978b. Metabolism of cytokinin; Ribosylation of cytokinin base by adenine phosphorylase from wheat germ. Plant Physiol. 62:871-874

Chapman GP and AS Sadjadi 1981. Exogenous growth substances and internal competition in *Vicia faba* L. Z. Pflanzenphysiol. 104:265-273

Clifford PE 1981. Control of reproductive sink yield in mung beans. Z. Pflanzenphysiol. 102:173-181

Crosby KE 1979. Cytokinins on Soybean *Glycine max* (L.) Merr. fruit and seed development. MS Thesis, Virginia Polytechnic Institue and State UNiversity, Blacksburg, VA

Crosby KE, LH Aung, GR Buss 1978. Natural and synthetic cytokinins on soybean development and yield. 5th Proceedings Plant Growth Regulator Society, Blacksburg VA

Crosby KE, LH Aung, GR Buss 1981a. Influence of 6-benzylaminopurine on fruit-set and seed development in two soybean *Glycine max* (L.) Merr. genotypes. Plant Physiol. 68:985-988

Crosby KE, LH Aung, GR Buss 1981b. Plant Characteristics as determinants of yield in soybeans *Glycine max* (L.) Merr. Agron. Abstracts 1981, p83

Davey J and J Van Staden 1979. Cytokinin activity in *Lupinus albus* L. IV. Distribution in seeds. Plant Physiol. 63:873-877

Dua IS, KK Jindal, LJ Srivastava, CL Dinabandhoo, JR Thakur, R Jain 1978. Correlation of endogenous cytokinins with apical dominance in response to morphactin in soybean Glycine max L. Proc. Indaian Acad Sci. 87: 319-324

Duke CC, DS Letham, CW Parker, JK MacLeod RE Summons 1979. The complex of O-glucosylzeatin derivatives formed in Populus species. Phytochemistry 18:819-824

Eliot DC 1979. Analysis of variability in the Amaranthus betacyanin assay for cytokinins. Effects of "aging" excised cotyledons. Plant Physiol. 63:274-276

Englebrecht L 1972. Cytokinins in leaf cuttings of Phaseolus vulgaris L. during their development. Biochem. Physiol. Pflanzen. 163:335-343

Entsch B and DS Letham 1979. Enzymic glucosylation of benzylaminopurine. Plant Sci. Letters. 14:205-212

Fehr WR and CE Caviness 1977. Stages of soybean development. Iowa State Univ. Special Report 80.

Fox JE, J Cornette, G Deleuze, W Dyson, G Giersak, P Niu, J Zapata, J McChesney 1973. The formation, isolation and biological activity of a cytokinin 7-glucoside. Plant Physiol. 52:627-632

Hakamori SI 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem (Tokyo) 55:205-208

Heindl JC,WA Brun, M Brenner 1980. Developmental profiles of cytokinins in soybean root pressure exudate. Agron. Abstracts 1980,p 81

Henson IE 1978. Types, formation and metabolism of cytokinins in leaves of Alnus glutinosa (L.) Gaertn. J Exp.Botany 29:935-951

Holland JA, EH McKerrill, KJ Fuell, WJ Burrows 1978. Separation of cytokinins by reversed phase high performance liquid chromatography. J Chrom. 166:545-553

Horgan R, EW Hewitt, JG Purse, JM Horgan, PF Wareing 1973. Identification of a cytokinin in sycamore sap by gas chromatogrphy-mass spectrometry. Plant Sci. Letters 1:321-324

Horgan R 1978a. The nature and distribution of cytokinins. Phil. Trans Royal Soc. London B 284:239-247

Horgan R 1978b. Analytical procedures for cytokinins. In Isolation of Plant Growth Substances Ed. JR Hillman. Cambridge University Press, Cambridge

Horgan R and MR Kramers 1979. High performance liquid chromatography of cytokinins. J Chrom. 173:263-270

Koshimizu K, S Matsubara, T Kusaki, T Mitsui 1967. Isolation of a new cytokinin from immature yellow lupin seeds. Agric. Biol. Chem. 31: 795-801

Letham DS 1967. Regulators of cell division in plant tissues V. A comparison of the activities of zeatin and other cytokinins in five bioassays. Planta 74:228-242

Letham DS, MM Wilson, CW Parker, ID Jenkins, JK MacLeod 1975. Identity of an unusual metabolite of benzylaminopurine. Biochem. Biophys. Acta. 399:61-70

Lindoo SJ and LD Nooden 1978. Correlation of cytokinins and abscisic acid with monocarpic senescence in soybeans. Plant and Cell Physiol. 19:997-1006

Nesling FAV and DA Morris 1979. Cytokinin levels and embryo abortion in interspecific Phaseolus crosses. Z. Pflanzenphysiol. 91:345-358

Morris RO, DA Regier, EMS MacDonald 1981. Analytical procedures for cytokinins; Application to Agrobacterium tumefaciens. p 3-16, In Metabolism and molecular activities of cytokinins. Eds. J Guern and C Peaud-Lenoel. Springer Verlag, Berlin.

Palmer MV, R Horgan, PF Wareing 1981a. Cytokinin metabolism in Phaseolus vulgaris L. I. Variations in cytokinin levels in leaves of decapitated plants in relation to lateral bud outgrowth. J Exp. Botany 32:1231-1242

Palmer MV, IM Scott, R Horgan 1981b. Cytokinin metabolism in Phaseolus vulgaris L. II Comparative metabolism of exogenous cytokinins by detached leaves. Plant Sci. Letters 22:187-195

Parker CW and DS Letham 1973. Metabolism of zeatin by radish cotyledons and hypocotyls. Planta 114:199-218

Peterson CM and MW Folsom 1979. The effect of cytokinin on flower and pod abscission of soybeans, Glycine max (L.). J Alabama Acad. Sci. 50:110

Ramina A 1979. Aspects of 8-^{14}C benzylaminopurine metabolism in Phaseolus vulgaris L. Plant Physiol. 63:298-300

Reda AF and O Rasmussen 1975. A modified Amaranthus betacyanin test for cytokinin bioassay. Biol. Plant. 17:368-370

Skoog F and RY Schmitz 1979. Biochemistry and physiology of cytokinins In Biochemical Actions of Hormones. Ed. by G. Litwack, Academic Press, New York

Summons RE, B Entsch, DS Letham, BI Grollnow, JK MacLeod 1980. Regulators of cell division in plant tissue. XXVIII Metabolites of zeatin in sweet corn kernels: Purification and identification using high performance liquid chromatography and chemical ionization mass spectometry. Planta 147:422-434

Summons RE, B Entsch, CW Parker, DS Letham 1979. Mass spectrometric analysis of cytokinins in plant tissue. III Quantitation of the cytokinin glucoside complex of lupin pods by stable isotope dilution. FEBS Letters. 107:21-25

Thomas TH, JE Carroll, FMR Isenberg, A Pendergrass, L Howell 1975. Thin-layer chromatography of cytokinins on a mixed layer of polyvinyl-pyrrolidone and calcium sulphate. J Chrom. 103:211-215

Terrine C, M Laloue 1980. Kinetics of N^6- (Δ^2-isopentenyl)adenosine degradation in tobacco cells. Evidence of a regulatory system under control of cytokinins. Plant Physiol. 65:1090-1095

Van Staden J and JE Davey 1977. The metabolism of zeatin and zeatin riboside by soya bean callus. Ann. Botany 41:1041-1048

Van Staden J and AP Papaphillipou 1977. Biological activity of O-β-D-glucopyranosylzeatin. Plant Physiol. 60:649-650

Wang TL, SG Thompson, R Horgan 1977. A cytokinin glucoside from the leaves of Phaseolus vulgaris L. Planta 135:285-288

Whitty CD and AH Hall 1974. A cytokinin oxidase in Zea mays. Can. J. Biochem. 52:789-799

Yokota T, J Ueda, N Takahashi 1981. Cytokinins in immature seeds of Dolichos lablab L. Phytochemistry 20: 683-686

Young H 1977. Identification of cytokinins from natural sources by gas liquid chromatography-mass spectrometry. Anal. Biochem. 79:226-233

APPENDIX I

Calculation of endogenous cytokinin content of Essex fruits (10-25 mm)

Because of variability between individual bioassays, an overall standard curve was not used. Instead, a series of zeatin standards were included in each assay and a standard curve plotted from the regression equation of log concentration of zeatin versus bioassay response.

Fraction	Regression equation	Zeatin equivalent conc.	Total zeatin equiv. (ng)
I	$y=0.04x+0.493$, $r^2=0.83$	6.3×10^{-8}	105
II		1.5×10^{-9}	0.2
III	$y=0.045x+0.499$, $r^2=0.91$	1.5×10^{-8}	1.8
IV		6.0×10^{-8}	100
V		6.0×10^{-8}	100
VI		3.0×10^{-9}	5
VII	$y=0.021x+0.244$, $r^2=0.61$	4.5×10^{-8}	75
VIII		4.5×10^{-8}	75
IX	$y=0.033x+0.383$, $r^2=0.67$	1.0×10^{-8}	16

APPENDIX II

Calculation of the amount of BAP applied per fruit by a 2.0 mM spray

Assumptions

1. 0.5 ml of 2.0 mM BAP applied per inflorescence
2. 50% of spray actually raeches fruit.
3. There are an average of 10 fruits per inflorescence
4. Average fruit weight is 10 mg

Calculations

1. BAP concentration- 2.0 mM = 450 mg L^{-1} = 450 µg ml^{-1}

2. Amount BAP sprayed - 0.5 ml $inflorescence^{-1}$ x 450 µg ml^{-1} = 225 µg $inflorescence^{-1}$

3. Amount of BAP reaching inflorescence -

   225 µg $inflorescence^{-1}$ x (0.5) = 112.5 µg

4. Amount of BAP per fruit -

   112.5 µg BAP $inflorescence^{-1}$ x 10 fruits $inflor^{-1}$

   = 11.25 µg BAP $fruit^{-1}$

5. Amount of endogenous cytokinin = 30 ng g^{-1}

6. Weight of one fruit = 10 mg, therefore there is 0.3 ng endogenous cytokinin per fruit

7. The BAP exceeds the endogenous cytokinin by $\frac{11250}{0.3}$ or 37500 times.

APPENDIX III

Thin Layer Chromatography

Thin layer chromatography is a powerful technique that can rapidly separate several cytokinins. The small amounts of cytokinins in soybean tissues were generally below the detection limit of the thin layer plates used in this study, therefore the technique was not used extensively in this study.

Part A) Preparation of PVP thin layer plates.

Thin layer chromatography using PVP plates was described by Thomas *et al.* (197) but the procedure described did not discuss the size of the PVP for use. PVP available from Sigma Chemical Co. (St. Louis) has many large particles (greater than 80 mesh) that are not suitable for thin layers. Therefore only material passing through a 200 mesh sieve was used. The small PVP particles were ground in a mortar and pestle and thoroughly mixed with reagent grade $CaSO_4$ in a 1:1 volume ratio. This mixture was ground again and sieved through a 200 mesh sieve. A fluorescent indicator was added to 2% by weight and the mixture slurried in enough water to make a smooth suspension. Plates were made using a Brinkmann TLC spreader. The plates were air dried then eluted with 16 mM KH_2PO_4(25% acetone) to remove UV absorbing interferences.

APPENDIX III continued

Part B) Chromatographic behavior of zeatin, dihydrozeatin and zeatin riboside on different thin layers and solvents.

Thin layer	Solvent[b]	Z	ZR	DZ
Silica gel- hand made	A	0.60±0.05	0.41±0.04	0.58±0.03
Silica gel- Whatman LK6DF	A	0.74±0.01	0.52±0.01	0.74±0.01
Silica gel- Whatman LK6DF	B	0.26±0.02[a]	0.46±0.02[a]	---------
PVP- hand made	C	0.52±0.05	0.60±0.01	---------

[a] Permethylated cytokinins

[b] Solvent systems

A = water saturated n-butanol
B = chloroform:methyl ethyl ketone (1:1, v:v)
C = 0.016 M KH_2PO_4:acetone (3:1, v:v)

The vita has been removed from
the scanned document

IDENTIFICATION OF SOYBEAN, GLYCINE MAX (L.) MERR., CYTOKININS

by

Kevin Edward Crosby

(ABSTRACT)

Soybean, Glycine max (L.) Merr., fruits 10-25 mm long contained nine fractions having cytokinin-like activity detectable by the Amaranthus bioassay. These fractions were derived from purification of extracts from 15 g of immature fruits. The fractions were purified by polyvinylpyrrolidone (PVP), paper, Sephadex LH-20 and high performance liquid chromatography. The fractions separated on the basis of polarity into polar, moderately polar and relatively non-polar groups, compared to zeatin. The polar fraction was the single most bioactive fraction and the active substance was tentatively identified as a zeatin glucoside or zeatin ribosylglucoside. The moderately polar fraction contained bioactive substances that co-eluted with zeatin and zeatin riboside in four chromatographic procedures. The relatively non-polar fraction contained two bioactive components, one chromatographically resembling 2iPA, and the second less polar. A conjugated cytokinin was released from the original aqueous phase by alkaline phosphatase hydrolysis. This substance co-eluted with zeatin riboside on Sephadex LH-20. Further purification and analysis by multiple ion monitoring GC-MS did not yield conclusive

identification of the cytokinins. Based on bioassay, the biological estimates of cytokinin-like substances present in the extracts were below the detection limit of the GC-MS. Obtaining sufficiently large samples of soybean fruits at the proper stage of physiological interest complicated definitive structural identification based on the purification and identification techniques used.