INFLUENCE OF SENESCENCE RETARDANTS ON STORED CABBAGE AND ISOLATION OF CYTOKININS FROM CABBAGE

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

John Kort

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

Department of Plant Science

April 1985

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ABSTRACT

Kort,J., M.Sc., The University of Manitoba, April,1985.

Title: Influence of Senescence Retardants on Stored Cabbage and Isolation of Cytokinins from Cabbage.

Major Professor: Dr. M.K. Pritchard.

Laboratory and field trials were conducted to compare the activity of known, physiologically active compounds in delaying colour loss of winter cabbage (Brassica oleracea L. var. capitata L.). Subsequent to pre-screening tests conducted in the laboratory with cabbage leaf discs, several compounds were selected and sprayed on field-grown winter cabbage prior to harvest. Colour loss of the cabbage, as determined by Hunterlab colorimeter, was reduced by N^6-benzyladenine, silver nitrate, zeatin, and gibberellic acid (GA_3) after six months of storage. N^6-benzyladenine, silver nitrate, and zeatin also reduced fungal incidence in the cabbage.

Attempts to quantify natural zeatin, zeatin riboside and their respective O-glucosides from cabbage tissue by high pressure liquid chromatography (HPLC) or by the Amaranthus betacyanin bioassay were unsuccessful. Coextracted impurities, such as phenols, interfered with their quantification by HPLC and, possibly, with the Amaranthus bioassay. Since standard zeatin and zeatin riboside, which were added to one of the cabbage extract samples, were recovered at 45.7% and 58.8%, respectively, the results also indicate that the cytokinin levels were very low.

On the basis of subjective evaluations made during the extraction procedure, it was concluded that an additional, preliminary purification step needed to be included to reduce the content of impurities such as

lipids and phenols. The use of a polyvinylpyrrolidone (PVP)-packed column was not found to selectively remove all phenols from the tissue extract. The amount of coextracted impurities made the use of thin-layer chromatography (TLC) somewhat impractical as the residue physically impeded solvent movement up the plate.

The procedures used in the extraction, purification and quantification of endogenous cytokinins were also evaluated with standard, aqueous solutions of zeatin and zeatin riboside. Reduction of solvent volume by rotary evaporation was found to contribute to cytokinin losses. The use of a cation exchange resin was suitable for the purification of zeatin and zeatin riboside if the resin was used in the NH_4^+ form and if the adsorption and elution of cytokinins was conducted at 0^oC to prevent hydrolytic breakdown of zeatin riboside.

It was concluded that routine, physical quantification of zeatin, zeatin riboside and their respective O-glucosides from cabbage was possible with some modifications to the procedures used in these studies. The Amaranthus bioassay was found to be both sensitive and convenient, making it a useful bioassay for the confirmation of cytokinin activities in HPLC eluates. It was concluded that identification and quantification of cytokinins from cabbage tissue could be accomplished by the use of HPLC and the Amaranthus betacyanin bioassay.

ACKNOWLEDGEMENTS

To my wife Linda and my children Greta, Peter and Mary, whose constant love and support was essential to the completion of this work.

To Dr. Merv Pritchard for his advice, encouragement and patience.

To Betty Lowey for all the hours she spent over the typewriter.

To Dr. L. Lacroix and Dr. B. Irvine whose advice and insights I found especially valuable.

To Mr. J. Itzke who was very generous in allowing me the use of his storage facilities and donated the produce used in the study.

To Mr. J. Mosiewich who also made his storage facility available to me.

To Dr. C.E. Palmer whose advice was highly valued and who was very good to allow me the use of his laboratory facilities.

To Dr. B. McConnell who made his facilities at the Department of Food Science available to me.

To Mr. Doug Durnin, Mr. George Luther and Mr. Lorne Adam whose help, advice and generous sharing of their laboratory facilities was very important.

To the faculty, technical staff and fellow students who made the working atmosphere so pleasant.

FOREWORD

This thesis is arranged in a paper style format as described in "A Guide to Thesis Preparation for Plant Science Graduate Students" approved by the University of Manitoba Plant Science Department Council on April 29, 1976. Section 1, "Effects of Pre-Harvest Applications of Senescence Retardants on the Quality of Stored Winter Cabbage (Brassica oleracea L. var. Capitata L.)" will be submitted to the "Journal of the American Society for Horticultural Science" for publication, while Section 2, "Cytokinin Extraction from Cabbage (Brassica oleracea L. var. Capitata L.)" will not be submitted for publication, but may be used as background material for future publications based on continuing research to be conducted in this area.

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INTRODUCTION

Senescence in stored winter cabbage is a limiting factor to the storage life of the cabbage, particularly when storage conditions are less than ideal. Primarily, senescence involves the yellowing of the outer leaves which necessitates excessive trimming or makes the cabbage unmarketable. A secondary effect of senescence is a loss of integrity of the cabbage tissue which can lead to fungal infestation.

Senescence retardants, including plant hormones, have previously been demonstrated to be effective in various plant tissues. To overcome the limitation of senescence to the storage life of winter cabbage, information concerning the relative effectiveness of some of these compounds in cabbage tissue and their usefulness as pre-harvest treatments will be required.

In order to guide future studies regarding senescence retardants, it is necessary to have a basic understanding of the natural senescence process. As cytokinins have been implicated as major controlling factors in senescence, it is desirable to determine endogenous levels of active and storage forms of cytokinins in stored cabbage.

Laboratory and field studies were carried out to find senescence retardants which are effective in extending the storage life of winter cabbage. Laboratory studies were also conducted to measure endogenous levels of cytokinins in cabbage tissue.

LITERATURE REVIEW

Senescence

Introduction

Senescence has been defined by Thimann (1980) as "... the deteriorative processes that are the natural causes of death". In detached leaves, these deteriorative processes include primarily a loss of Fraction I protein (RuBP carboxylase), loss of chlorophyll, loss of RNA, and increase in hydrolytic enzymes such as proteases and ribonuclease, followed by ultrastructural deterioration and climacteric increases of CO_2 and ethylene (Thimann, 1980). The most frequently used measure of senescence has been chlorophyll loss, (Thomas, 1977; Beevers, 1976) and the terms 'senescence', 'colour loss', and 'chlorophyll loss' are frequently used interchangeably in the literature.

It is generally accepted by plant physiologists that senescence is controlled by endogenous plant hormone levels (Letham and Palni, 1983; Thimann, 1980; Beevers, 1976).

Cytokinins

Although endogenous cytokinin levels are not strictly correlated with degree of senescence in leaves as measured by chlorophyll levels, (Even-Chen *et al.*, 1978; Thomas, 1977) it is clear from studies involving exogenous applications of synthetic cytokinins, that cytokinins have a

major effect in delaying senescence in leaves (Even-Chen et al., 1978; Biddington and Thomas, 1978). Plant hormone physiologists such as Letham and Palni (1983) and Beevers (1976) now agree that endogenous cytokinins play a role in regulating the natural senescence of plants.

Henson and Wareing (1977) showed that when buds and leaves of Xanthium strumarium L. were detached from the plant, their endogenous cytokinin activity was decreased by 80% within 24 hours and concluded that the cytokinin found in the buds and leaves of intact plants were not synthesized there but were imported from the roots.

Smith (1965) and Went and Bonner (1943), working with wheat (Triticum aestivum L.) apices and tomato (Lycopersicon esculentum Mill.) stem cuttings, respectively, found that root development was necessary for the growth of the shoot tissue, indicating that some root-produced growth factor was required for normal shoot growth.

However, Lee et al. (1974) showed that hadacidin, (N-formyl, N-hydroxy glycine) an inhibitor of adenine synthesis and, they presumed, of cytokinin synthesis could inhibit bud development when locally applied. Since the inhibition was reversible by the local application of kinetin, the authors concluded that synthesis of cytokinins in the bud was indicated. While the issue of site of synthesis of cytokinins is not completely resolved, it is safe to say (Letham and Palni, 1983) that cytokinins are mainly produced in the roots.

Endogenous cytokinins have been isolated from xylem root exudates (Kende, 1965; Morris et al., 1976; Carnes et al., 1975). Kende (1965) showed that cytokinins obtained from sunflower (Helianthus annuus L.) root exudate had activity in maintaining chlorophyll in barley (Hordeum vulgare L.) leaf segments.

Carnes et al. (1975) showed that tomato root exudate contained at least eight different cytokinins and were able to identify the principle cytokinins as cytokinin nucleotides, zeatin, and zeatin riboside. This confirmed the conclusion of Carr and Reid (1967) that, from a wide variety of plant species, the three mobile forms of cytokinins consist of a purine (zeatin), its nucleoside (zeatin riboside), and its nucleotide (zeatin ribotide) (Appendix 1a and 1b).

Letham and Palni (1983) have suggested that the purines, purine nucleosides and purine nucleotides, which were identified by Carr and Reid (1967) in root exudate, represent the translocation forms of cytokinins. In particular, the nucleotide has seldom been found in leaf tissue but commonly occurs in the root exudate. This implies that its function is as a translocation form of cytokinin and that it is quickly converted to other forms when it enters the leaf. Laloue and Pethe (1982) showed with tobacco (Nicotiana tabacum L.) callus, that added nucleotides are quickly degraded or converted to the nucleoside.

The active forms of cytokinin are not conclusively known but Letham and Palni (1983) have suggested that they may consist of the cytokinin free bases and/or their ribosides. Laloue and Pethe (1982), working with tobacco callus, concluded that conversion from the riboside to the free base was necessary for activity.

O-glucosides of cytokinins (Appendix 1b) have been detected at significant levels in leaf tissue (Davey and Van Staden, 1978; Duke et al., 1979). Due to their susceptibility to enzymatic breakdown by β-glucosidase to zeatin riboside and zeatin, O-glucosides were postulated by Duke et al. (1979) to be storage forms of cytokinin. Further evidence

for a storage role for these compounds is cited by Letham and Palni (1983) including the conversion, in plant tissues, of the O-glucosides to cytokinin free bases and their ribosides. Van Staden and Dimalla (1978) found a decline in endogenous O-glucosides accompanied by an increase in zeatin riboside during the breaking of dormancy in potato (Solanum tuberosum L.) tubers.

The final major category of endogenous cytokinins consists of the 7- and 9-glucosides (Appendix 1b) which have been identified in significant quantities in roots and cotyledons of radish (Gordon et al., 1974). When zeatin was applied exogenously to tobacco callus, Laloue and Pethe (1982) identified zeatin 7-glucoside as a primary metabolite. Similarly, Tao et al. (1983) found that zeatin was metabolized primarily to zeatin 9-glucoside when it was applied to oat (Avena sativa L.) leaf segments. These glucosides are very stable and are considered by Letham and Palni (1983) and Laloue and Pethe (1982) to be deactivated forms of cytokinins and are not believed to be important to cytokinin action. In particular, they have little or no activity in delaying senescence in leaves (Tao et al., 1983; Letham et al., 1983).

Letham et al. (1983) found zeatin to have far less activity in delaying senescence in oat leaf segments than N^6-benzyladenine. N^6-benzyladenine was also more effective than zeatin in delaying colour loss when applied to broccoli (Brassica oleracea L. var. botrytis L.) as a dip. This is due to the metabolism of zeatin to the inactive compounds adenosine, adenine nucleotides, zeatin 9-glucoside, and others (Tao et al., 1983). N^6- benzyladenine is not rapidly metabolized or deactiviated, as Tao et al. (1983) found that when it was added to oat leaf segments, free N^6-benzyladenine and a senescence-delaying metabolite were recoverable

after four days.

Letham et al. (1983) found that the O-glucoside of zeatin was the most active natural cytokinin in delaying oat leaf senescence. The authors suggested that this was because the O-glucosyl moiety made the molecule impervious to sidechain cleavage until it reached its site of action in the cell where the glucosyl group was hydrolyzed to release the active molecule, zeatin. The study showed, however, that zeatin O-glucoside was considerably less effective as a senescence retardant than N^6-benzyladenine.

Rapid metabolism of free zeatin, as concluded by Tao et al. (1983) and Manos and Goldthwaite (1975), explains studies such as those of Henson and Wareing (1977) and Even-Chen et al. (1978) who found a rapid decrease of active cytokinin levels in detached leaves of Xanthium strumarium L. and Nicotiana rustica L., respectively. Of particular relevance are the results of Thomas (1977) and Isenberg et al. (1974) who found the same rapid decrease of active cytokinins in leaves of detached brussels sprouts (Brassica oleracea L. var. gemmifera Zenker) and winter cabbage (Brassica oleracea L. var. capitata L.), respectively.

In winter cabbage, which naturally resists senescence, since the cabbage head is an overwintering storage organ, it may be that the levels of O-glucosides are of more importance than free zeatin or zeatin riboside. Dekhuijzen (1980) found in turnip (Brassica campestris L. var. rapa L.) tissue that the level of O-glucosides was eight times that of zeatin and zeatin riboside.

Based on the studies cited, it is likely that zeatin, zeatin riboside and their respective O-glucosides would be the endogenous cytokinins

which affect or regulate the natural senescence and loss of colour of stored cabbage tissue.

Ethylene

Senescence studies with detached tobacco leaves have shown production of ethylene to undergo a climacteric increase during the late stage of senescence (Aharoni and Lieberman, 1979; Even-Chen et al., 1978). Even-Chen et al. (1978) found that the rise in ethylene production coincided with the loss of chlorophyll.

Applied ethylene has been found to accelerate senescence in flowers (Halevy and Kofranek, 1977) and fruit (Saltveit et al., 1978) and there have been some reports of ethylene-induced senescence in leaves (Pendergrass et al., 1976; Burg, 1968). Other studies, however, have shown little effect of applied ethylene on colour loss so that Thimann (1980) concluded that "...ethylene production of the leaves is not a factor in their senescence".

Pendergrass et al. (1976) reported that, in early cabbage stored in an ethylene-containing atmosphere, chlorophyll loss and leaf abscission was increased relative to air-stored cabbage.

Wang (1977) showed that the aminoethoxy analog of rhizobitoxine (aminovinyl glycine) which is known to be a potent inhibitor of ethylene synthesis, was effective in retarding loss of chlorophyll in heads of broccoli when applied as a post-harvest dip.

Silver ion is known to inhibit the action of ethylene (Beyer, 1976). Aharoni et al. (1979) reported an initial increase in ethylene synthesis in tobacco leaf discs which had been treated with silver

nitrate but found that the climacteric rise in ethylene production was delayed and decreased. Aharoni et al. (1979) showed that yellowing of tobacco leaf discs was delayed after they had been floated on a solution of silver nitrate.

Gibberellic acid

Gibberellic acid was found to be an effective inhibitor of colour loss in a few plant species (Beevers, 1966; Fletcher and Osborne, 1965; Goldwaite and Laetsch, 1968; Aharoni et al., 1975). In nasturtium (Tropaeolum majus L.) (Chin and Beevers, 1970) and dandelion (Taraxacum officinale Weber) (Fletcher et al., 1969) which are species in which gibberellic acid delays yellowing, the endogenous gibberellic acid levels decreased during senescence while no such correlation was found in stored cabbage (Isenberg et al., 1974).

Back and Richmond (1969) found that kinetin and gibberellic acid acted similarly in delaying senescence in three gibberellin-sensitive species and suggested that both hormones may act in the same way by binding to a common senescence-inducing protein.

Several studies have been unable to show an effect of exogenously applied gibberellic acid in retaining colour of stored cabbage (Isenberg et al., 1971; Kasukawa et al., 1969).

Abscisic acid

Endogenous abscisic acid levels in leaves of tobacco and nasturtium rise soon after leaf detachment (Even-Chen et al., 1978; Chin and Beevers, 1970). Even-Chen et al. (1978) found the level of abscisic acid in detached tobacco leaves to reach its maximum level within three days and to begin decreasing after the sixth day. The increase corresponded with a decrease in endogenous cytokinins while the ethylene climacteric peak occurred at the seventh day after the abscisic acid level had decreased.

A very similar pattern was found by Isenberg et al. (1974) in stored winter cabbage. Cytokinins decreased in the first four weeks after harvest. Abscisic acid increased between four and eight weeks after harvest and declined to its original level by sixteen weeks. Although the authors did not measure ethylene, they found an auxin peak at twenty-four weeks after harvest, similar to the peak reported by Even-Chen et al. (1978) which accompanied the climacteric ethylene peak.

In the study by Even-Chen et al. (1978), rapid chlorophyll loss began on the fourth day while the abscisic acid level was at its maximum and ethylene production was increasing.

Abscisic acid accelerated senescence in leaf discs of all of eighteen plant species screened by El-Antably et al. (1967).

Abscisic acid is best known for its effectiveness in causing rapid stomatal closure in leaves (Mittelheuser and Van Steveninck, 1969) and Thimann and Satler (1979a) concluded "...that stomatal aperture is the principal controlling agent in leaf senescence," thus implying that the acceleration of senescence in leaves by exogenously applied abscisic acid is mediated through its action in causing stomatal closure.

Interactions among plant hormones

In plant species in which cytokinins and gibberellic acid are active as senescence retardants, their effects are similar. Back and Richmond (1969) showed that gibberellic acid and kinetin were equally active in delaying chlorophyll loss in leaves of dandelion and nasturtium while Beevers (1976) reported equal effects of these hormones in rentention of chlorophyll and protein in nasturtium leaves and Aharoni et al. (1975) showed an additive effect of gibberellic acid and the cytokinin isopentenyladenine in retarding senescence of romaine lettuce. Livne and Vaadia (1965) showed that kinetin and gibberellic acid were both able to increase the transpiration rate in barley leaves. Back and Richmond (1969) proposed the existence of a common acceptor for these hormones to explain their parallel effects.

Cytokinins and abscisic acid appear to be antagonistic in most areas of activity including loss of protein and RNA (Thimann, 1980), chlorophyll loss (Thimann and Satler, 1979a) and stomatal closure (Thimann and Satler, 1979b). Cytokinins inhibit or delay these effects while abscisic acid promotes them. Endogenous cytokinins decreased prior to an increase in abscisic acid in senescing leaf tissue (Even-Chen et al., 1978; Isenberg et al., 1974), and Even-Chen et al., (1978) reported that cytokinins inhibited the production of abscisic acid.

Climacteric ethylene production by senescing tobacco leaves was reported by Even-Chen et al. (1978) to be decreased by kinetin and gibberellic acid but abscisic acid did not affect ethylene levels.

Applied ethylene in stored cabbage, however, did result in a decrease in abscisic acid (Pendergrass et al., 1976). Even-Chen et al.,

(1978) showed that ethylene applied to detached tobacco leaves as Ethrel (2-chloroethane phosphonic acid) also decreased endogenous cytokinin activity but Van Staden and Dimalla (1980) were unable to show that inhibition of ethylene-induced senescence in cut carnations (Dianthus caryophyllus L.) with silver thiosulphate resulted in a maintenance of endogenous cytokinins.

Analytical Techniques for Cytokinin Extraction

Extraction and purification procedure

The traditional method of extracting cytokinins from plant tissue consists of homogenizing the tissue in cold 80% methanol (Horgan, 1978) or ethanol (Miller, 1974). When [^{14}C] zeatin was added to soybean callus, Horgan (1978) reported 100% recovery of the radioactivity as zeatin, zeatin riboside, and zeatin ribotide using 80% methanol as the extracting solvent.

Solvent evaporation is a necessary part of an extraction procedure (Horgan, 1978; Arteca et al., 1980; Dekhuijzen and Gevers, 1975). Little attention, however, has been given to losses that may occur during evaporation even in quantitative studies (Arteca et al., 1980). Dekhuijzen and Gevers (1975) showed that kinetin could be substantially degraded during lengthy evaporative steps and recommended reducing large volumes by freeze-drying or by rapid in vacuo evaporation at high temperatures by rotary evaporation.

Partitioning aqueous solutions with immiscible organic solvents is one method which is commonly used to purify or isolate cytokinins from

plant extracts. The behaviour of the cytokinins depends on their relative solubilities in the two phases and can be characterized by the 'partition coefficient' which is defined by the following equation (Horgan, 1978):

$$\text{partition coefficient} = \frac{\text{conc. of cytokinin in the organic phase}}{\text{conc. of cytokinin in the aqueous phase}}$$

Aqueous cytokinin solutions were shown by Horgan (1978) to have very low partition coefficients when partitioned against petroleum ether. These coefficients indicated that no measurable cytokinins would be transferred to the organic phase during normal partitioning.

Partition coefficients for aqueous cytokinin solutions at pH 8.2 when partitioned against n-butanol were reported by Horgan (1978) to be 11 and 2.1 for zeatin and zeatin riboside, respectively. Letham (1974), using labelled cytokinins, found the partition coefficients of zeatin and zeatin riboside to be 6.26 and 2.2, respectively, when partitioned against n-butanol at pH 7.0. Assuming partitioning of the aqueous solution against four equal volumes of n-butanol, a coefficient of 2.2 would yield 99% recovery of the cytokinin in the organic phase.

Glenn et al. (1972) reported the use of insoluble polyvinyl pyrrolidone (PVP) for the removal of phenols and other impurities from plant extracts. These authors found a 96% recovery of zeatin from a PVP column eluted at pH 8.0 while Biddington and Thomas (1973a) found a 95% recovery of zeatin from a PVP column eluted at pH 6.4 and found that zeatin riboside eluted from the column prior to zeatin. Glenn et al. (1972) reported 60-70-fold reductions in dry weight of Phaseolus and Pisum seedling extracts which had been passed through a PVP column. Thomas et al. (1975) used a PVP column for final separation of cytokinins from extracts of winter cabbage.

Cation exchange resins have been used to separate cytokinin bases and their ribosides from non-polar and negatively charged compounds at acidic pH (Miller, 1974; Van Onckelen and Verbeek, 1972). Miller (1974) reported "...slightly less than complete recovery of activity", upon elution with an ammonium hydroxide-ethanol mixture while Van Onckelen and Verbeek (1972) reported no loss of zeatin or zeatin riboside after adsorption and elution from a cation exchange column. Miller (1974), however, cautioned that hydrolysis of zeatin riboside to zeatin was possible if adequate precaution was not taken to prevent heating during the elution step.

Thin-layer chromatography (TLC) is a frequently used purification step prior to final, analytical chromatography (Miller, 1974; Parker and Letham, 1973; Dekhuijzen and Gevers, 1975; Horgan, 1978). Horgan (1978) reported that TLC of cytokinins on silica gel plates had excellent resolving power but that recoveries of only about 80% should be expected for zeatin. Dekhuijzen and Gevers (1975) found a recovery of 90% of labelled zeatin from silica gel TLC plates.

Quantification by high-pressure liquid chromatography (HPLC)

High-pressure liquid chromatography (HPLC) has been shown to yield excellent resolution of standard cytokinins in nanogram quantities (Stahly and Buchanan, 1982; Kannangara et al., 1978). For final quantification of endogenous cytokinins by HPLC, however, thorough sample purification is necessary. Stahly and Buchanan (1982) reported successful quantification of endogenous cytokinins in pear fruit extracts and Arteca et al. (1980) quantified zeatin and zeatin riboside in potato leaf extracts. Kannangara et al. (1978) were able to find zeatin and zeatin riboside peaks in HPLC-analyzed extracts of Sorghum bicolor L. leaves but

were unable to eliminate relatively large peaks of U V absorbing impurities which eluted close to the cytokinins. Thomas et al. (1975) concluded however, that HPLC quantification of cytokinins from cabbage tissue was not practical using their procedures and relied on the Amaranthus betacyanin bioassay for quantification.

Quantification by bioassay

A thorough study of the activity of a number of naturally-occurring cytokinins by Letham et al. (1983), in the radish (Raphanus sativus L.) cotyledon, Amaranthus betacyanin, tobacco pith callus, soybean (Glycine max Merr.) callus, and leaf senescence bioassays was conducted. The authors found that the Amaranthus betacyanin bioassay could detect 2 ng of zeatin and 8 ng of zeatin riboside while the soybean callus bioassay could detect 3 ng and 9 ng of the respective cytokinins. The sensitivity of the Amaranthus test combined with the short assay time required (Biddington and Thomas, 1973b) make this a very attractive bioassay for measuring zeatin and zeatin riboside. Modifications to this bioassay by Elliott (1979b) including optimum levels of phosphate salts in the incubation medium, optimum incubation temperatures, and aging pretreatments were found by the author to increase sensitivity and reduce variability.

SECTION 1

EFFECTS OF PRE-HARVEST APPLICATIONS OF SENESCENCE RETARDANTS ON THE QUALITY OF STORED WINTER CABBAGE (*BRASSICA OLERACEA* L. VAR. *CAPITATA* L.)

ABSTRACT

Several compounds with senescence-retardant characteristics were evaluated in laboratory and field studies for their effect on retarding colour loss in winter cabbage (Brassica oleracea L. var. capitata L.). Chemicals were screened for their ability to reduce chlorophyll loss in leaf discs and to retain colour in field-grown cabbage after six months of commercial storage. Colour loss of commercially stored cabbage (c. v. Winterkeeper), as measured by Hunterlab colorimeter 'L' and 'b' values, was delayed most by pre-harvest treatments of N^6-benzyladenine and silver nitrate while zeatin was somewhat less effective. Gibberellic acid (GA_3) affected only the Hunterlab 'b' value while benomyl and 2 chloroethyl trimethylammonium chloride (Cycocel CCC) had no effect on cabbage colour. N^6- benzyladenine, silver nitrate, and zeatin reduced fungal infestation in stored cabbage.

INTRODUCTION

Senescence of plants is a deteriorative condition in the tissue involving losses of protein, chlorophyll, and membrane integrity and other related symptoms. Colour loss of leaf tissue can be easily measured and has been frequently used as a measure of senescence (Letham et al., 1983; Thimann and Satler, 1979a; Even-Chen et al., 1978). In cabbage, colour loss is an important aspect of senescence as it influences product salability (Tsujita and Andrew, 1967). A variety of physiologically active compounds have been found to be effective in delaying senescence of leaves of various plant species.

Cytokinins, a class of plant hormones which are involved in cell division and expansion, pigment synthesis, breaking of bud dormancy, and stomatal opening, have been found to be generally effective in delaying all aspects of senescence. N^6-benzyladenine, a synthetic cytokinin, has been shown to effectively delay colour loss when applied exogenously in a variety of vegetable crops (Zink, 1961), including a number of cruciferous crops (Fuller et al., 1977; Dennis et al., 1967; Zink, 1961). Some studies have demonstrated the effectiveness of N^6-benzyladenine in maintaining colour in stored cabbage (Isenberg et al., 1969; Kasukawa et al., 1969; Tsujita and Andrew, 1967).

The natural cytokinin, zeatin, has been shown to delay senescence in leaves but has been found to be less effective than kinetin and N^6-benzyladenine in a number of plant species (Fuller et al., 1977; Varga and Bruinsma, 1973; Letham, 1967). However, Letham (1967) showed that, when Chinese cabbage leaf discs were incubated on solutions of

zeatin or kinetin, the two compounds had similar activity in delaying colour loss, and the author stated that, with radish leaf discs, these compounds were equally active. Fuller et al. (1977) reported that post-harvest dips in zeatin solutions extended the salable storage life of broccoli.

The systemic fungicide, benomyl, was reported by Skene (1972) to behave as a cytokinin in the promotion of growth in soybean callus and the expansion of radish cotyledons. Pressman and Palevitch (1974) showed that benomyl prolonged the storage life of broccoli by maintaining colour when applied as a post-harvest dip.

Compounds other than cytokinins have also been investigated for their senescence retarding properties. Silver nitrate delays senescence when applied to the stem end of cut carnation flowers (Halevy and Kofranek, 1977), as a foliar spray applied to cuttings of geranium (Carow and Bahnemann, 1980), and in tobacco leaf discs floated on a silver nitrate solution (Aharoni et al., 1979). Cycocel (CCC) was reported by Isenberg et al. (1969) and Kasukawa et al. (1969) to maintain colour in stored cabbage when applied as a foliar spray or as a stem end treatment, respectively, but Halevy and Wittwer (1966) found CCC to be ineffective in retarding senescence in cut broccoli when applied as a post-harvest dip. Gibberellic acid (GA_3) has been reported to delay leaf senescence in a number of plant species (Aharoni et al., 1975; Manos and Goldthwaite, 1975; Beevers, 1966; Fletcher and Osborne, 1965) but was reported to be ineffective when applied to stored cabbage (Isenberg et al., 1969; Kasukawa et al., 1969).

This study included a laboratory segment in which a number of reported senescence retardants were pre-screened to determine their effect on colour loss of leaf discs from field-grown winter cabbage. Chemicals which maintained colour in the laboratory tests were applied as foliar sprays to field-grown cabbage immediately prior to harvest and their activity in maintaining colour was evaluated after long-term storage of the cabbage.

MATERIALS AND METHODS

Leaf Disc Pre-Screening

A number of cultivars of winter cabbage were seeded in the greenhouse on April 27, 1981 and were watered and fertilized regularly. On June 2, 1981 the plants were transplanted into the field at the Fort Garry Campus, University of Manitoba, Winnipeg. Cultural conditions in the field included weekly irrigation, periodic applications of the insecticide Thiodan 2E to control flea beetles, and one mid-season application of side-banded fertilizer.

A number of the largest outer leaves were obtained from the cabbage plants at each pre-screening test date. As the pre-screening tests were conducted between May 30, 1981 and July 15, 1981 the leaf material used varied considerably in maturity. Due to the limited amount of material available, especially in the early part of the growing season, it was necessary to gather leaves from a number of cultivars.

In the laboratory, leaf discs 1 cm in diameter were cut from the leaves with a cork borer taking care to avoid major leaf veins. Discs were surface-sterilized for 30 sec in 10% commercial grade sodium hypochlorite and thoroughly rinsed in distilled water prior to being placed on the treatment solutions. The treatment solutions consisted of various concentrations of the senescence retardants in aqueous solution with .05% (v/v) Tween-20, a surfactant. Initial screening (Experiment 1) included compounds in Table 1. The tests were repeated (Experiment 2) using the compounds found to be most effective for reducing colour loss. The compounds and rates used are given in Table 2. Five ml of the

Table 1. Influence of various concentrations of reported senescence retardants on the chlorophyll content (% of control) of cabbage leaf discs incubated in the dark for 14-15 days at 23°C in a preliminary, non-replicated trial (Experiment 1).

Part I (May 29 - June 12)			Part II (June 10 - June 25)			Part III (June 19 - July 2)		
Compound	Conc. (mg/l)	Chlorophyll (% of cont.)	Compound	Conc. (mg/l)	Chlorophyll (% of cont.)	Compound	Conc. (mg/l)	Chlorophyll (% of cont
N^6-benzyl-adenine	25	181	N^6-benzyl-adenine	10	281	N^6-benzyl-adenine	10	304
	50	106		25	180		25	329
				50	108		50	308
GA_3	5	142	Cyclo-heximide	0.5	105	Silver nitrate	2	183
	10	107		1	117		10	217
	25	170		5	179		50	203
				10	185		200	128
Phenazine Methosulfate	1	124	Calcium nitrate	.2g/l	113	L-arginine	25	105
	5	113		2g/l	164		100	113
	10	123		5g/l	111		500	159
	25	121		10g/l	33		1750	46
B-nine (SADH)	10	154	Strepto-mycin	10	78	Benomyl	25	420
	50	194		50	106		50	384
	100	144		100	88		100	418
	500	144		500	108		500	420
Zeatin	1	171	CCC	10	85	Sodium benzoate	5g/l	88
	5	191		50	116		10g/l	303
	10	177		100	136		25g/l	167
				500	171		50g/l	218
Benzimid-azole	25	108	Spermine	5	71	2,4-D	5	31
	50	151		10	89		20	senesced
	100	141		25	60		50	early
	500	deteriorated		50	44		100	"
Kinetin	0.25	126						
	0.5	132						
	5	168						

Table 2. Compounds and their concentrations used in a replicated pre-screening trial (Experiment 2) and storage trials conducted to determine their effects on senescence of winter cabbage.

Compound	Concentration used (mg/1)		
	Leaf disc pre-screening (Expt. 2)	Storage trial 1981-1982	Storage trial 1982-1983
N^6-benzyladenine	2.5, 5, 10, 25	10, 20, 40	40
Zeatin	1, 5, 10, 15	40	40
Benomyl	25, 50, 100, 500	40, 80, 160	80
Gibberellic acid	5, 10, 25, 50	20, 40, 80	80
Silver nitrate	2, 10, 25, 50	150, 300	150
Kinetin	.25, .5, 1.0, 5.0	-	-
CCC	125, 250, 500, 1000	1000, 2000	3000
L-arginine	25, 100, 250, 500	-	-
Sodium benzoate	5*, 10*, 25*, 50*	20*, 40*, 80*	-
Calcium nitrate	.5*, 1.0*, 2.5*, 5.0*	15*, 30*	-
Cycloheximide	2.5, 5, 10, 30	10, 20, 40	-
B-Nine	10, 25, 50, 100	-	-

* g/1

treatment solutions were added to 9 cm Petri dishes containing one disc each of Whatman #1 filter paper. Ten leaf discs were placed in each dish, adaxial surface downwards. Discs were distributed so that, within each test, all dishes were identical with respect to the origin of leaf tissue. As tissue varied in maturity and cultivar from test to test, comparisons between tests could not be made.

The dishes were covered and incubated in the dark at room temperature (23°C) for 13-15 days. In the initial screening (Experiment 1) one dish was incubated for each of four concentrations of each test compound with four control dishes. When the more effective treatments were repeated (Experiment 2), four dishes were incubated for each of four concentrations of each compound and four control dishes were included for each compound tested.

Chlorophyll content of the leaf discs was determined by grinding the tissue with a mortar and pestle with 20 ml of cold 80% acetone, centrifuging, and reading the absorbance of the supernatant at 652 nm on an Hitachi model 100-40 spectophotometer. Total chlorophyll was calculated as per Bruinsma (1963).

Storage Trial (1981-1982)

Winter cabbage (c.v. Houston Evergreen) was seeded in the greenhouse on April 28, 1981 and transplanted into the field in Red River clay soil at the Fort Garry Campus on June 3, 1981. The cabbage was planted in six ranges each consisting of twelve 6.0 m rows spaced at 1 m with 60 cm between plants. Cultural conditions included weekly irrigation, control of flea beetles with Thiodan 2E, and a mid-season fertilizer application.

Aqueous solutions of the senescence retardants listed in Table 2, at the concentrations listed, and an aqueous control, were applied to the cabbage heads 24 h prior to harvest on September 16, 1981. All solutions contained the surfactant Tween-20 at .05% by volume. The field was divided into three blocks consisting of two ranges each. The 23 treatments were randomized within each block, with each row receiving a different treatment.

A bicycle-type sprayer pressured by a compressed air cannister was used to spray the heads to run-off with the appropriate test solution. On September 17, 1981, four uniformly mature cabbage heads were harvested per row, put into nylon mesh bags, weighed, and placed immediately in storage. Bags were separated in storage by replicate.

Storage conditions were maintained as nearly as possible at 0°C and 100% R.H. in storage facilities at the Department of Food Science, Univ. of Manitoba. The storage period was from September 17, 1981 to February 12, 1982. Cabbage quality was evaluated intermittently throughout the storage period. Visual evaluation consisted of a colour rating (1-10) and a rating for decay (0-5). Chlorophyll content was determined by cutting out 1 cm leaf discs at five positions on the head for each evaluation date and extracting in 20 ml of cold 80% acetone as described. Heads were also trimmed and weighed.

Storage Trial (1982-1983)

Due to crop failure at the Fort Garry Campus, field-grown winter cabbage of the cultivar Winterkeeper were obtained from a commercial grower at St. Eustache, Manitoba in 1982-1983, where they were grown on a clay loam soil. Cool growing conditions prevented maturation of the cultivar Houston Evergreen.

A section of field 30 m long and 5.5 m (eight rows) wide was divided lengthwise into five equal blocks 6 m long. Spacing between rows was 60 cm and in-row spacing between plants averaged 1.5 m but was very variable due to poor emergence. Heads of uniform size were selected for treatment and treatments were randomized within each block. In each block, four cabbage heads for each of six treatments were sprayed to run-off while only two heads per block were sprayed with zeatin due to limited availability of the compound. Spraying was by a hand-held sprayer pressured by compressed carbon dioxide. Treatments were aqueous solutions of the compounds listed in Table 2 and a control sprayed with water only.

Spraying was completed on October 15, 1982 and heads were harvested on October 16, 1982. All treated heads were harvested and trimmed to two wrapper leaves. The heads were individually labelled and gently placed in a 1.5 m x 1.5 m x 1 m storage bin with heads from each block being placed together in the bin. The treated cabbage was stored in commercial storage from October 16, 1982 until April 4, 1983 and at the University's Department of Food Science until final evaluation on April 22, 1983. Storage conditions varied from 3-7^{o}C and from 70-80% R.H.

Evaluation was conducted only three times toward the end of the storage period on March 10, April 5, and April 22, 1983 so that handling was minimal to reduce damage. Evaluation on March 10 was a visual colour and decay rating. Visual evaluations for decay and colour were conducted on April 5 and April 22, as well as measurement of weight and reflected colour. Colour measurement was by a Hunterlab colorimeter as described below and proved to be a fast, non-destructive means of objective colour

measurement which was well correlated with visual colour rating, especially on the final evaluation on April 22. As differences between treatments were more pronounced on the final evaluation date, only these results are reported.

The colorimeter was standardized with a standard plate having the values L=65.4, a=-16.2, and b=6.9. Cabbage colour was determined by placing the head over the viewport in five different orientations so that one reading was taken from the top of the head and four readings were taken around its circumference. 'L', 'a', and 'b' values were determined at each orientation and the five readings per head were averaged to give a value for the head.

RESULTS AND DISCUSSION

Leaf Disc Pre-Screening

Experiment 1 was conducted as a non-replicated, preliminary trial of a number of reported senescence retardants (Table 1). Based on the chlorophyll-retaining activity of the compounds, all of the chemicals except phenazine methosulphate, benzimidazole, streptomycin, spermine, and 2,4-D were retained for further, replicated testing.

Leaf discs which were incubated in N^6-benzyladenine, zeatin, kinetin, or GA_3 began curling at the edges shortly after the beginning of the incubation period. All of these compounds delayed chlorophyll loss and since Back and Richmond (1969) showed common effects of kinetin and gibberellic acid in the delay of senescence in leaf discs of Taraxacum officinale Weber and Tropaeolum majus L., the curling of leaf discs may be due to a common mode of action of cytokinins and gibberellins.

Browning of margins of leaf discs treated with high concentrations of N^6-benzyladenine, kinetin, zeatin, silver nitrate, and phenazine methosulphate was observed. This may be related to the ability of a number of chemicals including kinetin and silver nitrate to increase the production of ethylene (Aharoni et al., 1979) which accelerates senescence and is normally produced in large amounts in wounded plant tissue such as the cut surfaces of the leaf disc margins. Early yellowing of leaf discs treated with 50 mg/l N^6-benzyladenine occurred in a clear pattern, beginning at the leaf disc margins and progressing inwards, and may be explainable in the same way.

Silver nitrate treatment at 200 mg/l was found to cause greying of

the leaf disc margins and leaching of chlorophyll and anthocyanins from the leaf discs into the incubation medium was noted. This is likely due to toxicity caused by the high concentration used.

Leaf discs incubated on sodium benzoate solutions showed marked overall browning and physical deterioration although acetone-extractable compounds which absorb at 652 mm remained much higher than that of untreated discs. Sodium benzoate appears to be toxic to cabbage leaf tissue at 10-50 g/l.

In Experiment 2, a replicated test for chlorophyll retention by compounds selected from Experiment 1, it was possible to determine which compounds significantly delayed chlorophyll loss in cabbage leaf discs. Meaningful comparison could not be made among the compounds tested since leaf tissue for each part of the experiment varied with respect to cultivar as well as leaf age, position, and size. Within each part of the experiment, however, it was possible to determine optimal concentrations for use in the field in the subsequent storage trials (Figures 1a-1c).

N^6-benzyladenine, benomyl, zeatin, cycloheximide, GA_3, sodium benzoate, calcium nitrate, silver nitrate, and CCC were selected for further testing in the 1981-1982 storage trial.

Storage Trial (1981-1982)

Fungal infection was evident on the stored cabbage within three months. On December 15, 1981, fungus appeared to be more extensive in cabbage which had been pre-treated with sodium benzoate. This infection spread quickly through the storage so that all heads were more or less infected when the final evaluation was completed on February 12, 1982

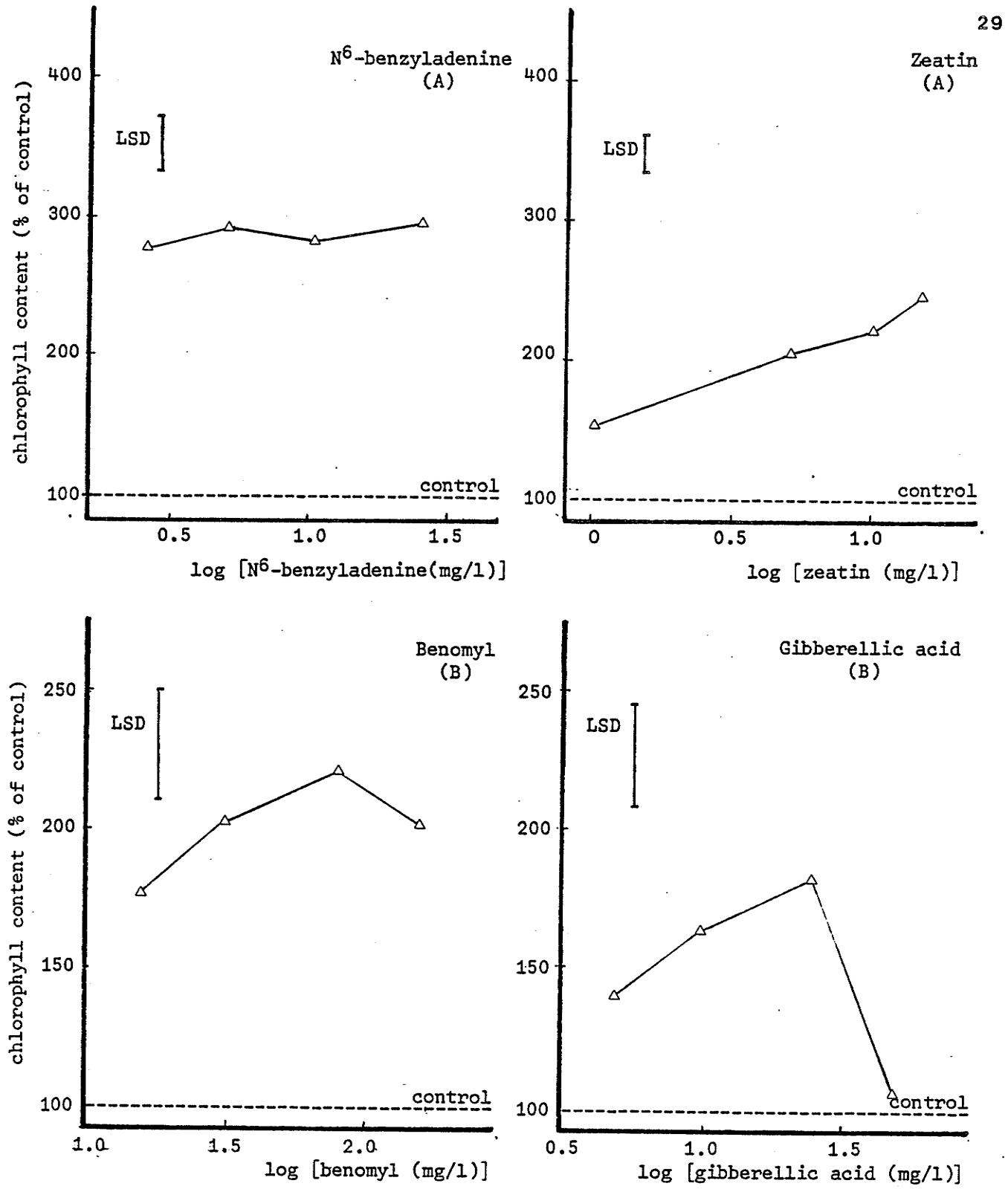

Figure 1a. Chlorophyll content of cabbage leaf discs incubated in the dark at 23°C for 13-15 days on different concentrations of N^6-benzyladenine, zeatin, benomyl or gibberellic acid. Graphs with the same letter (A-F) were generated in the same trial with similar plant material.

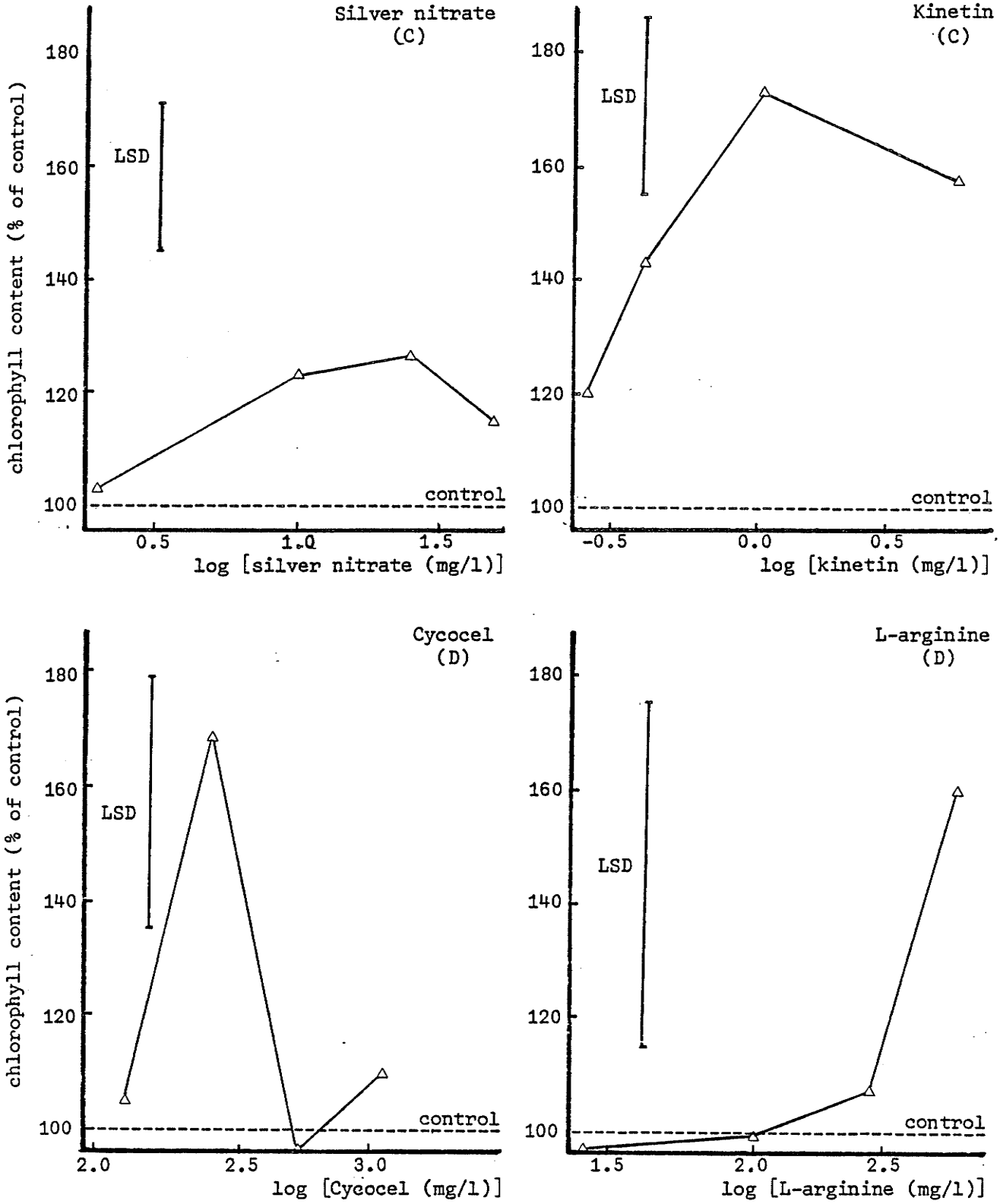

Figure 1b. Chlorophyll content of cabbage leaf discs incubated in the dark at 23°C for 13-15 days on different concentrations of silver nitrate, kinetin, Cycocel and L-arginine. Graphs with the same letter (A-F) were generated in the same trial with similar plant material.

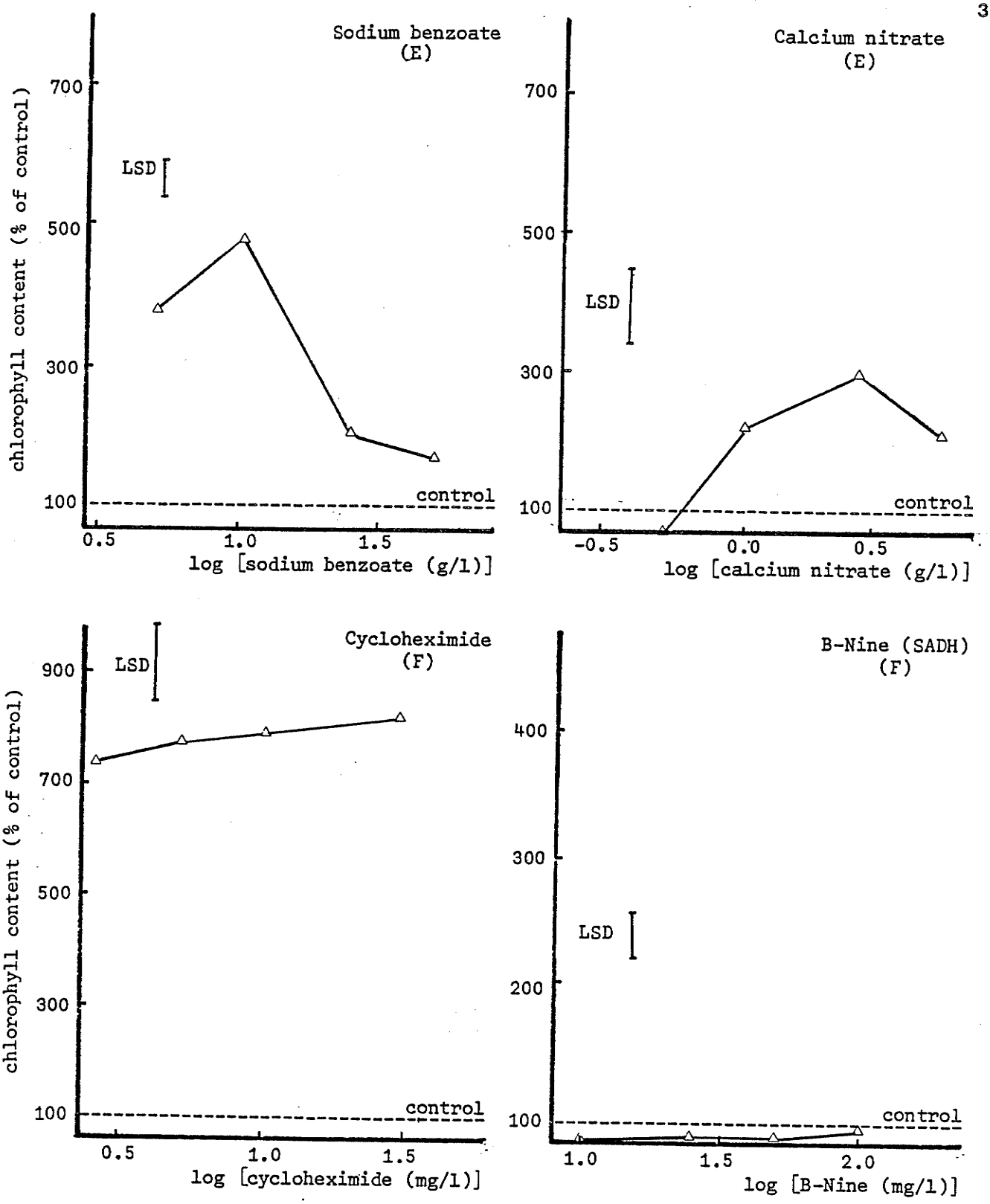

Figure 1c. Chlorophyll content of cabbage leaf discs incubated in the dark at 23°C for 13-15 days on different concentrations of sodium benzoate, calcium nitrate, cycloheximide, or B-Nine. Graphs with the same letter (A-F) were generated in the same trial with similar plant material.

and no treatment differences were observable (Table 3). This problem was attributed to a number of factors. The nylon mesh bags were very abrasive to the cabbage tissue and this, combined with frequent handling necessary for evaluation, resulted in surface wounding of the tissue and in debris collecting in the bags. A relative humidity of near 100% has been found to be optimal for the storage of healthy cabbage tissue (Van den Berg and Lentz, 1973) but in wounded tissue, it is likely a contributing factor to the spread of hydrophilic pathogens such as Botrytis cinerea Pers. (Yoder and Whalen, 1975).

Visual colour rating on February 12, 1982 was found to correlate well with the acetone-extractable chlorophyll levels measured on January 13, 1982 with a correlation coefficient of .5697 so that chlorophyll levels measured on January 13 were used for statistical analysis of colour differences between treatments in the stored cabbage (Table 3).

Statistical analysis of chlorophyll levels by Duncan's multiple range test at the 5% level showed only cabbage treated with 300 mg/l silver nitrate to have significantly higher chlorophyll levels than the control by the end of the trial.

Visual evaluation of colour and decay also showed silver nitrate-treated heads to be somewhat superior to the control. Although chlorophyll levels were not significantly different from the control for any other treatment, cabbages treated with N^6-benzyladenine at 40 mg/l had a higher visual colour rating, a higher level of extracted chlorophyll, and a lower decay rating than the control. GA_3 at 80 mg/l appeared to improve colour retention as measured by visual rating and chlorophyll levels, but had no visible effect on decay.

Table 3. Influence of pre-harvest spray of senescence retardants on weight, colour, and decay of winter cabbage (c.v. Houston Evergreen) stored near 0°C and 100% R.H. in 1981-1982.

Treatment		Visual colour	Chlorophyll	Visual decay score (0-5)[2]		Wt. loss after trimming (Feb 12)
Compound	Conc. (mg/l)	score (1-10)[1] (Feb 12, 1982)	(mg/g fr. wt.) (Jan 13, 1982)	(Dec 15)	(Feb 12)	(kg)
Control	-	3.3	.049	1.8	4.2	.58
N^6-benzyl-adenine	10	3.5	.036	2.8	4.2	.60
	20	4.3	.045	1.8	3.3	.48
	40	5.1	.071	1.3	3.1	.40
Benomyl	40	3.8	.049	1.4	3.4	.57
	80	3.3	.056	2.3	3.5	.52
	160	3.1	.035	2.2	3.9	.54
Zeatin	40	3.6	.052	1.6	3.7	.53
Cycloheximide	10	2.6	.040	2.5	4.3	.54
	20	3.6	.051	2.6	4.3	.46
	40	3.5	.047	2.3	3.7	.49
GA_3	20	3.5	.046	1.9	4.1	.50
	40	4.4	.054	2.1	3.2	.66
	80	5.0	.068	2.1	3.8	.54
Sodium benzoate	20 g/l	2.8	.044	2.6	4.4	.51
	40 g/l	2.7	.048	3.2	4.3	.52
	80 g/l	2.6	.041	3.9	4.5	.55
Calcium nitrate	15 g/l	2.6	.033	2.6	4.1	.77
	30 g/l	2.7	.044	2.7	4.3	.59
Silver nitrate	150	5.6	.066	1.4	2.9	.49
	300	5.3	.081**	1.5	3.3	.57
CCC	1000	4.2	.014	1.9	3.8	.46
	2000	3.3	.038	2.2	4.1	.48

**Significantly different from control at 5% level by Duncan's multiple range test.

[1]Visual colour score: 1=yellow, no evident green colour
10=dark green, colour of fresh cabbage.

[2]Visual decay score: 0=observable decay
5=outer leaves completely decayed.

Based on the results of this storage trial and the pre-screening test, the compounds silver nitrate, N^6-benzyladenine, zeatin, GA_3, benomyl, and CCC were carried forward to the 1982-1983 storage trial.

Storage Trial (1982-1983)

The visually assessed colour of cabbage which had been pre-treated in the field with N^6-benzyladenine, silver nitrate, zeatin, or GA_3 was better than that of the control cabbage after storage from October 15, 1982 to April 22, 1983 (Table 4). Pre-treatments with benomyl at 80 mg/l or CCC at 3000 mg/l had no apparent effect on cabbage colour.

Objective measurement of cabbage colour with a Hunterlab colorimeter (Figure 2) yielded 'L', 'a', and 'b' values. Statistical comparisons of treatment means for each of these colour values, or functions of them, by the Duncan's multiple range test are reported in Table 4. Correlations were conducted between each colour function and visual colour rating for the April 5 and April 22 evaluation dates (Table 5).

The Hunterlab 'b' value was found to be the most meaningful objective measure of colour. The 'b' value was best correlated with visual colour assessment with correlation coefficients of -.6998 and -.8489 for April 5 and April 22, respectively. Since the 'b'-axis measures blueness at the negative end and yellowness at the positive end (Figure 2) this negative correlation indicates that, in winter cabbage, visible colour loss involves an increase in yellowness and/or a decrease in blueness. Both of these effects were observed by Isenberg *et al.* (1971) in winter cabbage stored in modified atmospheres.

Duncan's test at the 5% significance level yielded the greatest number of differences among treatment means when the 'b' value was

Table 4. Comparison of Hunterlab values for determining differences among chemical treatments as they influenced post-storage colour of winter cabbage after storage from October 15, 1982 to April 22, 1983.

Treatment	Mean* visual rating	Hunterlab colour functions** L	a	b	Lxb	a/b
N^6-benzyladenine	8.3	49.8 a	-10.4 a	18.6 a	930 a	-.560 a
Silver nitrate	7.4	52.1 b	-10.3 a	20.3 b	1063 b	-.514 ab
Zeatin	5.6	54.5 c	-10.3 a	21.5 c	1179 c	-.484 bc
GA_3	4.5	54.7 c	-9.5 ab	21.4 c	1175 c	-.451 c
Benomyl	4.0	56.2 cd	-8.9 b	22.9 d	1292 d	-.397 d
Control	4.0	56.5 cd	-8.8 b	23.0 d	1298 d	-.385 d
CCC	3.6	57.0 d	-9.1 b	23.0 d	1313 d	-.395 d

*On a scale of 1 to 10: 1=very pale, 10=dark green.

**Duncan's Multiple Range Test: Values followed by the same letter within each column are not significantly different at the 5% level.

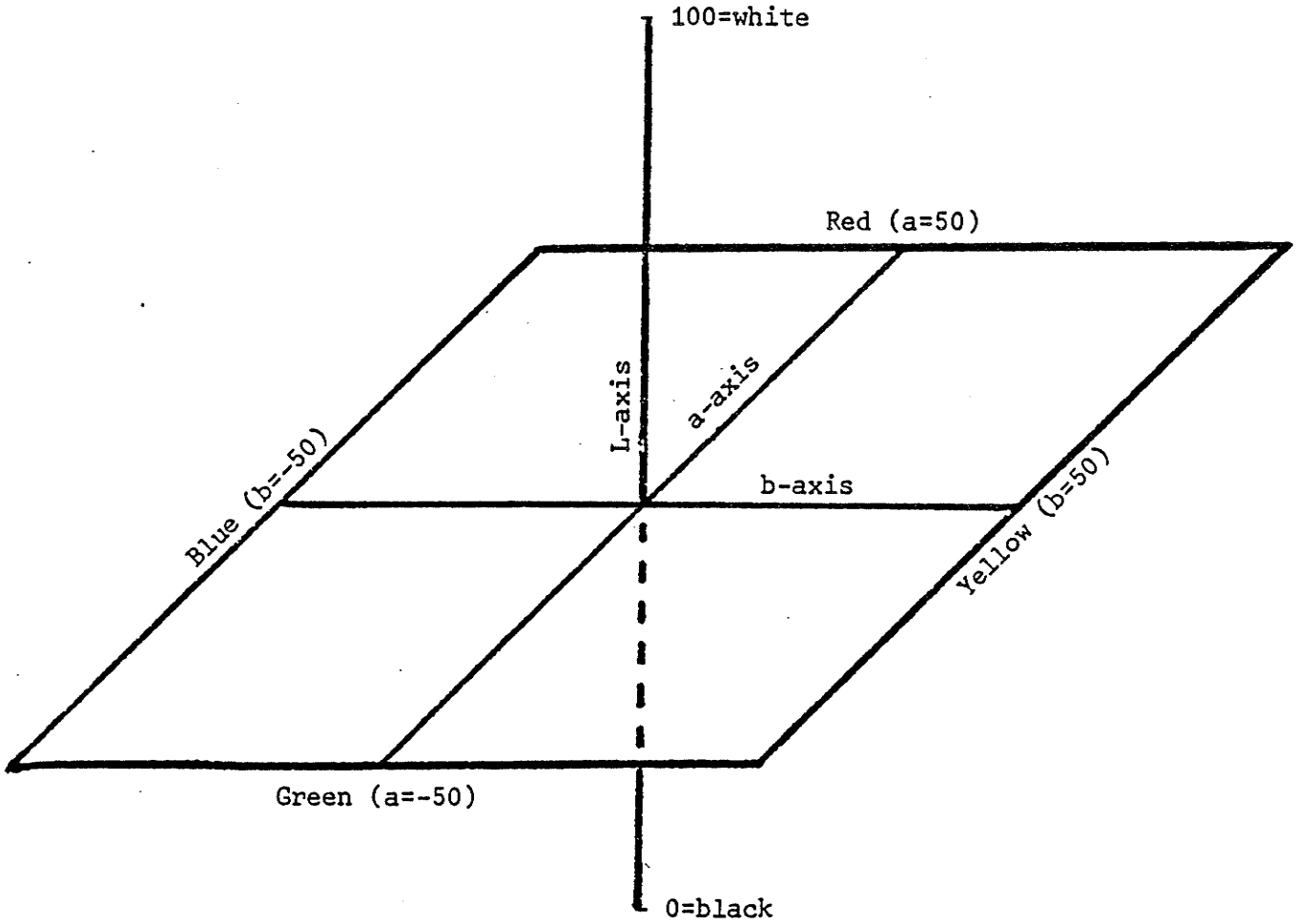

FIGURE 2. Spatial representation of the Hunterlab colour axes.

Table 5. Correlation coefficient (r) between visual colour rating and Hunterlab colorimeter colour functions for stored winter cabbage for April 5 and April 22 evaluation dates.

Colour function	Evaluation date	
	April 5/83	April 22/83
L	-.6586	-.8232
a	-.1966	-.5724
b	-.6998	-.8489
a/b	-.6965	-.8350
Lxb	-.7025	-.8484
$(a^2+b^2)^{1/2}$	-.6303	-.7614

considered (Table 4). N^6-benzyladenine, silver nitrate, zeatin, and GA_3 treatment means for the 'b' values were higher than the control mean. Cabbages treated with these compounds were therefore bluer and/or less yellow than untreated cabbage. Isenberg et al. (1971) found higher 'b' values of cabbage which had been stored in a modified atmosphere (MA) of 5.0% CO_2 and 1.0% O_2 as compared to cabbage stored in air and suggested that this was due to a retention of the bluish waxy bloom in MA storage.

The 'L' value was negatively correlated with visual colour ratings with coefficients of -.6586 and -.8232 for the April 5 and April 22 evaluations, respectively (Table 5). The 'L' axis measures darkness on a scale of 0 (black) to 100 (white) so that poorer visual ratings were given to lighter coloured heads. Isenberg et al. (1971) found that use of the 'L' value in cabbage which had been stored in different modified atmospheres resulted in the most significant differences among treatment means and concluded that the 'L' value "... is probably as good a measure of senescence in mathematical units as can be determined." Analysis of treatment means with the 'L' value as measurement showed that N^6-benzyladenine and silver nitrate prevented fading in stored winter cabbage (Table 4) while no other treatments affected the 'L' value of cabbage relative to the control.

The Hunterlab 'a'-axis measures greenness when negative and redness when positive. Measured 'a' values for stored cabbage were correlated negatively with visual colour assessment although the correlation coefficient was very low on April 5 with a value of -.1966 (Table 5). The coefficient for the April 22 evaluation was much higher with a value of -.5724 but was still considerably lower than that of either the 'L' or

'b' values. This correlation means that a poor visual colour assessment is associated with a decrease in greenness and/or increase in redness.

The 'a' value was found by Isenberg et al. (1971) to show more differences between treatment means in chemically pre-treated, stored cabbage than the 'L' or 'b' values, but Isenberg and Sayles (1969) found the 'L' and 'b' values to be the most useful in measuring colour differences in cabbage which had been stored in modified atmospheres. Cabbage which had been pre-treated with N^6-benzyladenine, silver nitrate, or zeatin had lower 'a' values than the control cabbage and thus were greener and/or less red.

Although carotenoids are known to accumulate in senescing fruits (Britton 1976), Thimann (1980) reported a decrease of carotenoids in senescing oat leaves. Therefore, the changes in the 'L', 'a', and 'b' values were interpreted to reflect changes in chlorophyll content and in the bluish waxy bloom which is normally found on winter cabbage at harvest rather than changes in carotenoid levels. Chlorophyll levels affect all three Hunterlab values, so that functions such as 'a/b' which combine these values have been recommended as valid measures of chlorophyll content (Francis and Clydesdale, 1975). However, in this study no function of the Hunterlab values was better correlated to visual colour rating than the 'b' value alone (Table 5) and no function showed more differences among treatment means than did the 'b' value (Table 4).

With the 'b' value as objective measure of cabbage colour, the following treatment effects were observed. N^6-benzyladenine-treated cabbage retained colour more than any other treatment. Although treatment with zeatin also improved colour retention, it was less effective than the synthetic cytokinin, N^6-benzyladenine. This may reflect a difference in

metabolism of the two compounds in the cabbage as Tao et al. (1983) reported rapid metabolism of zeatin in oat leaf tissue while N^6-benzyladenine was not as rapidly metabolized.

Silver nitrate effectively reduced colour loss in stored cabbage. Silver ions were shown by Beyer (1976) to inhibit ethylene action. Thimann (1980) stated that ethylene was not an important factor in the normal senescence of leaves, but Pendergrass et al. (1976) reported that storage of early cabbage in 100 ppm ethylene accelerated colour loss. The ability of silver nitrate to retard colour loss in stored cabbage implies that ethylene is normally an important factor in the natural senescence of leaves.

GA_3 was somewhat effective in retaining colour of stored cabbage as it was in cabbage leaf discs. This is in contrast to the results of Isenberg et al. (1971) who reported no effects of GA_3 on stored cabbage, and Kasukawa et al. (1969) who reported accelerated senescence and increased leaf damage in cabbage due to GA_3. This may be a concentration dependent effect since Isenberg et al. (1971) used 500 and 1000 mg/1 GA_3 and Kasukawa et al. (1969) used 200 mg/1 GA_3. In this study, 80 mg/1 GA_3 was used on the basis of the pre-screening test (Figure 1b).

Benomyl had no significant effect on colour but this may be because the concentration used was too low. The level used in this study was 80 mg/1 which corresponded to the optimum level for cytokinin-like activity reported by Skene (1972) and was indicated by the pre-screening test (Figure 1b). However, Pressman and Palevitch (1974) reported colour retaining activity of benomyl when applied to broccoli at 2000 mg/1.

CCC applied at 3000 ppm had no effect on colour in stored cabbage.

This contrasts with the results of Kasukawa et al. (1969) who reported anti-senescence activity of CCC at 500 ppm and Isenberg et al. (1971) who found an effect of CCC at 500 ppm and 1000 ppm. A concentration of 3000 ppm may be too high as the pre-screening test showed a significant effect of CCC at only one concentration (Figure 1b). However, the storage and pre-screening test results indicate that the effects of CCC are variable and it may be that the differences between these results and those cited above depend on cultivar, method of application, or storage conditions.

Although chemical treatments did not affect head weight, silver nitrate, zeatin, and N^6-benzyladenine reduced fungal infestation (Table 6). It is possible that this effect is a secondary one resulting from the senescence-retarding activity of these compounds since none of them have been reported to be fungicidal.

It is notable in this regard, however, that benomyl, a commercially available systemic fungicide, is reported to have cytokinin-like properties (Skene, 1972). Possibly, some of its anti-fungal activity is attributable to its effect on senescence. The lack of activity of benomyl in this study is likely attributable to the low concentration used.

In summary, colour loss of commercially stored cabbage was substantially reduced under normal storage conditions by pre-harvest applications of N^6-benzyladenine or silver nitrate. These results point out the importance of cytokinins and ethylene, respectively, as controlling factors in the senescence process. Zeatin had some effect in reducing colour loss but was less effective than N^6-benzyladenine, likely due to its metabolism by the tissue. GA_3 was also somewhat effective in maintaining colour.

Table 6. Effect of pre-harvest chemical treatments on post-storage decay and weight of winter cabbage in 1982-1983.

Treatment	Decay[1] (visual rating) (April 22/83)	Wt. increase*(kg/head)** (April 5 - April 22/83)
Silver nitrate	0.1	.14 a
Zeatin	0.1	.13 a
N^6-benzyladenine	0.2	.12 a
Benomyl	1.0	.12 a
Gibberellic acid	1.1	.11 a
CCC	1.1	.13 a
Control	1.2	.13 a

[1]Based on a scale of 0=no decay to 5=poor.
**Duncan's Multiple Range Test: Value followed by the same letter are not significantly different at the 5% level.
*Head weight increase over this period is likely due to a higher R.H. on April 22, as the cabbage heads had water droplets on them at that time.

Zeatin, silver nitrate, and N^6-benzyladenine reduced fungal infection of cabbage to a large degree, but this effect may be secondary, resulting from their anti-senescence activity.

SECTION 2

CYTOKININ EXTRACTION FROM CABBAGE (*BRASSICA OLERACEA* L. VAR. *CAPITATA* L.)

ABSTRACT

Quantification of endogenous cytokinins from cabbage tissue by high pressure liquid chromatography (HPLC) and the Amaranthus bioassay was undertaken to determine whether a relationship existed between endogenous cytokinin levels and cabbage storage quality. Although actual quantification was not successful due to interference from coextracted impurities, recovery of added zeatin and zeatin riboside standards was determined by HPLC to be 45.7% and 58.8%, respectively.

In this study, techniques for the extraction, identification and quantification of zeatin, zeatin riboside and their respective O-glucosides from cabbage tissue were evaluated and modified to improve their applicability. It was concluded that reduction of extract volume by rotary evaporation should be minimized to reduce possible cytokinin losses and breakdown. Additional steps need to be included in the extraction and purification procedure to reduce the levels of impurities such as lipids and phenols which are coextracted. Coextracted impurities which were UV absorbent at 254 nm and coeluted with zeatin and zeatin riboside from an HPLC reversed phase column, presumed to be phenols, interfered with physical quantification of cytokinins by HPLC. Selective removal of phenols by adsorption on insoluble PVP was not found to be sufficiently effective to prevent such compounds from interfering with final cytokinin quantification. Purification of zeatin and zeatin riboside by cation exchange chromatography was found to be suitable if the cation exchange resin was initially in the NH_4^+ form and if adsorption and elution of cytokinins were conducted in a slurry at 0°C to prevent hydrolytic breakdown of zeatin riboside. The effectiveness of TLC chromatography in

preliminary cytokinin separation was reduced by the large amount of impurities coextracted and may need to be substituted with another separatory step such as column chromatography. In general, it was concluded that HPLC quantification of endogenous zeatin, zeatin riboside, and their respective O-glucosides from cabbage tissue is likely with some improvement in purification procedures.

INTRODUCTION

Endogenous cytokinins have been implicated as controlling factors of senescence in intact plants (Letham and Palni, 1983; Beevers, 1976).

Exogenous applications of a number of natural cytokinins, including zeatin, zeatin riboside and dihydrozeatin (Appendix 1a) have been shown to delay yellowing of green plant tissue (Fuller et al., 1977; Manos and Goldthwaite, 1975; Letham et al., 1983). These compounds, however, are less effective in retaining leaf colour than the synthetic cytokinins, kinetin and N^6-benzyladenine (Appendix 1a) (Varga and Bruinsma, 1973; Letham, 1967). Manos and Goldthwaite (1975) showed that the colour retention in leaf discs of Rumex obtusifolius L. incubated on a zeatin-containing medium was greater if the medium was replaced periodically during the experiment. They concluded that zeatin was rapidly deactivated metabolically in this tissue.

Studies regarding the metabolism of cytokinins have recently been reviewed by Letham and Palni (1983). Zeatin and zeatin riboside are reported to be metabolized primarily to glucosides. Parker and Letham (1973) identified zeatin 7-glucoside (7G -Z) (Appendix 1b). The principle metabolite of zeatin in cotyledons of derooted radish (Raphanus sativus L.) seedlings, and found that 25% of the added zeatin was metabolized into this form within fifteen hours. Dekhuijzen (1980) found that the majority of bound cytokinins in turnip (Brassica campestris L. var. rapa L.) root tissue were β-glucosidase labile O-glucosides (Appendix 1b).

Letham and Palni (1983) suggested that [7G]-Z may be a primary deactivation form of cytokinin, naturally lowering endogenous levels of active cytokinins. They did not believe it to represent a storage form of active cytokinin due to its stability and insensitivity to enzymatic attack. Letham et al. (1983) found [7G]-Z to be inactive in any standard cytokinin bioassay.

By contrast, Letham and Palni (1983) supposed O-glucosides to be storage cytokinins, normally releasing active cytokinins when required by the plant. O-glucosides are susceptible to hydrolysis by non-specific β-glucosidases and are active in standard cytokinin bioassays (Letham et al., 1983).

Naturally occurring cytokinins in cabbage (Brassica oleracea L. var. capitata L.) tissue decrease soon after harvest and remain low throughout the storage period (Isenberg et al., 1974). On the basis of the previously cited metabolic studies, this decrease can be explained by the metabolism of the active cytokinin free bases. In view of the high levels of O-glucosides reported by Dekhuijzen (1980) to occur in turnip tissue, it may be that zeatin and zeatin riboside were metabolized to O-glucosides in the stored cabbage tissue as well.

The purpose of this study was to isolate and quantify endogenous zeatin, zeatin riboside and their respective O-glucosides in cabbage tissues by purification, high pressure liquid chromatography (HPLC), and bioassay in an attempt to understand their role in the control of senescence.

Techniques for the extraction and isolation of natural cytokinins

from plant tissue have been reviewed by Horgan (1978). The quantification of cytokinins by HPLC and bioassay have been discussed by Stahly and Buchanan (1982) and Letham et al., (1983) respectively. The procedure used in any particular extraction depends on the plant material used and the cytokinins under study so that few studies in the literature report the use of identical extraction, identification and quantification procedures.

Extraction of endogenous cytokinins in cold 80% methanol or ethanol was recommended by Horgan (1978). Extraction volumes should be minimal since Dekhuijzen and Gevers (1975) found that major kinetin breakdown resulted from extended evaporation.

Solvent partitioning of aqueous cytokinin solutions with petroleum ether did not remove any significant amounts of zeatin, zeatin riboside or zeatin 9-glucoside ([9G] -Z) (Appendix 1b) from the aqueous phase at pH 2.5 or 8.2 (Horgan, 1978). This procedure would, therefore, be a safe way of removing non-polar compounds from aqueous plant extracts. Based on reported partition coefficients, solvent partitioning with water-saturated n-butanol at pH 7.0 or more could be considered a suitable way of purifying zeatin, zeatin riboside and other free cytokinins (Horgan, 1978; Letham, 1974). The partition coefficient for [9G] -Z was too low at pH 8.2 (Horgan, 1978), however, to justify n-butanol partitioning to purify this cytokinin, and no partition coefficients were reported for other, naturally-occurring cytokinin glucosides.

Loomis and Bataille (1966) found that insoluble polyvinylpyrrolidone (PVP) selectively adsorbed phenols from plant extracts by hydrogen bond formation. Glenn et al., (1972) reported the use of columns of PVP to

remove the majority of impurities from crude plant extracts from a number of plant species while known amounts of standard zeatin were quantitatively recovered. Kannangara et al., (1978), Palni et al., (1983) and other researchers have found PVP chromatography to be suitable means of purifying cytokinins.

Cation exchange purification of cytokinins by strong cation exchange resins was reported by Horgan (1978) to give consistently high cytokinin recoveries (≥90%) with the cautions that the resin be used in the NH_4^+ form rather than the H^+ form to prevent cytokinin breakdown and the elution be done with 1N NH_4OH in 50% methanol rather than in aqueous solution to optimize recovery efficiency. Miller (1974) recommended the use of precooled elution solvent to prevent heating due to cation elution which had been found to cause hydrolysis of zeatin riboside to zeatin.

Horgan (1978) reported thin-layer chromatography (TLC) to give excellent resolution of cytokinins. Stahly and Buchanan (1982) found good separation of zeatin and zeatin riboside on a silica gel TLC plate using water-saturated n-butanol as the developing solvent. They recovered 80.4 ± 4.4% and 78.5 ± 6.1% of standard zeatin and zeatin riboside, respectively, when the silica gel was thoroughly washed with 90% methanol.

High-pressure liquid chromatography (HPLC) has been used for final quantification of endogenous cytokinins from pear (Pyrus communis L.) fruit (Stahly and Buchanan, 1982) and from potato (Solanum tuberosum L.) leaves (Arteca et al., 1980) but Thomas et al., (1975) had to use bioassay to quantify cytokinins from cabbage tissue due to the UV-absorbing impurities present in the HPLC trace.

Routine quantification of cytokinins from cabbage tissue using the Amaranthus betacyanin bioassay (Biddington and Thomas, 1973b)was

reported by Isenberg et al., (1974) and Thomas et al., (1975). Letham et al., (1983) were able to measure smaller cytokinin quantities with this bioassay than with the tobacco (Nicotiana tabacum L.) and soybean (Glycine max Merr.) callus growth bioassay.

MATERIALS AND METHODS

Extraction of Endogenous Cytokinins from Cabbage Tissue

The procedure for the extraction of endogenous cytokinins summarized in the flow chart in Figure 3 was followed in the case of cabbage tissue. Chronologically, this extraction followed the evaluation and modification of the individual steps in the procedure as described in the section following this one. Although the extraction was conducted only once exactly as described here, the results of this extraction followed a number of preliminary attempts which led to the refinement of the procedure to the form described here.

Storage cabbage of unknown cultivar was commercially obtained and 500 g of tissue from the centres of two heads was removed. The tissue was homogenized in 500 ml of 80% ethanol at room temperature (22°C) and, after 10 hours, the homogenate was vacuum filtered on Whatman No. 1 filter paper. The solid residue was homogenized in 500 ml of fresh 80% ethanol and was refiltered after a further 10 hours. The filtrates were combined and divided into three equal volumes which were labelled S, E and B which were treated as described below. To sample S, 70µg of standard zeatin and 40 µg of standard zeatin riboside were added (i.e. 2 ml each of 35 mg/l zeatin and 20 mg/l zeatin riboside), while no standards were added to samples E or B.

Ethanol was evaporated from all three samples by rotary evaporator at 50°C leaving an aqueous volume of about 100 ml. Since a large amount of water insoluble substances came out of solution during this step, the aqueous extracts were filtered through Whatman No. 1 filter paper. The

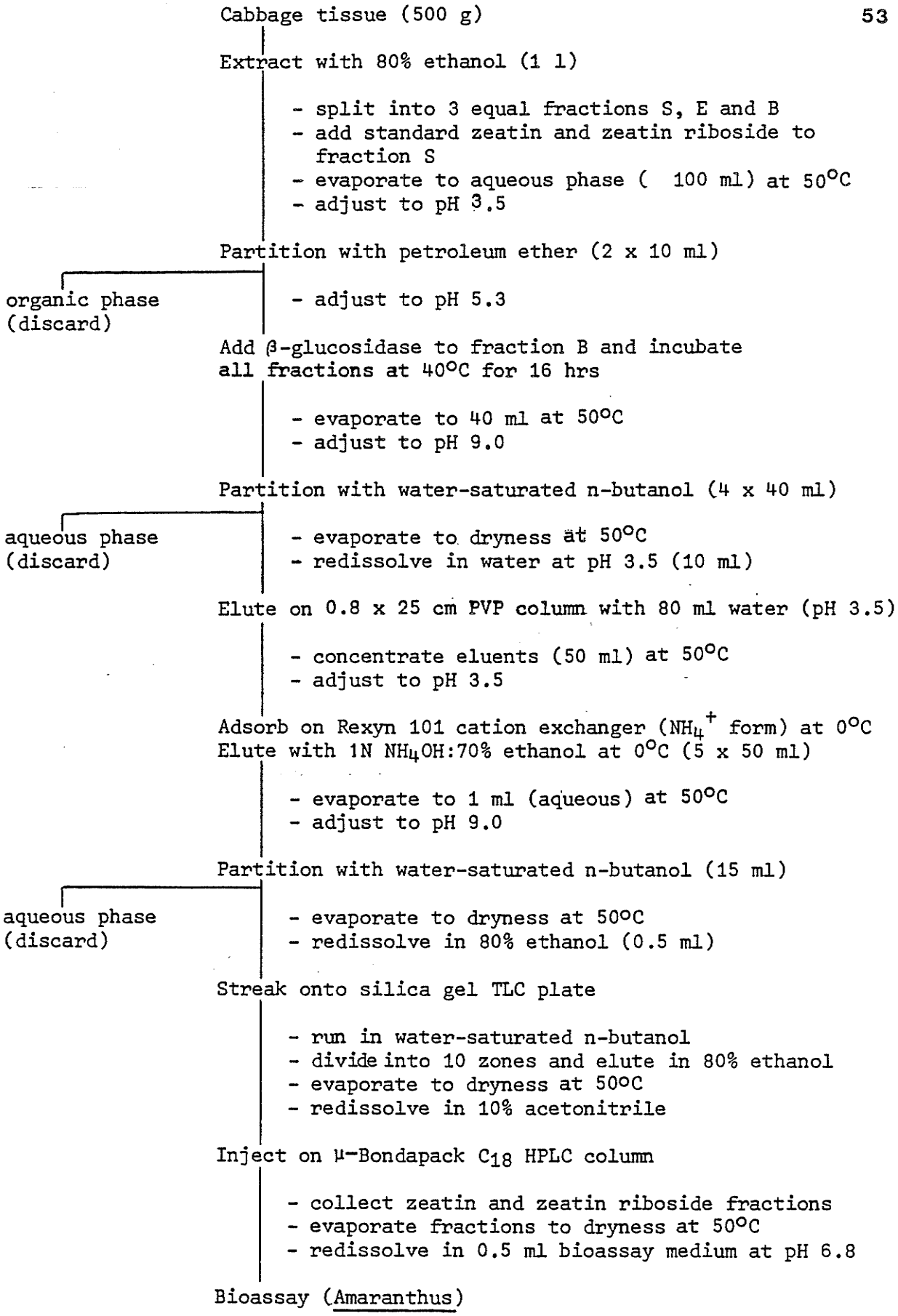

Figure 3. Flowchart for extraction and purification of cytokinins from cabbage tissue.

filtrates were then adjusted to pH 3.5 and partitioned with 2 x 10 ml of petroleum ether to remove any remaining, water insoluble, petroleum ether soluble, non-polar compounds.

The extracts were adjusted to pH 5.3 and 20mg of β-glucosidase were added to sample B to convert any O-glucosides of zeatin or zeatin riboside into their active or free forms. All three samples were incubated in a water bath at 40°C for 16 hours. The samples were then evaporated to 40 ml by rotary evaporator at 50°C and adjusted to pH 9.0. They were partitioned with four equal volumes (4 x 40 ml) of water-saturated n-butanol. The combined n-butanol volumes were evaporated to dryness at 50°C and redissolved in 10 ml of distilled water.

The 10 ml aqueous samples were adjusted to pH 3.5 and loaded onto 0.8 x 25 cm columns packed with 7.0 cm^3 of insoluble PVP[1]. The columns were then washed with 80 ml of water at pH 3.5 to ensure complete elution of zeatin and zeatin riboside. The eluent samples of 90 ml each were evaporated by rotary evaporator at 50°C to final volumes of 50 ml each.

The procedure used in the cation exchange step is based on preliminary tests using aqueous standard zeatin riboside solutions as described on page 60. Small differences in procedure were adopted to deal with increased volumes of the cabbage extracts and to ensure maximum adsorption and elution of endogenous cytokinins. The 50 ml aqueous samples from the previous step were adjusted to pH 3.5. Three 250 ml Ehrlenmeyer flasks containing 40 cm^3 each of the cation exchange resin

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[1]The insoluble PVP was obtained from Sigma Chemicals Ltd. and fine particles were removed by slurrying some PVP in distilled water and allowing the larger particles to settle before pouring off the supernatant with all the particles still in suspension.

Rexyn 101[2] in the NH_4^+ form which had been previously washed with water at pH 3.5, were placed in an ice bath prior to cytokinin adsorption. The 50 ml aqueous extracts were pre-cooled in an ice bath and then added to the Rexyn 101 slurry. The mixture was stirred in the ice bath for 1.5 hours to permit total adsorption of the cytokinins. The cation exchange beads were allowed to settle and the aqueous supernatant solutions were poured off and discarded. The Rexyn 101 was then washed with 80 ml of cold distilled water which was also poured off. Forty ml of cold distilled water were added to each flask to just cover the cation exchange resin to absorb the heat of elution. These slurries were stirred in an ice bath while 50 ml of ice cold 1N NH_4OH in 70% ethanol was added dropwise over a 15 min interval. After stirring for an additional 15 min the supernatants were poured off and retained. The cation exchange packing was then washed with four more consecutive 50 ml volumes of ice cold 1N NH_4OH in 70% ethanol to ensure maximum elution of the cytokinins. The supernatants for each sample were combined for a total volume of about 280 ml per sample, vacuum filtered on Whatman No. 42 filter paper to remove any fine cation exchange particles, and evaporated to 1 ml aqueous solutions by rotary evaporator at 50°C.

The 1 ml samples were adjusted to pH 9.0 and were shaken in a centrifuge tube with 15 ml of water- saturated n-butanol. The mixtures were centrifuged and the aqueous phase was discarded. The organic phase was evaporated to dryness by rotary evaporator at 50°C.

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[2]Rexyn 101 is a strong cation exchange sulfonated polystyrene-divinyl-benzene copolymer produced by Fisher Scientific Ltd. similar to Dowex 50W-X8.

The residues were redissolved in 0.5 ml of 80% ethanol and streaked onto fluorescent silica gel TLC plates. The plates were then run in water-saturated n-butanol, dried, and the chromatograms were divided into ten equal fractions. Since simultaneously run chromatograms of standard zeatin and zeatin riboside showed Rf values of .70 and .62, respectively, only fractions 5 to 8 (Rf = .40 to .80) were selected for further analysis. These fractions were scraped from the TLC plates into separate centrifuge tubes. Three ml of 80% ethanol were added to each tube and the slurries were thoroughly mixed and then centrifuged. The supernatant ethanol eluate was poured off and retained and the procedure was repeated four times. The combined supernatants for each fraction (15 ml) were evaporated to dryness by rotary evaporator at 50°C.

Fractions 5 to 8 of sample S, the standard cytokinin treated cabbage extract, were redissolved in 2 ml of 10% acetonitrile and the recovered levels of standard zeatin and zeatin riboside were determined by quantification by HPLC. The procedure for cytokinin quantification by HPLC is described in a later section and the set up is depicted in Figure 4. The UV absorbance trace at 254 nm was recorded for compounds eluting from the column.

Two runs each of the original standard cytokinin solution and of the recovered fractions from sample S, the cytokinin treated extract, were made and compared to determine percentage recovery of zeatin and zeatin riboside. The zeatin and zeatin riboside peaks were collected separately from sample S as they eluted from the HPLC column to further

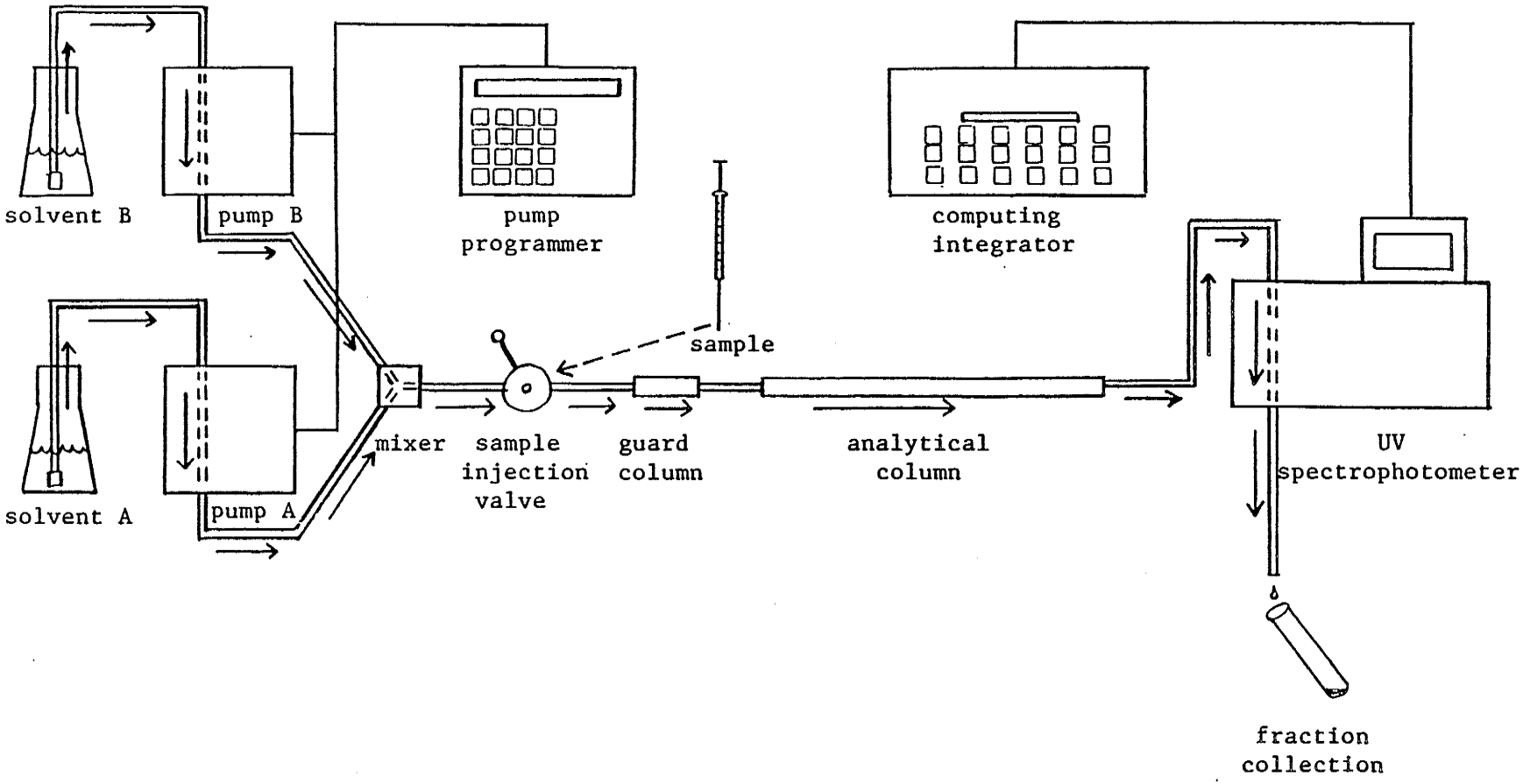

Figure 4. Schematic representation of HPLC system for separation and quantification of cytokinins.

isolate them. These fractions were evaporated to dryness by rotary evaporator. Since the HPLC sample loop had a volume of 20 µl, a total of 40 µl was injected onto the column for each fraction. To maintain the same cytokinin concentrations in the injected samples after this purification step, the residue was taken up in 40 µl of 10% acetonitrile and rerun on the HPLC column. The zeatin and zeatin riboside peaks were again collected as they eluted from the HPLC column, evaporated to dryness, redissolved in 1.5 ml of the Amaranthus incubation medium and were tested in triplicate by the Amaranthus bioassay as described in a later section.

Since only TLC fraction 6 (Rf = .50 to .60) from sample S, the cytokinin treated cabbage extract, contained zeatin or zeatin riboside, only fraction 6 from sample E, the untreated extract, and sample B, the β-glucosidase treated extract, were redissolved in 100 µl of 10% acetonitrile for further analysis. Two runs of each fraction were made on the HPLC and fractions corresponding to the retention times of zeatin and zeatin riboside were collected, evaporated to dryness, and taken up in 40 µl of 10% acetonitrile. These purified samples were rerun on the HPLC. As with sample S, the cytokinin treated cabbage extract, the fractions corresponding to zeatin and zeatin riboside with respect to elution time, were again collected, evaporated, redissolved in 0.5 ml of the Amaranthus incubation medium, and bioassayed. Each fraction was bioassayed in triplicate.

Extraction of Standard Cytokinins from Aqueous Solution

To test the suitability of the individual procedures employed in the extraction of cytokinins from cabbage tissue, aqueous solutions of zeatin and zeatin riboside standards (20 mg/l) were made up, extracted by the given procedure and recoveries were determined by HPLC as discussed below.

The possible loss of cytokinins by evaporation was evaluated. Two ml of 20 mg/l zeatin or zeatin riboside were added to 800 ml of 80% ethanol. The samples were evaporated to dryness by rotary evaporator at 35°C and redissolved in 2 ml of distilled water. The recovered cytokinins were then quantified by HPLC and compared against the original cytokinin levels.

Petroleum ether partitioning was evaluated by adding 2 ml of 20 mg/l zeatin or zeatin riboside to 38 ml of distilled water adjusted to pH 2.5. Each sample was partitioned twice with petroleum ether. Both the organic and aqueous phases were evaporated to dryness at 35°C by rotary evaporator, redissolved in 2 ml of distilled water, and cytokinin recoveries were determined by HPLC.

The efficacy of butanol partitioning was checked by adding 2 ml of 20 mg/l zeatin or zeatin riboside to 38 ml of distilled water adjusted to pH 10 and partitioning with four equal volumes (40 ml) of water-saturated n-butanol. The organic and aqueous phases were both evaporated to dryness at 35°C by rotary evaporator and redissolved in 2 ml of distilled water. The cytokinin recoveries from the two phases were determined by HPLC.

The recovery of cytokinins from a PVP column was determined by adding 2 ml of zeatin or zeatin riboside to a 2.16 x 22 cm column containing

40 cm^3 of insoluble PVP which had been previously equilibrated at pH 3.0. The cytokinins were eluted with 400 ml (10 bed volumes) of distilled water at pH 3.0. The eluent aqueous samples were evaporated to dryness at 35°C by rotary evaporator and redissolved in 2 ml of distilled water. Recovery of cytokinins was then determined by HPLC.

The use of the cation exchange resin, Rexyn 101 for the purification and recovery of zeatin and zeatin riboside was evaluated. Forty cm^3 of Rexyn 101 which had been equilibrated in the H^+ form at pH 2.5 were packed into each of two 1.9 x 20 cm columns. Two ml of zeatin or zeatin riboside were added to 40 ml of 50% ethanol at pH 2.5 and run through the columns at 0.5 ml/min. The columns were then washed with 200 ml (5 bed volumes) of 50% ethanol at pH 2.5 and eluted with 200 ml of 3N NH_4OH in 50% ethanol at 0.5 ml/min. The eluate was evaporated to dryness at 35°C, redissolved in 2 ml of distilled water and analyzed by HPLC.

Since substantial hydrolysis of zeatin riboside to zeatin occurred when the above cation exchange purification procedure was used (see Results and Discussion), a further study on the improved extraction and recovery of cytokinins from Rexyn 101 was initiated. One ml of standard zeatin riboside was added to each of eight flasks containing 40 ml of water at pH 2.5. Rexyn 101 was equilibrated in either the H^+ or the NH_4^+ form by stirring 40 cm^3 of packing in either 2N HCl or 5N NH_4OH, respectively, for 30 min. The resin was equilibrated at pH 2.5 and each batch was divided into four equal 10 cm^3 portions and just covered with 10 ml of water at pH 2.5 in eight 125 ml Ehrlenmeyer flasks. Two of the flasks in each equilibrium form were kept at 22°C during subsequent adsorption and elution and two flasks in each form were kept at 0°C in an ice bath. The aqueous solutions

of zeatin riboside standards were added slowly to the flasks while stirring. This slurry was then stirred for 15 min and the supernatant was poured off. The packing was washed with 2 x 50 ml of neutral distilled water which was subsequently poured off. Ten ml of distilled water were added to each flask to just cover the Rexyn 101 beads and the zeatin riboside was desorbed by adding 50 ml of ice cold 1N NH_4OH in 70% ethanol in a dropwise fashion over a 15 min period while stirring the cation exchange slurry. After all the eluent had been added, the slurry was stirred for a further 15 min and then allowed to settle. The supernatant containing the eluted zeatin riboside was poured off and retained. Two more 50 ml volumes of 1N NH_4OH:70% ethanol were added consecutively to the cation exchange resin. The slurry was stirred for 15 min and the eluent poured off and added to the first volume. The zeatin riboside containing eluent was then filtered on Whatman No. 42 filter paper to remove any fine particles of Rexyn 101 which had remained in suspension, and then evaporated to dryness at 35°C by rotary evaporator. The residue was taken up in 1 ml of 10% acetonitrile and the recovered zeatin riboside was quantified by HPLC.

A combination of the extraction and purification steps described above was used to determine the recovery of zeatin and zeatin riboside from standard aqueous solution (Figure 5). Two ml of 20 mg/l zeatin or zeatin riboside were added to 800 ml volumes of 80% ethanol which were then evaporated at 35°C by rotary evaporator to 40 ml aqueous and adjusted to pH 2.5. Each sample was partitioned with 2 x 10 ml of petroleum ether and the aqueous phase was retained and adjusted to pH 3.0. The aqueous solution was run through a 2.16 x 22 cm column containing 40 cm^3 of insoluble PVP and was followed with an additional 400 ml (10 bed volumes)

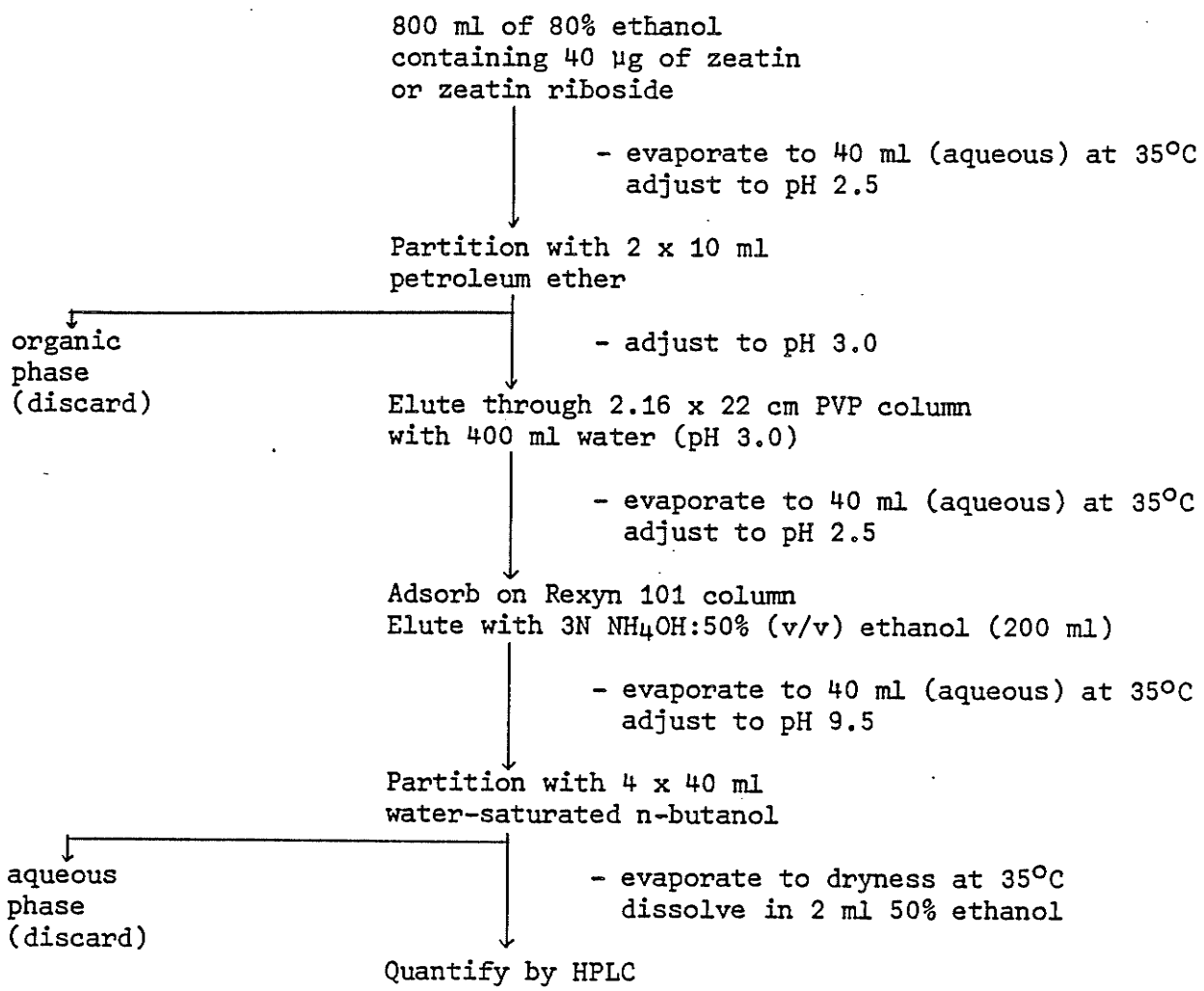

Figure 5. Flowchart for the extraction of standard zeatin and zeatin riboside from aqueous solution.

of water at pH 3.0. The eluate was evaporated to 40 ml at 35°C by rotary evaporator and adjusted to pH 2.5. This solution was then run slowly (0.2 ml/min) through a 1.9 x 20 cm column containing 40 cm^3 of Rexyn 101 cation exchange resin in the H^+ form. The column was washed with 3 bed volumes (120 ml) of neutral distilled water and then eluted at 0.2 ml/min with 200 ml of 3N NH_4OH in 50% ethanol. The eluate was evaporated to 40 ml (aqueous) and adjusted to pH 9.5. The aqueous solution was partitioned with 4 x 40 ml of water-saturated n-butanol and the organic phase was evaporated to dryness at 35°C by rotary evaporator. The residue was redissolved in 2 ml of 50% ethanol and the cytokinin recovery was determined by HPLC.

HPLC Analysis of Zeatin and Zeatin Riboside

The HPLC set-up for the analysis of zeatin and zeatin riboside is depicted in Figure 4. Samples were injected through an Altex U6 universal injection valve fitted with a 20 µl sample loop, and eluted under pressure from a Beckman model 420 double piston solvent pump (pump A) and a Beckman model 110A single piston solvent pump (pump B) programmed to regulate elution speed at 1.4 ml/min with a Beckman model pump programmer. Solvent A was 5% acetonitrile and solvent B was 100% acetonitrile. To both of these solvents, glacial acetic acid was added at a rate of 1 ml/100 ml of solvent. Eluting solvent concentration was adjusted to between 10% and 12% acetonitrile by programming differential pump outputs. Separation of compounds was by means of a 4 mm x 30 cm Waters µBondapak reversed phase analytical column containing microoctyldecylsilica beads 10 µm in diameter preceded by a Waters guard column packed with Bondapak C18/Corasil packing (particle size 37-50 µm). UV absorbing (254 nm) compounds were detected as they eluted from the column by a Hitachi model 100-40 spectrophotometer with a flow

through cell. The absorbing peaks were recorded and quantified by an attached Spectra Physics SP4100 computing integrator.

Amaranthus Bioassay

The Amaranthus bioassay used in these studies was according to the procedures recommended by Elliott (1979a and 1979b) with some modifications. Amaranthus seeds (0.2 g) were germinated in 5 cm plastic dishes on 1 cm thick foam pads wetted with 9 ml of distilled water. Germination was at 26°C for 92 h in the dark. The cotyledons with attached hypocotyls were removed and placed in a petri dish which contained 10 ml of distilled water for 2.5 h at 23°C in the dark. Seedlings were then quickly dried on filter paper, 10 at a time, and added to 2 ml plastic vials which contained 0.1 ml of the bioassay incubation medium. The incubation medium contained the standard cytokinin or plant extract dissolved in distilled water with 5 mM KH_2PO_4, 5 mM Na_2HPO_4 and 1 mg/ml tyrosine at pH 6.8. The plastic vials were incubated for 24 h at 30°C in the dark, after which they were immediately frozen at -20°C. The seedlings were defrosted by adding 1 ml of distilled water at 23°C to the vials and they were then refrozen at -20°C. The red pigment, betacyanin, was leached from the seedlings by exposing them to two additional freeze-thaw cycles. The aqueous betacyanin solutions were withdrawn from the vials and their absorbance at 537 nm and 620 nm was measured on an Hitachi model 100-40 spectrophotometer. The difference between the two readings represented the absorbance due to betacyanin. A calibration curve which plotted the absorbance due to betacyanin against the concentration of standard zeatin in the incubation medium was prepared and used to estimate the levels of cytokinins extracted from cabbage tissue.

RESULTS AND DISCUSSION

Extraction of Endogenous Cytokinins from Cabbage Tissue - Discussion of Procedures and Problems

Extraction of compounds such as cytokinins from various tissues and plant species can be expected to yield unique extraction problems due to differences in tissue structure, and chemical composition. Such differences inevitably lead to modifications of accepted extraction techniques (Van Staden and Drewes, 1975; Dekhuijzen and Gevers, 1975; Stahly and Buchanan, 1982). In this study, the procedures used for the extraction and purification of endogenous cytokinins from cabbage leaf tissue presented some difficulties. Some of the problems encountered were adequately solved while others require further modifications to improve cytokinin extraction.

It was not possible to determine the initial extraction efficiency for endogenous zeatin or zeatin riboside by tissue homogenization in 80% ethanol since cytokinin standards were in aqueous solution and were added after homogenization of the tissue. However, if it is assumed that the endogenous cytokinins of interest are in free solution and that thorough homogenization of the tissue releases all of the zeatin, zeatin riboside and their O-glucosides into the extraction solvent, the factor limiting complete cytokinin extraction is the extent to which the extracting solvent can be recovered from the homogenate. In the case of cabbage, the tissue pulp was very absorbent and vacuum filtration did not totally recover the extraction solvent. By measuring the volumes of added solvent and the recovered extract, it was determined that, after the first extraction, 62.9% of the extracting solvent (including the original tissue water) was recovered, and that 58.4% of the total extraction solvent was

recovered after the second extraction. Using these figures, a cytokinin extraction efficiency of 84.6% should be possible. This figure could be increased to 93.6% by homogenizing and extracting a third time. However, this would also increase the total extraction volume, thus increasing the time required for solvent evaporation.

The use of fresh tissue for extraction also caused some difficulty. The tissue water content diluted the 80% ethanol extracting solvent, slowed subsequent solvent evaporation, and resulted in a larger final aqueous volume after evaporation. In future work, it is recommended that cabbage tissue be freeze-dried before cytokinin extraction.

Evaporation of the ethanolic plant extracts to the aqueous phase resulted in a large amount of water-insoluble substances coming out of solution. In view of the waxiness of cabbage leaf tissue surface, it is supposed that structural lipids made up much of this material. This material made the samples physically difficult to handle and, due to the unknown nature of the substances, there was a concern that some cytokinins might be bound in this fraction and thus be lost when the solids were removed by filtration. In future studies, it would therefore be desirable to remove these substances before evaporation by some cleanup procedure such as chromatography or dialysis which would not affect the cytokinins in solution.

Evaporation of the organic phase after butanol partitioning also left a large amount of solid residue which was difficult to redissolve in water. Preliminary cleanup as was mentioned above would also minimize this problem.

Although elution of the extract on a PVP column was intended to selectively remove phenols, the column eluent was slightly yellow in

colour at pH 3.5 and became lemon yellow when the pH was increased to over 7.0. It was concluded that a number of phenols, including a nitro phenol, had not been quantitatively removed by the PVP column. Removal efficiency of such compounds may be improved in future studies by the use of the commercially available PVP chromatography packing Polyclar AT (General Aniline and Film Corp. N.Y.) or by increasing the total column volume.

The evaporation of the aqueous eluent from the PVP column may be an unnecessary step resulting in excessive cytokinin loss. It was assumed that increased cytokinin concentration resulting from such evaporation would improve cytokinin uptake in the subsequent cation adsorption step. However, cation adsorption on Rexyn 101 resin is a physically active process and, as such, may be independent of cation concentration as long as the slurry is mixed long enough to allow maximum adsorption.

The use of silica gel TLC for preliminary separation of cytokinins from impurities posed some physical difficulties since the large amount of impurities present in the plant extracts formed a physical barrier to solvent movement up the plate. Uneven solvent movement up the plate resulted in poor compound separation. Improved preliminary cleanup of samples in earlier steps could reduce this problem. Alternatively, this preliminary compound separation technique could be replaced with another separatory procedure such as the use of a Sephadex LH-20 (Horgan, 1978), Sephadex G-10 (Kannangara *et al.*, 1978), PVP (Thomas *et al.*, 1975) or μSpherogel (Crozier *et al.*,1980) column.

In spite of carefully carried out preliminary extraction, purification and separation of cytokinins, some UV-absorbing impurities inevitably are found in the HPLC trace. These impurities need to be separated from the cytokinins of interest in order to permit physical

cytokinin quantification. In such a case, extensive testing to determine the nature and behaviour of such impurities is required. Such testing may include the use of several different eluting solvents such as acetonitrile, methanol or ethanol or the manipulation of solvent pH and concentration. Identification of the major impurities may be helpful in further improving isolation and purification procedures.

The Amaranthus bioassay was found to offer a fast and sensitive way to detect and quantify cytokinins. It is not known, however, how much impurities such as phenols inhibit betacyanin formation. Care must also be taken to keep the Amaranthus betacyanin solutions in the dark and on ice to prevent colour breakdown.

In general, a number of the cytokinin extraction and detection techniques can be improved to permit physical cytokinin quantification by HPLC or cytokinin detection and quantification by HPLC or cytokinin detection and quantification by the Amaranthus bioassay.

Extraction of Endogenous Cytokinins From Cabbage Tissue - Results

The recovery of standard cytokinins from sample S, the cytokinin treated extract, after purification by the described procedure and preliminary quantification by HPLC (Figure 3) was determined to be 16.0 mg/l or 45.7% of the added zeatin and 11.8 mg/l or 58.8% of the added zeatin riboside (Figure 6a). These figures were derived by calculating the mean cytokinin peak areas from three runs of the TLC fraction in which they were found (between Rf. = .5 and .6) and dividing these values by the respective mean peak areas from twenty runs of the aqueous standard cytokinin solutions. When the zeatin and zeatin riboside containing fractions were collected as they eluted from the column and rechromatographed, no loss of either

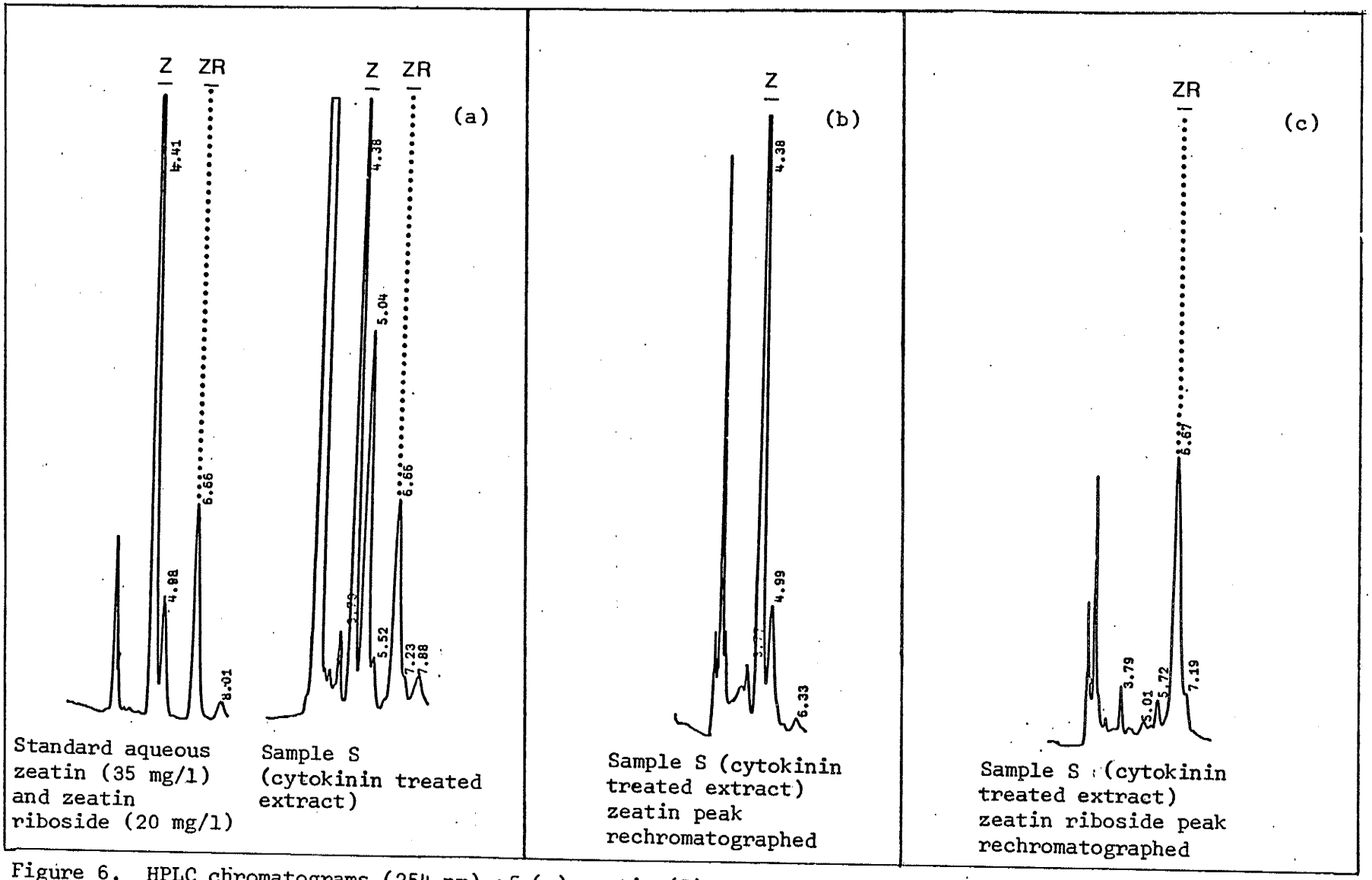

Figure 6. HPLC chromatograms (254 nm) of (a) zeatin (Z) and zeatin riboside (ZR) recovered from cabbage tissue which had been pretreated with standard cytokinins prior to extraction and purification (sample S), (b) zeatin rechromatographed from eluate collected from (a), (c) zeatin riboside rechromatographed from eluate collected from (a). Elution conditions were 1.4 ml/min with 10% acetonitrile.

cytokinin was found (Figure 6b). The recovery percentages reported above were assumed in the quantification of endogenous cytokinins from sample E, the untreated extract, and sample B, the β-glucosidase treated extract.

The HPLC trace of TLC fraction 6 from sample E showed a small peak occurring at the zeatin retention time (Figure 7a) and a large peak occurring slightly after the zeatin riboside retention time. These peaks were collected and rechromatographed at slightly lower solvent concentrations to separate the peaks from nearby peaks. There was no clear peak coeluting with zeatin (Figure 7b) and no zeatin riboside peak was observable due to major impurities coeluting with it (Figure 7c).

Fraction 6 from sample B, the β-glucosidase treated extract (Figure 8a) revealed a small but distinct peak coeluting with zeatin, while a shoulder on a following major peak was visible near the zeatin riboside retention time. Rechromatography of the putative zeatin peak showed a relatively large peak very nearly coeluting with zeatin (Figure 8b). The putative zeatin riboside peak, however, was not better resolved upon rechromatography (Figure 8c).

From the HPLC traces, it was apparent that there are major UV-absorbing impurities which are extracted from the cabbage tissue with the cytokinins which have HPLC retention times similar to those of zeatin and zeatin riboside under the conditions used in this study. Although the nature of these impurities was not fully resolved, it was noticed, after the PVP purification step (Figure 3), that the eluate turned yellow at pH ≥7.0. Chromatography by TLC (Figure 3) also showed clearly visible yellow streaks coeluting with the cytokinins in fraction 6. Despite the activity of the PVP column in selectively removing phenols, these observations suggest that phenols, including nitrophenols were extracted

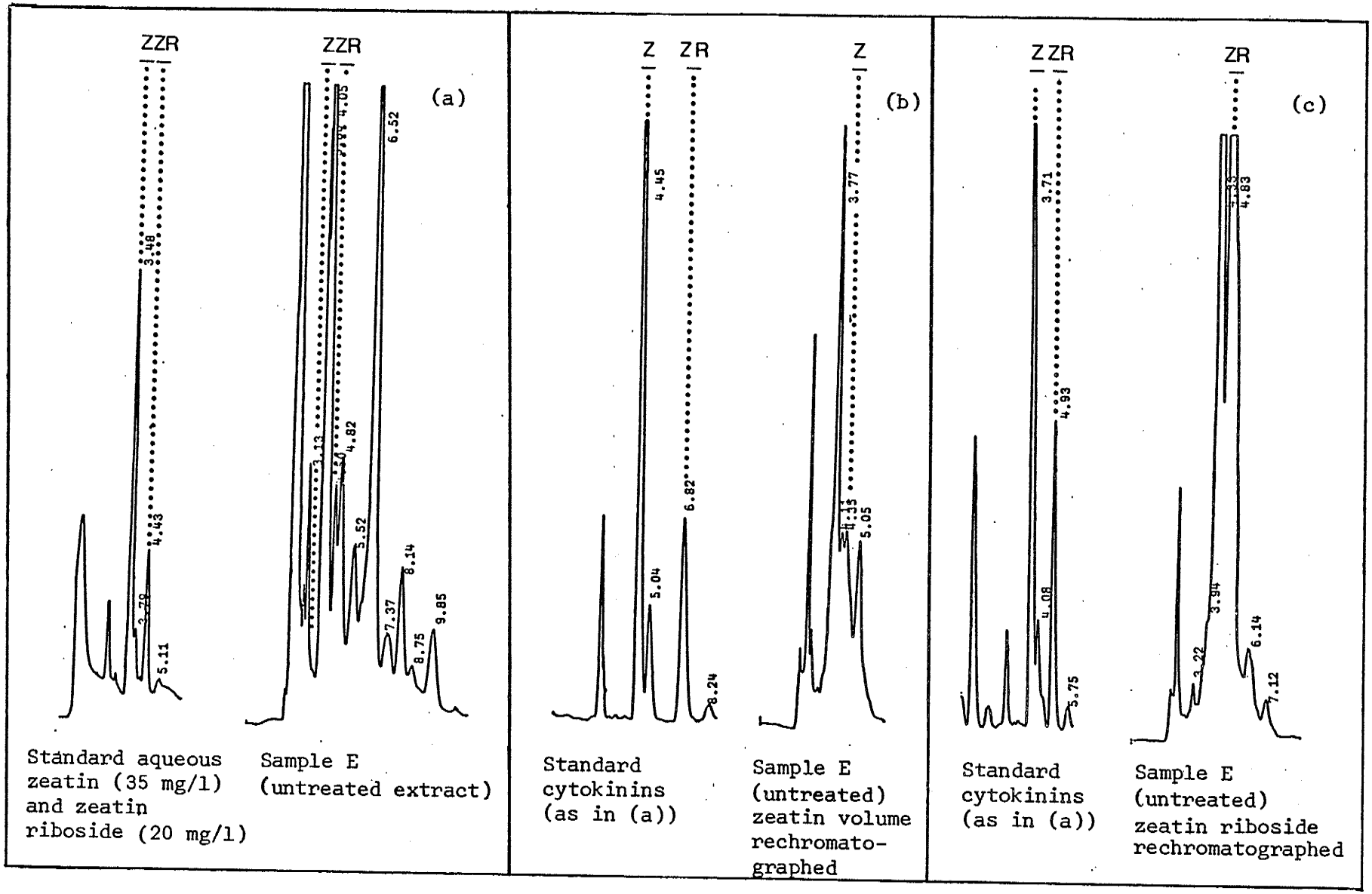

Figure 7. HPLC chromatograms (254 nm) of (a) purified cabbage extract, (b) zeatin containing volume rechromatographed from eluate collected from (a), (c) zeatin riboside containing volume rechromatographed from eluate collected from (a). Elution at 1.4 ml/min with 10-12% acetonitrile.

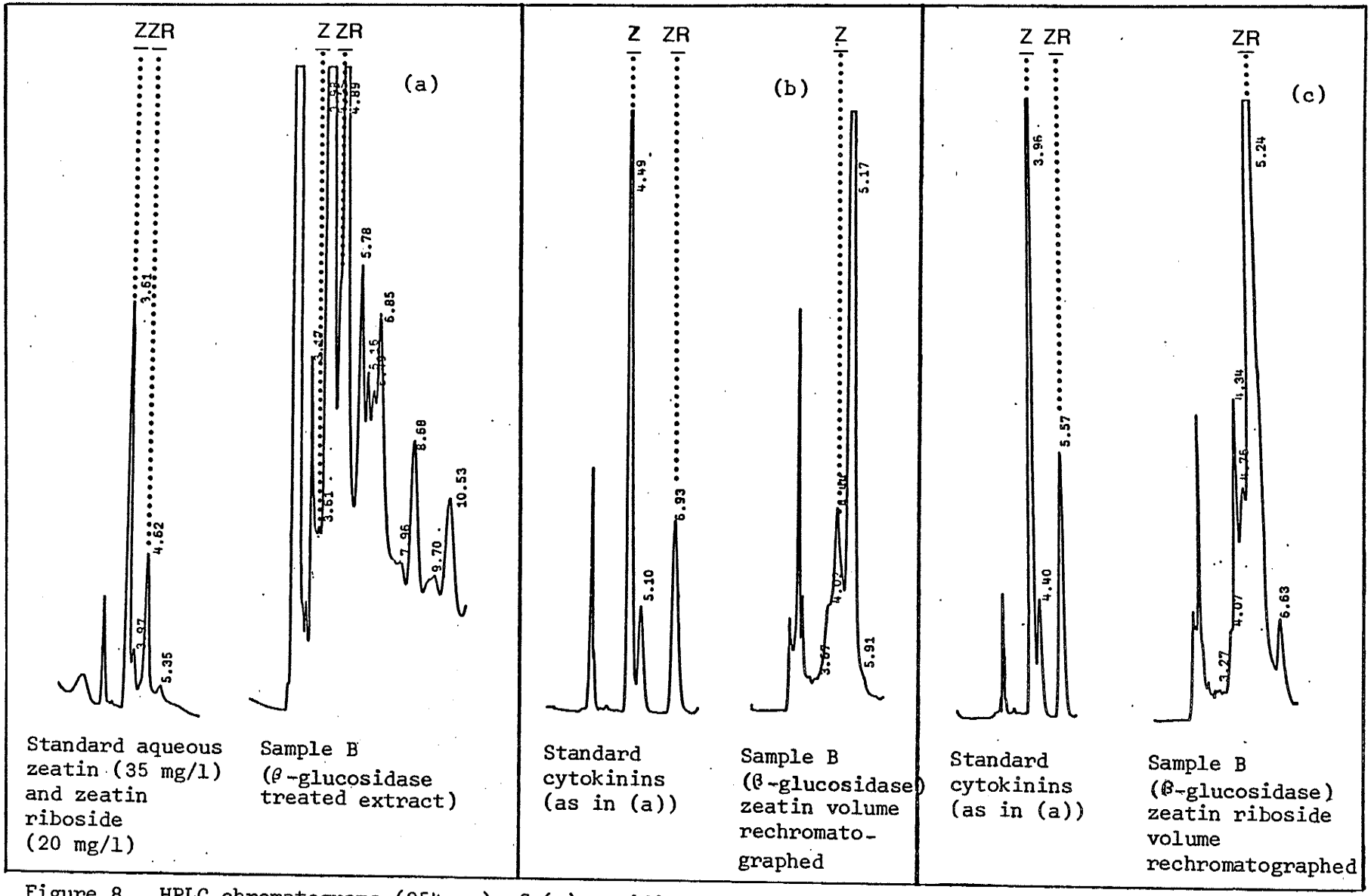

Figure 8. HPLC chromatograms (254 nm) of (a) purified, β-glucosidase treated cabbage extract, (b) zeatin containing volume rechromatographed from eluate collected from (a), (c) zeatin riboside containing volume rechromatographed from eluate collected from (a). Elution at 1.4 ml/min with 10-12% acetonitrile.

with the cytokinins as impurities and interfered with cytokinin quantification by HPLC (Figures 7a and 8a). Rechromatography was able to screen out a large number of UV absorbing impurities (Figures 7b,7c,8b and 8c) but did not sufficiently remove closely coeluting major peaks, especially in the case of zeatin riboside, (Figures 7c and 8c) to permit physical cytokinin quantification.

The zeatin and zeatin riboside fractions from sample S, the cytokinin treated extract, collected from the HPLC column showed significant cytokinin activity in the Amaranthus bioassay while neither fraction from sample E, the untreated extract, or sample B, the β-glucosidase treated extract, differed from the cytokinin-free control (Table 7). Due to the relatively high variability of the bioassay results, the HPLC quantification of the recovered zeatin and zeatin riboside from sample S, the cytokinin treated extract, was considered more reliable. A bioassay calibration curve was generated using standard aqueous zeatin solutions (Figure 9). Taking into account the variability of the bioassay, it was determined from this curve that .043 mg/l was the lowest zeatin concentration which would show activity significantly higher than the control. Under the extraction procedures used in this study, and assuming a 50% recovery rate, this level would be obtained from cabbage tissue having an endogenous zeatin level of 1.3 ng per gram fresh weight.

The lack of cytokinin activity by the cabbage extracts may indicate that endogenous cytokinin levels were less than this figure. If this was the case, levels could be measured by improving the sensitivity or decreasing the variability of the bioassay or by using larger amounts of plant tissue.

An alternative explanation for the lack of activity is that inhibiting substances such as phenols may have been coextracted as was

Table 7. Activities of zeatin (Z) and zeatin riboside (ZR) fractions from cabbage extracts in the Amaranthus bioassay for samples S) standard Z and ZR added, E) untreated, and B) incubated with β-glucosidase.

Sample	Cytokinin fraction	Mean absorbance* (537 nm-620 nm)
S	Z	.133 c
E	Z	.103 ab
B	Z	.104 ab
S	ZR	.135 c
E	ZR	.081 a
B	ZR	.111 bc
control	none	.089 ab

*Note: Figures followed by the same letter do not differ significantly at the 5% level by Duncan's Multiple Range Test.

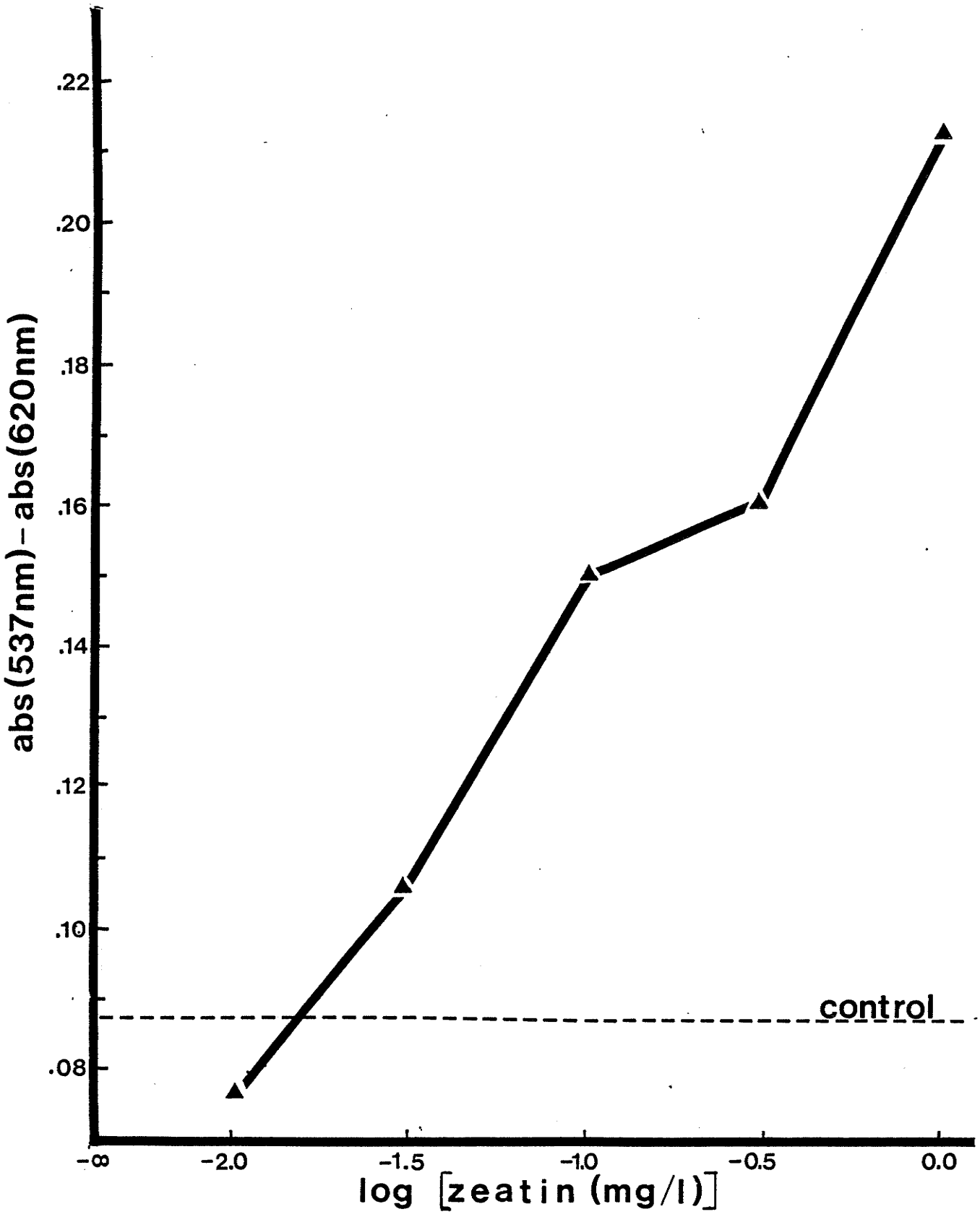

Figure 9. Calibration curve for zeatin activity in the Amaranthus betacyanin bioassay.

noted in the discussion on the HPLC traces. Phenols are known plant growth inhibitors (Glenn et al., 1972) and may affect the Amaranthus bioassay.

Extraction of Standard Cytokinins from Aqueous Solution

Analysis by HPLC of zeatin and zeatin riboside after evaporation of 800 ml of 80% ethanol showed that 46% of the zeatin and at least 10% of the zeatin riboside was not recovered after evaporation (Table 8). Dekhuijzen and Gevers (1975) reported serious breakdown of kinetin in n-butanol or aqueous solution under extended, low temperature evaporation and recommended use of higher evaporation temperatures to accelerate this process. Minimal solvent volumes and a higher evaporation temperature were used in cabbage tissue extraction and evaporation to dryness was avoided, where possible, to minimize such breakdown.

Petroleum ether partitioning of aqueous zeatin and zeatin riboside showed that none of either cytokinin was recoverable from the organic phase (Table 8). The cytokinin losses from the aqueous phase were presumed to be due to evaporation.

Partitioning aqueous zeatin and zeatin riboside solutions with four equal volumes of water saturated n-butanol resulted in 56% and 66% of the respective cytokinins being recovered from the organic phase (Table 8). Since only 3% and 4% of the respective cytokinins were recovered from the aqueous phase, evaporative losses of 41% of the added zeatin and 30% of the added zeatin riboside were indicated.

Zeatin and zeatin riboside were recovered from a PVP column as indicated by Thomas et al., (1975) within the first ten column volumes (Table 8). Losses of 70% for zeatin and 27% for zeatin riboside were attributed to evaporation since a large volume of water (400 ml) had to be evaporated in this step.

Table 8. Analysis of standard zeatin and zeatin riboside recovery from commonly used purification procedures conducted separately or in combination.

Procedure	Phase or Fraction	% Recovery** Zeatin	% Recovery** Zeatin riboside
Evaporation of 800 ml of 80% ethanol		54	90*
Partitioning of 40 ml aqueous standard with 2 x 10 ml petroleum ether at pH 2.5	Aqueous	60	83
	Organic	0	0
Partitioning of 40 ml aqueous standard with 4 x 40 ml water-saturated n-butanol at pH 10.0	Aqueous	3	4
	Organic	56	66
Elution of 40 ml aqueous standard from 40 cm^3 PVP column at pH 2.5 with 400 ml water	Eluent	30	73
Elution of 40 ml aqueous standard from 40 cm^3 Rexyn 101 cation exchange column (H^+ form)	Effluent	0	0
	Eluent	75	49 (28% recovered as zeatin)
Combination of above purification procedures (Figure 5)		59	53 (42% recovered as zeatin)

*An impurity coeluting with zeatin riboside from the HPLC column interfered with this quantification so that the determined recovery percentage (90%) is likely too high.

**Each value is based on duplicate extractions with the exception of the combined procedure for which the recovery values were determined from one extraction only.

Zeatin and zeatin riboside were quantitatively adsorbed from aqueous solution on Rexyn 101 cation exchange columns since neither compound was detected in the initial effluent.(Table 8). Desorption from the zeatin-containing column resulted in the recovery of 75% of the added zeatin. The zeatin riboside-containing column,when eluted,yielded a 21% recovery of the added zeatin riboside as zeatin riboside and 28% was recovered as zeatin. This hydrolysis of zeatin riboside to zeatin was likely due to heating of the column upon elution. Miller (1974) warned that such hydrolysis was possible unless precautions were taken against heating of the cation exchange packing during elution.

Hydrolysis was minimized and recoveries were improved when the cation exchange packing was in the NH_4^+ form (Table 9). Elution of cytokinins at 0°C is recommended as a precaution against heating. The eluting solvent was changed from 3N NH_4OH in 30% ethanol to 1N NH_4OH in 70% ethanol to reduce the heat of elution and the evaporation time required. It was also observed that the Rexyn 101 was more prone to physical breakdown in the H^+ form than it was in the NH^+_4 form.

Some workers in the area continue to ignore this problem of hydrolysis in their extraction procedures (Even-Chen et al., 1978; Van Staden and Dimalla, 1980). This is important whether the focus of the work is to quantify endogenous cytokinins as in the former case since zeatin and zeatin riboside vary in their activity in a number of bioassays (Letham et al., 1983). It is also important in the latter case in which zeatin was identified as a naturally-occurring cytokinin in leaf tissue of carnations since the zeatin found may in fact have originated from hydrolyzed zeatin riboside.

Table 9. Recovery and hydrolysis of zeatin riboside adsorbed on Rexyn 101 cation exchange packing at pH 2.5 and eluted with 1N NH_4OH:70% ethanol as a slurry at controlled temperatures.

Cation exchange packing form	Temp. of adsorption and elution (°C)	Cytokinin Recovery (%)*			
		Zeatin	Zeatin Riboside	Total	Mean
H^+	22	2.5	66.9	69.4	73.3
	0	0.5	76.7	77.2	
NH_4^+	22	0.0	88.5	88.5	86.5
	0	0.0	84.4	84.4	

* Each value is the mean of two repititions of each procedure.

A combination of the procedures described above was evaluated to determine the total zeatin and zeatin riboside recovery. Recovery of zeatin was found to be 59% (Table 8). Hydrolysis of zeatin riboside to zeatin during elution from the cation exchange column was thought to explain the recovery of only 11% of the added zeatin riboside in its original form while an additional 42% of the added zeatin riboside was recovered as zeatin. On the basis of these recoveries and refinements of the separate recovery procedures as discussed previously, it was felt that the recovery percentage of endogenous cytokinins from cabbage tissue would be high enough to permit routine quantification if impurities were more thoroughly removed.

Since the recoveries of 59% and 53% determined for zeatin and zeatin riboside, respectively, from the combined procedure are much higher than those which would be expected from the product of the recovery percentages reported for the individual purification procedures (Table 8) (4% and 18% for zeatin and zeatin riboside respectively) the discrepancy was concluded to be due to the greater amount of evaporation which was required to analyze each procedure separately. It was felt that evaporation to dryness was a particularly hazardous step. Although the combined procedure required the evaporation of 220 ml (31%) less water and 100 ml (14%) less ethanol than the procedures taken separately, only one evaporation to dryness was required for the combined procedure while each of the five procedures, when analyzed separately, required evaporation to dryness. The reason for the cytokinin loss may be breakdown as reported by Dekhuijzen or incomplete redissolution of the dried cytokinins. Such problems in determining recovery efficiencies could have been circumvented with the use of radioactive standard cytokinins.

GENERAL DISCUSSION

The storage quality of cabbage was improved by the synthetic cytokinin N^6-benzyladenine and the natural cytokinin zeatin which significantly delayed colour loss, tissue decay, and fungal infestation. Attempts to quantify endogenous levels of the naturally occurring cytokinins zeatin and zeatin riboside, and their respective storage forms, zeatin O-glucoside and zeatin riboside O-glucoside, were made in order to determine whether storage quality of cabbage could be attributed to available natural cytokinins but quantification was not successful in this study.

Isenberg *et al.* (1974), however, reported that the cytokinin level in storage cabbage cv. Green Winter decreased sharply after harvest and remained at a constant low level for the first eighteen weeks of storage but decreased to negligible levels for two or three weeks thereafter. This latter period also included major increases in gibberellin and auxin levels and was followed by the breaking of dormancy and the commencement of apical meristem development. Cytokinin levels rose sharply with apical meristem development, presumably due to the novo synthesis. Even-Chen *et al.*, (1978) found a similar pattern of cytokinin level decreases upon detachment and subsequent senescence of tobacco leaves. In this case, the second decrease of cytokinins to negligible levels was accompanied by major increases in auxin and ethylene levels and rapid chlorophyll loss.

It appears, then, that the presence of a stable, low level of endogenous free cytokinins is associated with both a maintenance of

dormancy and a delay of senescence. The increase of auxins may relate to the onset of senescence, and possibly to the breaking of dormancy in cabbage as well. Even-Chen et al., (1978) found that addition of exogenous kinetin to detached tobacco leaves inhibited the increases in auxin and ethylene levels and maintained chlorophyll while the addition of naphthalene acetic acid (NAA), a synthetic auxin, greatly increased ethylene production.

Manos and Goldthwaite (1975) showed that free zeatin is rapidly inactivated in Rumex leaf tissue. Since zeatin was significantly less effective in delaying senescence in cabbage leaf tissue in the study than N^6-benzyladenine it is likely that such rapid metabolism also occurs in cabbage tissue. To maintain a stable level of free cytokinins in stored cabbage as found by Isenberg et al., (1974), a storage pool of cytokinins such as that found by Dekhuijzen (1980) in turnip tissue would therefore be necessary for long-term storage. Exhaustion of this storage pool would result in the final decrease of cytokinin levels reported by Isenberg et al. (1974) and the initiation of senescence.

Techniques for the quantification of cytokinin O-glucosides need to be improved to find whether such a relation actually exists. Such knowledge and techniques could conceivably be used to routinely predict storability of freshly harvested cabbage or to determine cabbage maturity prior to harvest. Breeding for more storable cabbage varieties could also be based on cytokinin O-glucoside levels.

The storage life and quality of cabbage was also significantly improved by exogenous application of silver nitrate. Although the most probable explanation for this delay of senescence is that of Aharoni

et al. (1979) who suggest that the silver ions bind to the ethylene receptor site, thus inhibiting the senescence-inducing effect of ethylene. This suggestion is reasonable since Pendergrass et al. (1976) showed that ethylene induced chlorophyll loss in stored cabbage.

Another possible effect of silver nitrate is suggested by the recovery of zeatin from corn extract by precipitation with silver nitrate reported by Letham (1966). Since silver nitrate forms a precipitate with zeatin in solution, it is likely that a similar process occurs when silver nitrate is applied to intact cabbage tissue. If the silver-zeatin precipitate is slightly water soluble, such a precipitate within the tissue could serve as an alternate storage pool to the cytokinin O-glucosides, slowly releasing free zeatin into solution as metabolism of existing zeatin progresses. The mode of action of silver nitrate in delaying senescence will become more clear when analysis of endogenous zeatin and zeatin riboside bases and their O-glucosides becomes routine so that the effect of silver nitrate on cytokinin levels can be monitored.

The similar activity of cytokinins and silver nitrate treated cabbage in resisting fungal infestation is suggested to be a secondary effect resulting from the anti-senescence activity. In stored cabbage, the knowledge of endogenous cytokinin status may provide an early warning system for the susceptibility of the cabbage to fungal storage problems. The activity of benomyl, a commercially available fungicide, as a cytokinin, may at least partially account for its fungicidal activity.

In general, the ability to efficiently and accurately quantify and characterize endogenous hormones may be important in future strategies

to improve techniques of keeping storable commodities fresh. Improvements in ths area may lead to increased self-sufficiency in such commodities and thus improve local economies. Improvements to the procedures used in this study may make physical quantification of zeatin, zeatin riboside and their respective O-glucosides from cabbage tissue a possibility. The capability to monitor endogenous cytokinins may be an important step in the elucidation of the mode of action of senescence retardants such as silver nitrate.

SUMMARY AND CONCLUSIONS

Senescence of winter cabbage in long-term storage can be effectively delayed by N^6-benzyladenine and silver nitrate and, to a lesser extent, by zeatin and GA_3. The effectiveness of N^6-benzyladenine can be attributed to the fact that, as a synthetic cytokinin, it is more slowly metabolized or inactivated than zeatin, a natural cytokinin. Silver nitrate is reported to act through its inhibition of ethylene action although it may also interact with endogenous cytokinins. GA_3 has been reported to delay senescence by an interaction with cytokinins. The delay of senescence by N^6-benzyladenine, silver nitrate and zeatin coincide with a reduction of fungal infestation. It is presumed that this effect is a result of the anti-senescence action of these compounds.

The extraction, purification and quantification of endogenous cytokinins from cabbage tissue was complicated by the presence of a number of impurities which were coextracted by the procedure used and interfered with HPLC quantification since they eluted from the column with the cytokinins and were UV-absorbent. The lack of activity of HPLC eluates in the Amaranthus bioassay may indicate inhibition by these interferants of betacyanin synthesis or may simply indicate that endogenous cytokinins in stored cabbage are extremely low. The success of future attempts to quantify cytokinins from cabbage tissue will depend on the modification of some of the extraction and purification methods used in these studies and the addition or deletion of others as previously discussed.

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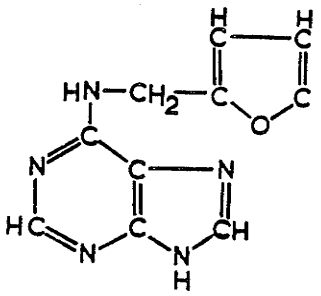
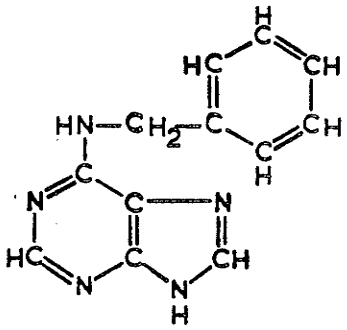
APPENDIX 1a. Chemical structures of cytokinins

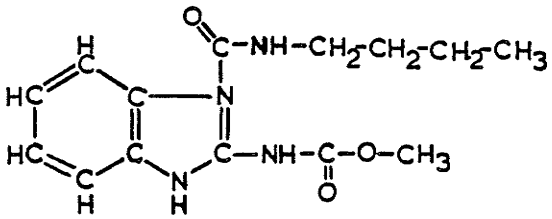
I. Synthetic cytokinins



kinetin



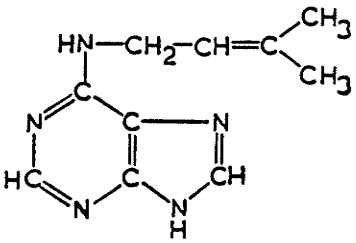
N^6-benzyladenine



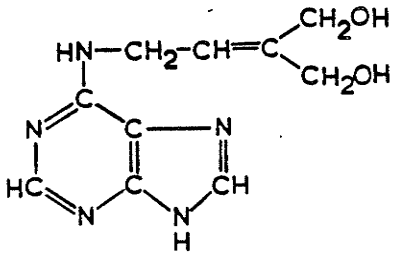
benomyl

II. Naturally occurring cytokinins

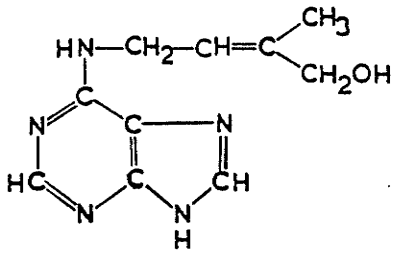
A. Free bases



isopentenyl adenine



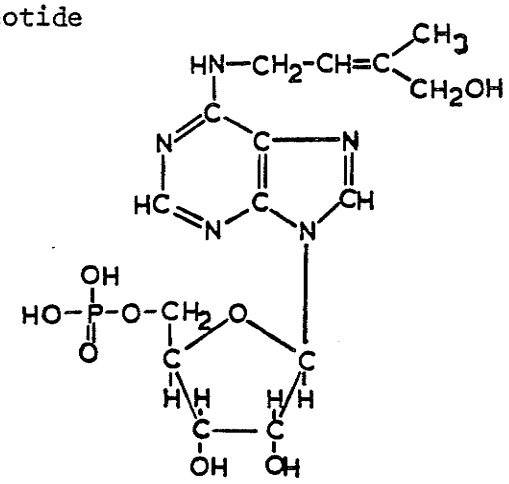
dihydrozeatin



zeatin

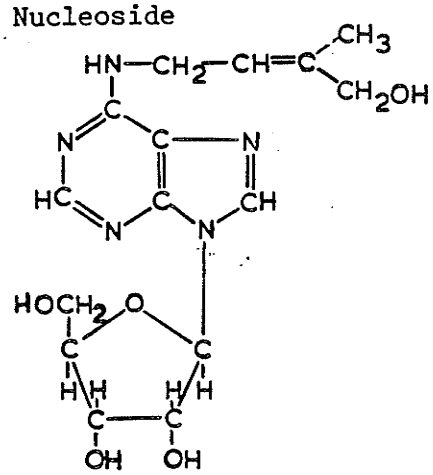
APPENDIX 1b. Chemical structures of cytokinins

B. Nucleotide



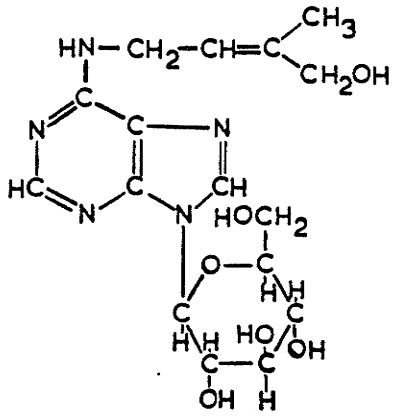
zeatin ribotide

C. Nucleoside

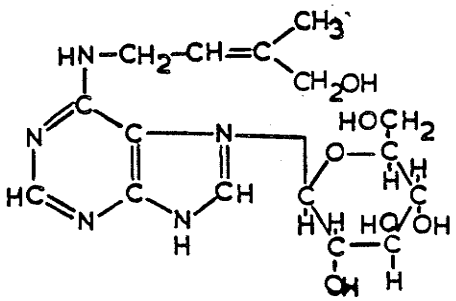


zeatin riboside

D. Deactivation glucosides

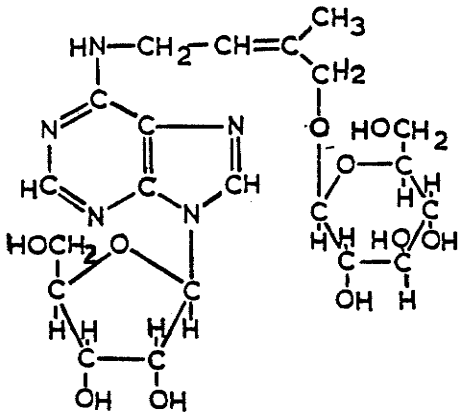


zeatin 9-glucoside ([9G]-Z)

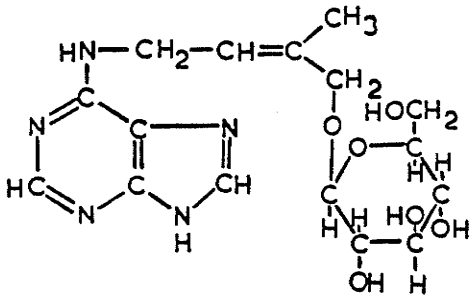


zeatin 7-glucoside ([7G]-Z)

E. Storage glucosides



zeatin riboside O-glucoside



zeatin O-glucoside