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Title: A Technique for the Measurement of Gibberellins and its Application to the Xylem Sap of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco]

Abstract Approved: _____
Joe B. Zaerr

An understanding of the gibberellins present in Douglas-fir is essential to understanding and controlling the growth and development of this species.

A technique was developed which permits the separation of certain gibberellins with high performance liquid chromatography and their identification with mass spectrometry. An extraction technique for the isolation of gibberellins from plant material was then developed. These techniques were demonstrated to be successful in identifying some of the gibberellins present in wild cucumber seeds, a plant system known to contain gibberellins, but no gibberellins were found in Douglas-fir xylem sap during the spring. However, an increase

in growth inhibitor levels was observed which could have interfered with the detection of the gibberellins. The increase in the level of inhibitors during June and July coincided with the decrease in shoot growth, suggesting that the cessation of developmental processes is the result of an increased level of growth inhibitors rather than a change in concentration of free gibberellins. Another explanation for the lack of evidence for gibberellins in Douglas-fir xylem sap is that the native gibberellins may occur in a form other than the free acid and therefore could not be detected with procedures used in this investigation.

A Technique for the Measurement of Gibberellins and its Application to the Xylem Sap of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco]

by

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INTRODUCTION

The most important tree species in the Pacific Northwest is Douglas-fir. As the demand for this wood increases, the requirement for highly productive forests becomes more acute. Regeneration of forests is dependent upon the successful establishment of new seedlings, and the physiologist can improve the probability of seedling survival and vigor by regulating the internal and external factors that are involved in tree growth, factors such as nutrient balance, water supply, amount of light, competition from other species, and hormonal interactions.

Gibberellins have been demonstrated to be a vital link in the growth and development of plants (Leopold and Kriedemann, 1975), and a better understanding of their ability to control the many physiological responses of a plant to its environment is necessary before this growth regulator can be manipulated to our advantage. However, before the endogenous functioning of these compounds can be elucidated, it is necessary to learn more about the kinds of gibberellins that are present and their metabolism during plant development. Gibberellins have been implicated in shoot elongation, cambial growth, apical control, and reproductive and metabolic processes of many coniferous species (Pharis and Kuo, 1977). An understanding of the gibberellins in Douglas-fir could provide insight into the production and utilization of these hormones during

the critical stages of a tree's development and thus contribute to a better understanding of how tree growth might be managed.

The major objective of this research was to identify certain gibberellins present in the xylem sap of Douglas-fir during the spring. This involved developing a method for isolating and identifying unambiguously certain gibberellins and then applying this method to Douglas-fir sap.

LITERATURE REVIEW

The history of the discovery of the class of compounds known as gibberellins is a fascinating story, in which the participants were plant scientists from all over the world, and in which economics and politics played a role. The isolation and characterization of the original 'gibberellin' and the subsequent realization of its significance had ramifications throughout the realm of plant biology.

The events leading to the discovery of gibberellins have their roots in Japanese agriculture. Diseases which attack the rice crop had been studied for centuries out of economic necessity. One disease in particular, given the name Bakanae disease, or 'foolish seedling,' by Shotaro Hori in 1898 (Stodola, 1958), was known to be caused by the fungus Fusarium moniliforme Sheldon. In 1925 a Japanese plant pathologist, E. Kurosawa, was investigating the control of the bakanae disease. In 1926 he published the paper recognized as the original work about gibberellins, in which he describes a toxin secreted by the fungal mycelium that is responsible for the unusual hyperelongation of the affected rice seedlings (Kurosawa, 1926). The substance was given the name gibberellin in 1935 by Yabuta (Stowe et al., 1959) from the name of the perfect stage of the fungus responsible, Gibberella fujikuroi (Saw.) Wollenw., and by 1950 the Japanese had published 15 papers on gibberellin isolation and chemistry.

Communication between Japan and the western world was disrupted by World War II; consequently, it was not until the 1950's that the

Americans and the British, simultaneously and in separate laboratories, isolated gibberellin from cultures of the fungus (Mitchell and Angel, 1950; Stoddola et al., 1955; Curtis and Cross, 1954). Stoddola and coworkers isolated two compounds; one identical to that isolated by the Japanese and known as gibberellin A, and another unknown which they named Gibberellin X. At the same time Cross and Curtis in England, attempting to isolate Gibberellin A, found another compound of similar properties which they called Gibberellic Acid and which, it turns out, was identical to Gibberellin X. In 1955 Takahashi reported that the original Gibberellin A was actually three components, which he called GA_1, GA_2, and GA_3 (Gibberellic Acid) (Takahashi et al., 1955). Shortly thereafter, the fourth gibberellin was identified, and by 1961 nine gibberellins had been isolated, five from the fungus, three from a higher plant, Phaseolus multiflorus, and one from both sources (Gould, 1961). At the present time 52 compounds have been isolated which can be classified into the group of plant hormones known as gibberellins (Hedden, MacMillan, and Phinney, 1978); these compounds have been designated GA_1, GA_2, GA_3, ... GA_{52}, and their structures are shown in Figure 1.

About the same time gibberellins were arousing interest, the cytokinins were being studied, and scientists began to realize that there were compounds other than auxins controlling the growth of plants. Mitchell and Angel, the first group of Americans to isolate the compound from Fusarium moniliforme (Mitchell and Angel, 1950), found that the isolate stimulated elongation of bean plants and termed it a 'plant growth regulating substance.' Henceforth,

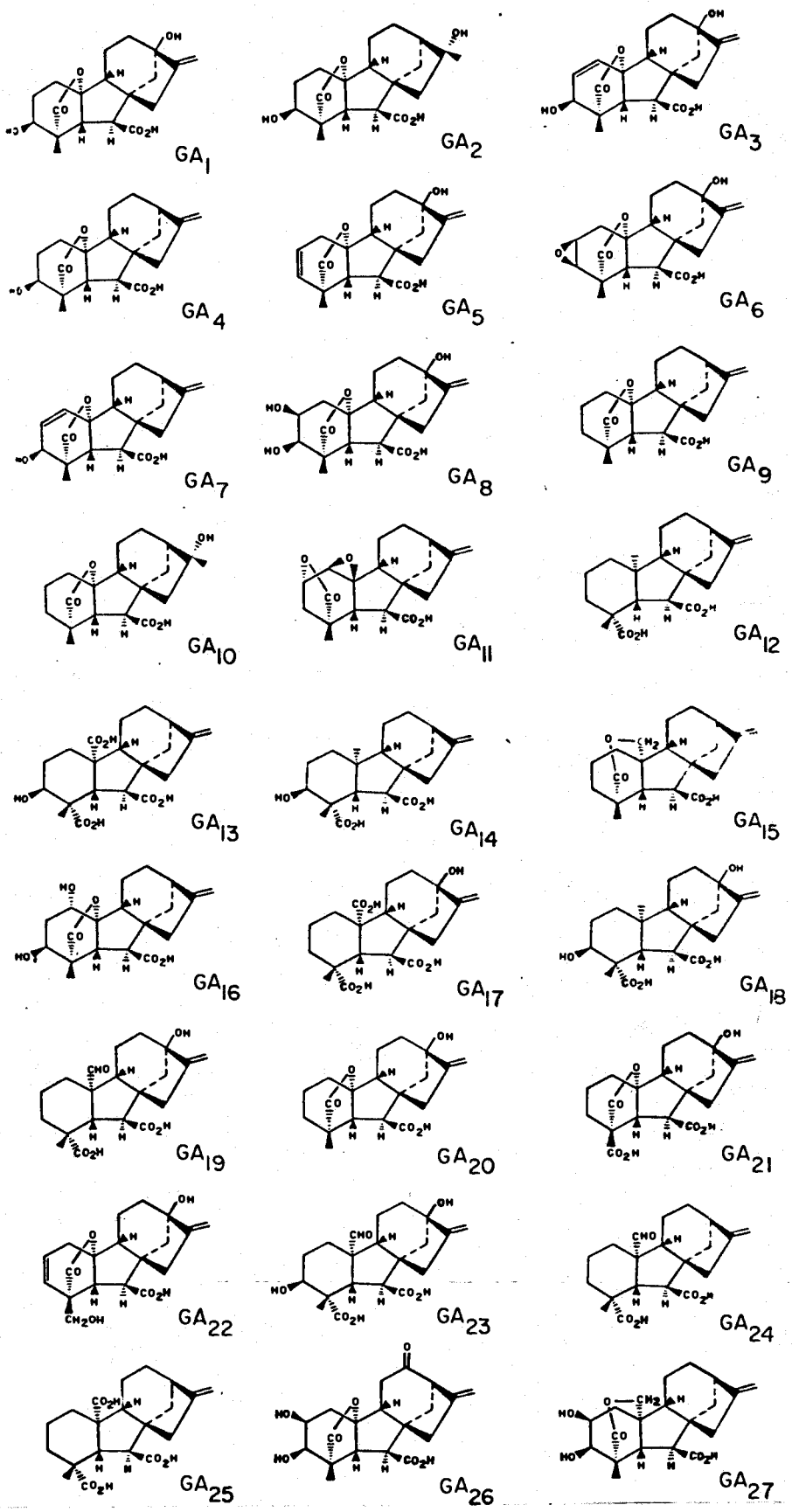


Figure 1. Structures of the presently known gibberellins (Hedden, MacMillan, and Phinney, 1978).

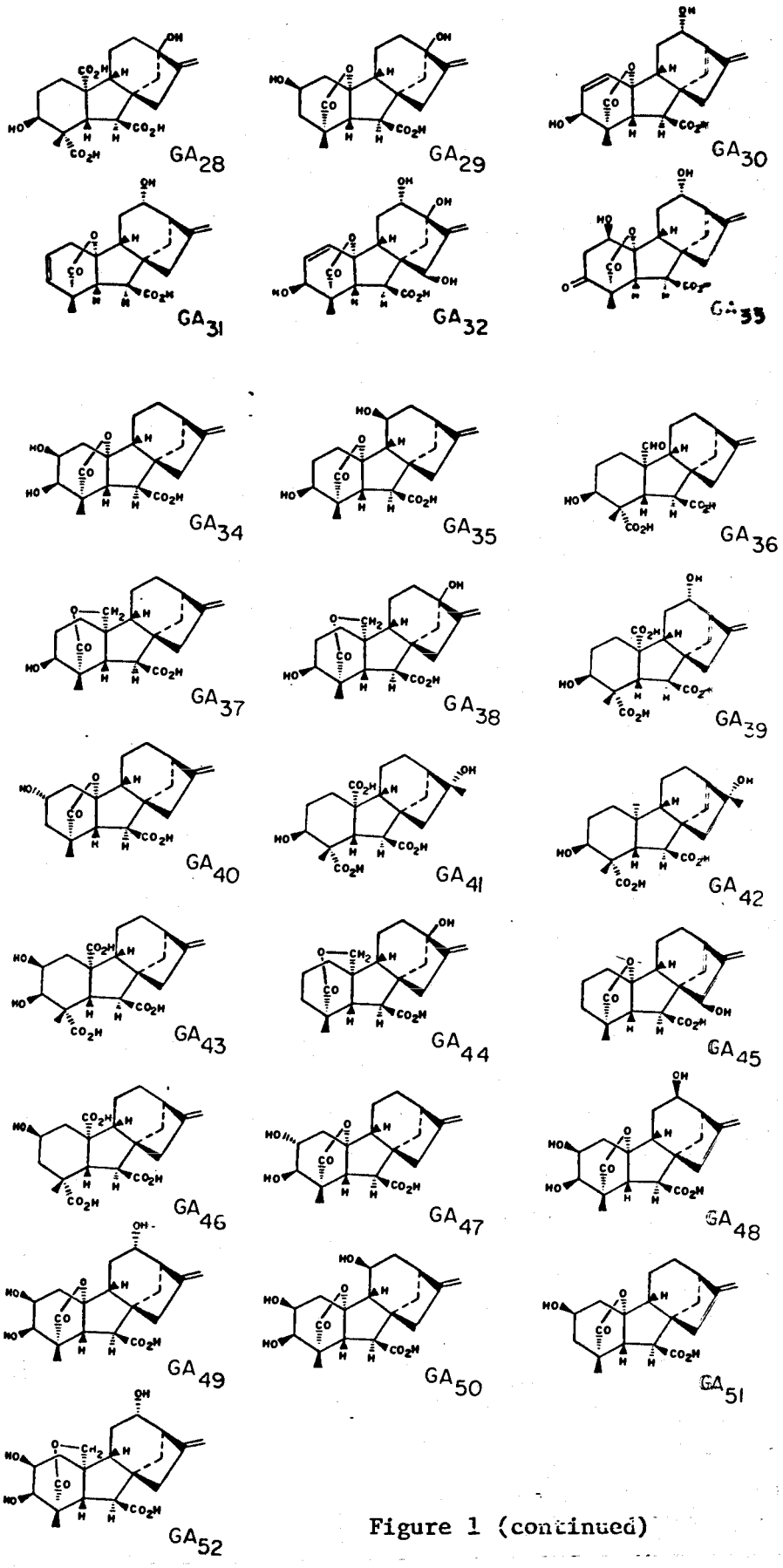


Figure 1 (continued)

experiments to elucidate the various responses of plants to gibberellins, to determine their mode of action, their biochemistry and synthesis, and their presence in higher plants have led to a plethora of papers on the subject. Gibberellins have been implicated in every aspect of plant growth and development from stem elongation to initiation of flowering. The first comprehensive review on gibberellin (GA) research was published in 1957 (Stowe and Yamaki, 1957); since that time there have been several others (Lang, 1970; Paleg, 1965; Hedden, MacMillan, and Phinney, 1978; and Pharis and Kuo, 1977).

The numerous responses of higher plants to gibberellins prompted attempts to manipulate their physiology by exogenous applications of GA's. A review by Stuart and Cathey (Stuart and Cathey, 1961) discusses the effects of exogenous GA's in many plant species that were observed when testing the nine gibberellins that were known to be in existence at that time. As the research continued, the different kinds of gibberellins present in higher plants began to mount, and plant biologists questioned the physiological significance of so many gibberellins. Investigations into the biosynthesis of the gibberellins were thus conducted in order to determine their interconversions in higher plants. The effects of 2-chloroethyltrimethyl ammonium chloride (CCC) and 2'[Isopropyl-4'-(trimethylammonium chloride)-5'-methylphenylpiperidine carboxylate] (AMO-1618), known inhibitors of gibberellin biosynthesis in fungal cultures (see Figure 2), were observed in pea, bean, and balsam seedlings to determine if these compounds caused a parallel decrease

in growth (Reid and Carr, 1967; Reid and Crozier, 1970; Crozier et al., 1973). None of the authors could state conclusively that the growth inhibition observed was a direct result of blocked GA biosynthesis. A cell-free system from Cucurbita maxima was successful in converting mevalonate to GA_{12} aldehyde via the series of kaurene derivatives and further to GA_{15}, GA_{24}, GA_{26}, and GA_{27} (Graebe et al., 1974). Other studies on both the fungal system and plant systems have determined the proposed mechanism of GA biosynthesis shown in Figure 2.

Concurrently, testing of the known GA's in bioassay systems revealed that some gibberellins were more active than others (Crozier et al., 1970). The same gibberellin can also induce different responses from different bioassays. Pharis reported a wide spectrum of responses to GA_{24} in ten bioassay systems that gave a relatively balanced response to GA_3 (Pharis et al., 1968).

The relationships between chemical structure and biological activity had been determined by Japanese researchers in 1959 and later confirmed by Brian (Sumiki et al., 1959; Brian et al., 1967). In general, the intact gibbane ring (see Figure 3) appears necessary for activity, and fission of ring A or D results in loss of activity. The lactone ring confers high activity, as does the exocyclic methylene on the D ring. If the free carboxyl is masked by methylation or some other esterification, physiological activity is reduced or lost. The isolation of a gibberellin glucoside, GA_8 O(3)β, D-glucopyranoside (Smebdner, 1968), led to speculation that esterification may be a deactivation process. Using

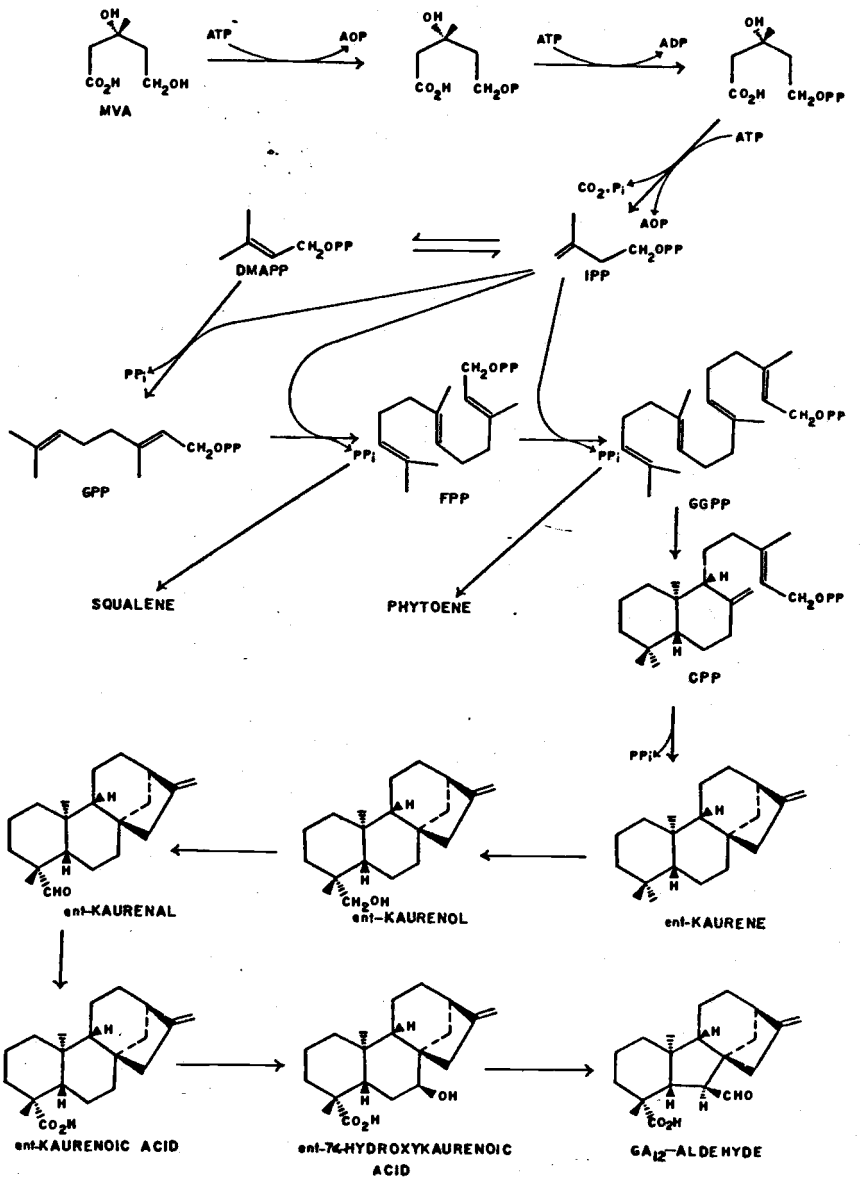


Figure 2. The biosynthetic pathway from mevalonic acid (MVA) to GA_{12} aldehyde. The inhibitors CCC and AMO-1618 block the conversion from transgeranyl geranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP). Other abbreviations: isopentenyl pyrophosphate (IPP); dimethyl allyl pyrophosphate (DMAPP); geranyl pyrophosphate (GPP); farnesyl pyrophosphate (FPP) (Hedden, Macmillan, and Phinney, 1978).

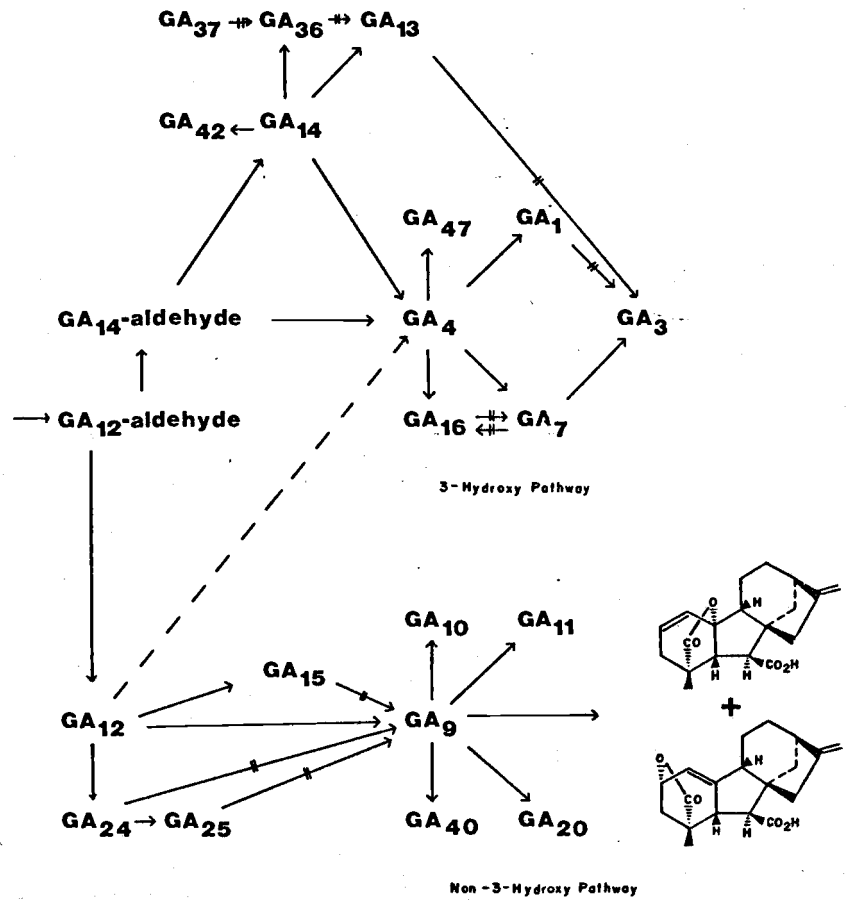
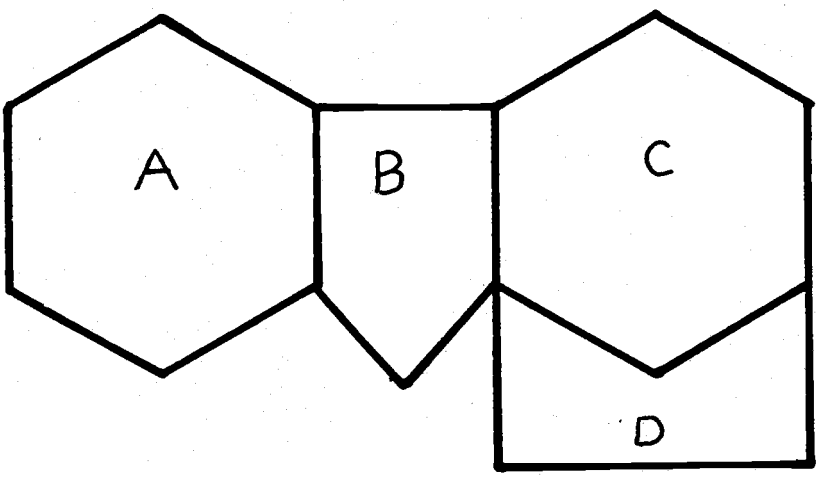
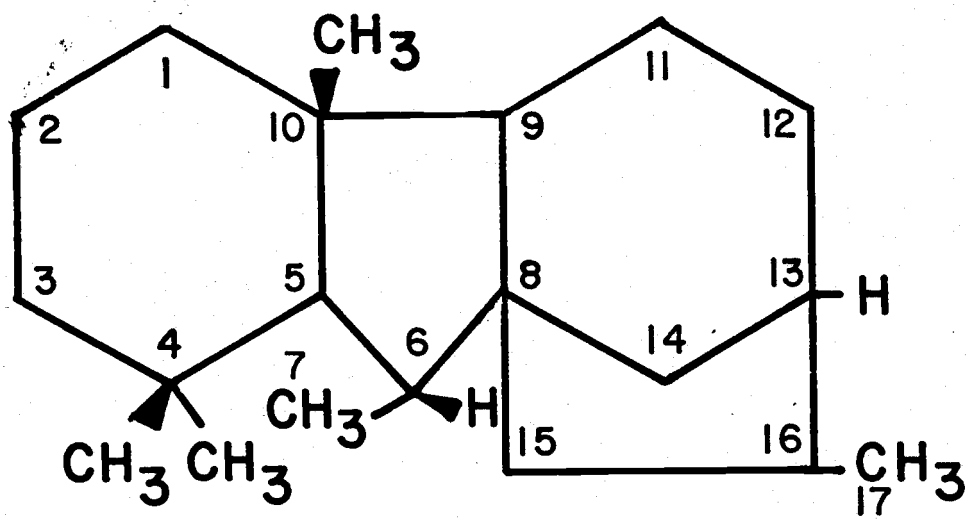


Figure 2. (continued). Interconversions of gibberellins beyond GA_{12} aldehyde established for cultures of the fungus Gibberella fujikuroi. (Hedden, MacMillan, and Phinney, 1978).



Gibbane



Gibberellane

Figure 3. Structures of gibbane and gibberellane, with numbering of the carbon atoms indicated.

radiolabelled GA_1, Barendse established the formation of 'bound' GAs in Pharbitis nil, and suggested that they were synthesized as free acids and glucosylated later when they were no longer in demand (Barendse, 1971).

Generally, studies on the metabolism of GA's in higher plants reveal that exogenous gibberellins are β-hydroxylated to the less active structure. Railton and others found GA_1 converted to its β-hydroxy counterpart GA_8 in Oryza sativa (Railton et al., 1973), and observed the hydroxylation of GA_{20} to GA_{29} in Pisum sativum (Railton et al., 1974). This decrease in GA_{20} and subsequent increase in GA_{29} has been correlated with levels of maturation of pea seeds (Frydmann, Gaskin, and MacMillan, 1974). The general tendency of C_{19} gibberellins to undergo 2β-hydroxylation has been demonstrated to be ubiquitous with regard to species and plant part: the conversion of GA_1 to GA_8 in bean seedlings, along with an unknown metabolic fate of GA_4 (Reeve et al., 1974); the metabolism of GA_4 to GA_2 and GA_{34} by vegetative shoots of Douglas-fir (Wample et al., 1975); and the conversion of GA_4 to GA_{34} via GA_1 by the pollen of three Pinus species (Kamienska and Pharis, 1975; Kamienska, Durley, and Pharis, 1976a; Kamienska, Durley, and Pharis, 1976b).

These metabolic alterations of free gibberellins to deactivated compounds suggest a possible reason for the seemingly superfluous number of GA's that have thus far been isolated from higher plants. The alteration of highly active gibberellins to their inactive β-hydroxy forms have occurred at different stages in development.

In Douglas-fir shoots the metabolism was most rapid during bud set and bud break and least rapid during shoot elongation (Wample et al., 1975). The more polar GA's also appeared as germination progressed in pine pollen (Kamienska and Pharis, 1975). These results suggest that, as the need for hormone production disappears, the active GA's become deactivated.

Since a glucosylated gibberellin is more water soluble than the free acid, the glucosylations may be necessary as a form of transport between roots and shoots. Although both roots and shoots have been convincingly demonstrated as sites of GA synthesis, a number of researchers have suggested that certain GA's may be synthesized in one location and translocated to another for conversion to a more useful form. Evidence for the synthesis of GA's in the roots of pea, lupin, and balsam plants and their subsequent translocation to the shoots was provided by Carr (Carr et al., 1964). Since that time several gibberellin-like substances have been detected in the spring xylem sap of sycamore and birch (Reid and Burrows, 1968) and apple and pear trees (Jones and Lacey, 1968). Gibberellic acid labelled with ^{14}C was implicated in a two-way exchange between xylem and sieve tube sap (Wareing and Bowen, 1969).

Various researchers have examined decapitated and rootless seedlings to determine the metabolism and translocation of GA's between roots and shoots. Crozier and Reid reported a disappearance of GA_1 in the leaves and apical buds of de-rooted Phaseolus coccineus seedlings, with a concomitant increase in GA_{19} levels, the major GA in control plants being GA_1 (Crozier and Reed, 1971). From these

results they theorized that shoots were the site of GA_{19} biosynthesis, and roots were the site of conversion to GA_1. Thus when the roots were removed from the plants, the locale for GA interconversion disappeared and the GA_{19} level in the shoots increased. The sap of defoliated Douglas-fir seedlings was measured for GA content (Sweet et al., 1973) and exhibited high levels of two gibberellin-like substances not found in control and girdled plants, suggesting that the leaves may have been a sink for gibberellins produced by the roots, and their removal resulted in a buildup of GA's in the sap. Recent studies using Phaseolus coccineus, however, seem to indicate that root to shoot transport of GA's in this species is unlikely (Railton, 1979).

These conflicting results suggest a need for more accurate and sensitive methods of detection for gibberellins extracted from higher plant tissues. The earliest chemical identifications of gibberellin structures were confirmed by physical and chemical properties such as melting point, optical activity, and ester formation (Kurosawa, 1926; Stowe et al., 1959; Mitchell and Angel, 1950; Curtis and Cross, 1954).. Separation of components from plant tissues was often effected by paper chromatography (Ogawa, 1963); hence authors reported the isolation of GA-like substances that induced a response to various bioassays (Krugman, 1967; Crozier and Audus, 1968). MacMillan discussed the lack of resolution obtained with paper chromatography and reported the adequate separation of the nine existing gibberellins (MacMillan and Suter, 1963) using thin layer chromatography (TLC); however, this technique still was not reliable

enough for conclusive identification. The development of liquid chromatographic techniques that employed a number of different separation mechanisms improved the purification of plant extracts, which is necessary as a preliminary step prior to bioassay. These included Sephadex columns and silica gel partition columns (Powell and Tautvydas, 1967; Crozier et al., 1969; Durley et al., 1972).

With the application of combined gas chromatography-mass spectrometry (GC-MS) techniques to gibberellin research, it became possible to identify components of plant extracts that had been rigorously purified. The mass spectra of the methyl esters and trimethylsilyl ethers of the methyl esters of GA_1 through GA_{24} were published in 1969 (Binks and MacMillan, 1969). These two derivatives enhance the volatility of the gibberellins, and thus increase the ease of identification by MS. Since that time other researchers have published MS-confirmed identifications of many of the known GA's that have been isolated from higher plants (Durley et al., 1971; Crozier et al., 1971; Bowen et al., 1973; Yokota et al., 1975). By far, the most potent sources of gibberellins have been immature seeds. A review by Gaskin and MacMillan detailing the GC-MS technique as it applies to gibberellins appeared in 1977 (Gaskin and MacMillan, 1977).

Any compounds which are to be analyzed by mass spectrometry must be high in purity or the spectrum will be so inundated by various masses that identification will be virtually impossible. The variety of High Performance Liquid Chromatography (HPLC) systems available today improves that requisite purity, and therefore allows

for optimum use of the mass spectrometer. High performance gel permeation chromatography is based upon separation via molecular size differences and offers the advantages of a finite elution volume and an isocratic solvent system (Krishen, 1977). Reeve and Crozier applied this technique to the purification of plant extracts for hormone analysis, and demonstrated the separation of gibberellins as a group rather than individuals (Reeve and Crozier, 1976b). Other types of separatory mechanisms include both straight phase and reverse phase chromatography (Crozier and Reeve, 1978). The greater the number of different fractionating methods that can be used in purification of extracts, the purer the extracts will become, since compounds that co-chromatograph with GA's on one system may have unlike retention times on another and thus would be separated out. A discussion by Reeve and Crozier on the quantitative analysis of plant hormones explains the theoretical basis of this premise (Reeve and Crozier, 1979).

This review has dealt with some of the early history of gibberellin research as it applies to this study. Many of the techniques described here have been used in this research to develop a method for identifying gibberellins isolated from plant extracts, in this case, Douglas-fir xylem sap.

MATERIALS AND METHODS

Development of Derivatization Technique

If High Performance Liquid Chromatography (HPLC) is to be used for separating gibberellins, the gibberellin (GA) molecules must be detectable by the available method, in this case, absorbance of light in the ultraviolet (uv) wavelength range. Gibberellins as free acids do not absorb appreciable quantities of uv light, therefore, esters which do absorb in the uv were produced.

The derivatization procedure was modified from that used by Durst in the esterification of fatty acids (Durst et al., 1975). It was performed by adding an equimolar amount of KOH in ethanol and a catalyst, 18-Crown-6 (Regis), to a given quantity of gibberellin. After the addition of $KHCO_3$ (less than 1 µgm), the solvents were evaporated in a stream of nitrogen. The reagent, para-bromophenacyl bromide (pBPB) in acetonitrile, was then added in an equimolar amount, and the mixture was heated for 30 minutes at 75 C with occasional stirring. After completion of the reaction, the acetonitrile was evaporated and the products were partitioned in chloroform and water. The gibberellins, which became more nonpolar with the addition of the pBPB moiety, partitioned into the chloroform. The water portion was discarded. After evaporation of the chloroform, the gibberellin derivatives were stored in ethanol at -20 C. A detailed diagram of the chemical reaction is provided in Figure 4.

Experiments were performed using GA standards to determine: a) the length of time in minutes necessary to complete the reaction;

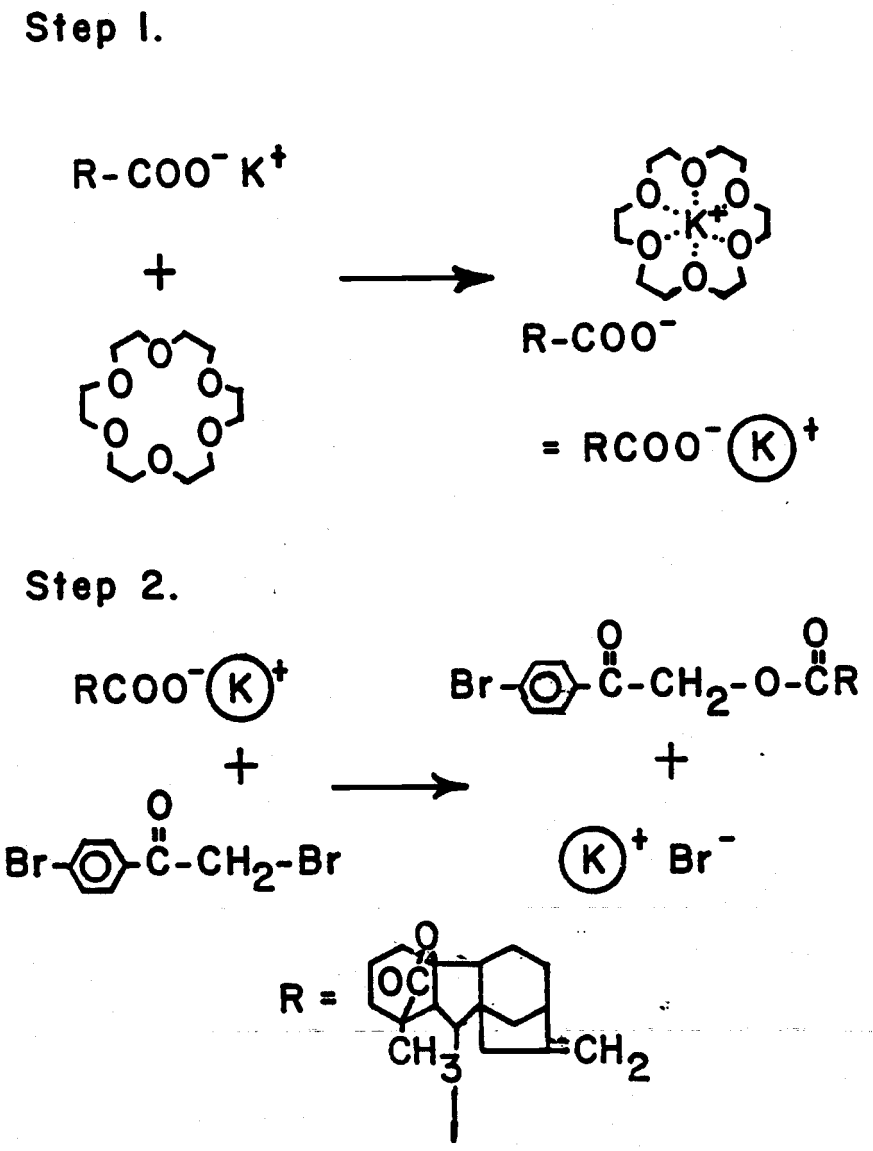


Figure 4. Reaction mechanism of parabromophenacyl derivatization. Step 1 is the chelating of the gibberellin potassium salt to the catalyst. Step 2 is the esterification of the reagent, parabromophenacyl bromide, to the gibberellin.

b) the extent of the derivatization expressed as a percentage; and c) the probability of methyl ester formation instead of para-bromo phenacyl ester formation. The time study was accomplished by derivatizing a known quantity of GA_3 using the above procedure. The reaction was continued for three hours; every 30 minutes an aliquot was chromatographed on a Waters octadecyl silica reverse phase column at a flow rate of 2.0 milliliters per minute (ml/min) over a ten minute gradient from 50 to 80% ethanol (ETOH) in 0.02 Molar NH_4OAc pH 3.5. The resultant peak areas were compared using a Hewlett Packard model 3370A integrator.

The extent of the derivatization was determined by esterifying tritiated GA_9 by the above procedure. After completion of the reaction, the levels of radioactive label were measured in the chloroform and water portions using a Packard tricarb liquid scintillation spectrometer. The percent derivatization was calculated as the counts per minute (cpm) in the chloroform divided by the total counts per minute.

The test for methyl ester formation was performed by first fractionating tritiated GA_1 methyl ester standard and non-labelled GA_1 on a reverse phase column. An isocratic system of 60% ETOH/40% buffer[1] running at 1.0 ml/min was determined to be optimum for compound separation. Detection of the methyl ester was via the distribution of the tritium label in collected fractions, whereas the pBPB ester was evident from the uv absorbance. Esterification

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[1]Buffer refers to 0.02 M NH_4OAc pH 3.5 unless otherwise stated.

of 100 µl of non-labelled GA_1 plus 100 µl tritiated GA_1 followed. At ten-minute intervals, aliquots were removed from the vial and partitioned in cold chloroform and water. In order to stop the reaction completely, this operation was performed on ice. Each aliquot was then chromatographed under the same conditions as the GA standard esters and fractions were collected. The main peak of radioactivity and the main peak of uv absorbance were then compared to those obtained previously with the standard esters.

Collection of Plant Materials

a) Sap Collection. Upon exposure to an oxygen atmosphere, certain compounds that are present in plant tissues appear to oxidize to a form that can selectively bind other compounds such as amino acids and proteins. Since the presence of these compounds can interfere with the detection of gibberellins that may be present, a procedure precluding their formation was necessary. A singular collection method was thus developed in this laboratory to prevent possible oxidation of the components of the sap; this initial, preventative procedure was done to maintain the natural quality of the sap, thus obviating measures to remove any artifacts in subsequent steps.

The xylem sap used in these experiments was obtained from Douglas-fir saplings growing wild in MacDonald Forest, near Corvallis. Predawn harvesting of saplings of an appropriate diameter was carried out from the time of bud break through summer. The harvested trees were bound by string into a cylindrical shape to permit their

introduction into a pressure apparatus, wetted down, and stored in a black plastic bag to insure their freshness until they could be transported to the laboratory. The length of time between the cutting of the trees and cold storage was in all cases no more than one hour. At the laboratory, the trees were stored at a temperature of approximately 3 C for up to eight hours, until the xylem sap could be extracted.

The extrusion of the sylem sap was accomplished with a pressure chamber apparatus. The particular instrument used for these saplings was a chamber five inches in diameter and five feet long into which the tree can be placed with the cut end protruding through a rubber seal. With the application of ~ 20 bars to the chamber, the sap in the xylem is forced out the open end of the bole. The sap so obtained, termed extrudate, was collected into a 250 ml bottle via a funnel, with a stream of nitrogen directed onto it, and set into a box filled with dry ice. Upon acquisition of 200 to 250 ml of sap, the bottle was capped, stored at -80 C initially and then at - 40 C.

The lack of an oxygen atmosphere and exposure to room temperature successfully prevented the oxidation of the sap components, as evidenced by the transparency of the sap and the total lack of brownish color upon thawing.

b) Cucumber Seed[2] Collection. The cucumber seeds, of the species Marah oreganus T. and G., were collected locally along the roadside in late May. They were placed in cold storage for a few hours until the fleshy husk could be removed. The seeds were then weighed, and the endosperm removed for immediate extraction.

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[2]The wild cucumber seeds will hereafter be referred to as cucumber.

Trace Enrichment. Initial attempts to extract gibberellins from the requisite quantity of xylem sap via the solvent partitioning method (see below) met with no success; when dealing with 500 to 1000 ml of sap, the technique became cumbersome and unwieldy, resulting in heavy losses of the gibberellin radio-tracer. The method of trace enrichment, first developed by Morris and associates for cytokinins, was used here with some modifications in an attempt to concentrate the gibberellins that may be present in xylem sap (Morris et al., 1976).

Approximately 500 mls. of sap, collected during the months of March, April, June, and July, were examined. The frozen xylem sap was first thawed and then filtered through a Millipore apparatus with a 0.45 micron acetate filter that had been washed previously with two liters of distilled, deionized water to remove a saponaceous deposit on the filter. After lowering the pH of the sap to 3.5 with concentrated HCl, a radiotracer was added. Using a peristaltic pump, the sap was forced at a rate of approximately 100 ml/hour through a glass column packed with octadecyl silane (ODS), a silaceous support material with retentive properties. A 200 ml rinse with buffer pH 3.5 followed. Aliquots of both the sap eluate and the buffer rinse were kept for determining uv absorbance and radiotracer levels; the remainder of both was discarded. Elution of the adsorbed sap was accomplished with the following concentrations of ETOH in ammonium acetate buffer pH 6.0: 10%, 30%, 50%, and 100%; in each case the elution volume was 25 to 30 ml. The eluates were stored at -20 C until solvent partitioning. Before another

application of sap to the column, the packing material was regenerated by washing with 50 ml aliquots of a succession of solvents: ethanol, tetrahydrofuran, dimethylformamide, and ethanol. The column was then reequilibrated with 100 mls of ammonium acetate buffer.

The endosperm and embryos were removed from 100 grams of immature wild cucumber seeds and homogenized in 50 ml distilled water. After buffering to 0.02 M NH_4OAc pH 3.5, the extract was trace enriched in an identical manner to the xylem sap.

Solvent Partitioning

This technique has been the preferred method for the extraction of gibberellins from various types of plant parts (Russell, 1976). In these experiments the trace enriched fractions were first evaporated to dryness in vacuo, and then reconstituted with approximately 30 ml of 1.0 M phosphate buffer pH 8.0. After insuring that the pH remained at 8.0, each collection from the trace enrichment column was partitioned three to four times against an equal volume of distilled toluene. Cross-linked polyvinyl pyrrolidone, obtained from Sigma as polyvinyl polypyrrolidone (PVPP), was added to the buffer portion, and the toluene was discarded. PVPP had been demonstrated to be instrumental in removing the artifacts which arise from oxidation of phenolic compounds and their subsequent combination with proteins and amino acids (Loomis, 1974; Loomis and Battaile, 1966). The mixture was stirred occasionally over a period of 20 to 30 minutes, then the PVPP was filtered off through a washed bed of α-floc (α cellulose powder) with a Buchner funnel. After

filtering, the bed of α-floc was rinsed with five to ten ml of 1.0 M phosphate buffer. Adjusting the pH of the buffer fraction to 2.5 protonates the acids present, thus increasing their affinity for an organic solvent over an inorganic one. The buffered extract was partitioned five times with a 2/5 volume of ethyl acetate (ethyl acetate:buffer = 2.5) so that the final volume was twice the initial. At this point, if desired, an aliquot of each fraction was taken to determine the distribution of radiotracer; the buffer portion was discarded and the EtOAc fraction was stored at a temperature of -20 C to allow the dissolved water to freeze out of the EtOAc. The radioactivity was counted only at this step of the entire extraction because previous experiments indicated that the bulk of the losses incurred with this method were at this point, while losses to toluene, PVPP, or α-floc were negligible. Usually the extract was frozen overnight, then the water was filtered off in a -20 C room. The addition of anhydrous sodium sulfate (Na_2SO_4) was necessary to remove completely the water from the extract. After filtering, the EtOAc was evaporated <u>in vacuo</u> and the samples stored at -20 C until High Performance Liquid Chromatography.

The following instruments and equipment were used in this study:

Instruments:

Varian Model 5000 high performance liquid chromatograph (HPLC)

Altex HPLC, programmer model 420, pumps model 110A

Water's ultraviolet absorbance spectrometer model 440

Savant speed vac concentrator model SVC100

HPLC Columns:

Reverse Phase	
10μ Waters μBondapak C_{18}	3.9 x 300 mm
5μ Varian micropak MCH·5 (#108)	4 x 250 mm
5μ Altex ultrasphere ODS (#VE551)	46 x 250 mm
Gel Permeation	
Perkin Elmer Shodex (#A 8025)	8 x 250 mm
Altex 50Å μSpherogel (#255-80)	4 x 300 mm

High Performance Liquid Chromatography

a) Gel Permeation Chromatography (GPC). To each of the dried samples was added 100 μl of HPLC-grade tetrahydrofuran (THF). Each sample was then injected into a Varian model 5000 liquid chromatograph and pumped onto an Altex 50 Å μSpherogel gel permeation column. The isocratic solvent system consisted of 100% THF pumping at a flow rate of 1.0 ml/min over a period of 15 minutes. The uv absorbance was monitored by a Waters model 440 uv spectrophotometer and recorded on a Linear chart recorder set at 1.0 cm/minute. A collection was taken between seven and ten minutes for each of the injected samples. This fraction was previously determined to contain compounds of a molecular weight and size comparable to gibberellins by injecting tritiated GA standards, collecting half-minute fractions, and monitoring the radioactivity on a scintillation counter. The collected fractions were stored at -20 C until they could be further fractionated on a reverse phase system.

b) Reverse Phase Chromatography. After evaporating the THF from each of the samples, 100 µl of distilled ethanol (95%) was added in order to separate the compounds present on a reverse phase system. For this fractionation a Varian micropak MCH-5 column was eluted with a buffer/ETOH gradient consisting of a 30 minute program beginning with 100% of the buffer (0.02 M NH_4OAc pH 3.5) and finishing at 30 minutes with 100% ETOH. A flow rate of 0.8 ml/min was necessary to maintain a column head pressure below 350 atmospheres during the course of the gradient. Thirty fractions were collected at one minute intervals from minute one to minute 31. These fractions were evaporated to dryness with a Savant rotary evaporator and stored at -20 C until bioassay.

Bioassay

Of the various gibberellin bioassays available, the Tan-ginbozu dwarf rice microdrop bioassay was selected in order to test the HPLC fractions for GA activity, because of the sensitivity and range of the assay (Crozier et al., 1970). This particular strain of rice is deficient in endogenous gibberellins, hence a growth response is observed when GA's are applied (Reeve and Crozier, 1976a).

The assay required one week to complete, beginning with the soaking of the rice seeds in an excess of distilled water. The seeds were soaked for 45 hours under continuous light at 32 C and as near as possible to 100% relative humidity. The humidity was maintained by placing the seeds in a tightly closed plexiglass box equipped with a screen as a shelf on which to place the seeds over a water reservoir.

After 45 hours of soaking, the coleoptiles had emerged and the seeds were planted in two-inch by two-inch wide-mouth bottles which had been filled with a solidified 0.8% agar in distilled water solution as a support. Ten seeds were planted per bottle. After permitting the seedlings to grow for 48 hours, at which time the second leaf had emerged, five uniform seedlings were selected and the remainder were discarded. The HPLC fractions were reconstituted with ten µl of 50% ETOH/distilled H_2O, and one µl was applied to each seedling at the intersection of the second leaf and the leaf sheath. Elongation of the second leaf sheath was measured after 75 hours, and the fractions which induced a growth response that was significantly above that of the control were judged to contain gibberellin-like substances.

Derivatization of Plant Extracts by parabromophenacylation

Those fractions which appeared biologically active in the bioassay were derivatized by the method described previously. One µmole of reactants were used to carry out the procedure; this quantity was determined to be sufficient from the results of the bioassay.

Methyl-TMS Derivatization of Plant Extracts

Half of each fraction to be derivatized was used to make methyl tri-methyl silyl (Me-TMS) derivatives to confirm the results of the mass spectrometry of the pBPB derivatives. The samples were dried thoroughly under nitrogen, and an excess ($\sim$ 2 ml)

RESULTS

The objectives of this research were to develop a method for isolating and identifying certain gibberellins and to apply this technique to Douglas-fir sap. The results section describes the development of the technique using gibberellin standards; some preliminary attempts to extract GA's from Douglas-fir sap which resulted in the development of trace-enrichment; the use of wild cucumber seeds to determine the feasibility of the revised technique for a biological system; and the application of the technique to Douglas-fir sap.

Technique Development

a) Derivatization of Gibberellin Free Acids

High Performance Liquid Chromatography (HPLC) of the aliquots from the derivatized GA_3 standard resulted in two peaks of uv absorbance, the first corresponding in retention time to GA_3 parabromophenacylbromide (GA_3pBPB) standard. Comparison of the peak areas (Table 1) indicates the degree to which the GA_3 free acid was converted to the GA_3 ester. The reaction, although continued for 180 minutes, was essentially complete within 30 minutes (Figure 5).

The extent of the derivatization of GA free acid to GApBPB was calculated as a percent of the total tritiated GA_9 involved in the reaction. 1.6 ng of tritiated GA_9 (^{3}H-GA_9) corresponding to 220,000 cpm were derivatized according to the standard procedure. After partitioning with 100 µl $CHCL_3$ against 100 µl H_2O, the partition layers were counted in separate scintillation vials. Since the

Table 1: Comparison of peak areas of chromatographed GA_3 aliquots from time study

Injected sample	Peak 1		Peak 2	
	R_t	$area_5$ x 10^5	R_t	$area_5$ x 10^5
GA_3pBPB standard	3.4	---	---	---
Time 0	3.2	---	4.8	---
Time 30	3.0	6.4	5.0	2.0
Time 60	3.0	5.9	5.0	1.7
Time 90	3.0	5.3	5.4	1.6
Time 120	3.2	6.0	6.0	1.7
Time 150	3.1	6.0	5.4	2.0
Time 180	3.0	5.8	5.5	1.7

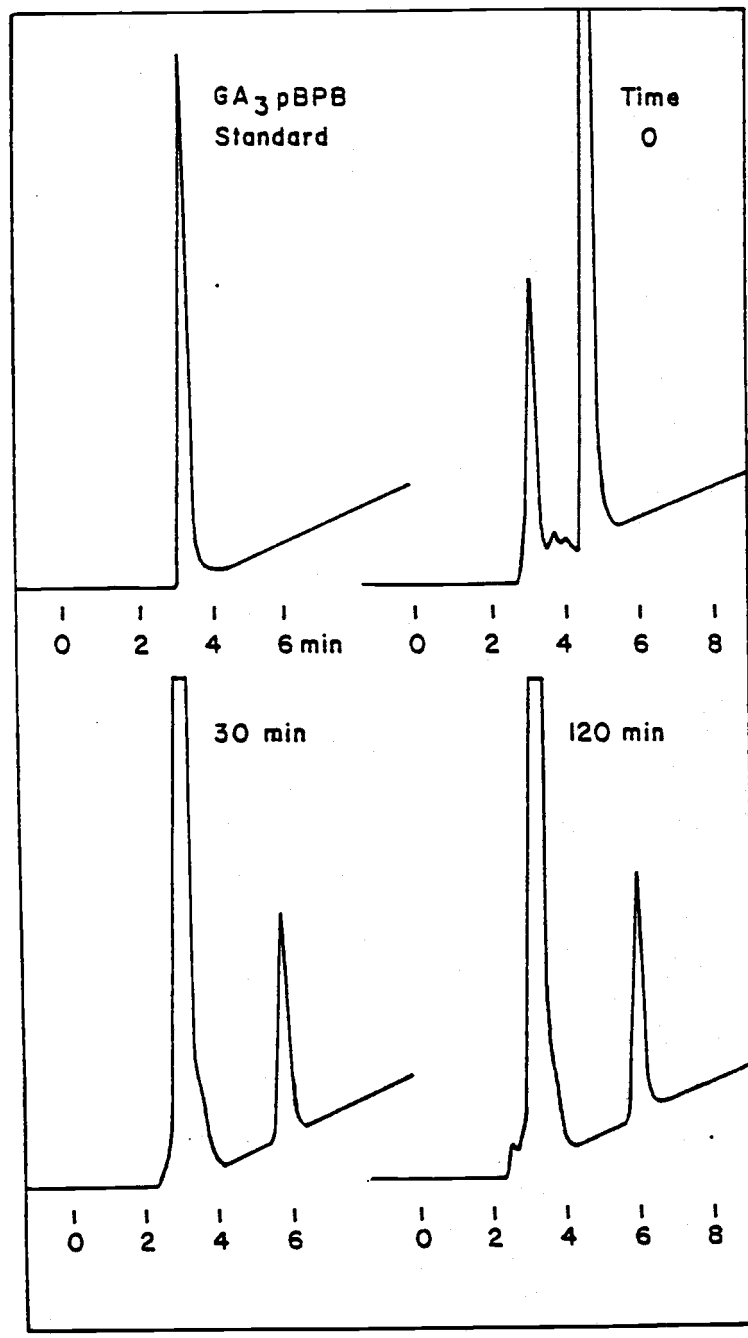


Figure 5: HPLC of derivatization time study. Waters μC_{18} column, 50-80% EtOH/buffer, 10 minutes, 20 ml/min. The derivatization, evident at time 0, is essentially complete by time 30.

esterified GA_9 partitioned into the chloroform layer and the free acid into the aqueous layer, the percent derivatization can be estimated as the chloroform-soluble cpm divided by the total cpm. Of the original 220,000 cpm, 195,000 were counted in the chloroform, indicating 88% derivatization.

Reports of GA methyl ester contaminants during the formation of other GA derivatives (A. Crozier, personal comm.) indicated that an investigation of the derivatization was warranted. Since the methyl ester of a gibberellin cannot be detected via uv absorbance, the methyl ester of GA_1 (GA_1ME) was produced using tritiated GA_1. A mixture of GA_1ME and GA_1pBPB was then fractionated on an octadecyl silyl (ODS) column. The GA_1ME had a retention time of 3.5, while the GA_1pBPB had a retention time of 10.6. A time course was performed using tritiated GA_1 for the derivatization to GA_1pBPB, and aliquots at 0, 10, 20, 30, 40, 50, and 60 minutes were injected into a Shandon ODS column. The radioactivity in collected fractions was shown to be eluting with the GA_1pBPB rather than the GA_1ME. I therefore concluded that there are no detectable levels of GA_1ME, and that the tritiated GA_1 is being exclusively converted to a compound with retention properties similar to GA_1pBPB.

b) HPLC of Standards

Gibberellin standards obtained from Dr. J. MacMillan (University of Bristol, Bristol, England) were derivatized and chromatographed to observe their retention characteristics, both individually and combined with other standards. A list of their retention times on

octadecylsilica is given in Table 2. In reverse phase chromatography, the order of elution is governed by decreasing polarity. With gibberellins, the polarity is most strongly influenced by the number of hydroxyl groups and carboxyl groups. Of the monocarboxylic GA's, GA_8pBPB elutes first, since, having three hydroxyl groups, it is the most polar. It is followed by GA_3pBPB and GA_1pBPB, which have two hydroxyl groups each, followed by GA_5pBPB, GA_{20}pBPB, GA_7pBPB, and GA_4pBPB with one hydroxyl each. GA_9pBPB, lacking any hydroxyl functions, is the least polar and hence the last of the monocarboxylic GA's to elute. Of the gibberellin esters sharing a like number of carboxyl and hydroxyl groups, the polarity is affected by such things as the distribution of the hydroxyl groups on the gibberellane (Fig. 3) skeleton, and the amount and location of unsaturated bonds. For example, GA_3pBPB and GA_1pBPB are identical except for the double bond between carbons 1 and 2 on GA_3. This unsaturation accounts for the earlier elution of GA_3pBPB from the ODS column. GA_5pBPB and GA_{20}pBPB are analogous, as are GA_7pBPB and GA_4pBPB. The importance of hydroxyl distribution is evident in the fact that GA esters 5 and 20, with the hydroxyl at C-13, elute sooner than GA esters 7 and 4, which have their hydroxyls attached to carbon 3.

Because their free carboxyls have been esterified with bulky, nonpolar parabromophenacyl groups, the esters of GA's 13, 14, 25, and 36 require almost 100% EtOH to remove them from the nonpolar stationary phase. GA_{25}pBPB, tricarboxylic and lacking hydroxyls, is the last to elute. GA_{36}pBPB, GA_{14}pBPB. amd GA_{13}pBPB each have one hydroxyl group and elute in that order. GA_{13}pBPB is the last of the three because it also is tricarboxylic. GA_{36}pBPB and

Table 2: Retention times (R_t) in minutes of individual GApBPB standards by reverse phase liquid chromatography

GApBPB	# OH	# COOH	structural differences between similar GAs	R_t	polarity
8	3	1	----	4.00	most polar
3	2	1	unsaturation C1-C2 (GA_1)	4.25	
1	2	1	saturation C1-C2 (GA_3)	5.75	
5	1	1	unsaturation C2-C3 (GA_{20})	7.50	
20	1	1	saturation C2-C3 (GA_5)	8.50	
7	1	1	unsaturation C1-C2 (GA_4)	9.00	
4	1	1	saturation C1-C2 (GA_7)	9.25	
9	0	1	----	10.50	
36	1	2	CHO function C20 (GA_{14})	11.25	
14	1	2	CH_3 function C20 (GA_{36})	12.00	
13	1	3	----	12.10	
25	0	3	----	13.25	least polar

GA_{14}pBPB are dicarboxylic; GA_{36}pBPB is more polar due to the replacement of the lactone bridge by an aldehyde function, which in GA_{14}pBPB is merely a methyl group. The two molecules are otherwise identical.

Chromatograms which illustrate these properties are shown in Figure 6.

A 1.0 mg portion of GA_3pBPB was weighed out and dissolved in 1.0 ml of EtOH as an accurate standard of an esterified GA with a concentration of 1.0 µgm/µl. This was then chromatographed on a reverse phase system with a gradient from 50 to 100% EtOH in order to permit quantitation. A 1.0 µgm aliquot yielded a peak of uv absorbance (254 nm) that was 18.5 cm in height at a full scale deflection of 0.8.

Gel permeation chromatography (GPC) of these standards was also performed. Unlike reverse phase, retention on a GPC column is based upon size and shape of the molecules; consequently, the gibberellins will elute essentially as a group. Comparison of the retention times in Table 3 illustrates this. The di- and tricarboxylic GA esters, being somewhat larger and bulkier, elute slightly ahead of their monocarboxylic counterparts, because a gel permeation column retains smaller molecules longer. GA_9pBPB, lacking the jutting hydroxyl groups, is the last of the gibberellins to elute (Figure 7).

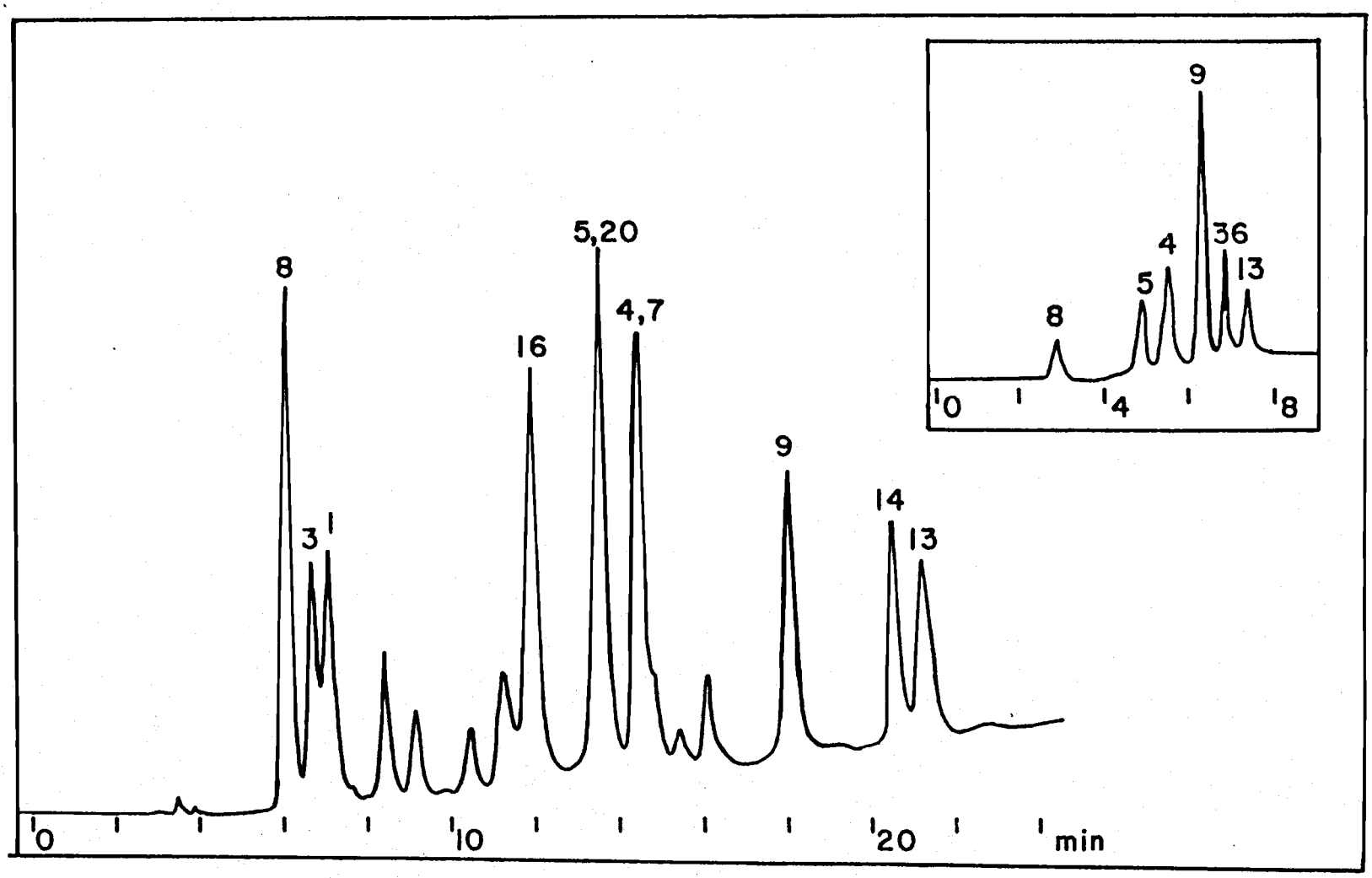


Figure 6: Reverse phase chromatography of GApBPB standards. The individual gibberellins are indicated above their corresponding peaks. Full size figure: Varian MCH-5 column, 60-100% EtOH/buffer, 20 minutes, 1.5 ml/min. Inset: Waters μC_{18} column, 50-100% EtOH/buffer, 10 minutes, 2 ml/min.

Table 3: Retention times (R_t) in minutes of individual GApBPB standards by gel permeation chromatography

GApBPB	R_t
1	8.3
3	8.3
4	8.7
5	8.8
7	8.8
8	8.4
9	9.2
13	8.2
14	8.6
25	8.4
36	8.5

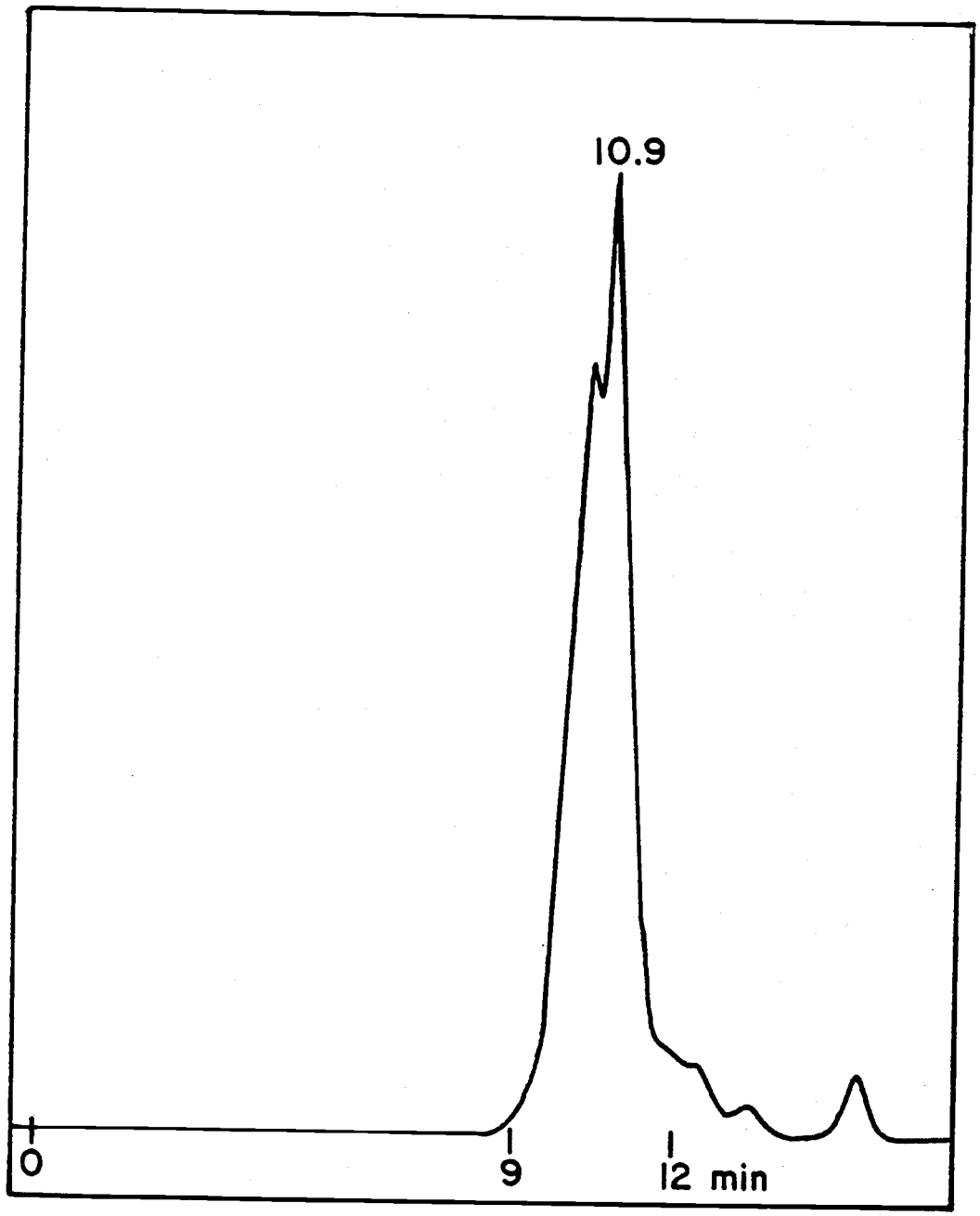


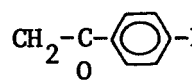
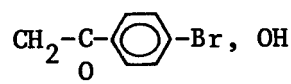
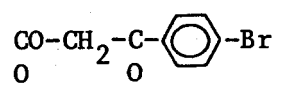
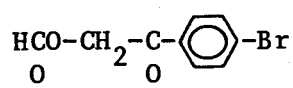
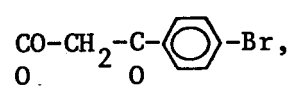
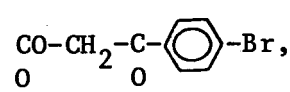
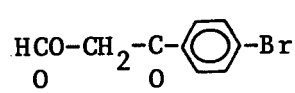
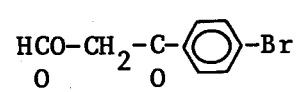
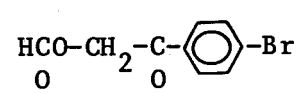
Figure 7. Gel permeation chromatography of CApBPB standards. The retention time of the main peak is at 10.9 minutes. Perkin Elmer Shodex column, 99% THF/1% acetic acid isocratic, 1.0 ml/min.

c) Mass Spectrometry of GApBPB Esters

HPLC-purified GApBPB standards were analyzed on the mass spectrometer. Table 4 lists the masses and structures of the fragments lost from the gibberellin molecules, and Table 5 lists the fragmentation patterns of some GApBPB standards.

The mass spectrum of GA_4pBPB is shown in Figure 8. The molecular ion, the large doublet at m/e 528,530, is the entire, unfragmented molecule minus one electron, and is represented by the symbol M^+ (McLafferty, 1966). Small peaks at m/e 510,512 and m/e 466,468 are due to the loss of H_2O (18) and H_2O and CO_2 (62) respectively. The loss of the parabromophenacyl moiety (pBP) (197) and the hydroxyl group (17) result in the peak at m/e 314 (M-214). With the loss of the entire ester fragment (pBP ester) (241) and a hydrogen atom, the free GA_4 sans carboxyl is evident at m/e 286 (M-242), and the further loss of the hydroxyl (17) yields the peak at m/e 269 (M-259). The largest peak in the spectrum, referred to as the base peak (McLafferty, 1966), is at m/e 224 (M-304) and is due to the loss of the pBP ester (241), COO (44), H (1) and H_2O (18); while the smaller peak at m/e 225 (M-303) is the base peak fragment that still retains a hydrogen atom. The doublet at m/e 198,200 corresponds to the protonated pBP ester, and the doublet at m/e 155,157 is phenyl bromide.

Table 4: Typical fragmentations of GApBPB standards by mass spectrometry*

Mass lost from M+	Structure of fragment	designated symbol
18	H_2O	H_2O
46	H_2O, CO	H_2O, CO
61	H_2O, CO, CH_3	H_2O, CO, CH_3
62	H_2O, COO	H_2O, CO_2
63	H_2O, COOH	H_2O, COO, H
197		pBP
214		pBP, OH
241		pBP ester
242		protonated pBP ester
259		pBP ester, H_2O
260		protonated pBP ester, H_2O
286		protonated pBP ester, CO_2
287		protonated pBP ester, COOH
304		protonated pBP ester, CO_2, H_2O

*The fragments, of necessity positive ions, have not been indicated individually as such.

Table 4 (continued): Some fragment ions typical to all GApBPB spectra

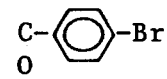
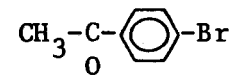
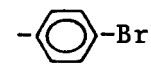
ion	structure
183/185	
198/200	
155/157	

Table 5: Fragmentation patterns of some GApBPB standards by mass spectrometry

GApBPB M+	Fragments
1 544/546	347 (M-197); 330 (M-214) base peak; 302 (M-242); 285 (M-259); 284 (M-260); 240 (M-304); 183/185 198/200
3 542/544	524/526 (M-18); 496/498 (M-46); 479/481 (M-63); 345 (M-197); 301 (M-241); 283 (M-259); 256 (M-286); 238 (M-304); 237 (M-305) base peak
4 528/530	510/512 (M-18); 466/468 (M-62); 314 (M-214); 286 (M-242); 269 (M-259); 225 (M-303); 224 (M-304) base peak; 198/200; 155/157
5 526/528	508/510 (M-18); 464/468 (M-62); 312 (M-214); 284 (M-242); 285 (M-241); 266 (M-260); 239 (M-287) base peak; 222 (M-304); 221 (M-305)
7 526/528	498/500 (M-28); 480/482 (M-46); 465/467 (M-61); 301 (M-225); 284 (M-242); 239 (M-287); 256 (M-274); 329 (M-197); 312 (M-214); 222 (M-304) base peak
8 560/562	542/544 (M-18); 363 (M-197); 346 (M-214) base peak; 319 (M-241); 318 (M-242); 300 (M-260); 256 (M-304)
9 512/514	494/496 (M-18); 466/468 (M-46); 315 (M-197); 298 (M-214); 271 (M-241); 270 (M-242) base peak; 225 (M-287); 226 (M-286); 210 (M-302)
14 740/742/744	526/528 (M-214) base peak; 498/500 (M-242); 480/482 (M-260); 465/467 (M-275); 301 (M-439); 284 (M-456)
20 528/530	331 (M-197); 314 (M-214) base peak; 286 (M-242); 241 (M-287); 198/200; 183/185; 510/512 (M-18)

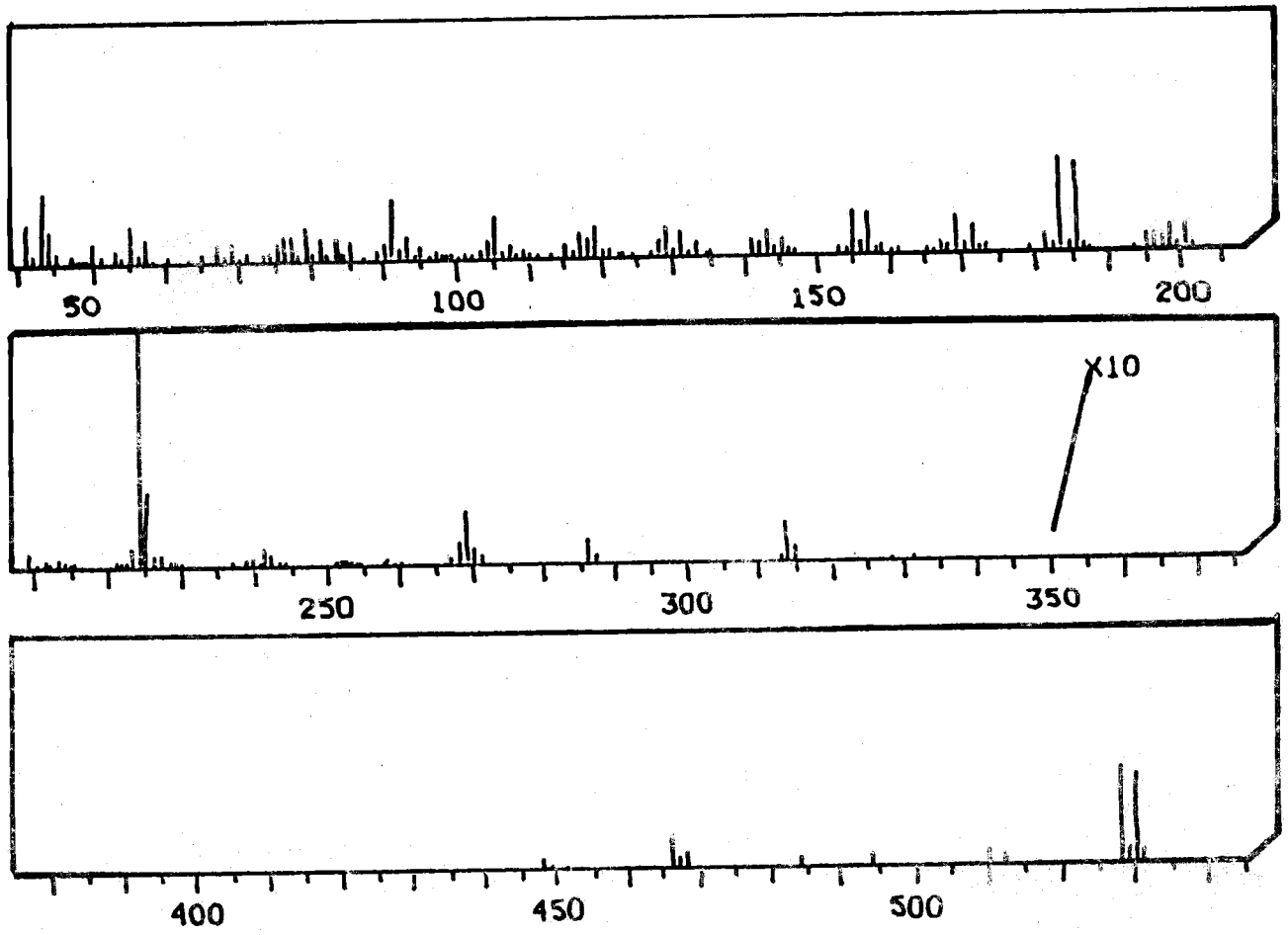


Figure 8. Mass spectrum of GA_4pBPB. The molecular ion is the doublet at m/e 528, 530, and the base peak is m/e 224 (M-304). The major peaks are m/e 466, 468 (M-62); m/e 314 (M-214); m/e 269 (M-259); and m/e 198/200 (protonated pBP).

Preliminary Studies on Gibberellin Isolation

a) Solvent Partitioning of Sap

The first attempt to isolate GA's from Douglas-fir sap utilized a technique which required partitioning the buffered sap between various solvents as described earlier. Table 6 lists the collection dates and volumes of the sap used in this extraction. Since at this time the trace enrichment procedure had not been developed, the sap had not been concentrated and the method differs slightly from that described previously.

The sap was thawed and ^{3}H-GA_1 was added as an internal standard. An equal volume of 1.0 M phosphate buffer at pH 8.0 was added to the sap prior to filtering through a bed of α-floc. After restandardizing the pH at 8.0, the buffered sap was partitioned against toluene. Hereafter the procedure is identical to that described earlier.

The tritiated GA was followed throughout the partitioning procedure to account for losses at each step. The losses in radiotracer due to the extraction are virtually negligible prior to the ethyl acetate partition (Table 7). However, due to the large volume of buffered sap extracted, the sap could not be partitioned against EtOAc more than twice. The nature of the partitioning coefficient of GA's between EtOAc and buffer requires four to five partitions to obtain substantial yields. Consequently, at this step there were considerable losses of gibberellin, at times as high as 40%. The losses could also be due in part to the inherent problems associated with trying to partition 2000 mls of solvent in a separatory funnel.

Table 6: Collection dates and volumes of xylem sap

collection date	volume
March 20, 1979	800 ml
April 5, 1979	1000 ml
April 17, 1979	1000 ml
May 4, 1979	600 ml
May 17, 1979	1000 ml
May 30, 1979	1000 ml
June 14, 1979	800 ml
June 27, 1979	900 ml
July 13, 1979	1000 ml

Table 7: Radiotracer levels at each step of solvent partitioning

Step	Aqueous			Organic		
	solvent	cpm ($\times 10^6$)	%	solvent	cpm ($\times 10^6$)	%
Addition of tracer	phosphate buffer pH 8.0	2.1	100	----	--	--
first partition buffer/toluene	"	2.0	95	toluene	*	*
PVPP	"	2.0	95	----	--	--
α-floc	"	1.97	94	----	--	--
second partition buffer/EtOAc	phosphate buffer pH 2.5	1.7	81	EtOAc	0.4	19

* cpm no greater than background

b) HPLC of Extracts

GPC

Each of the sap extracts was chromatographed on a Perkin Elmer Shodex gel permeation column that was 25 cm long with an internal diameter of eight mm. The collection band had been determined previously by chromatographing ^{3}H-GA_1 and ^{3}H-GA_9, collecting fractions, and counting the fractions on a scintillation counter to determine the retention times of free gibberellins (Figure 9).

A representative chromatogram of the GPC of the sap extract collected on April 5, 1979 is shown in Figure 10. The dotted line represents a 1/100th aliquot of the extract at an increased sensitivity. The solid line represents the entire extract at the full scale deflection of 2.0 on the detector, the highest available. The collection band began at 6-1/2 minutes and continued through 9-1/2 minutes.

Reverse Phase

Results of the GPC indicated that the extracts were still quite impure; therefore, as a preliminary purification step, each extract was chromatographed on an ODS column and a large band collected corresponding to the retention times of the free GA's. This collection band was determined by separating ^{3}H-GA_8 and ^{3}H-GA_9 on a 50 to 100% gradient of EtOH in buffer for 20 minutes. Since GA_8 is one of the most polar GA's and GA_9 is one of the least polar GA's, the bulk of the gibberellins would chromatograph between those two.

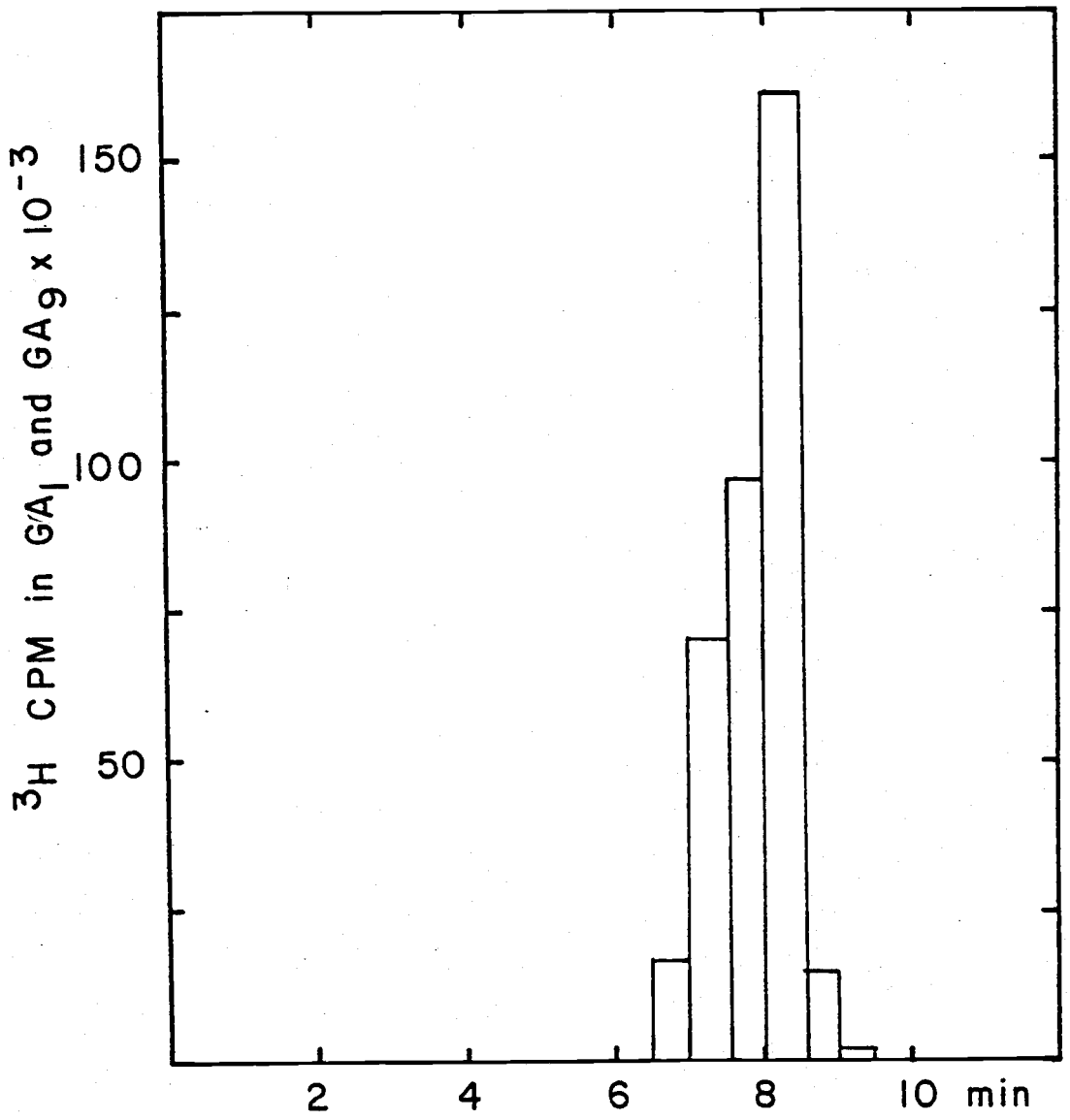


Figure 9: Gel permeation chromatography of ^{3}H-GA$_1$ and ^{3}H-GA$_9$. The gibberellins elute between 7 and 9 minutes. Perkin Elmer Shodex column, 99% THF/1% acetic acid, isocratic, 1.0 ml/min.

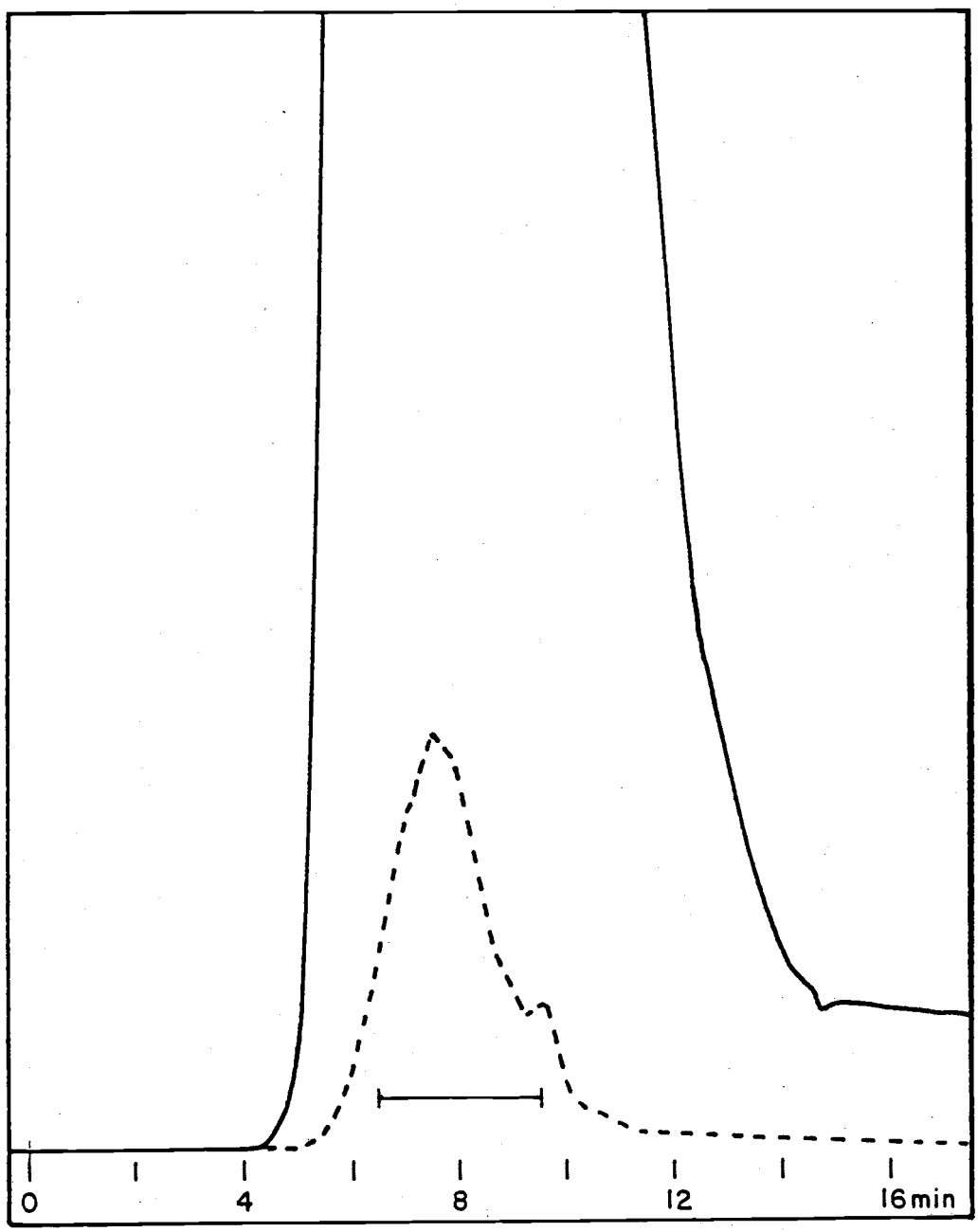


Figure 10. Gel permeation chromatography of Douglas-fir Xylem sap extract collected 4/5/79. The dotted line represents a 1/100th aliquot at an increased sensitivity; the solid line is the uv absorbance of the entire extract. The collection band is from 6-1/2 to 9-1/2 minutes. Conditions - see figure 9.

Figure 11 illustrates these results, and a representative chromatogram is shown in Figure 12.

Prior to the bioassay for GA activity, the extracts were re-fractionated on the ODS column. The gradient was from 0 to 100% EtOH in buffer over 30 minutes, with a flow rate of 0.8 ml/min. One minute fractions were collected, beginning at minute one and continuing through minute 30. The chromatogram for the April 5th collection can be seen in Figure 13.

c) Bioassay of Extracts

Thirty fractions from each of the collection dates were assayed for gibberellin activity by the dwarf rice microdrop bioassay. None of the fractions elicited a growth response from the rice seedlings (Figure 14). Possible reasons for this lack of activity could be that the concentration of gibberellins in the fractions was too low or that the concentration of inhibiting substances was high enough to mask any GA effect. The degree of inhibition is visibly substantial, and appears to increase as time progresses. This increase in inhibiting components is also evident in the HPLC spectra; the July extracts contained a much larger quantity of uv-absorbing components than did the March extracts (Figures 13,15). In order to alleviate the losses incurred during the solvent partitioning and as another step in purification, the trace enrichment technique was developed.

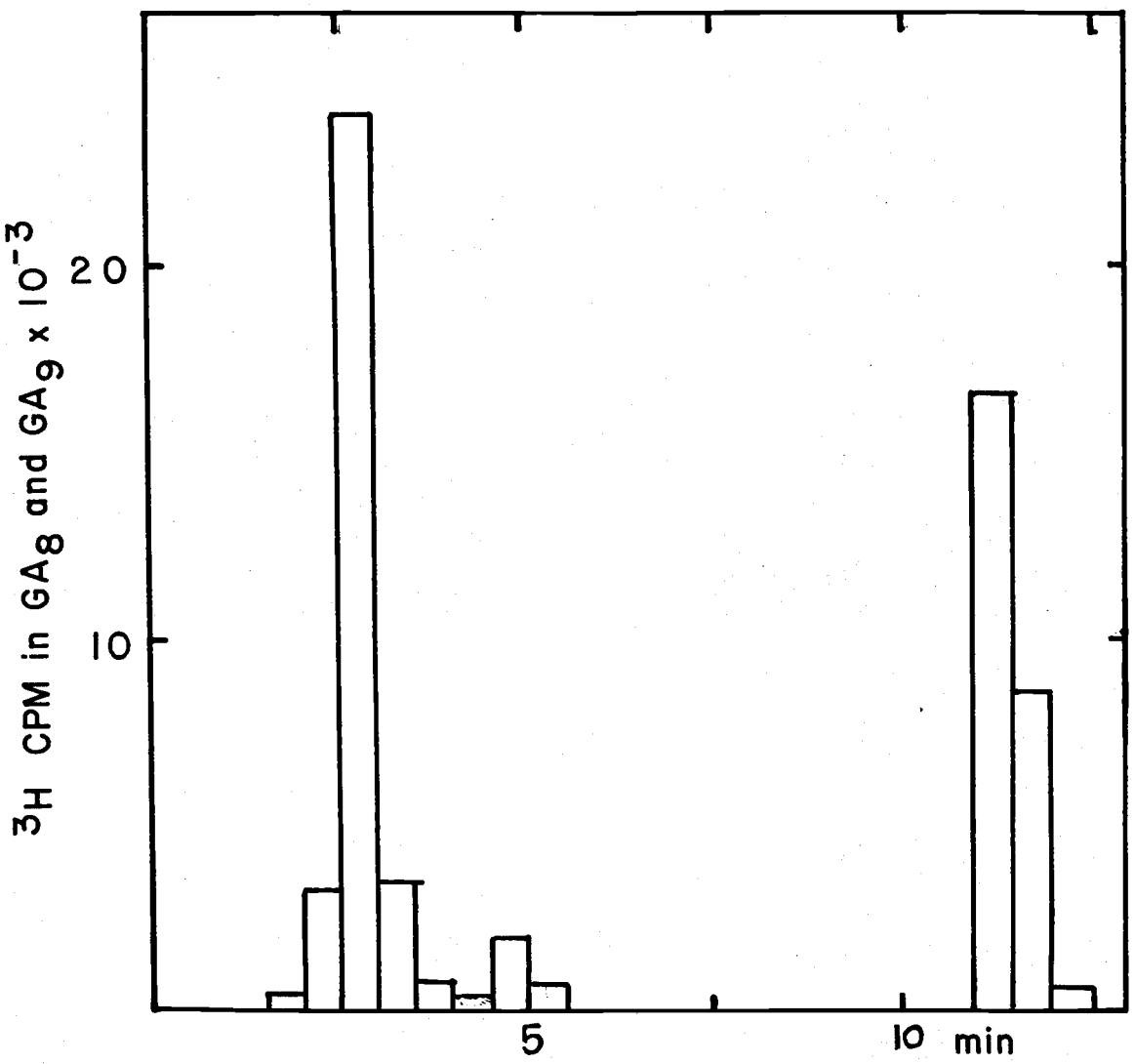


Figure 11. Reverse phase chromatography of ^{3}H-GA$_8$ and ^{3}H-GA$_9$. ^{3}H-GA$_8$ elutes at minute 2.5 and the ^{3}H-GA$_9$ elutes at minute 11.0. Varian MCH-5 column, 50 - 100% EtOH/ buffer, 20 minutes, 0.8 ml/min.

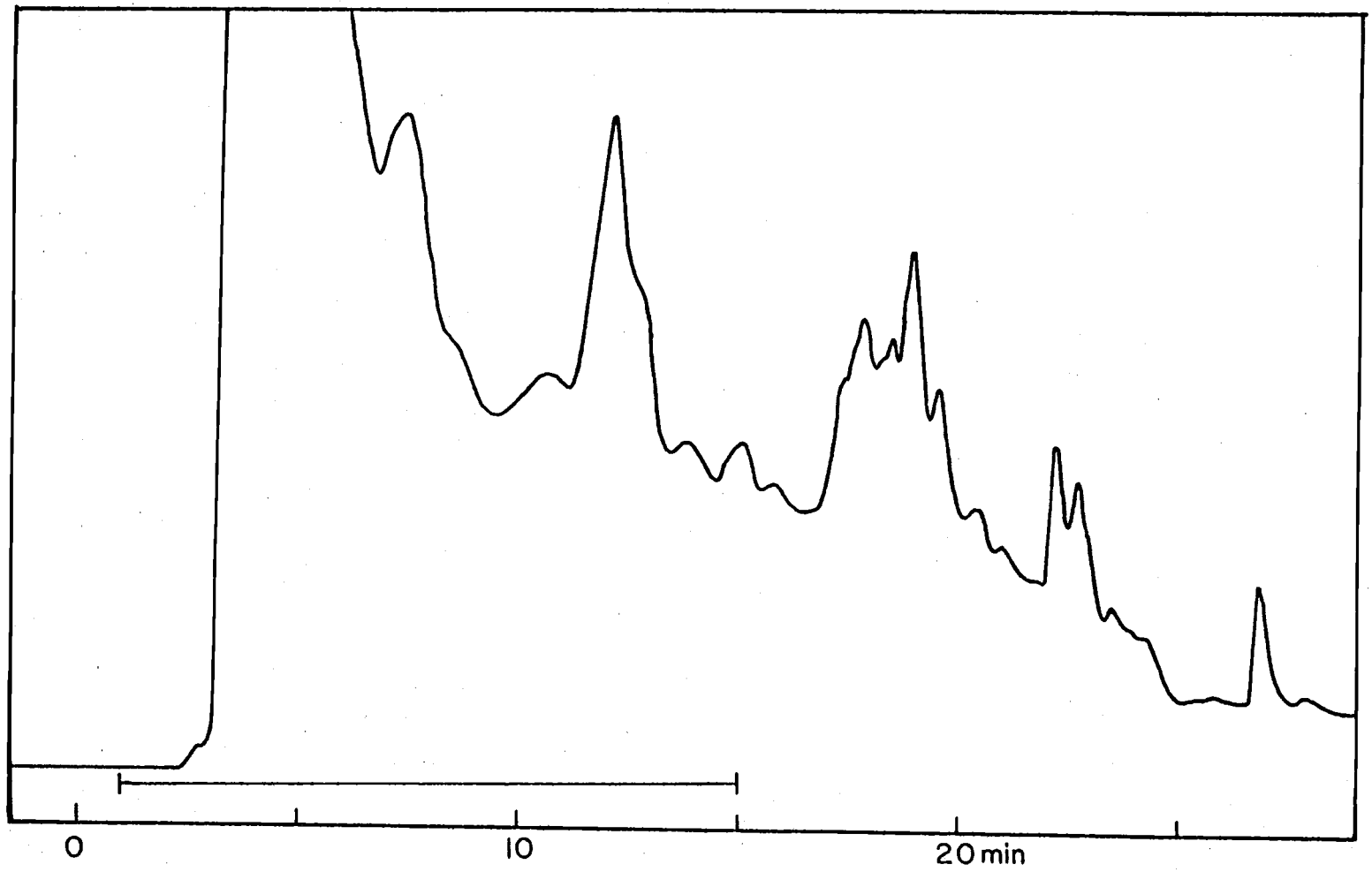


Figure 12. Reverse phase chromatography of Douglas-fir xylem sap extract collected 4/5/79. Collection band was between minute one and minute fifteen. Conditions - see figure 11.

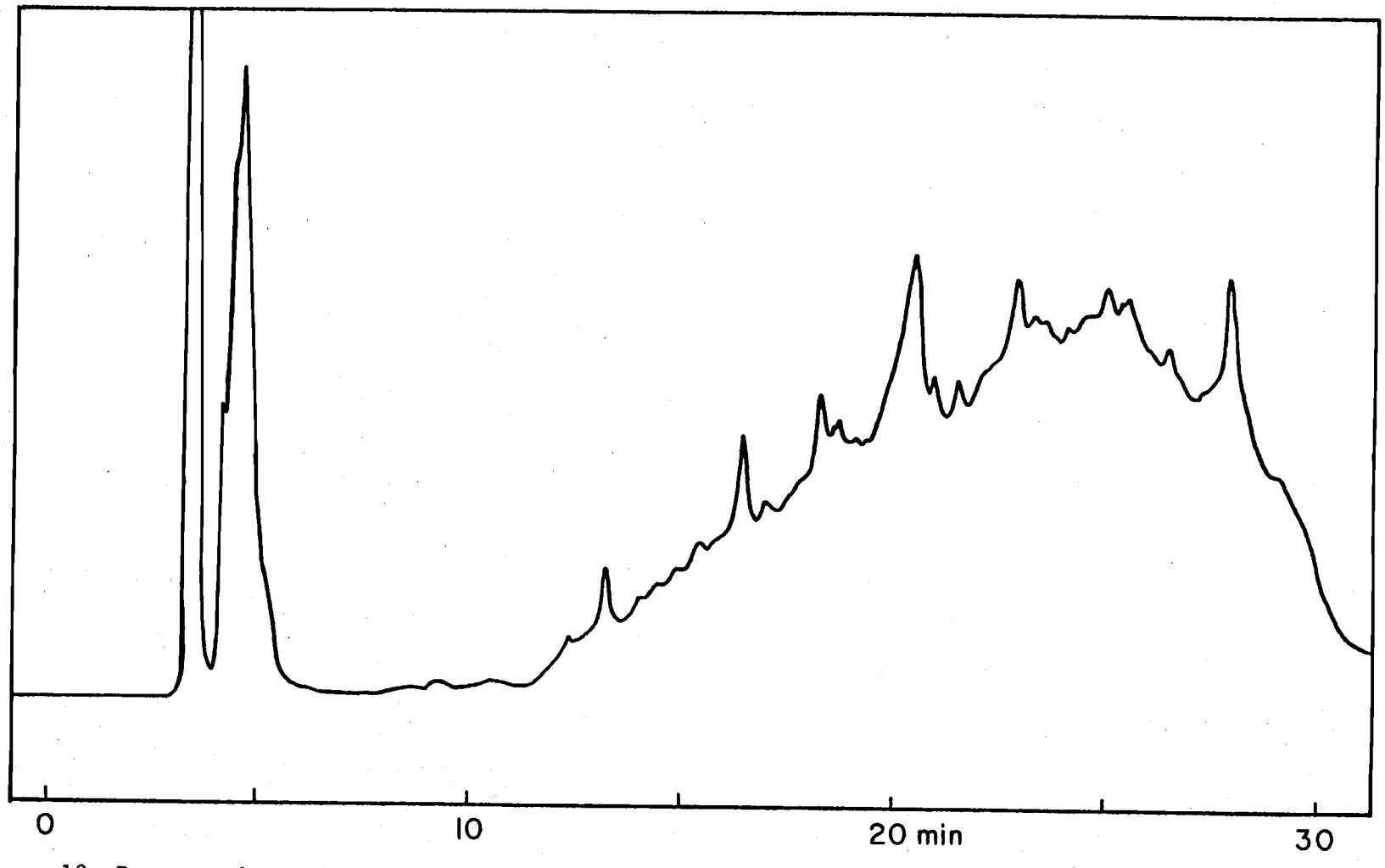


Figure 13. Reverse phase chromatography of prechromatographed Douglas-fir xylem sap extract collected 4/5/79. Fractions collected every minute from minute one through minute thirty and bioassayed (Figure 14). Varian MCH-5 column, 0 - 100% EtOH/buffer. 30 minutes, 0.8 ml/min.

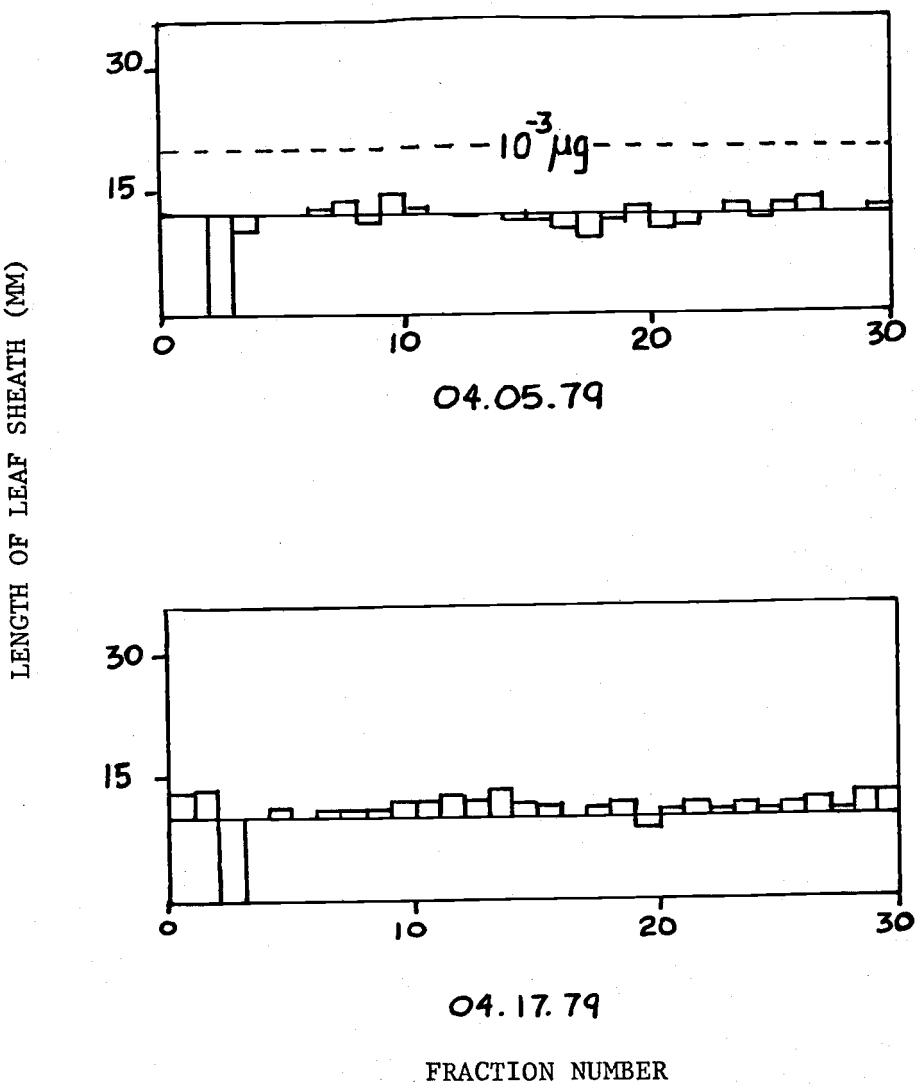


Figure 14: Dwarf rice microdrop bioassay of Douglas-fir xylem sap extracts with the sap collection dates indicated below each bar graph. There is an increase in the degree of inhibition by sap extracts collected during June and July compared to those collected during April.

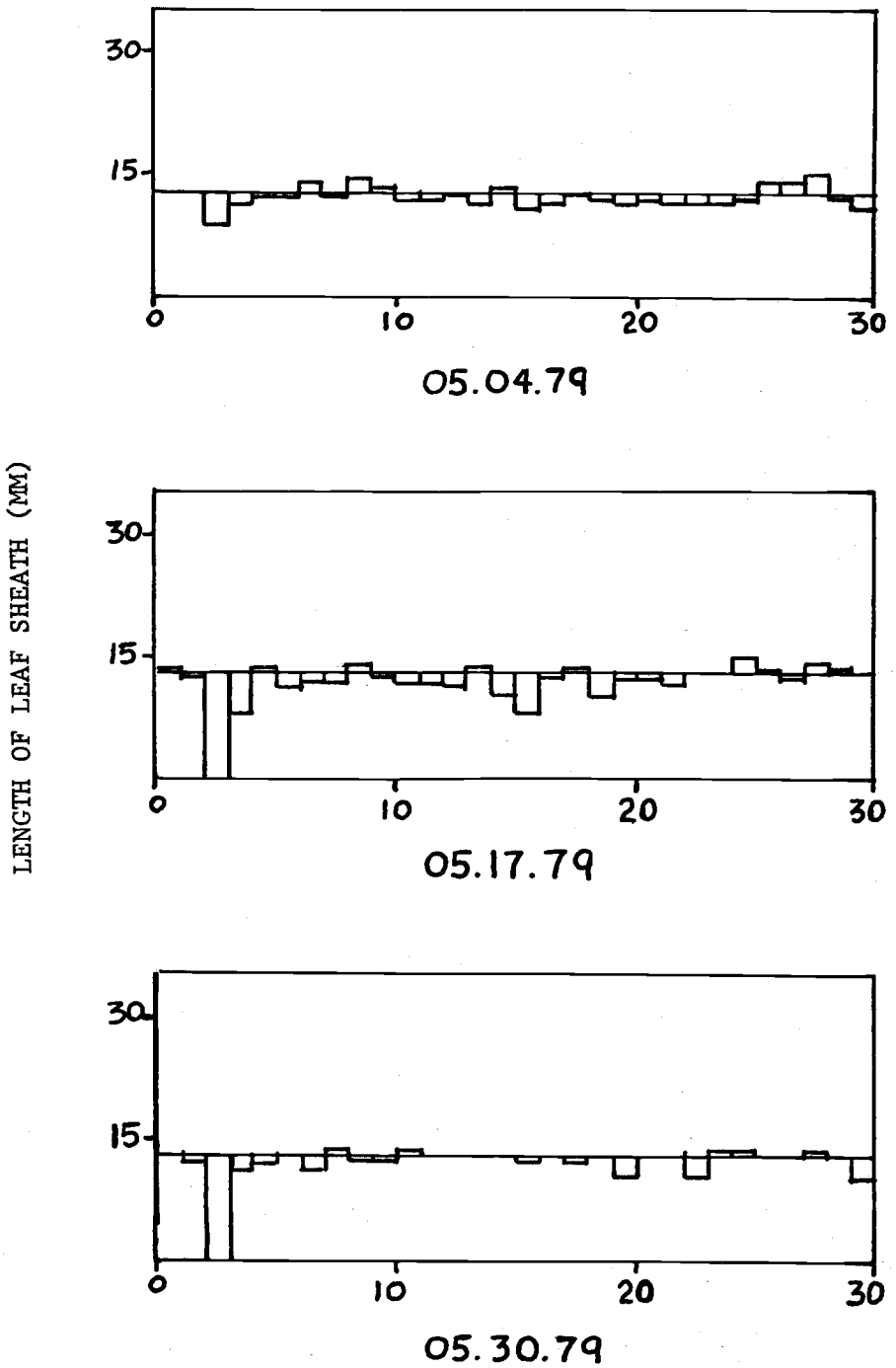


Figure 14 (continued)

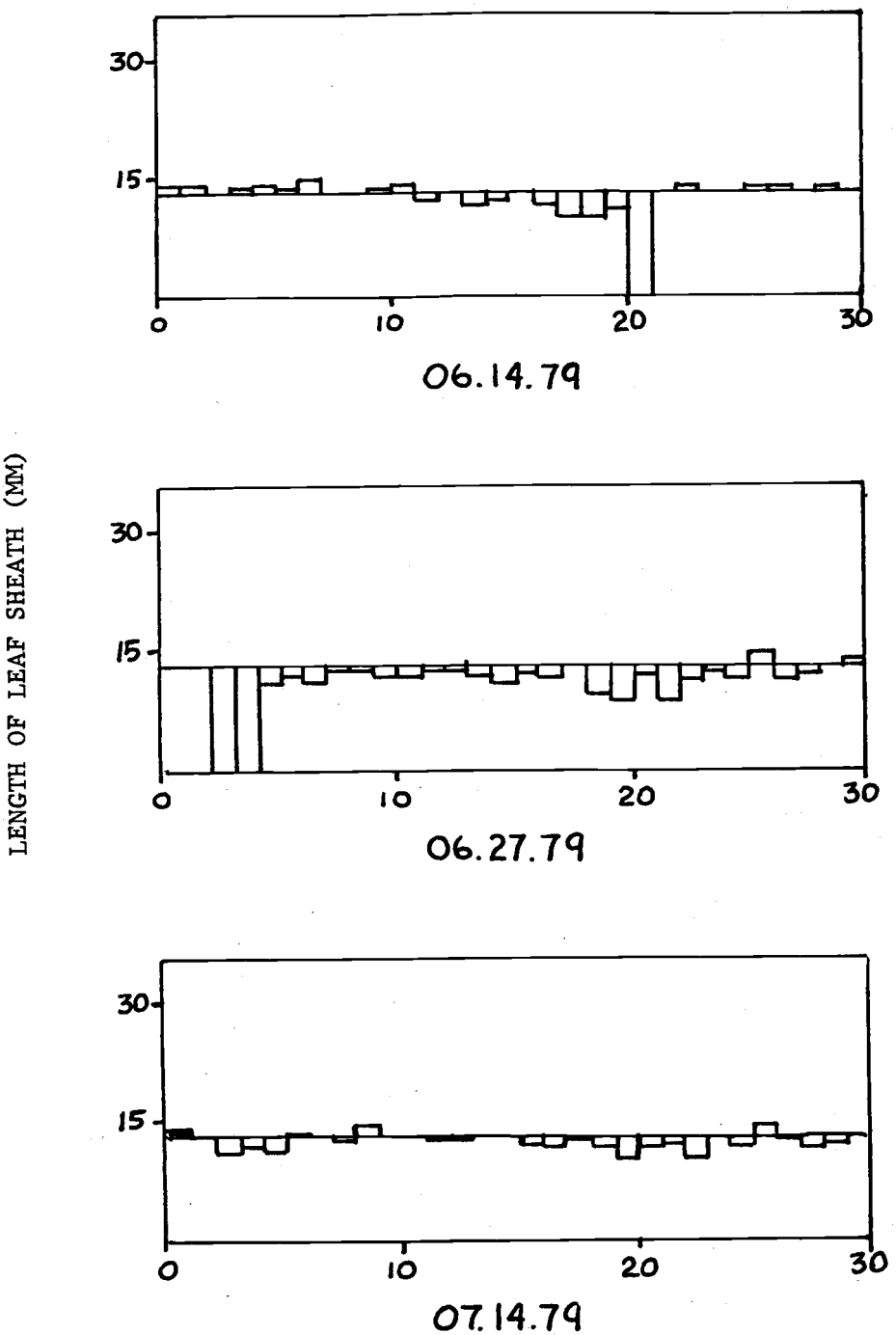


Figure 14 (continued)

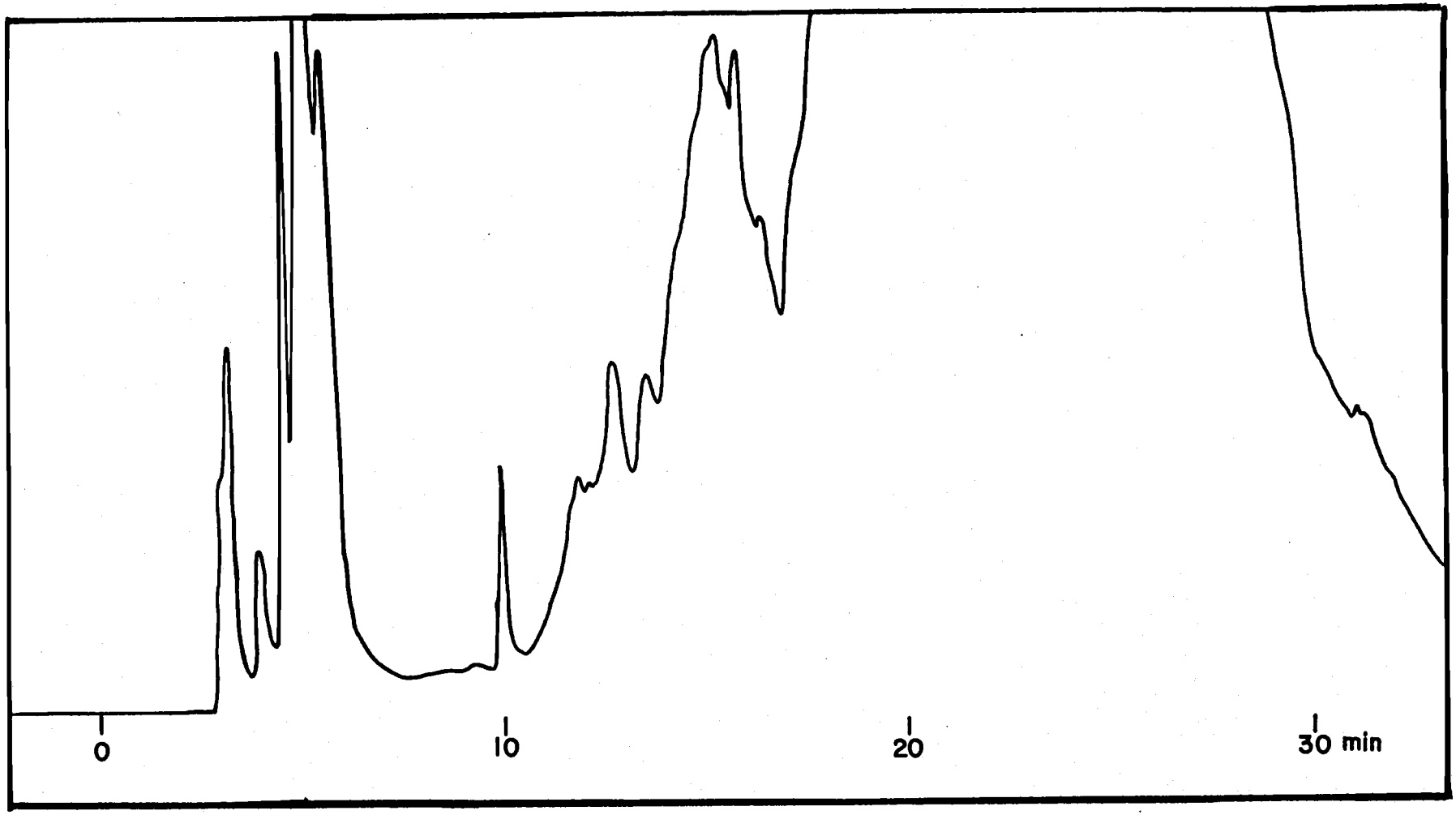


Figure 15. Reverse phase chromatography of Douglas-fir xylem sap extract collected 7/14/79. Bioassay (Figure 14). Conditions (see Figure 13).

d) Development of Trace Enrichment

During the initial stages of the purification of a plant extract, it is desirable to employ a group separatory procedure, in this case a process that would separate GA's as a group. An alternative to the solvent partitioning method that has been studied in this laboratory by Dr. David R. DeYoe is based upon the fact that free GA's can be equipped with a charge, thus are subject to electrophoresis. While experimenting with this method, a simpler, less time-consuming technique of trace enrichment emerged that was as quantitative as the electrophoretic elution. Tests were performed to determine the utility of this method using Douglas-fir sap to which had been added various tritiated gibberellin standards (Table 8).

Since the column used for trace enrichment consists of an octadecylsilane support material, the retention characteristics are similar to those occurring during reverse phase chromatography. The more polar GA_1 required only 10% EtOH to obtain 90 to 100% recovery, while GA_4, in the mid-polar range, eluted with 30% EtOH. The nonpolar GA_9 was more difficult to remove under these conditions; approximately 75% was recovered with 30% EtOH, with little more recovery at 50% and 100%. Since the tritiated standards were not purified by HPLC previously, the ion recovery rates of GA_9 and GA_{43} are most likely reflections of tritiated impurities and not a lack of recovery of gibberellins per se.

Table 8: Radiotracer levels of various ^{3}H-GAs tested in trace enrichment

$$\% \text{ recovery} = cpm_{elution}/cpm_{total}$$

^{3}H-GA	10% EtOH Elution	30% EtOH Elution	50% EtOH Elution	100% EtOH Elution	Total Recovery	% in Eluate & Wash	Total
1	81.1	8.8	2.7	1.5	94.1	8.3	102.4
4	14.0	73.3	1.9	0.7	89.9	6.7	96.6
9	2.6	73.5	1.8	0.2	78.1	12.0	90.1
43	60.8	7.1	---	---	67.9	46.4	114.3

Since most of the gibberellins are eluted from the column with only 30% EtOH, many nonpolar components that may be present in the sap remain on the column until washed with a stronger solvent, such as THF. Also, many uv absorbing components do not adhere to the column at all and are eluted with the buffer wash (Table 9).

Trace enrichment, when used as an initial purification step, can thus eliminate many of the losses incurred when dealing with large quantities of sap.

Isolation of Gibberellins from Plant Material

a) Cucumber Seeds

Earlier results on the isolation of gibberellins from Douglas-fir sap via solvent partitioning suggest that the concentration of GA's may be negligible or too low to detect by the available methods. To assure ourselves that the procedures work with a biological system, the entire isolation procedure was conducted using a plant material known to be sufficient in gibberellins. The seeds of wild cucumber (Marah oreganus T. and G.) were chosen for this purpose because of their availability and their abundance of free GA's (Corcoran and Phinney, 1962).

Table 10 shows the recovery data of the labelled standard throughout the trace enrichment and solvent partitioning steps. After partitioning, the cucumber extracts were chromatographed on a gel permeation system (Figure 16). Collections were made from eight to eleven minutes, the retention times of free GA's having been determined

Table 9: Losses in uv contaminants by using trace enrichment technique on Douglas-fir sap

sample measured for uv absorbance	% of total uv absorbance (A254)	% uv contaminants left on column
untreated sap	100.0	-----
column eluate	8.3	91.7
column wash	28.8	62.9
10% EtOH elution	5.1	57.8
30% EtOH elution	3.6	54.2
50% EtOH elution	0.7	53.5
100% EtOH elution	0.4	53.1

Table 10: Radiotracer levels in trace enrichment and solvent partitioning of cucumber extract

Trace Enrichment

elution	% of added ^{3}H-GA_9
10%	5.0
30%	54.0
50%	21.5
100%	0.5
total recovery	81.0

Solvent Partitioning

elution	% of added ^{3}H-GA_9
10%	3.8
30%	59.8
50%	17.4
total recovery	80.6

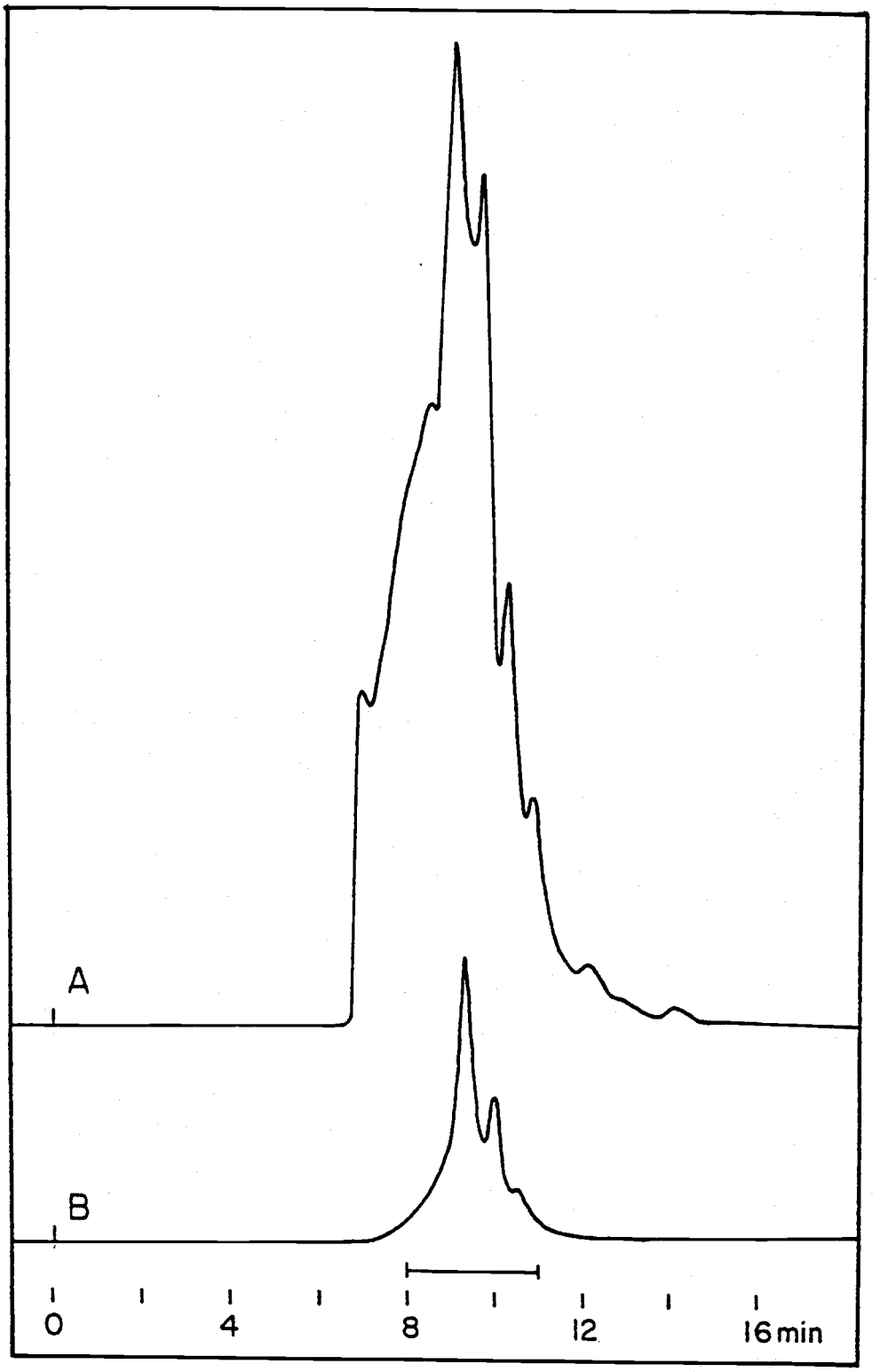


Figure 16: Gel permeation chromatography of trace enriched cucumber seed extracts. Curve A is the chromatogram of the 30% elution from trace enrichment; Curve B: 10%. Collection band between 8 and 11 minutes.

as previously. These collected fractions were then chromatographed on a reverse phase system prior to bioassay (Figures 17, 18, and 19). Collections from minute one through minute thirty, each one minute in duration, were bioassayed (Figure 20). From the 10% trace enrichment elution, a significantly active peak can be seen in fractions 16, 17, and 18. This peak has also been carried over into the 30% elution, accompanying new peaks at fractions 13, 20, and 24, 25, and 26. The large peak at 24, 25, and 26 is also evident in the 50% elution. The 10% elution should contain more polar gibberellins; this is borne out by the early peak that evoked a growth response in the bioassay. The 50% elution should be comprised of the less polar gibberellins, and is evidenced by the location of the active fractions in the bioassay. A standard curve using GA_3 was calculated to determine the sensitivity of the bioassay. Results, shown in Figure 21, indicate that the concentration of gibberellins in the cucumber active fractions approximate those given in Table 11.

The fractionated cucumber extracts which showed activity in the bioassay were combined in the manner shown in Table 12; these combined fractions were then divided into two equal portions, one of which was derivatized by parabromophenacylation, and the other which was derivatized by methyl trimethyl silylation.

The parabromophenacylated fractions were chromatographed on a Varian MCH·5 reverse phase column eluted with a gradient of 50% EtOH/buffer to 100% EtOH over 20 minutes at a flow rate of 0.8 ml/min. The uv-absorbing components were collected, and those corresponding to GApBPB standards (Table 13) were analyzed on the mass spectrometer.

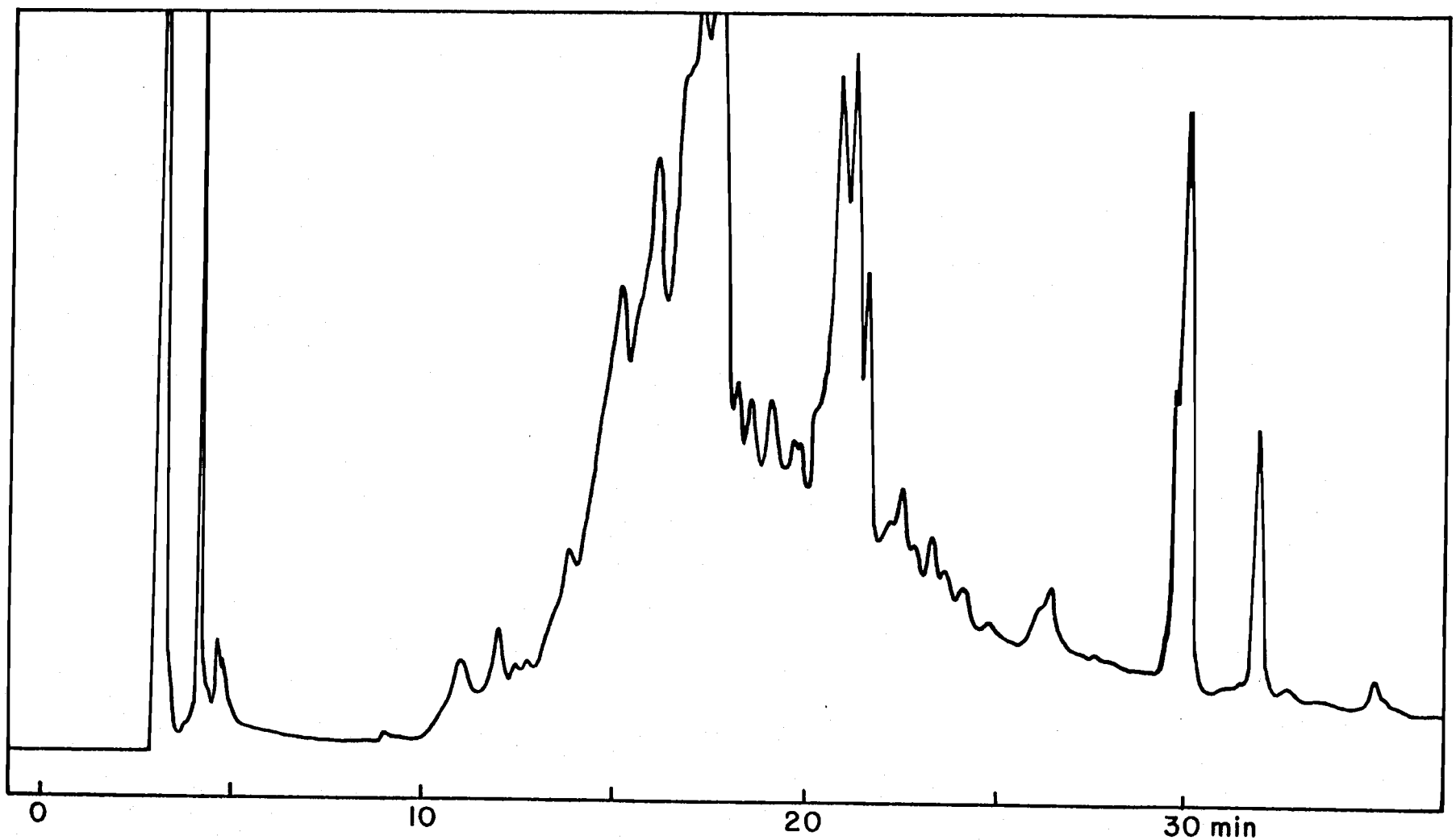


Figure 17: Reverse phase chromatography of trace enriched cucumber seed extract, 10% elution. For bioassay see Figure 21. Varian MCH-5 column, 0-100% EtOH/buffer, 30 minutes, 0.8 ml/min

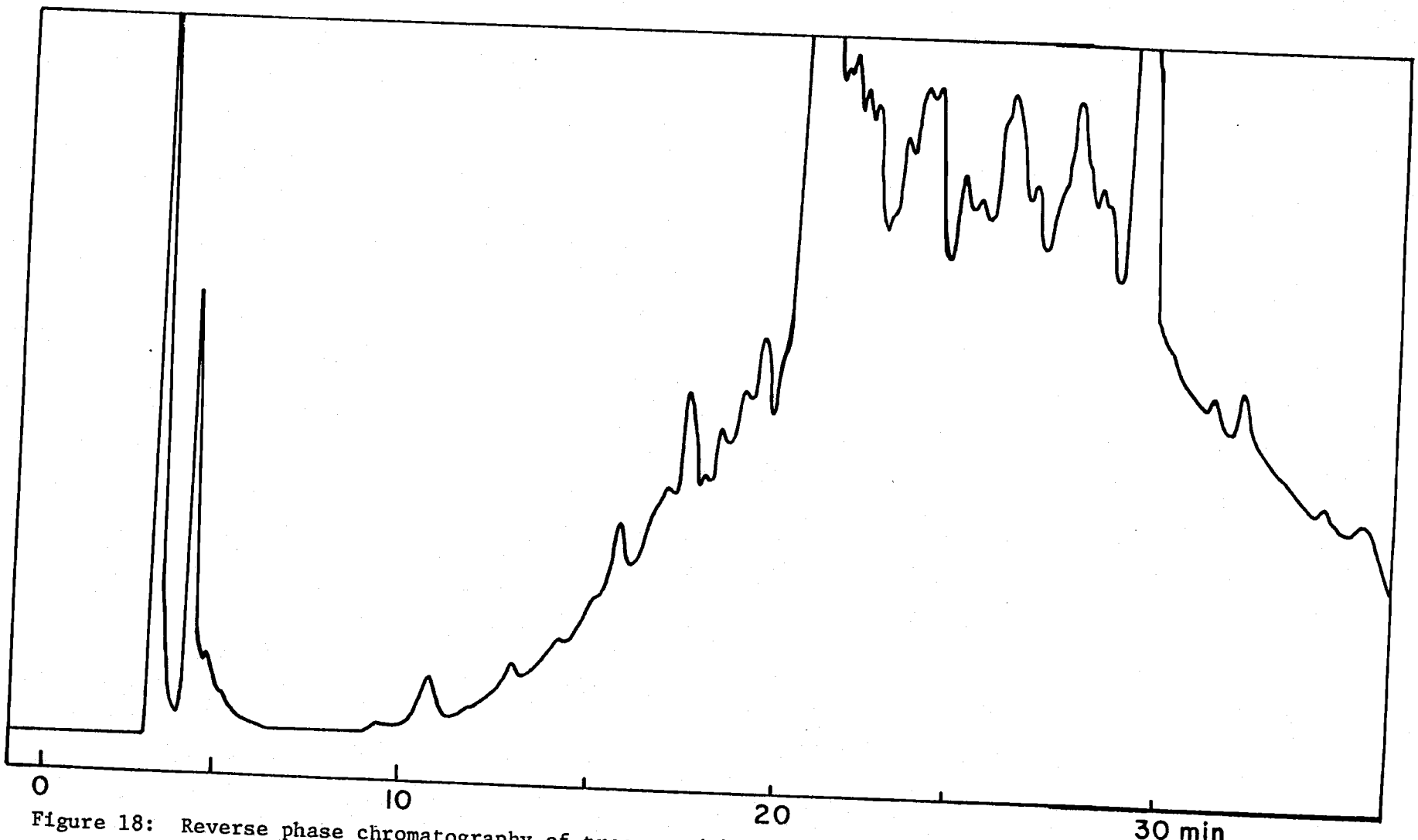


Figure 18: Reverse phase chromatography of trace enriched cucumber seed extract, 30% elution. For bioassay see Figure 21. Conditions- see Figure 17

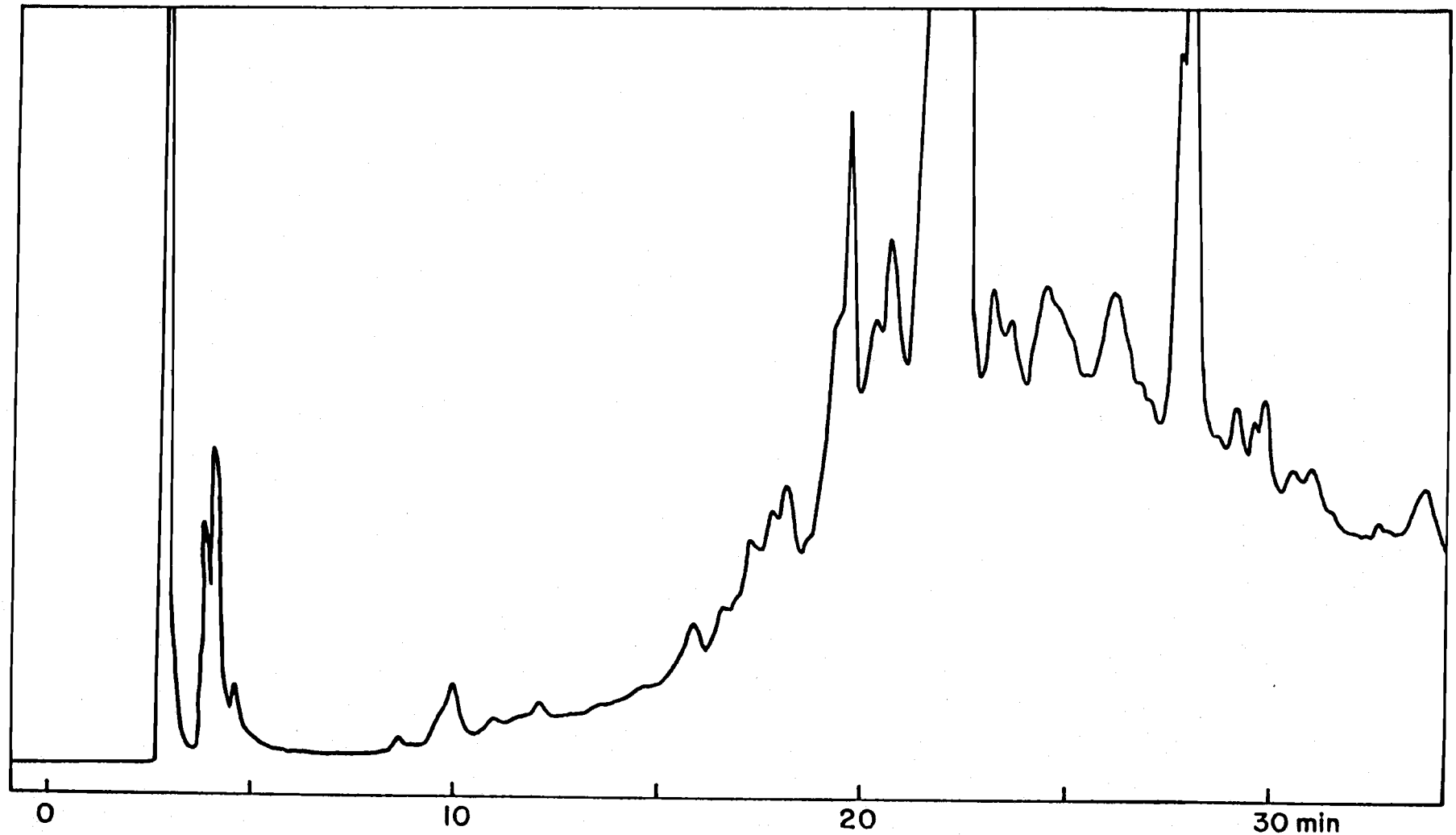


Figure 19. Reverse phase chromatography of trace-enriched cucumber seed extract, 50% elution. For bioassay see Figure 20. Conditions - see Figure 17.

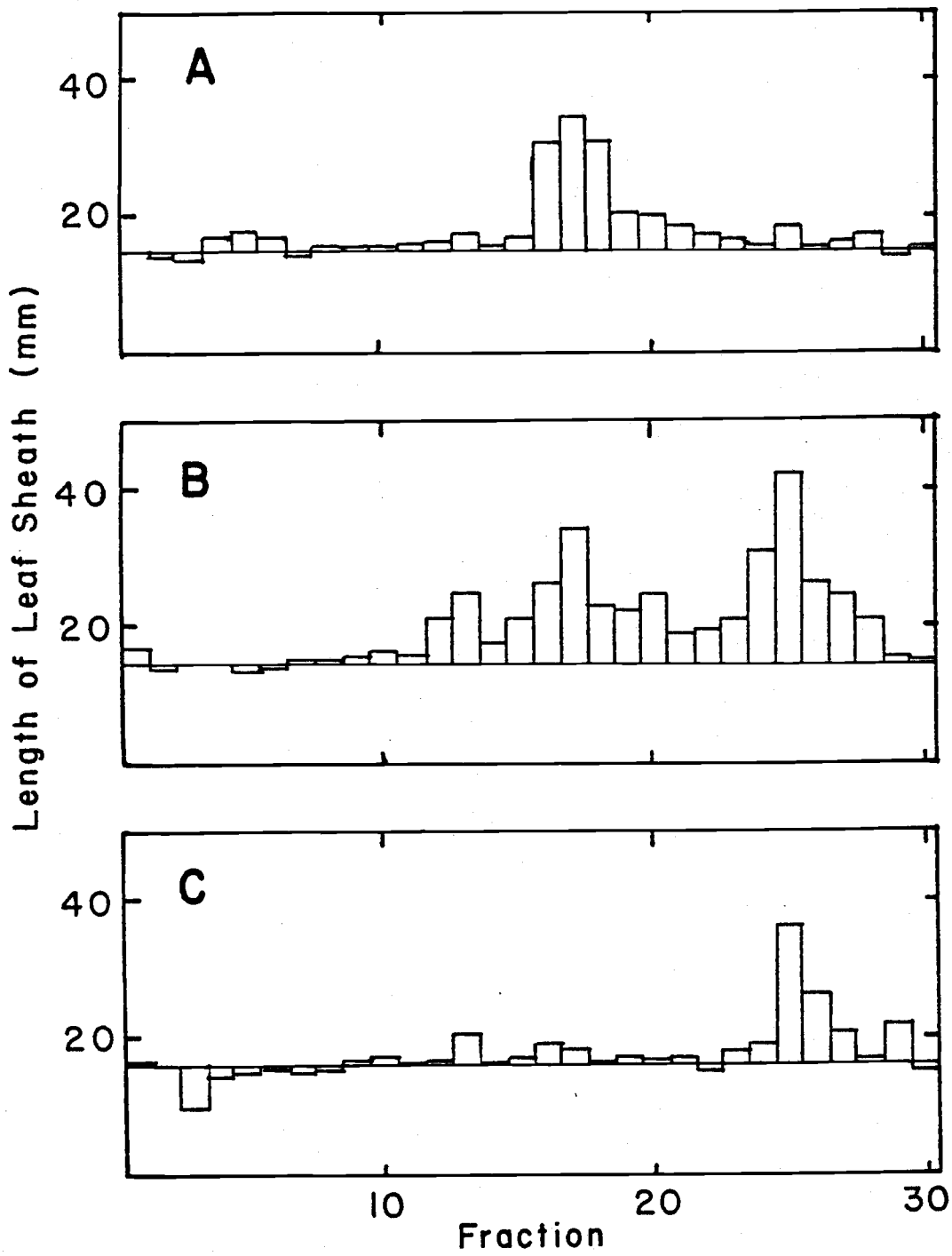


Figure 20. Dwarf rice microdrop bioassay of cucumber seed extracts: A - 10% elution from trace enrichment; B - 30% elution; C - 50% elution. Significant peaks listed in Table 11.

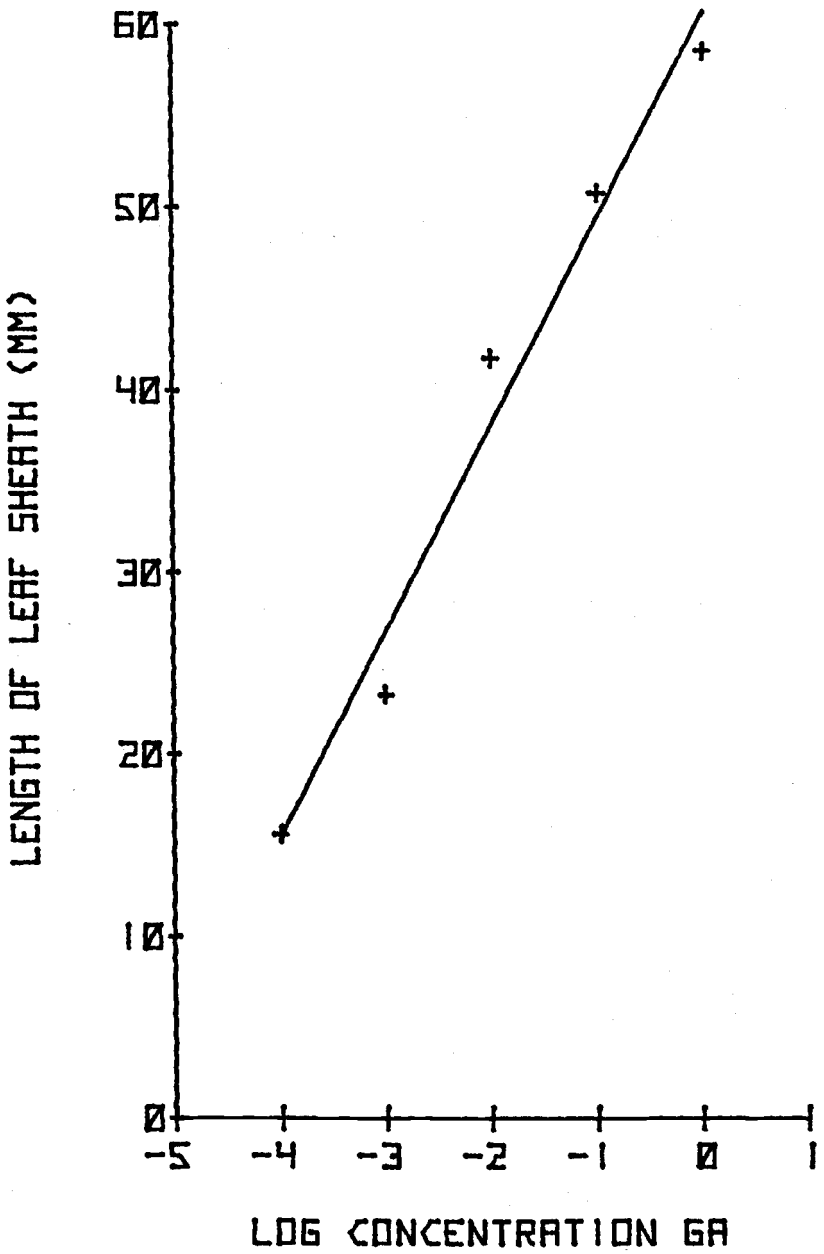


Figure 21. Regression of growth response of dwarf rice to GA_3 standard (µgm/µl)

Table 11: Approximate concentrations of GA equivalents in active fractions of cucumber extract as determined from regression equation of standard curve

fraction number	length of leaf sheath	µg/µl GA_3 equivalents*
10-13	17.2	1.5
10-16	30.4	21.3
10-17	33.4	39.2
10-18	30.6	22.2
10-19	20.2	2.7
10-20	20.0	2.6
10-25	18.4	1.9
30-12	21.2	3.3
30-13	24.8	6.9
30-14	17.8	1.7
30-15	21.2	3.3
30-16	26.4	9.5
30-17	34.2	46.1
30-18	23.4	5.3
30-19	22.4	4.2
30-20	25.0	7.1
30-23	21.2	3.3
30-24	31.0	24.1
30-25	42.4	243.0
30-26	27.6	11.2
30-27	25.2	7.4
30-28	21.4	3.4
50-14	20.6	2.9
50-16	19.2	2.2
50-17	18.6	2.0
50-24	19.8	2.5
50-25	37.0	81.0
50-26	26.6	9.9
50-27	21.2	3.3
50-29	22.6	4.4

* x 10^{-4}

Table 12: Description of active fractions of cucumber extracts which were derivatized and chromatographed

designated fraction number	% EtOH elution from trace enrichment	comprised of these active fractions*
13	30%	12, 13
17	10%	16, 17, 18
18	30%	16, 17, 18
25	30%	24, 25, 26
26	50%	25, 26

*see bar graphs in Figure 21

Table 13: Retention times of GApBPB standards on Varian micropak MCH-5 column

gradient: 50-100% EtOH/buffer

elution time: 20 minutes

flow rate: 0.8 ml/min

GApBPB	R_t
8	8.4
3	9.5
1	10.7
7	15.8
4	16.4
9	18.8

These derivatives did not produce a mass spectrum that could be conclusively identified as a gibberellin by chemical ionization, the method by which the GApBPB standards were analyzed. However, when analyzed in the negative ion mode, an identification of GA_4 was made (Figure 22). The peak at m/e 331 corresponds to the gibberellin free acid that is produced by the loss of the pBP moiety. Since in this mode the mass spectrometer only analyzes negative ions, the pBP fragment is not visible. The mass spectrum of standard GA_4pBPB in the negative ion mode is shown in Figure 23.

These results were confirmed when the MeTMS derivatives were analyzed by GC-MS (Binks, MacMillan, and Pryce, 1969). The MeTMS derivatives produced spectra which matched those of GA_4, GA_7, and GA_9 standards (Figure 24). The selected ion chromatograms for $MeGA_4$TMS, $MeGA_7$TMS, and $MeGA_9$ are shown in Figures 25, 26, and 27 respectively. These chromatograms indicate the scan which contains the major fragment ions of the molecule in question. All the fragments must be contained in the same scan. The mass spectra of the appropriate scans for $MeGA_4$TMS, $MeGA_7$TMS, and $MeGA_9$ are shown in Figures 28, 29, and 30 respectively, and these compare positively with the standard spectra (Figure 24).

b) Douglas-fir Xylem Sap

The growth response observed from the bioassay of the cucumber extracts showed that the extraction technique was sound; therefore, 500 ml of sap collected at each of the following dates was trace enriched and extracted via solvent partitioning: March 20, 1979;

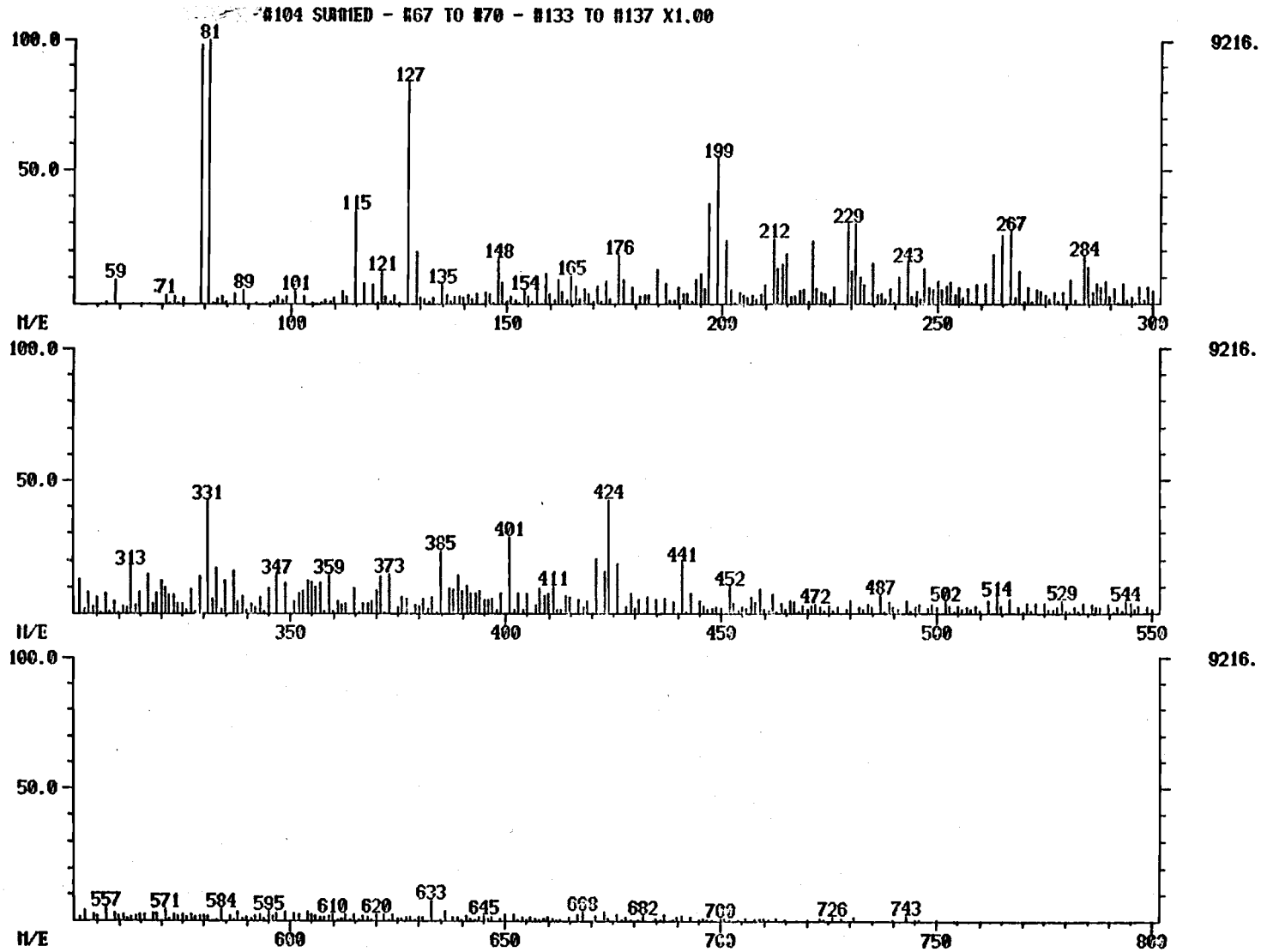


Figure 22. Mass spectrum of cucumber fraction 26-2 in negative ion mode. The GA_4 free acid ion is at m/e 331.

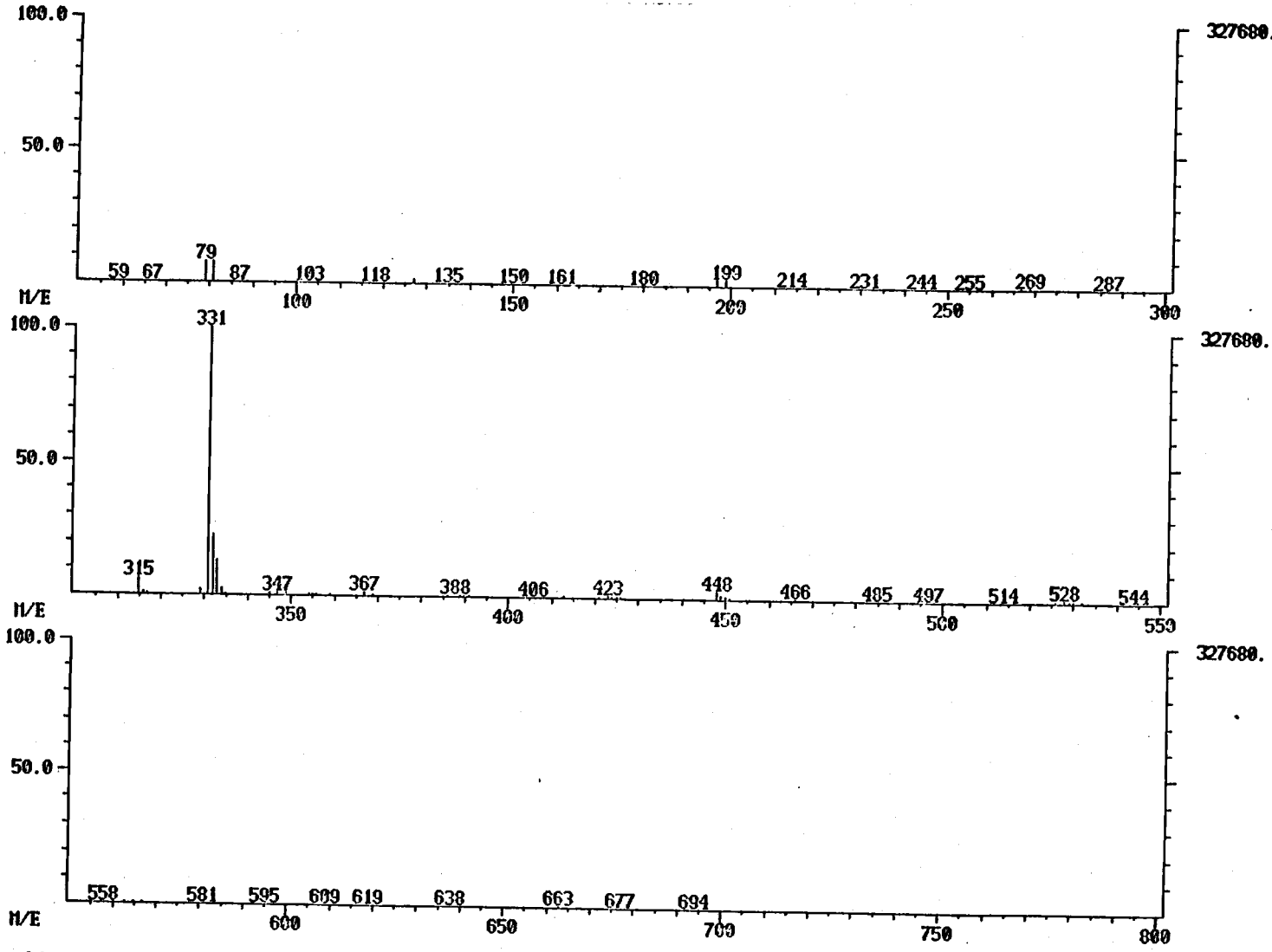


Figure 23. Mass spectrum of GA_4pBPB standard in negative ion mode. The GA_4 free acid ion is at m/e 331.

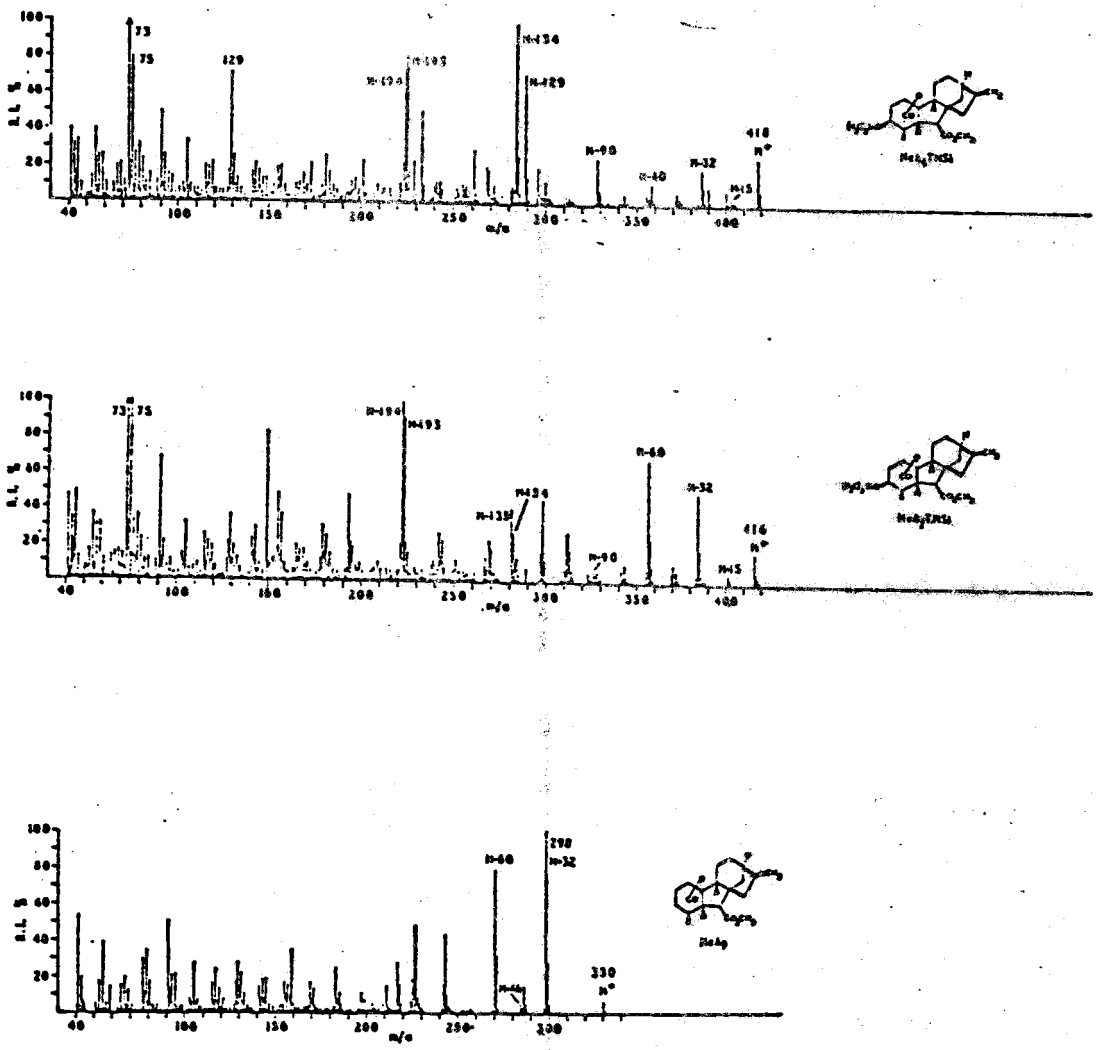


Figure 24: Mass spectra of methyl ester-trimethyl silyl ethers of GA_4, GA_7, and GA_9 standards (Binks, MacMillan, and Pryce, 1969).

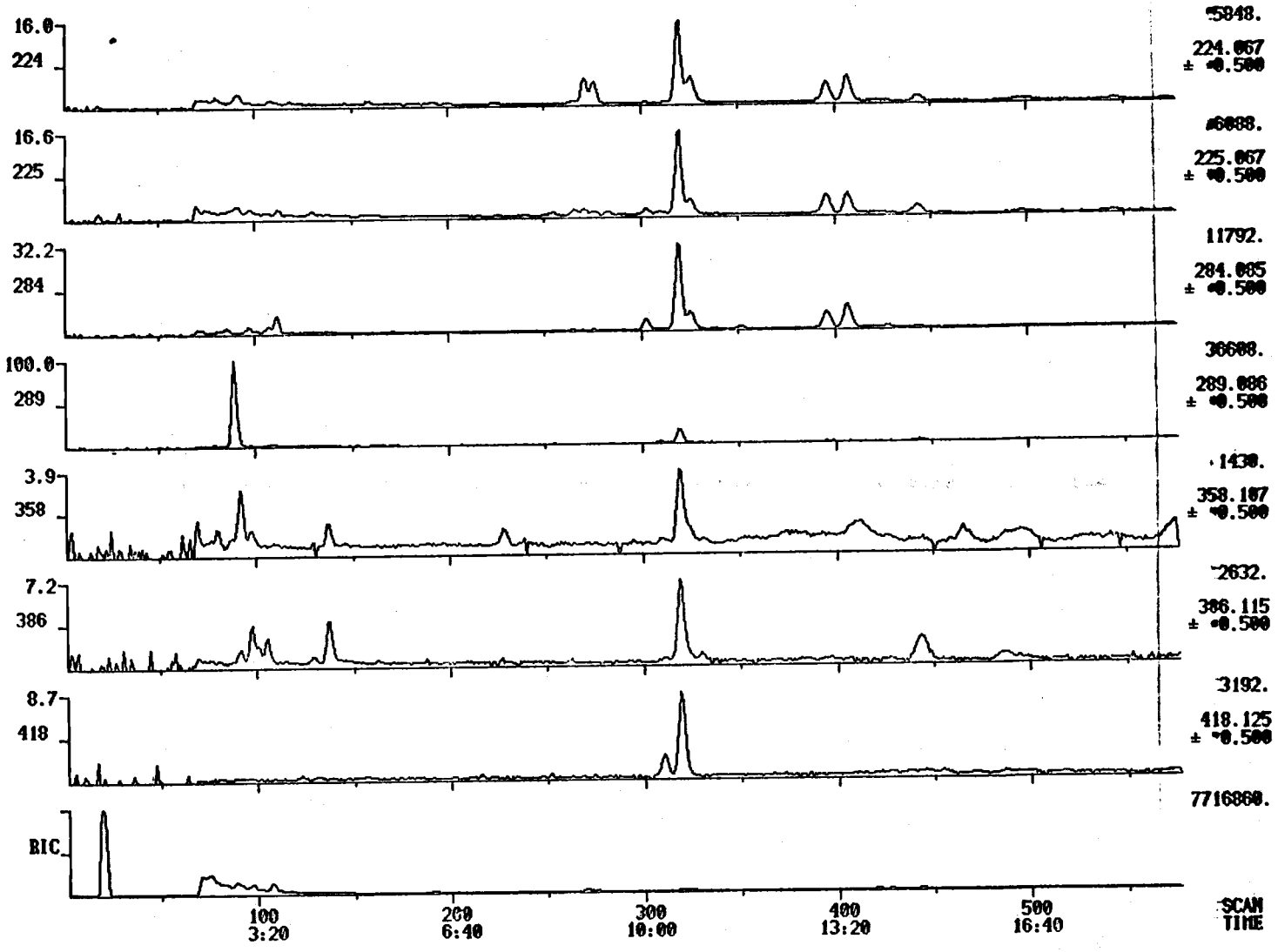


Figure 25. Selected ion chromatograms of the following MeTMS cucumber fraction 25 ions: 224, 225, 284, 289, 358, 386, 418 corresponding to GA_4 standard.

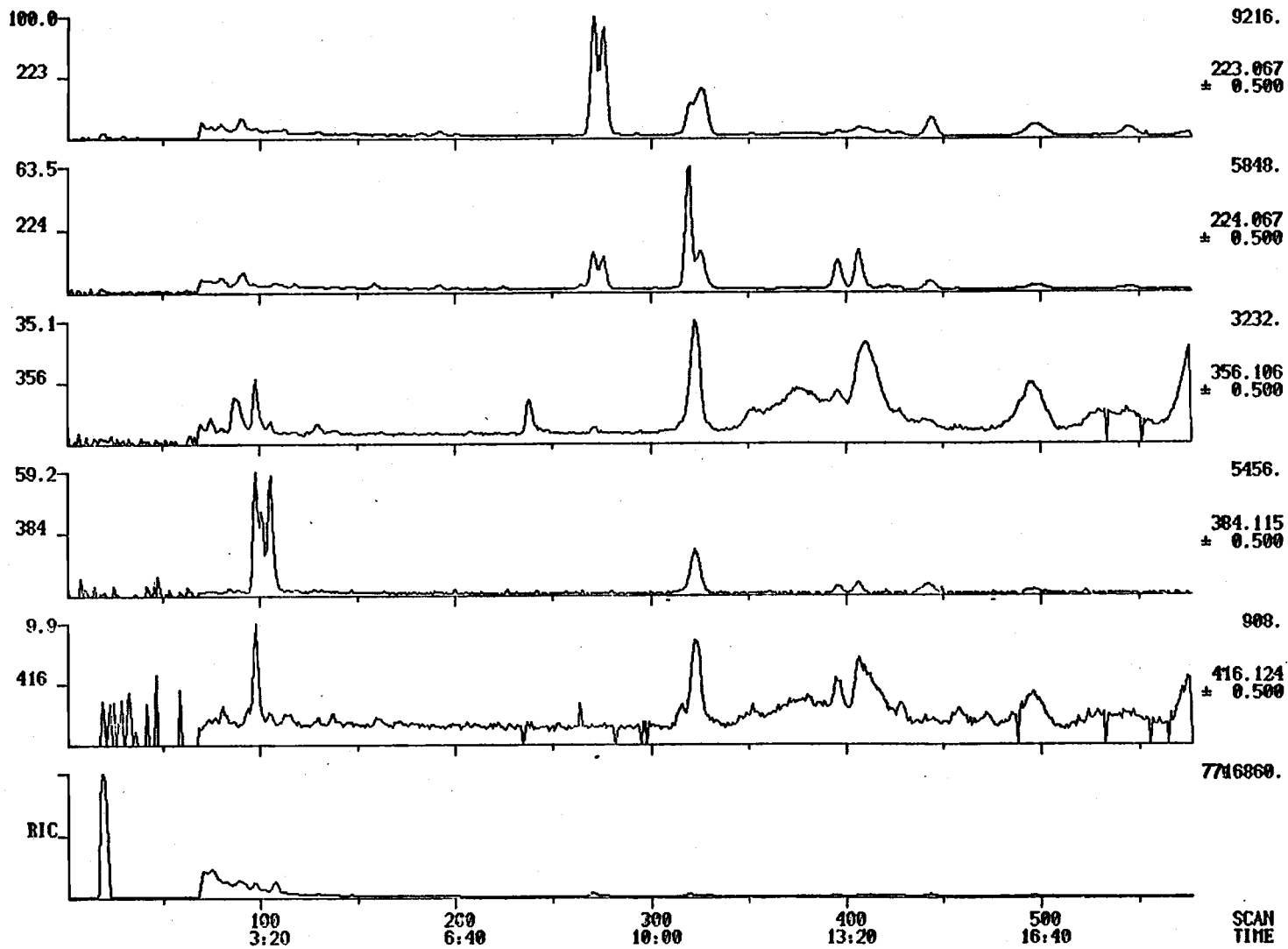


Figure 26. Selected ion chromatograms of the following MEtMS cucumber fraction 25 ions: 223, 224, 356, 384, 416 correspnding to GA_7 standard.

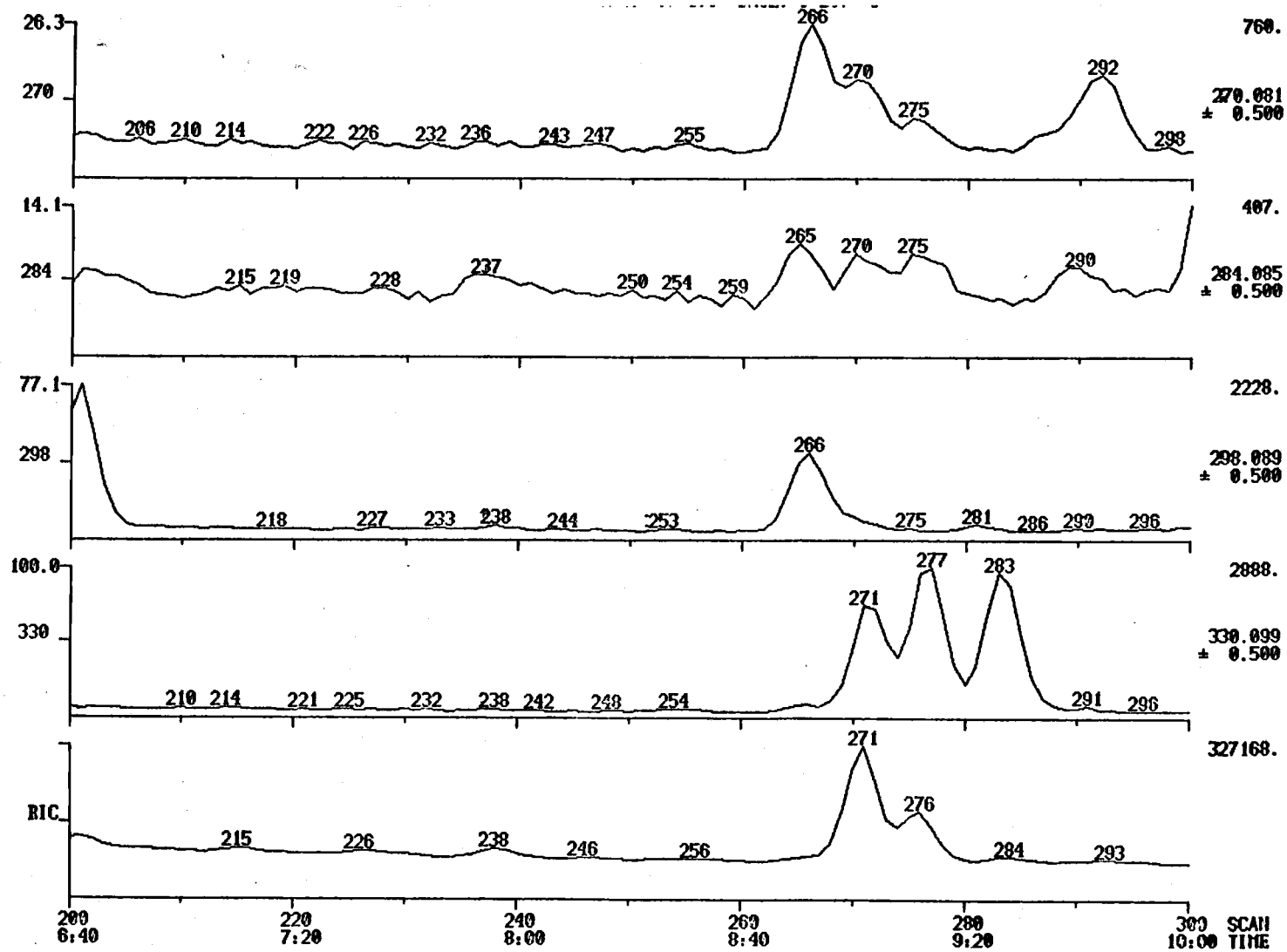


Figure 27. Selected ion chromatograms of the following MeTMS cucumber fraction 25 ions: 270, 284, 298, 330 corresponding to GA_9 standard.

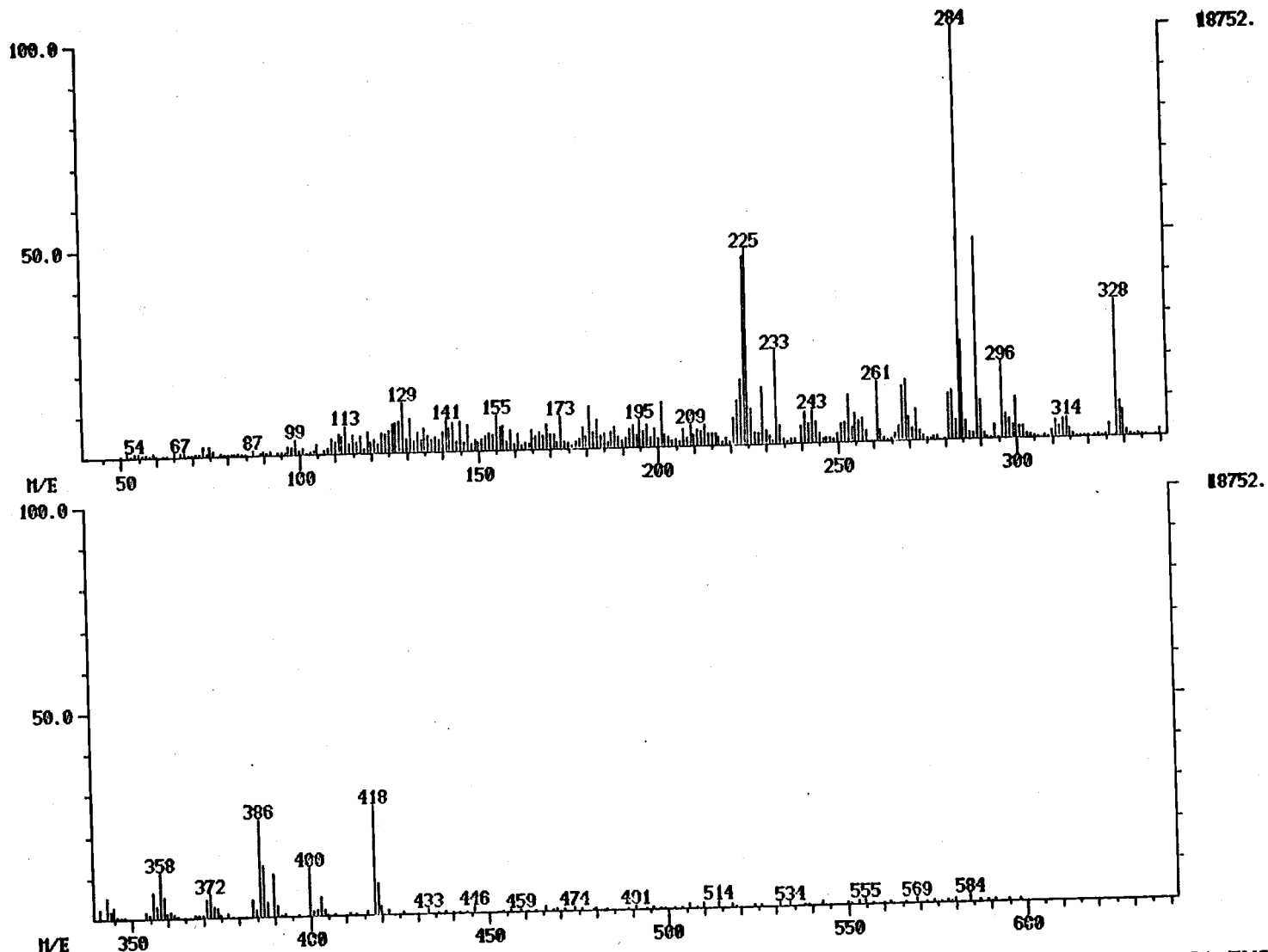


Figure 28. Mass spectrum of MeTMS cucumber fraction 25 scan 335 corresponding to $MeGA_4TMS$ standard (see figure 24).

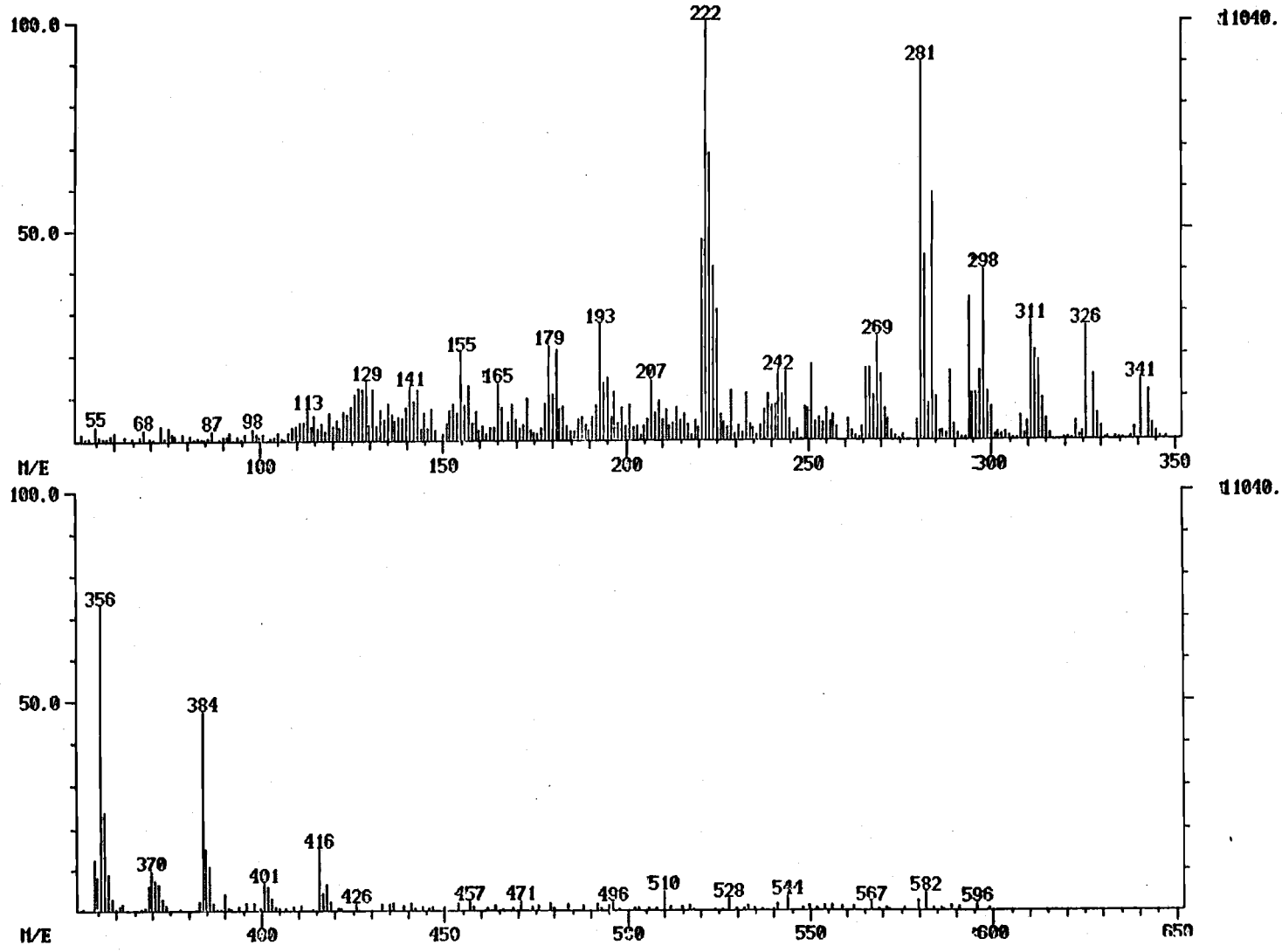


Figure 29. Mass spectrum of MeTMS cucumber fraction 25 scan 333 corresponding to $MeGA_7TMS$ standard (see figure 24).

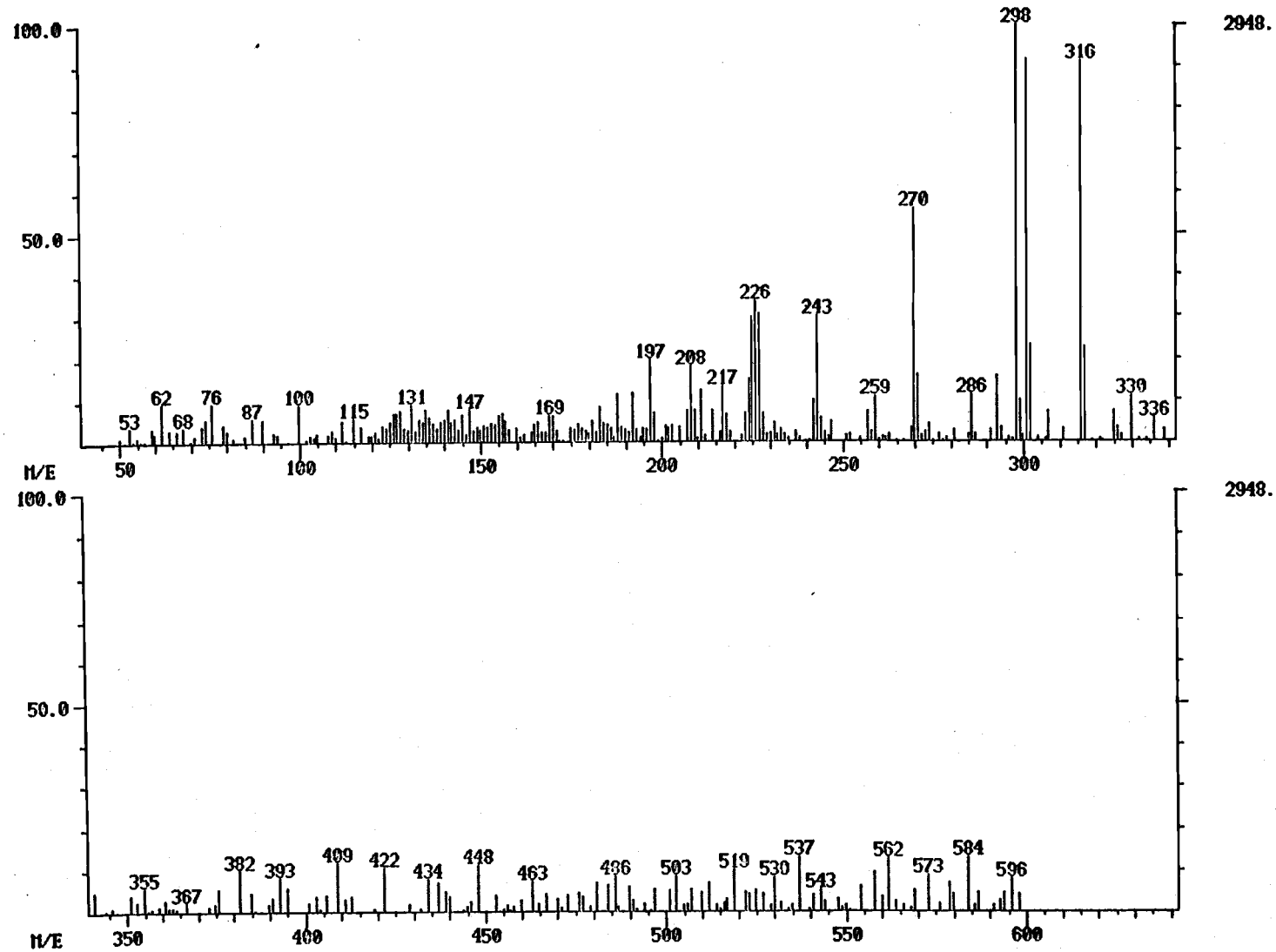


Figure 30. Mass spectrum of MeTMS cucumber fraction 25 scan 301 corresponding to $MeGA_9$ standard (see figure 24).

April 18, 1979; June 1, 1979; and July 18, 1979. A 10%, 30%, and 50% ETOH elution was collected from the trace enrichment column for each date and partitioned. Each of these extracts was subsequently chromatographed on a 50 Å Altex μSpherogel GPC column which was eluted with 100% THF at a flow rate of 1.0 ml/min (Figure 31). Collections were made at the retention times corresponding to those of free GA's, and these fractions were separated on an octadecyl-silica column eluted with a 0 to 100% ETOH/buffer gradient at 0.8 ml/min over 30 minutes. Fractions were collected as before (Figure 32) and bioassayed for gibberellin activity (Figure 33). The results of each bioassay are similar to those of the previous bioassays of Douglas-fir xylem sap extracts. These data, taken in conjunction with the earlier bioassay and with the assay of the cucumber extracts, indicate that the level of free GA's in Douglas-fir xylem sap is either lower than the limit of detection by the bioassay (0.1 ngm per seedling, or 1.0 ngm per extract) or that any GA's present are being masked by a substantial level of inhibiting compounds. The probability of the latter is increased when the trend of rising inhibition is considered. As before, the concentration of inhibiting components as determined by bioassay increased as time progressed, and the HPLC spectra became noticeably less pure (Figure 32, 34). The response of the rice seedlings to the March extracts appears positive but is not statistically significant; therefore, I cannot conclude that there are any gibberellins present in the xylem sap above the 1.0 ngm level. However, the July extracts are clearly inhibitory. These results suggest that the

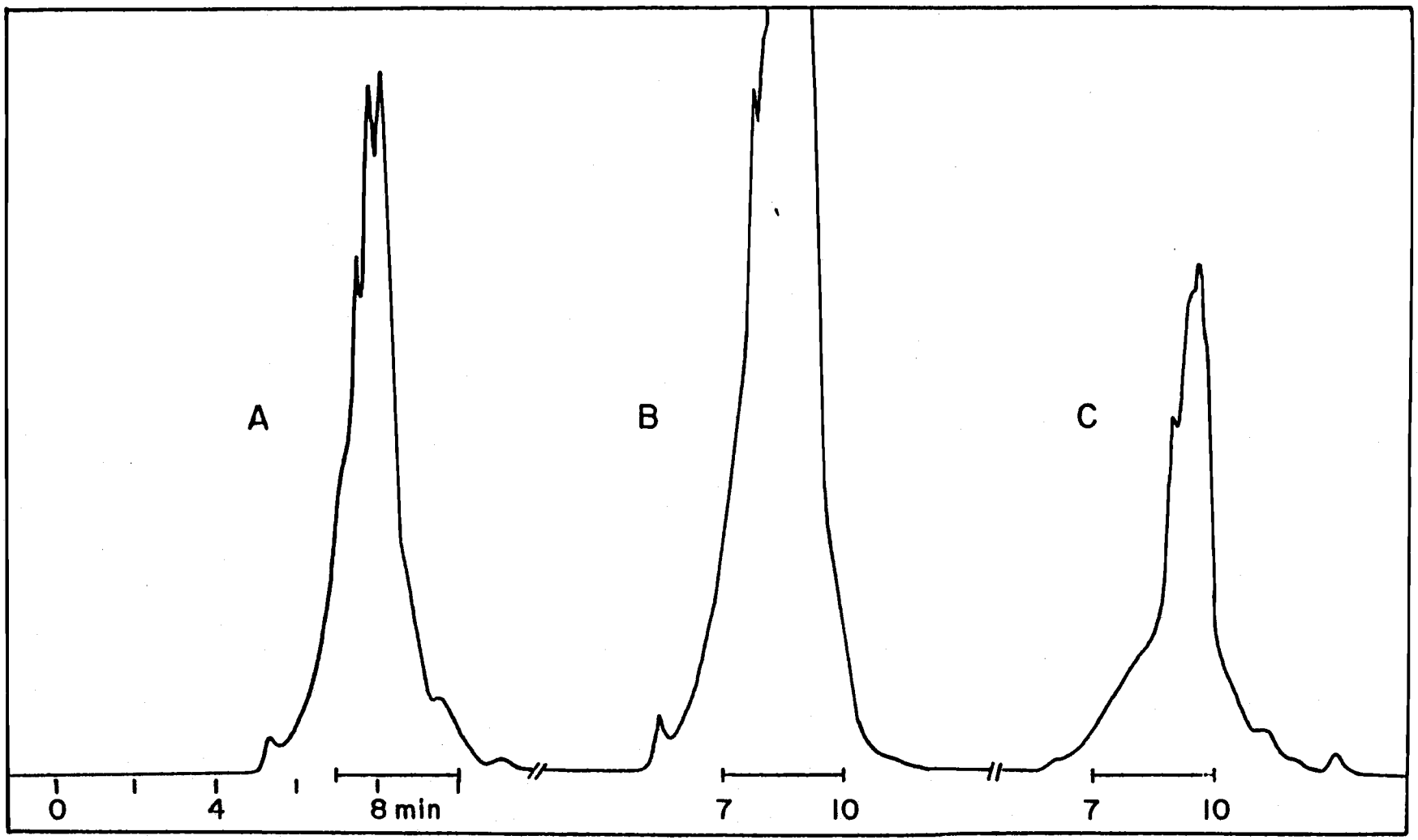


Figure 31: Gel permeation chromatography of March collection of trace enriched Douglas-fir xylem sap extracts. Altex μSpherogel column, 100% THF, isocratic, 1.0 ml/min. A: 10% elution from trace enrichment; B: 30% elution; C: 50% elution.

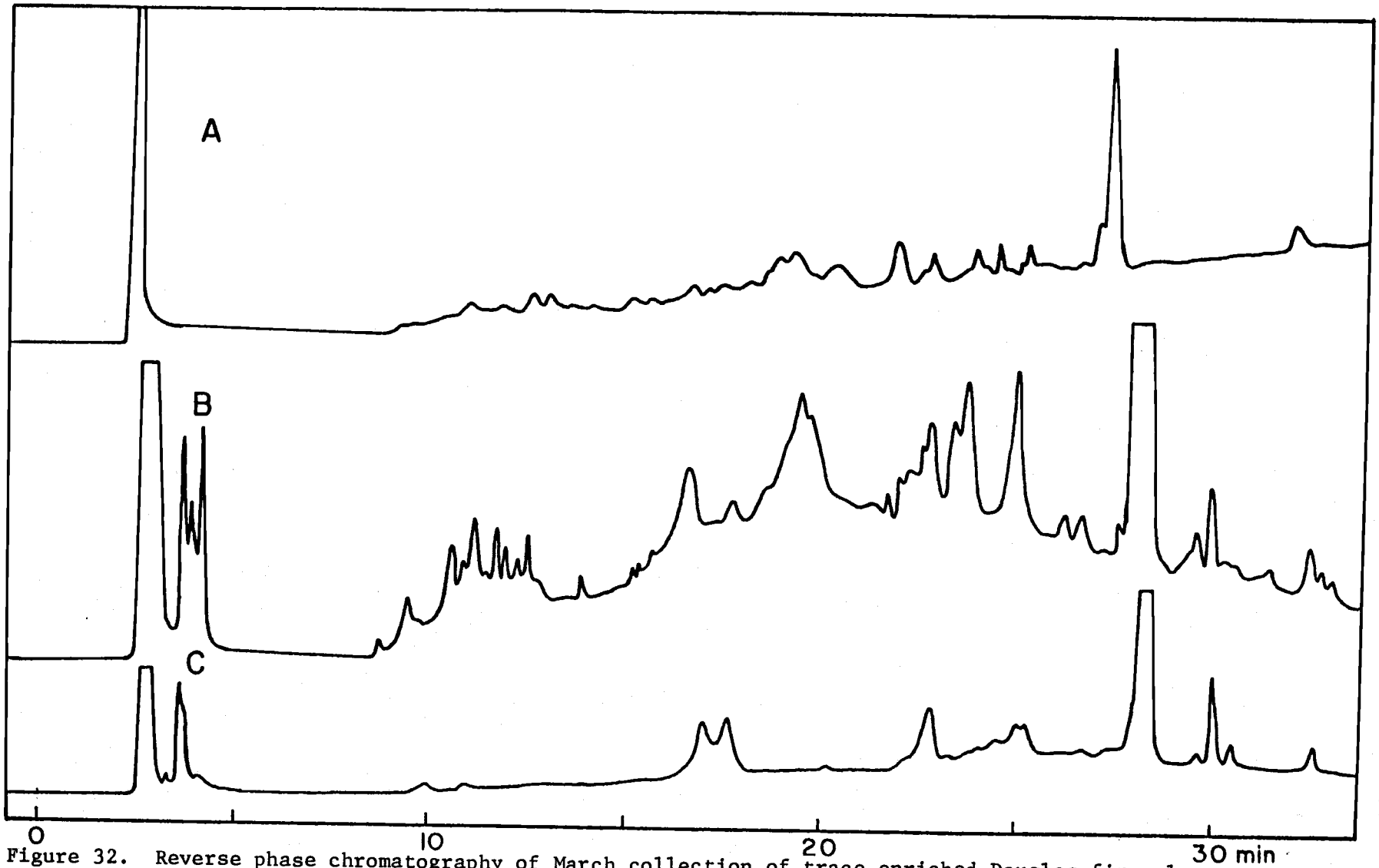


Figure 32. Reverse phase chromatography of March collection of trace enriched Douglas-fir xylem sap extracts. Varian MCH-5 column, 0 - 100% ETOH/buffer, 0.8 ml/min, 30 min. For bioassay see Figure 33. A: 10% elution from trace enrichment; B: 30% elution; c: 50% elution.

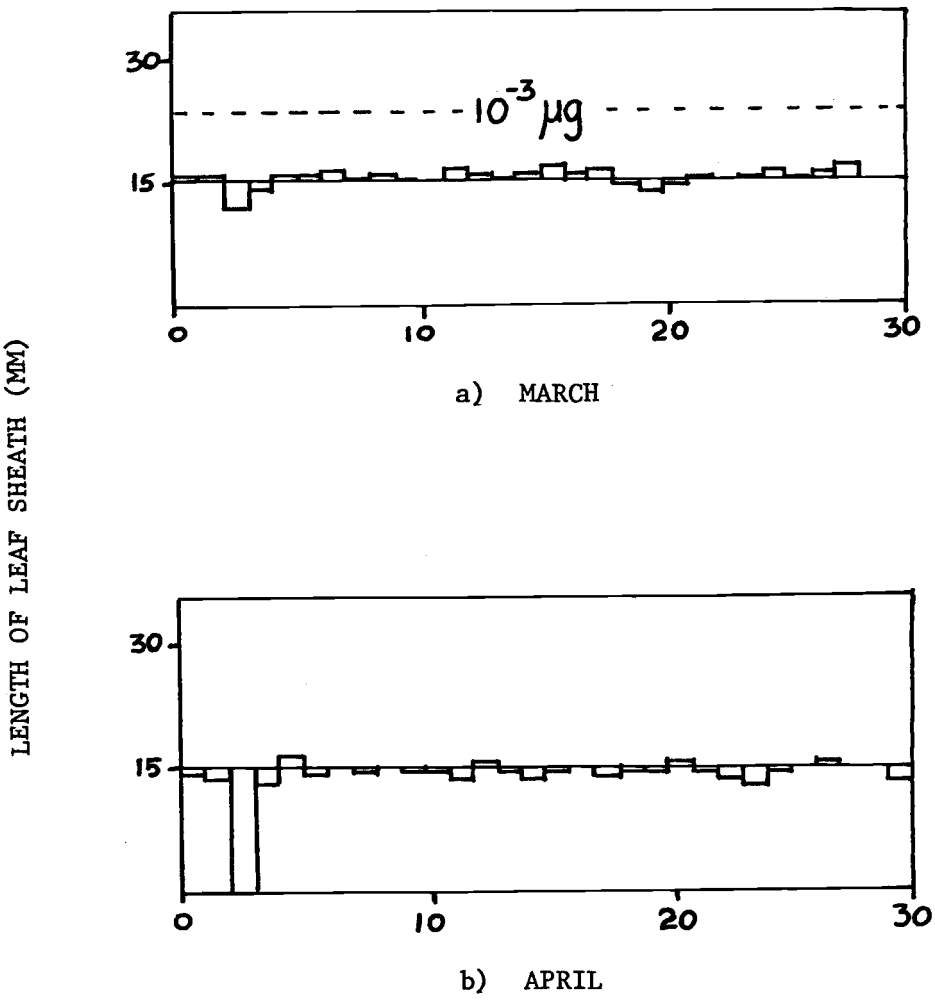
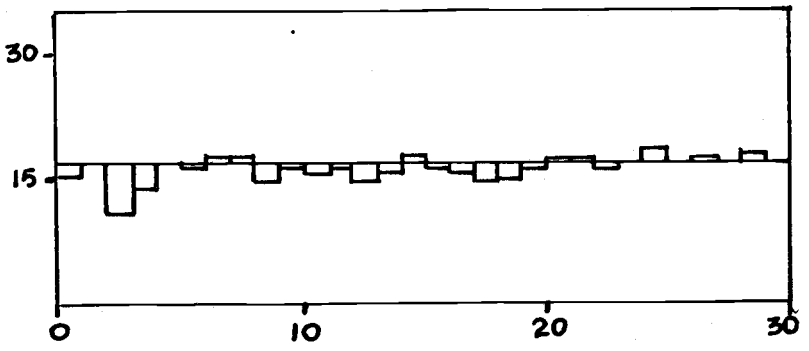
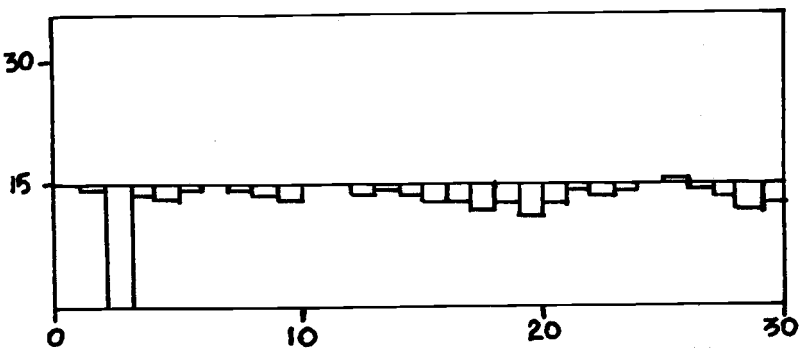


Figure 33. Representative results from Dwarf rice microdrop bioassay of trace enriched Douglas-fir xylem sap extracts. a: March 30% elution; b: April 30%; c: June 30%; d: July 30%; e: March 10%; f: April 10%; g: July 10%; h: March 50%. There is an increase in the degree of inhibition by sap extracts collected during June and July compared to those collected in March and April.

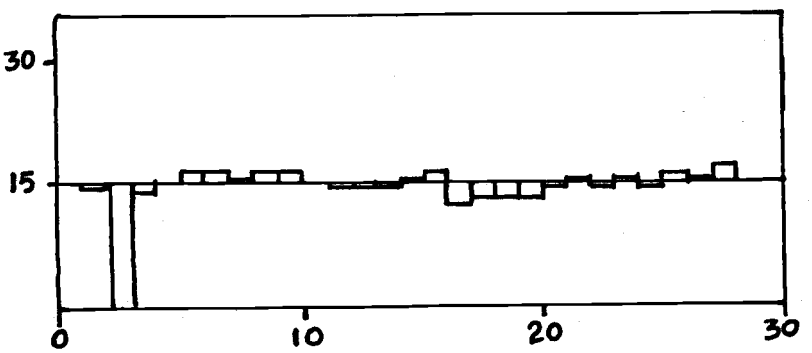


c) JUNE

LENGTH OF LEAF SHEATH (MM)



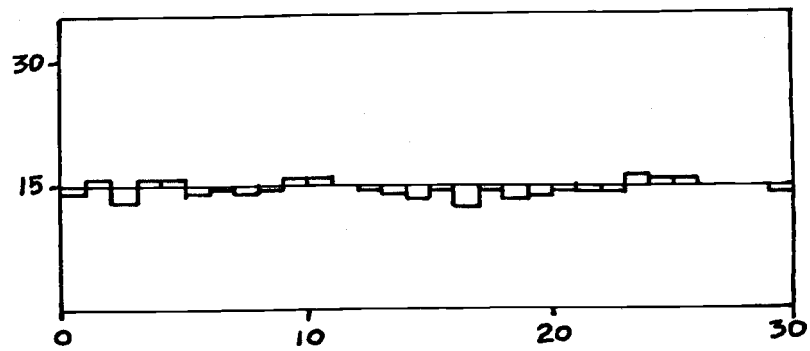
d) JULY



e) MARCH

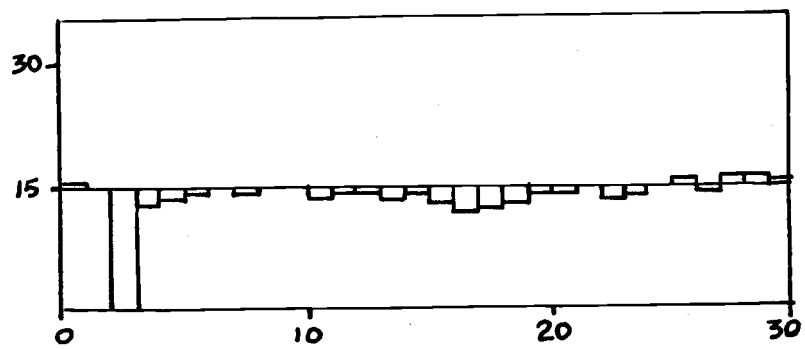
Figure 33 (Continued)

FRACTION NUMBER

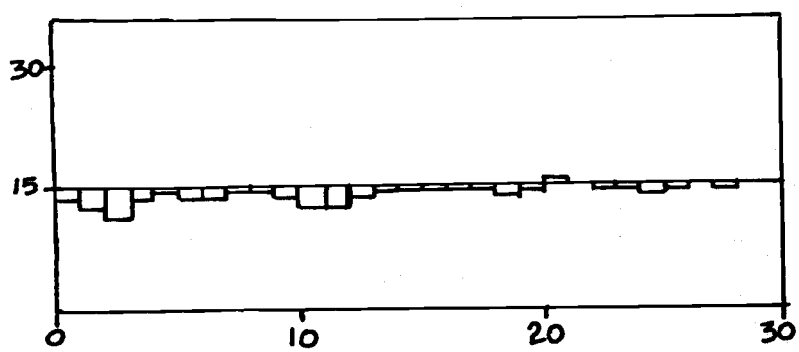


f) APRIL

LENGTH OF LEAF SHEATH (MM)



g) JULY



h) MARCH

Figure 33 (Continued) FRACTION NUMBER

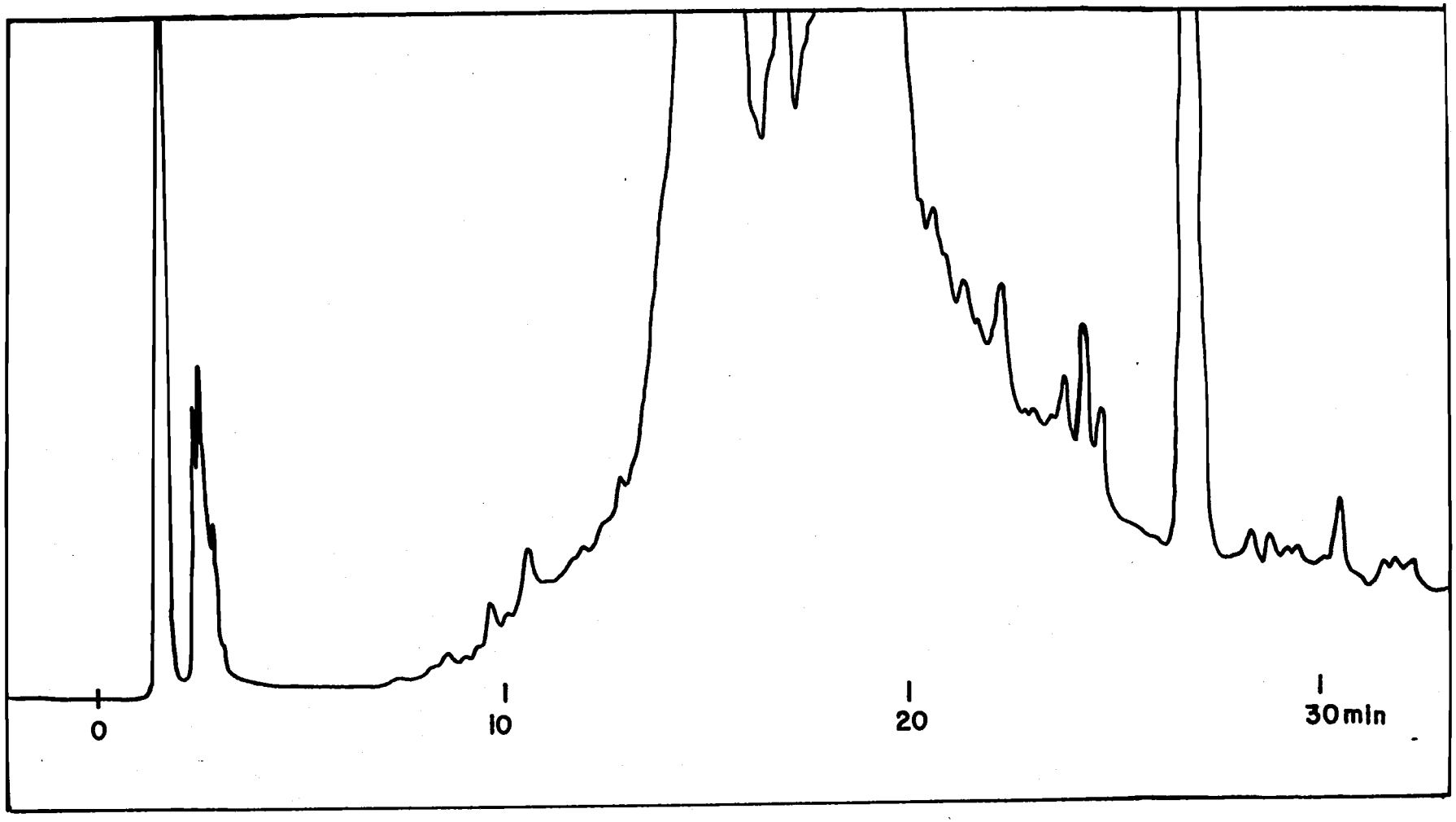


Figure 34. Reverse phase chromatography of Douglas-fir xylem sap extract collected June 1979. Conditions - see figure 32.

growth promoting compounds are present in sufficient quantities to overcome part of the effect of the growth inhibiting compounds during the period of active shoot growth, and the inhibitory compounds override the promoting effect as shoot growth decreases.

DISCUSSION

Before attempting a qualitative determination of the gibberellins present in the spring xylem sap of Douglas-fir, it was necessary to develop means by which the sap could be extracted and analyzed for gibberellin content. A simple yet quantitative technique of trace enrichment was developed which, although originally intended as a method for reducing the large volumes of sap required for the extraction of trace amounts of plant hormones, proved to be applicable as a concentrating technique for extracts of other tissue as well. Following their extraction from the sap and cucumber seeds, the gibberellins were separated from other components and from each other using high performance liquid chromatography. Gibberellins, however, do not absorb light appreciably in the ultraviolet wavelength range, and, because the most sensitive, convenient, and available method of detection in liquid chromatography was with a uv detector, it was necessary to modify the free gibberellin molecules chemically in such a way as to enhance their uv absorbance. The parabromophenacyl esters of the GA standards proved to be very highly uv absorbing (ε_{GA_3pBPB} = 12,200 l-moles^{-1}-cm^{-1}) and reliable in HPLC. This type of derivative has an added advantage in the attachment of the bromine atom; because there are two isotopes of bromine (^{79}Br and ^{81}Br) that are of nearly equal abundance in nature, a mass spectrum of a brominated compound will always display the characteristic doublet in any fragments still possessing the bromine

atom. This increases the probability of identifying fragments and fragmentation patterns and allows for the more positive identification of the compounds in question.

The techniques described in this paper resulted in the conclusive identification of GA_4, GA_7, and GA_9 from immature seeds of wild cucumber. This identification was based upon bioassay, retention times of both the underivatized and derivatized extract components, and mass spectra of the MeTMS derivatives. The GA_4 was identified further using the pBPB derivatives on the negative ion mode of the mass spectrometer. There was no conclusive evidence of any GA's above the 1.0 nanogram level in Douglas-fir sap. This result does not necessarily imply that there <u>are</u> no gibberellins present in Douglas-fir xylem sap at that particular time of year, however. A number of explanations are possible.

An obvious conclusion is simply that Douglas-fir xylem sap does not contain any gibberellins during the spring. In light of evidence of GA-like compounds in the spring sap of other species, however (Reid and Burrows, 1968; Jones and Lacey, 1968), it appears that other factors may be interfering in the extraction and/or detection of the GA's. Since the sap was collected from young trees during the growing season, it is likely that hormone biosynthesis mechanisms are operating. Douglas-fir trees of the same age used in this study were estimated from bioassay and chromatographic data to contain approximately 115 µgm GA_3 per kg of vegetative shoots (Crozier, <u>et al.</u>, 1970). The xylem sap from three year old seedlings of Douglas-fir was found by bioassay to contain GA-like substances in several

fractions from a gradient-eluted silicic acid partition column (Sweet, et al., 1973). Therefore, reasons for the lack of response to the dwarf rice bioassay, other than the statement that there are no GA's present in Douglas-fir xylem sap during the spring, would seem to be more plausible.

The bioassay data from seven year old trees used in this research versus three year old seedlings used by Sweet et al. seem to be in direct conflict, yet some interesting differences are noted.

The older trees are 5-6 times the size of the seedlings. It is possible that the concentration of gibberellins in the large vascular system of a sapling size tree is so dilute as to be unmeasurable by the currently available methods.

Differences in the manner of sap collection, extraction, and purification are also significant. For the experiments reported here, extreme care was taken to maintain the natural qualities of the sap during collection and extraction, and a number of different techniques were employed to enhance the purity of the extracts. However, Sweet et al. merely partitioned the sap against ethyl acetate and separated the compounds on a silicic acid partition column before subjecting the fractions to bioassay. While some bioassays can be extremely sensitive, they cannot be considered conclusive proof of a compound's existence. The number of compounds present in a plant extract is extremely large; the likelihood of compounds other than gibberellins evoking a response in a bioassay is quite possible. Also, when the biologically active compounds were analyzed by GLC, these compounds, suspected to be GA_3 and GA_7

because of their retention characteristics, did not match standards and could not be identified.

A more positive identification of GA_3 from trees of a similar age and size was made at the same time of year by Crozier et al. in 1970. Since these researchers found putative GA_3 in vegetative shoots of Douglas-fir, the presence of this compound in the xylem sap could be expected. Gibberellins are implicated in the breaking of dormancy and in shoot elongation, and transport between roots and shoots during the spring season is likely.

The following explanations could reconcile the presence of GA_3 in the shoots of Douglas-fir with the absence of GA_3 in the xylem sap. It is very probable that the gibberellins are present and are in reasonable concentration, yet are either: a) being masked by inhibitors, or b) in a form that either precludes their identification or separates them from the tritiated internal standards.

Both the bioassay data and the HPLC spectra indicate strongly that inhibiting compounds are present and are increasing later in the spring and summer. This increase coincides with the slowing down and eventual cessation of shoot growth. Thus, while a statement about the identity of gibberellins present in the xylem sap cannot be made at this time, it can be stated that the period of active shoot growth is correlated with a balance of growth promoters and growth inhibitors that reflect positive growth, whereas the cessation of shoot growth is correlated with a negative balance of growth promoters and growth inhibitors.

If the gibberellins are present in a form other than the free acid form, the likelihood of their extraction and detection would be severely reduced. Gibberellin glucosyl esters have been detected in higher plants and suggested as a transport form. If most of the GA's in the sap are glucosylated, they would not partition into the EtOAc fraction during the solvent extraction, and hence would be lost. Their retention characteristics on reverse phase and gel permeation columns would also be different from those of free GA's. More importantly, even if some esterified gibberellins were retained throughout the procedure, they would not exhibit significant activity in the bioassay.

The possibility that many of the gibberellins thus are isolated from plant material are bound to sugars and other polar molecules via an ester connection is not unlikely when the extraction procedures are considered. When extracting components of seeds, stems, and other plant tissues, the generally accepted practice is to macerate the tissue in a solvent such as methanol. Such a procedure destroys the compartmentalization inherent in an intact cell, and would permit the contents to intermix freely. Thus esterases would come into contact with their corresponding esters, and the carbon-oxygen bonds would be broken, leaving the free acid.

It would be possible to circumvent some of the difficulties outlined above. The trace enrichment process could be scaled up to accept a larger volume of sap. This change would increase the numbers of available adsorption sites on the column, and thus increase the amount of material that could be trace enriched. The

separation characteristics of the gibberellin glucosides could also be worked out if suitable standards were available. The conditions of the sap could be altered chemically in such a way as to induce de-esterification, and still maintain the intact gibberellin molecule in free acid form. The development of more sensitive detection methods, such as fluorescence spectroscopy, which is up to 1000 times more sensitive than uv absorption, would increase the probability of detection of trace quantities of plant hormones.

Although it was not possible to make a positive identification of any specific gibberellins in Douglas-fir xylem sap that was collected during the spring, there is evidence that the concentration of inhibitory compounds increases during the early summer when shoot growth is beginning to cease. This marked inhibition is not evident during March and April, and yet a significant growth response was not observed either. These results suggest that the gibberellins that may be present are being masked by the high level of inhibitory compounds. With some modifications, the techniques developed in this study could be used to determine the critical changes in the gibberellin content of Douglas-fir and how these changes relate to physiological development.

SUMMARY

Parabromophenacylbromide esters of gibberellins were prepared in order to enhance the suitability for detection of the gibberellin molecules by uv absorbance. High Performance Liquid Chromatography and mass spectrometry procedures were then developed using these standard derivatives.

Trace enrichment of underivatized gibberellins was developed and applied to the extraction of wild cucumber seeds, a plant material known to be sufficient in gibberellins. Using this extraction and the above-mentioned identification techniques, it was possible to identify GA_4, GA_7, and GA_9 in extracts of the endosperm of wild cucumber seeds.

These methods were applied to the xylem sap collected from seven year old Douglas-fir trees; however a conclusive identification of gibberellins could not be made. Some possible reasons for this result are: a) the concentration of gibberellins in sapling size Douglas-fir xylem sap is too dilute to be measured by the present methods; b) the gibberellins are present yet are in a modified form for which appropriate isolation procedures had not been employed; c) the level of inhibitors in the sap is high enough to mask the gibberellins. Any one or a combination of these alternatives could explain the results obtained in this study.

Before any studies can be undertaken to examine gibberellin metabolism during tree growth, it will be necessary to determine whether the native gibberellins occur in a modified form. The kinds

of inhibitors present and their interactions with the gibberellins will also require investigation.

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