THE EFFECT OF GIBBERELLIC ACID AND ETHEPHON ON ENZYMATIC BROWNING OF REDHAVEN PEACHES

by

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B.Sc.(Agr.), University of British Columbia, 1973

A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
Department of Food Science

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
March, 1978

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ABSTRACT

Redhaven peaches treated with gibberellic acid (GA, 100 ppm) and ethephon (75 and 150 ppm) 21 and 46 days after full bloom were evaluated for enzymatic browning in the ripe fruit. Treated fruit had less browning than untreated fruit, and fruit treated 46 days after bloom had less browning than fruit treated 21 days after bloom. Fruit pH and fresh weight were affected by treatment, but o-diphenol content and polyphenoloxidase (PPO) activity were not. Forward stepwise multiple regression on browning showed that 81% of the variation in browning was explained by differences in treatment, treatment application date, o-diphenol content, PPO activity, and fresh weight.

Twenty-one polyphenolic compounds from Redhaven peaches were separated by two-dimentional thin layer chromatography. Eight were oxidized by PPO, and were tentatively identified as four chlorogenic acid isomers, three leucoanthocyanidins, and catechin. No differences in qualitative distributions of phenolic compounds were observed in peaches receiving the different treatments.

Polyacrylamide disc gel electrophoresis of peach PPO preparations showed the presence of up to eleven isozymes with activity toward catechol. The isozymes had different substrate specificities and were present in different amounts. PPO from peaches treated 21 days after bloom appeared to have a catechol reactive isozyme not present in untreated peaches or peaches treated 46 days after bloom. One PPO isozyme from peaches treated 46 days after bloom with 150 ppm

ethephon appeared to have decreased substrate specificity toward pyrogallol.

Crude PPO preparations from untreated fruit and fruit receiving the 46-day treatments oxidized o-dihydroxyphenolic compounds only. The relative activities of the PPO preparations with these compounds varied with treatment. The same PPO preparations exhibited two pH optima; pH 4.4 and 6.2 for untreated and GA treated peaches (46-day treatment), and pH 4.4 and 6.6 for peaches treated with ethephon (75 or 150 ppm, 46-day treatment). PPO from the treated peaches had a lower proportion of total activity at pH 4.4 than PPO from untreated peaches. The Michaelis constant for PPO from untreated peaches was 9.1×10^{-3}M.

TABLE OF CONTENTS Page

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. J. Vanderstoep, Research Supervisor, for his help and encouragement during this study, to Dr. S.W. Porritt for assistance in the field work, and the Canada Agriculture Research Station, Summerland, B.C., for making available the Redhaven peach trees used in this study. Special thanks go to Dr. G.W. Eaton for his advice and assistance in the statistical analyses.

INTRODUCTION

The enzymatic browning of peaches is a result of the enzyme catalyzed oxidation of polyphenolic compounds to colored pigments. This reaction occurs when the fruit tissue has been disrupted during handling, packaging, or processing and may result in deleterious changes in color, odor, flavor, and nutritional value. The control of enzymatic browning has been the subject of much research, as the rejection of badly bruised or browned fruit products and the time and labor involved in browning control represent a large cost to the food industry.

Traditional methods of controlling enzymatic browning are based on controlling some aspects of its enzyme : oxygen : substrate system. These methods include heat denaturation of the browning enzyme, exclusion of oxygen by vacuum packing, addition of reducing agents such as ascorbic acid and sulfur dioxide, adjustment of pH, and freezing (Ponting, 1960; Mathew andParbia, 1971).

The use of plant growth regulators to control browning has been of more recent interest. In 1969 it was reported that "Early Amber" peaches sprayed two weeks after full bloom with the growth regulators gibberellic acid (GA) and ethephon (both at 50 ppm) had less enzymatic browning at harvest than untreated fruit (Buchanan et al., 1969). This was found to be due to a decrease in polyphenoloxidase, the browning enzyme, of over 90% (Knapp et al., 1970). Paulson (1973) found that "Redhaven" peaches sprayed with 100 ppm GA four weeks after

full bloom had decreased enzymatic browning at harvest and attributed this to a decrease in polyphenolic substrate. GA and ethephon at 50 ppm had no effect and "Fairhaven" peaches receiving the same treatment displayed no change in browning. Porritt (1974) however, found that "Redhaven" peaches receiving 75 ppm ethephon sprays 46 days after full bloom had decreased enzymatic browning at maturity. Italian prunes sprayed four weeks before harvest with GA (Proebsting and Mills, 1966) and with ethephon combined with GA (Proebsting and Mills, 1969) had lowered internal browning, and applies sprayed with ethephon ten days before harvest had lower levels of polyphenoloxidase and were more resistant to browning on cutting than untreated fruit (Sal'kova et al., 1977).

In view of these varying reports, the present study was undertaken to determine the effect of GA (100 ppm) or ethephon (75 or 150 ppm), applied at either of two application dates, on enzymatic browning of Redhaven peaches.

LITERATURE REVIEW

Enzymatic Browning Reaction

The fundamental step in the enzymatic browning of peaches is the oxidation of ortho-dihydroxyphenolic compounds (o-diphenols) to o-quinones catalyzed by the enzyme polyphenoloxidase (PPO) (Luh and Phithakpol, 1972). The reaction involves two substrates with o-diphenols serving as hydrogen donors and oxygen as the hydrogen acceptor. The order of binding of the substrates isn't known with certainty. Data from different sources have indicated a sequential mechanism with oxygen binding first, a sequential mechanism with oxygen binding second, or a random mechanism (Rivas and Whitaker, 1973; Lerner and Mayer, 1976). Studies of the mode of action of PPO's have suggested that they possess separate binding sites for oxygen and the phenolic substrate (Walker and Wilson, 1975).

The o-quinones produced by the oxidation reaction are themselves colored red to reddish-brown, but they are highly reactive and take part in non-enzymatic secondary reactions leading to the formation of more intensely colored secondary products. Such secondary reactions include 1) coupled oxidations of compounds that aren't PPO substrates or are oxidized with difficulty, 2) complexing with amino acids and proteins, and 3) condensation and polymerization with polyphenols to higher molecular weight more intensely colored compounds (Mathew and Parbia, 1971). In most food products the intense color of enzymatic browning occurs only after such complexing. PPO undergoes

"reaction inactivation" due to the formation of a covalent linkage between the enzyme molecule and a quinone at or near the active site (Whitaker, 1972).

It is assumed that browning doesn't take place in the intact cell because PPO and the polyphenolic substrates are spacially separated (Ponting, 1960). There has been little concrete evidence demonstrating the sub-cellular location of PPO and phenolic compounds however (Anderson, 1968). After the cells are damaged such as by impact or cutting, the enzyme and substrate are free to mix and in the presence of oxygen the browning reaction can proceed.

The function of the browning complex is uncertain. It has been suggested that PPO functions as a terminal oxidase in respiration (Boswell, 1963) but it has been found to compete poorly with the respiratory chain at low partial pressures of oxygen (Anderson, 1968). It has also been implicated in disease resistance of plants. Oxidized polyphenols are more potent anti-fungal agents than the unoxidized precursors (Walker, 1975).

The rate of browning in various foods has been related to PPO level, substrate level, and a combination of both factors (Kahn, 1975), but browning of peaches was most closely related to levels of polyphenols (Guadagni et al., 1949; Nakabayashi et al., 1963).

Polyphenoloxidase

Polyphenoloxidase (o-diphenol: oxygen oxidoreductase EC 1.14.18.1 also known as catechol oxidase, phenolase, diphenolase) contains copper

as its active prosthetic group. Lanzarini et al. (1972) demonstrated that the active enzymatic form is associated with Cu^{+} ions, with very little in the Cu^{++} form. They suggested that a $Cu^{+}-O_2$ interaction would activate molecular oxygen and the reaction would occur according to the scheme of Mason (1957). It also appears to involve an active site with a high affinity for the aromatic ring and a basic group which promotes phenol to phenolate ionization (Bright et al., 1963; Duckworth and Coleman, 1970). It is assumed, based on studies of the effects of inhibitors and ring substitution on reaction velocity that oxidation occurs via an electrophilic attack (Lanzarini et al., 1972).

PPO's from different sources usually have differences in such properties as substrate specificities, pH optima, and reaction kinetics. They also usually exist as isozymes which can be separated by electrophoretic and chromatographic means.

Peach PPO has been shown to oxidize o-diphenols almost exclusively, with negligible activity with monophenols (Luh and Phithakpol, 1972; Reyes and Luh, 1960). Slight activity with the p-diphenol quinol has been reported and varies with maturity (Reyes and Luh, 1960; Harel et al., 1970). PPO from some sources has the ability to catalyze both the hydroxylation of monophenols to o-diphenols and their subsequent oxidation to o-quinones (Mason, 1957). Constantinides and Bedford (1967) resolved mushroom PPO into nine isozymes, three of which had activity with monophenols. Taneja and Sarkar (1974) reported that the monophenolase and diphenolase activities of wheat were separable and reside in different enzymes.

The pH optimum of peach PPO ranges from 5.9 to 6.3 depending on the type of buffer used (Luh and Phithakpol, 1972; Reyes and Luh, 1960). Jen and Kahler (1974) found that as "Redhaven" peaches matured the PPO pH optimum changed from a single optimum at pH 6.2 to double optima at pH 6.0 and 6.5, suggesting the synthesis of new isozymes with maturity. Wong et al. (1971a) separated PPO from "Cortez" peaches into four isozymes which differed from each other in pH optima, substrate specificity, rate constants, and susceptability to inhibitors and heat. Harel and Mayer (1970) electrophoretically separated PPO from "Salvey" peaches into five bands, one of which was active with quinol, a characteristic of the enzyme laccase, not normally found in peaches.

The sub-cellular location of PPO is poorly understood. Sections of mature peach fruit stained for PPO with catechol showed the enzyme to be present in localized patches of parenchyma cells (Reeve, 1959). Harel et al. (1970) found peach PPO to be present in both the particulate and soluble form, which were present in differing amounts at different stages of maturity. The insoluble form of apple PPO has been found to be associated with both chloroplasts and mitochondria (Harel et al., 1965). Such particulate PPO can often be solubilized by treatment with various detergents, which is often accompanied by PPO activation (Sato and Hasegawa, 1976). Soluble PPO sometimes also exists in a latent form that can be activated by storage, temperature change, detergent treatment, or denaturing agents, contributing additional PPO activity (Kahn, 1977).

Due to the complexity of PPO it is difficult to ascribe any single role in cellular metabolism. Conn (1964) suggested a role for PPO in biosynthesis of phenolic compounds, but any explanation of function must take into account the demonstrated multiplicity of forms.

Browning Substrates

The ortho-dihydroxyphenolic configuration is essential for PPO activity, but not all compounds with such a configuration are oxidized, and those that are exhibit different rates of oxidation. Substituents on the benzene ring have been shown to influence reaction rate depending on position and electron donating or attracting character (Lanzarini et al., 1972).

Natural browning substrates found in food are usually cinnamic acid derivatives which arise from the shikimic acid pathway, and flavonoid compounds of which the "A" ring is derived from the acetate-malonate pathway and the "B" ring from cinnamic acid derivatives (Hess, 1975).

Of the cinnamic acid derivatives the most important in enzymatic browning is chlorogenic acid (3-caffeoylquinic acid) a 3-depside of quinic acid with caffeic acid. Chlorogenic acid content has been related to browning of several varieties of applies (Walker, 1962). Flavonoids involved in browning include catechins, leucoanthocyanidins, anthocyanins, and flavonols. Anthocyanins are not primarily significant

as PPO substrates but have been shown to be involved in secondary reactions (Mathew and Parbia, 1971).

The main polyphenols involved in enzymatic browning of peaches were found to be leucoanthocyanidins, chlorogenic acid isomers, and catechins (Craft, 1961; Fel'dman and Kostinskaya, 1970; Luh et al., 1967). Craft (1961) observed no qualitative change in polyphenolic pattern with ripening and the relative proportions remained constant. Phenolic compounds were seen to increase in concentration during the early stages of fruit growth, reach a maximum at pit-hardening, and then slowly decline until harvest (Craft, 1961; Harel et al., 1970). The amount of phenols on a whole fruit basis was seen to increase however (Craft, 1961).

Li et al. (1972) found that both flavor and color of peaches were negatively correlated with total phenols. Astringency of fruits has been associated with polyphenolic concentration, particularly catechins and leucoanthocyanidins (Goldstein and Swain, 1963; Craft, 1961). Peaches show a decrease in astringency during ripening (Craft, 1961), but the reasons for this are unknown.

The functions of plant phenols are obscure perhaps because they are secondary products and play no role in metabolism. Anthocyanins, flavones, and flavonols, due to their coloration, probably play a role in attracting insects (Salisbury and Ross, 1969). Hydroxylated cinnamic acids are believed to be important precursors of lignin (Van Buren, 1970). In peach, the highest total phenolic concentration is at pit-hardening. Phenolic compounds have also been suggested to

control auxin concentrations in some plants through their effects on the enzyme indole acetic acid (IAA) oxidase. Monophenols have been shown to enhance IAA oxidase activity while o-diphenols inhibit the enzyme (Nitsch, 1970).

Gibberellic Acid and Ethephon

Gibberellic acid (abbreviated GA or GA_3), best known for its stimulating effects on plant growth, is one of many different structural variations of the plant growth hormones known as gibberellins. As well as stimulating growth, gibberellins have been found to have regulatory effects on plant development. Plants have been found to have selectivity of response to the different forms. The term "gibberellin" is often used rather loosely in the literature as being synonymous with gibberellic acid (Stuart and Cathey, 1961). Recent reviews of the gibberellins have been made by Lang (1970) and Jones (1973).

Ethephon (2-chloroethylphosphonic acid) breaks down in the plant releasing the plant hormone ethylene (Yang, 1969). Ethylene is best known for its effect in triggering ripening of climacteric fruit but has also been found to be important in regulation of plant development. The physiology of ethylene has recently been reviewed by Abeles (1972). GA and ethylene often have similar effects (e.g. breaking of seed dormancy) as well as opposing effects (e.g. ethylene promotes but GA delays ripening and senescence in many types of fruit) (Leopold and Kriedmann, 1975).

Peach fruit growth is characterized by two stages of rapid growth (Stages I and III) separated by a period of slow growth (Stage II) during which lignification of the stone occurs (pit-hardening). Both fresh weight and dry weight growth follow similar double-sigmoid growth patterns (Chalmers and van den Ende, 1975). Growing fruit act as physiological sinks, attracting nutrients at the expense of the remainder of the tree. Both GA and ethylene are thought to be involved in this effect (Nitsch, 1970; Chalmers, et al., 1976). Jackson (1968) found gibberellin in the seeds of peaches immediately after full bloom, and later in the mesocarp and endocarp. Gibberellin was found to be closely associated with the rate of cell expansion in each tissue but not with cell division. Ogawa (1965) reported that gibberellins in peach seeds began to increase 35 days post-bloom, reached their maximum amount by 50 days post-bloom, and decreased rapidly thereafter. Looney et al. (1974) found an association between ethylene level and growth rate in Stage I of peach growth.

Applied GA and ethephon have varying effects on peach growth. GA has been shown to induce parthenocarpy (seedlessness), stimulate vegetative growth (Jerie and Taylor, 1971), achieve a thinning effect at flowering (Edgerton, 1966), and delay maturation and ripening (Leopold and Kriedmann, 1975; Paulson, 1973). Rom and Scott (1971) found that ethephon applied during the final swell of peach growth accellerated maturation. Byers and Emerson (1973) found that ethephon applied during Stage II of peach growth induced early onset of Stage III. Looney et al. (1974), however, found no effect of ethephon application

on length of Stage II. Other ethephon induced effects include fruit thinning and more uniform maturation (Stembridge and Raff, 1973; Rom and Scott, 1971).

The modes of action of GA and ethephon are unknown. Both have been seen to regulate enzyme formation, possibly by control of RNA directed protein synthesis, and both have been linked to alterations in cellular membranes (Leopold and Kriedmann, 1975).

The manner in which a single early season application of a growth regulator can induce a response months later is presently unknown. Byers et al. (1969) attributed the belated effects of ethephon to a higher rate of endogenous ethylene production, rather than ethephon degradation. Stembridge and Raff (1973) suggested that ethephon induces a lingering increase in ethylene level in immature peach fruit which persists until the fruit develop sufficiently to respond. Lavee and Martin (1974) however, showed that penetration of ethephon through peach exocarp into the mesocarp was very low; most of the ethephon accumulated on the exocarp and most of this could be removed by washing. They later found, however, that ethephon binds rapidly to sugars in the peach mesocarp and suggested that the formation of stable sugar-ethephon complexes may be involved in long-term responses, rather than release of ethylene at the fruit surface (Lavee and Martin, 1975). This is similar to the binding of GA as glycosides, which may be a storage form of this hormone (Leopold and Kriedmann, 1975).

EXPERIMENTAL

Application of Growth Regulators

Nine-year old "Redhaven" peach trees (Prunus persica) on Prunus tomentosa seedling roots were used in this study. Twenty-eight trees at the Canada Agriculture Research Station at Summerland, B.C. were randomly divided into seven groups of four trees each in the spring of 1975. Full bloom date was May 13, 1975. On June 3, twenty-one days after full bloom, gibberellic acid (as activol GA, Chipman Chemicals) at 100 ppm, and ethephon (as Ethrel, Anchem Products) at 75 ppm and 15 ppm were applied by hand sprayer to runoff (2.5 - 3 liters per tree) to three of the seven groups. 0.1% Rhodes R-11 spreader-activator was used as a wetting agent. Hand thinning of all trees to a desirable crop load was accomplished on June 24. On June 29, forty-six days after full bloom, the treatments were repeated on three of the remaining groups. The seventh group of trees served as the control.

Three picks were made as the fruit attained commercial picking maturity; Aug. 16, 22 and 28. Fruits from the second pick (Aug.22) were used for all analyses, and tree identity was maintained throughout. Fifteen fruits from each tree were weighed and the average weight per fruit recorded.

The fruits were placed in cold storage (0°C) immediately after picking. Five peaches from each tree were removed from cold storage and allowed to ripen at 21°C for determination of enzymatic browning and pH. The remainder of the fruits were transported to Vancouver, ripened at 21°C, halved, pitted, vacuum packaged with a nitrogen backflush in aluminum foil pouches, then placed in frozen storage (-35°C) until used.

Enzymatic Browning

One-third sectors of five ripe peaches from each tree were pureed for 20 seconds at half speed in a Waring blendor at 21°C. The degree of browning was measured with a Hunterlab D-25 Color Difference Meter as the difference in Rd (lightness) between readings taken at one minute and thirty minutes after blending. A yellow tile (Rd = 68.7, a =-3.8, b = 25.2) was used for standardizing the instrument. The pH's of the purees were measured by a Fisher glass-electrode pH meter.

Polyphenoloxidase Extraction and Assay

The methods used were modifications of those of Wong et al.(1971a). Unless otherwise indicated all extraction procedures were carried out at 4°C. Three frozen peach halves per tree were allowed to thaw at 4°C for 4 hours before extraction. Twenty-five grams from each of the 3 halves were blended with 150 ml cold acetone (-35°C) for 25 seconds at half speed plus an additional 5 seconds at full speed in a stainless steel Sorvall Omnimixer jar. The resulting slurry was allowed to stand

for 5 minutes in an ice bath and then suction filtered through Whatman no. 4 filter paper. The filter cake was washed with 500 ml cold acetone (-35°C) to remove pigments. The filter cake was suspended in 75 ml of 0.1 M Na phosphate buffer (pH 6.3) and shaken for 4 hr on a rotary shaker at 4°C, then centrifuged at 33,000 x G for 15 min at 0°C in a Sorvall RC-2 refrigerated centrifuge. The supernatant was collected as the crude enzyme solution for PPO assay and the pellet was discarded.

The spectrophotometric method of Wong et al. (1971a) was used for assay of PPO activities. The standard reaction mixture consisted of 0.1 ml crude PPO plus 2.9 ml 0.01 M catechol in 0.1 M Na phosphate buffer (pH 6.3). Preliminary investigation had found this to be the optimum pH of "Redhaven" peach PPO in Na phosphate buffer. To insure that oxygen was not limiting, the catechol solution was aerated by bubbling oxygen through it for 5 min. Reaction temperature was maintained at 25°C. The enzymatic formation of benzoquinone was followed at 420 nm (Ponting and Joslyn, 1948) with a Unicam SP-800 spectrophotometer with expanded scale recorder and a temperature controlled cuvette holder. One cm cuvettes were used. The initial increase in absorbance was used as a measure of PPO activity, and was recorded as ΔAbs_{420} 0.1ml PPO^{-1} min^{-1}. The reaction was performed in duplicate with PPO from each tree and the results averaged.

Extraction and Assay of o-Diphenolic Compounds

Twenty-five grams from each of 3 frozen peach halves per tree were macerated in 300 ml boiling 80% ethanol for 3 minutes at half speed

in an Osterizer blender under a nitrogen atmosphere. The slurry was suction filtered through Whatman no. 1 filter paper using "Hyflo Supercel" (Fisher) as a filter aid. The filter cake was washed with 2 aliquots of 300 ml boiling 80% ethanol then discarded. The filtrate was allowed to cool to room temperature, made up to 1 liter with 80% ethanol, then filtered through Whatman no. 4 filter paper to remove haze formed during cooling.

o-Dihydroxy and vicinal-trihydroxy phenolic compounds in the ethanol extract were determined by a modification of the method of Mapson et al. (1963). This method measures the yellow color resulting from a complex formed between 2 polyphenol molecules and one molybdate ion (Haight and Paragamian, 1960). One ml of 5% Na molybdate in 0.1% Na phosphate buffer (pH 7.0) was mixed with 3 ml of the same buffer. To this was added 1 ml of the ethanolic extract followed by immediate mixing. After 45 min at room temperature Abs_{375} was measured by a Beckman DB spectrophotometer using a blank consisting of 4.0 ml phosphate buffer plus 1 ml of the ethanolic extract. Preliminary experimentation had determined 375 nm to be the optimum wavelength for measuring this reaction. The reaction was performed in duplicate and the values averaged. The concentration of o-dihydroxy and vicinal-trihydroxy phenolics was determined from a standard curve prepared using 0.02-0.2 mg catechol/ml. The results were expressed as mg catechol/g peach tissue.

Statistical Methods

Data were analyzed by analysis of variance. To obtain information regarding specific treatment effects, the treatment sums of squares

and degrees of freedom were partitioned using the individual degree of freedom technique (Li, 1964a). The six treatments and the control were arranged as six treatment contrasts (Table I) and an orthogonal set of multipliers obtained (Table II). The facilities of the U.B.C. Computing Center Triangular Regression Package (Le and Tenisci, 1977) were used for multiple regression analyses.

Thin Layer Chromatography of Peach Phenolic Compounds

Qualitative analyses of peach phenolic compounds were accomplished by 2-dimentional thin layer chromatography (TLC). Peaches from one replicate of each treatment and control were used. To improve the possibility of detecting treatment effects, the replicate from each treatment was chosen on the basis of low browning and/or low o-diphenol content, and the untreated replicate was chosen on the basis of high browning and high o-diphenol content. The peaches used were from the second pick (Aug. 22, 1975) as were those analyzed for browning.

Twenty grams from each of 5 frozen peach halves were blended with 300 ml boiling 95% ethanol for 3 min under a nitrogen atmosphere in an Osterizer blender. The slurry was suction filtered through Whatman no. 54 filter paper using "Hyflo Supercel" as a filter aid. The filter cake was washed with an additional 300 ml boiling 95% ethanol. The yellow filtrate was evaporated on a flash evaporator at 32^{o} - 35^{o} C until an aqueous solution remained. This was extracted twice with an equal volume of hexane to remove carotenoids (Schaller and von Elbe, 1970), saturated with NaCl, then filtered through Whatman no. 4 filter

TABLE I

TREATMENT CONTRASTS

Symbol	Definition
a. C/Tr	Control vs. Treated
b. E/L	Early Treatment (21 days) vs. Late Treatment (46 days)
c. GA/Eth	Treatment with GA vs. Treatment with Ethephon
d. Lo/Hi	Low Ethephon Treatment (75 ppm) vs. High Ethephon Treatment (150 ppm)
e. B x C	E/L x GA/Eth
f. B x D	E/L x Lo/Hi

TABLE II

ORTHOGONAL MULTIPLIERS FOR TREATMENT CONTRASTS

Treatment	Application Time (days)	Treatment Contrast C/Tr	E/L	GA/Eth	Lo/Hi	BxC	BxD
Control	--	-6	0	0	0	0	0
Ethephon (75ppm)	21	1	-1	1	-1	-1	1
Ethephon (150ppm)	21	1	-1	1	1	-1	-1
Gibberellic Acid	21	1	-1	-2	0	2	0
Ethephon (75ppm)	46	1	1	1	-1	1	-1
Ethephon (150ppm)	46	1	1	1	1	1	1
Gibberellic Acid	46	1	1	-2	0	-2	0

paper. The filtrate was extracted three times with equal volumes of ethyl acetate, then dried by stirring over anhydrous sodium sulfate. The ethyl acetate extract was concentrated to 3 - 4 ml by vacuum evaporation at 32°C, centrifuged at 1000 x G for 1 min to remove insoluble material, then stored in the dark under a nitrogen atmosphere at 4°C until used.

Qualitative separation of peach phenolic compounds was carried out by ascending two-dimentional TLC on 20cm x 20cm plastic plates of 0.1-mm thick MN300 cellulose (M. Nagel and Co.). Three microliters of the concentrated phenol extract were applied 2 cm from the lower left corner of 5 thin layer plates. The chromatography tanks (27cm x 27cm x 7cm i.d.) were allowed to equilibrate with the developing solvent before each run. Development took place at room temperature. The chromatograms were developed in the first direction with butanol: acetic acid:water 12:3:5 v/v/v (BAW 12:3:5) until the solvent front was 1 cm from the top of the plates. The plates were removed from the tanks, air dried, then developed in the second direction with 5% acetic acid (5% HOAc) until the solvent front was 1 cm from the top of the plate.

The air dried chromatograms were observed under ultra-violet light before and after fuming with conc. ammonia. Phenolic compounds were visualized on one chromatogram with a spray of Folin-Ciocalteau reagent (diluted 3 times with water) followed by a spray of 10% aq. sodium carbonate (Krebs et al., 1969). The phenolic compounds were detected as blue spots on a light blue background. The intensity of the spot was proportional to the concentration of the phenol.

A second chromatogram was sprayed with diazotized p-nitroaniline (DPNA) reagent, prepared by mixing in an ice bath 0.5% p-nitroaniline in 2N HCl, 5% $NaNO_2$, and 20% sodium acetate (w/v) in a ratio of 1:10:30 (v:v:v) (Luh et al., 1967). This reagent gives characteristic colors with phenolics.

A third chromatogram was sprayed with freshly prepared vanillin reagent made by mixing a 10% ethanolic solution of vanillin with an equal volume of conc. HCl. This reagent gives pink to orange colors with leucoanthocyanidins and catechins (Swain and Hillis, 1959).

A fourth chromatogram was sprayed with a freshly prepared solution of 5% Na molybdate in 0.1 M Na phosphate buffer (pH 7.0). Phenolic compounds containing an o-dihydroxy or vicinal-trihydroxy configuration form yellow complexes with this reagent (Haight and Paragamian, 1960) and were visualized as yellow spots on the chromatogram.

The fifth chromatogram was sprayed with a crude preparation of PPO from control fruit (see Electrophoresis) and incubated in a humid chamber for 2 hours. PPO substrates showed up as yellow to brown spots (Siegelman, 1955).

R_f values of all spots were calculated and recorded.

Authentic samples of chlorogenic acid, caffeic acid, and l-epicatechin (all 5 mg/ml ethanol) were also chromatographed as described above and R_f values were determined.

Electrophoresis

Polyacrylamide gel electrophoresis of peach PPO preparation was

performed according to Davis (1964). Peach PPO has been shown to exist as isozymes separable by electrophoresis (Wong et al., 1971; Harel and Mayer, 1970). To determine the effect of growth regulator treatment on PPO isozymes, one tree from each treatment was selected on the basis of low browning and/or low PPO activity for electrophoresis, (Table III). The control replicate was chosen on the basis of high browning and high PPO activity.

For preparation of PPO for electrophoresis, 10 g of frozen peach tissue from each of 5 peach halves were blended with 100 ml cold acetone (-35°C) for 3 bursts of 10 seconds each at high speed in an Osterizer blender and allowed to stand at 4°C for 5 min. The slurry was suction filtered through Whatman no. 4 filter paper and then washed with 500 ml cold acetone (-35°C). The filter cake was suspended in 100 ml of 0.1 M Na phosphate buffer (pH 7.0) by stirring for 1.5 hr at 4°C. The suspension was centrifuged at 33,000 x G for 15 min in a Sorvall RC2 refrigerated centrifuge at 0°C. The supernatant (crude PPO preparation) was collected and the pellet discarded.

To precipitate pectins, 0.5 M $CaCl_2$ was added to the supernatant to a final concentration of 0.05 M. The solution was adjusted to pH 6.8 by addition of 0.1 M NaOH and the precipitate was removed by centrifugation at 15,000 x G and 0°C for 10 min in a Sorvall RC-2 centrifuge. Twenty ml of the supernatant was diluted to 50 ml with 0.1 M Na phosphate buffer (pH 6.8) and then concentrated to 6.5 ml by ultrafiltration (Diaflo PM-10 membrane, Amicon Corp.) under a pressure of 60 p.s.i. of nitrogen at 4°C. Protein concentration was determined

by the method of Lowry et al. (1951) as modified by Potty (1969) using crystalline bovine serum albumin as standard. This modification allows for the estimation of protein in the presence of phenolic compounds.

For electrophoresis, 150-200 µl of enzyme solution (containing approx. 65 µg of protein) was mixed with 1 drop of 40% sucrose and 1 drop of 0.001% bromophenol blue tracking dye and applied to the top of 5 geltubes containing 7% acrylamide running gel (5.0 cm long) and 1.25% stacking gel (1.0 cm long). All gels were run in a Pharmacia Model GE-4 electrophoresis apparatus. The starting pH was 8.3 and the running pH was 9.5. A current of 3.5 mA per tube was employed until the tracking dye migrated close to the end of the tube. The electrophoresis chamber was kept cool with circulating cold tap water. The position of the tracking dye was marked on the gel with a needle containing India ink.

One gel was stained for protein by immersion for 1.5 hr in 0.25% Coomassie blue dissolved in a mixture of 7% methanol and 5% acetic acid. The gel was destained by soaking in several changes of 7% methanol-5% acetic acid solution until a clear background was obtained. The position and intensity of the bands were recorded visually and then by densitometry using a Gelman Gelscan densitometer.

Four other gels were soaked for 30 min in a solution of 0.1% m-phenylenediamine (MPD) in 0.1 M Na phosphate buffer (pH 6.3) and then placed in a solution of catechol (0.03 M), pyrogallol (0.03 M), caffeic acid (0.03 M), or chlorogenic acid (0.01 M) all in Na phosphate buffer

(pH 6.3). The gels were vigorously aerated by bubbling oxygen through the solutions for 5 min. MPD is a coupling agent which reacts with the quinones produced at the site of substrate oxidation by PPO (van Loon, 1971; Harel et al., 1965). The color of the bands varied depending on the substrate used. Band development was complete in 1.5 hours and their position and intensity recorded. R_f values of the bands of all gels were calculated as

$$\frac{\text{distance of band migration}}{\text{distance of tracking dye migration}}$$

and averaged.

PPO Substrate Specificity

Several compounds with polyphenolic or monophenolic configurations were used for this study. 2.8 ml of catechol (10mM), pyrogallol (10mM), p-cresol (10mM), quinol (10mM), or chlorogenic acid (4.5mM) in 0.1M Na phosphate buffer (pH 6.3) was aerated and then rapidly mixed with 0.2 ml of a crude PPO preparation (see Electrophoresis). The temperature of the substrate solution was 25°C. The reaction was followed at 420 nm with a Beckman DB spectrophotometer with Photovolt recorder and the data for each substrate, with each PPO preparation, was recorded as activity relative to activity with catechol.

PPO pH Optima

Activity of the crude PPO preparations relative to pH was determined

as above by rapidly mixing 0.2 ml of crude PPO preparation with 2.8 ml of 10 mM catechol in 0.1 M citrate-0.2 M Na phosphate buffer over a pH range of 4.0 to 7.4. Activity at each pH was plotted as per cent of maximum activity attained.

Effect of Substrate Concentration

0.2 ml of crude PPO preparation from control peaches (see Electrophoresis) was rapidly mixed with 2.8 ml of catechol in 0.1 M Na phosphate buffer (pH 6.3) to final concentrations in the reaction mixture ranging from 9.3 mM to 28 mM, and PPO activity determined as above. The data were plotted as 1/substrate concentration (M) versus 1/initial velocity (v_o).

RESULTS AND DISCUSSION

Enzymatic Browning

Treated fruit had less browning at harvest than untreated fruit (Tables IV and V). Fruit treated 46 days after bloom had less browning than fruit treated 21 days after bloom ($P < .01$) regardless of the type of treatment.

Nakabayashi et al. (1963) correlated browning of peaches with polyphenol content, Grice et al. (1952) showed that the rate of browning of frozen peaches was influenced by both polyphenol and PPO contents of the fruit, while Guadagni et al. (1949) found initial browning tendency of peaches to be governed by original PPO activity but total amount of browning depended on polyphenol content.

The failure of "Early Amber" peaches to undergo enzymatic browning after early season applications of GA and ethephon was due to a reduction in PPO activity by over 90% in the treated fruit, with slight reductions in o-diphenol content (Knapp et al., 1970). Paulson (1973) attributed the reduction in browning of "Redhaven" peaches after a post-bloom application of GA to a reduction in available substrate. Sal'kova et al. (1977) found that apples treated with ethephon had lower levels of PPO, peroxidase (PRO), and ascorbic acid oxidase, and were more resistant to browning on cutting. GA applied to West Indian cherries was seen to cause a marked reduction in PPO and ascorbic acid oxidase activities (Srinivasan et al., 1973).

TABLE III ENZYMATIC BROWNING, O-DIPHENOL CONTENT, PPO ACTIVITY, FRESH WEIGHT, AND pH OF "REDHAVEN" PEACHES RECEIVING GROWTH REGULATOR TREATMENTS

Treatment	Application Time(days)	Tree No.	Browning ΔRd(29min)	o-diphenols mg catechol/g tissue	PPO Activity ΔAbs_{420} 0.1 ml^{-1} min^{-1}	Fresh Weight g/fruit	pH
Control	-	1	36.6	1.027	0.163	170.3	3.70
		2	33.6	1.253	0.160	148.4	3.80
		3	32.2	1.067	0.163	181.6	3.75
		4[ab]	36.2	1.840	0.183	177.6	3.70
Ethephon(75ppm)	21	1	33.6	1.507	0.135	164.6	3.85
		2[b]	32.1	1.053	0.280	201.7	3.70
		3[a]	31.0	1.347	0.145	191.4	3.80
		4	35.9	1.693	0.190	177.7	3.80
Ethephon(150ppm)	21	1	34.4	1.013	0.170	178.0	3.85
		2	33.7	1.600	0.145	150.3	3.80
		3[a]	34.4	1.000	0.150	151.3	3.85
		4[b]	32.8	0.840	0.230	154.2	3.80
Gibberellic Acid (100 ppm)	21	1	30.9	1.027	0.170	185.5	3.85
		2	34.5	1.507	0.178	189.2	3.75
		3[b]	32.6	0.987	0.175	198.5	3.85
		4[a]	35.3	1.533	0.155	162.1	3.60

Continued . . . . .

TABLE III (Continued)

Treatment	Application Time (days)	Tree No.	Browning ΔRd(29min)	o-diphenols mg catechol/g tissue	PPO Activity ΔAbs_{420} 0.1 ml^{-1} min^{-1}	Fresh Weight g/fruit	pH
Ethephon(75ppm)	46	1	31.6	1.640	0.165	141.9	3.75
		2[ab]	21.4	0.653	0.148	193.2	3.85
		3	27.4	0.747	0.125	158.9	3.90
		4	29.3	1.280	0.170	123.2	3.75
Ethephon(150ppm)	46	1	28.9	1.333	0.188	168.0	3.85
		2[ab]	22.1	0.840	0.138	232.4	3.80
		3	26.8	0.880	0.135	208.3	3.85
		4	29.4	1.667	0.133	185.2	3.75
Gibberellic Acid (100 ppm)	46	1	32.9	1.280	0.200	147.6	3.80
		2[a]	33.4	1.467	0.140	133.5	3.65
		3[b]	26.3	1.080	0.170	174.6	3.75
		4	36.2	1.600	0.255	160.2	3.70

[a]Trees chosen for PPO isozyme electrophoresis

[b]Trees chosen for TLC

TABLE IV

ANALYSIS OF VARIANCE OF BROWNING OF REDHAVEN PEACHES

Source of Variation	Degrees of Freedom	Mean Square	F-ratio
Treatment	6	40.30	**
Error	21	8.74	
Total	27		

**Denotes significance at $P < .01$

TABLE V

INDIVIDUAL DEGREES OF FREEDOM FOR THE EFFECTS OF TREATMENTS ON BROWNING

Contrast	Degrees of Freedom	Q^2	F-ratio
C/Tr	1	42.70	*
E/L	1	128.34	**
GA/Eth	1	32.34	n.s.
Lo/Hi	1	0.00	n.s.
B x C	1	36.75	n.s.
B x D	1	1.69	n.s.
Treatment	6	241.82	

**Denotes significance at $P < .01$

*Denotes significance at $P < .05$

Note:

$$Q^2 = \frac{(M_1T_1 + M_2T_2 + \ldots + M_kT_k)^2}{n(M_1^2 + M_2^2 \ldots + M_k^2)} = \frac{(\Sigma MT)^2}{n\Sigma M^2}$$

where M = Multipliers (from Table II)

Q^2 = An independent component of the treatment sum of squares

T = Treatment total

n = Number of observations per treatment

Analysis of variance of PPO and o-diphenol data from the present study revealed non-significant F-tests ($P > .05$). A significant F-test is not a pre-requisite for the partitioning of the treatment degrees of freedom (Li, 1964a) and the latter may show significance where the former does not. However, analysis of the individual degrees of freedom contrasts revealed no significant treatment effect.

PPO activity is pH dependent and control of browning by decreasing pH is well known (Eskin et al., 1971). Overall treatment effects on pH were not significant ($P > .05$) (Table VI). According to the individual degree of freedom contrasts, fruit treated with ethephon had higher pH values than fruit treated with GA ($P < .05$) (Table VII). Knapp et al. (1970) reported that neither GA nor ethephon treatment effected pH of "Early Amber" peaches.

Cell expansion of peaches (fresh weight growth) (Chalmers and van den Ende, 1975) has been well correlated with GA levels in the mesocarp (Jackson, 1968). Ethephon applied at stage I and II of peach growth has been known to result in increased fruit weight, presumably as a result of a thinning effect (Stembridge and Raff, 1973; Paulson, 1973). GA has also been evaluated for peach thinning (Corgan and Widmoyer, 1971; Edgerton, 1966). Chalmers et al. (1976) reported that exogenously applied GA and ethephon increased sink strength in developing peach fruits. Increased cell expansion could dilute the cell constituents involved in browning. Growth regulator treatment affected fruit weight ($P < .05$) (Table VIII). 150 ppm ethephon

TABLE VI

ANALYSIS OF VARIANCE OF pH OF REDHAVEN PEACHES

Source of Variation	Degrees of Freedom	Mean Square	F-ratio
Treatment	6	0.0062	n.s.
Error	21	0.0047	
Total	27		

n.s. Not significant at $P < .05$

TABLE VII

INDIVIDUAL DEGREES OF FREEDOM FOR THE EFFECTS OF TREATMENTS ON pH

Contrast	Degrees of Freedom	Q^2	F-ratio
C/Tr	1	8.57×10^{-3}	n.s.
E/L	1	4.17×10^{-4}	n.s.
GA/Eth	1	2.30×10^{-2}	*
Lo/Hi	1	1.41×10^{-3}	n.s.
B x C	1	2.56×10^{-3}	n.s.
B x D	1	1.41×10^{-3}	n.s.
Treatment	6	3.74×10^{-2}	

*Denotes significance at $P < .05$

treatment appeared to retard fruit growth if applied 21 days after bloom but enhanced fruit growth if applied 46 days after bloom (Table III and IX; Fig. 1). The reverse appeared to be true for the 75 ppm ethephon applications. The reasons for the differing effects on weight with ethephon concentration and application date are not clear. Cell division in peach fruit continues for about 30 days after pollination, after which growth is mostly due to cell enlargement (Nitsch, 1970; Jackson, 1968). The 21-day treatments were probably applied during the period of cell division, while the 46-day treatments were applied during the period of cell enlargement prior to the onset of stage II of "Redhaven" peach fruit growth (Looney, 1972).

Multiple Regression techniques (Le and Tenisci, 1977) were employed to identify the important experimental factors and measurements contributing to browning. Stepwise multiple regression of browning on o-diphenols, PPO, fruit weight, and pH showed that only o-diphenol content was a significant predictor of browning (Table X, column 1). The coefficient of multiple determination (R^2) of 0.32 indicates that there is appreciable variation in browning after pH, PPO, fruit weight, and o-diphenol data have been considered. This is similar to the results of Gajzago et al. (1976) who found that 30% of the variation in browning of apricots was explained by o-diphenol content, and 35% by a combined PPO and o-diphenol effect.

Qualitative variables can be analyzed by multiple regression through the use of dummy variable or contrast coding (Li, 1964b; Gujarati, 1970; Cohen, 1968). Dummy variables take account of the

TABLE VIII

ANALYSIS OF VARIANCE OF FRESH WEIGHT OF REDHAVEN PEACHES

Source of Variation	Degrees of Freedom	Mean Square	F-ratio
Treatment	6	1206	*
Error	21	410	
Total	27		

*Denotes significance at $P < .05$

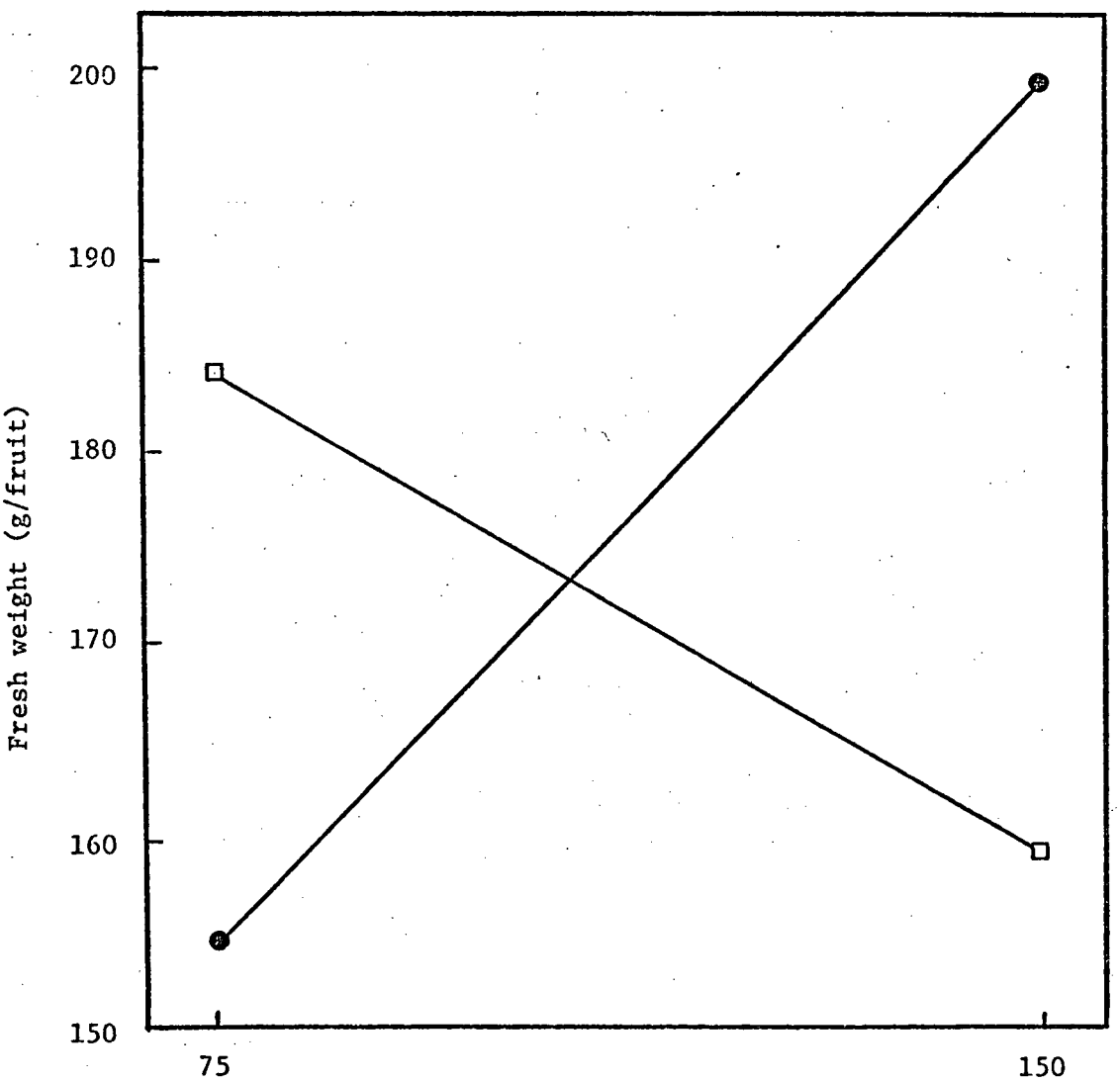
TABLE IX

INDIVIDUAL DEGREES OF FREEDOM FOR THE EFFECTS OF TREATMENTS ON FRESH WEIGHT

Contrast	Degrees of Freedom	Q^2	F-ratio
C/Tr	1	24.69	n.s.
E/L	1	250.26	n.s.
GA/Eth	1	126.43	n.s.
Lo/Hi	1	352.50	n.s.
B x C	1	1641.51	n.s.
B x D	1	4840.68	**
Treatment	6	7236.07	

**Denotes significance at $P < .01$

Figure 1. Effect of Ethephon Concentration and Application Date on Fresh Weight of Redhaven Peaches



● Fresh weight means of fruit treated with ethephon 21 days after bloom

□ Fresh weight means of fruit treated with ethephon 46 days after bloom

TABLE X

STEPWISE MULTIPLE REGRESSION ON BROWNING

Independent Variable	Regression coefficient 1	2	3	4	5
C/Tr	-	-0.504*	-0.447*	-0.439*	-0.452**
E/L	-	-2.313**	-2.146**	-2.364**	-2.205**
GA/Eth	-	n.s.	n.s.	n.s.	n.s.
Lo/Hi	-	n.s.	n.s.	n.s.	n.s.
B x C	-	n.s.	n.s.	n.s.	n.s.
B x D	-	n.s.	n.s.	n.s.	n.s.
o-Diphenol	6.965**	-	6.243**	4.791**	4.623**
PPO	n.s.	-	n.s.	-	24.879*
Weight	n.s.	-	-	-0.056**	-0.055**
pH	n.s.	-	-	-	n.s.
Constant	22.978	31.625	23.874	35.231	31.105
† Sy	3.319	3.189	2.449	2.103	1.915
††R^2	0.326	0.402	0.662	0.761	0.810

**Significant at $P < .01$

*Significant at $P < .05$

n.s. not significant ($P > .05$)

† Standard error of estimate

††Coefficient of Multiple Determination

separate deterministic effects of the treatments on the response, in addition to the variation that may occur due to other variables. The treatment contrasts and multipliers of Tables I and II were used for this purpose.

When treatment contrasts alone were considered as potential independent variables in the regression on browning, only C/Tr and E/L were significant (Table X, column 2). The physical meaning of the negative coefficients of these variables is that treated fruit brown less than control fruit, and fruit treated 46 days after bloom (late treatment) brown less than fruit treated 21 days after bloom (early treatment). The R^2 value of 0.402 indicates that 40.2% of the variation in browning is accounted for by treatment contrasts alone.

The result of adding PPO and o-diphenol content as potential independent variables along with the treatment contrasts is shown in Table X, column 3. Only o-diphenol, C/Tr, and E/L were significant. The R^2 value of 0.662 represents a further increase in explanation of variation in browning of 26% by inclusion of o-diphenol data.

The addition of fruit weight as a potential independent variable to treatment contrasts and o-diphenol content yields a R^2 value of 0.761 (Table X, column 4), a further increase in explanation of variation in browning of 9.9%. When all potential independent variables are included in the regression, C/Tr, E/L, o-diphenol, PPO, and weight are seen to be significant (Table X, column 5). Although the variable PPO was previously not significant, in stepwise multiple regression, the significance of a particular variable depends on the current regression equation (Le and Tenisci, 1977). pH was found to be non-significant.

The R^2 value of 0.810 represents a 40.8% increase in explanation of variation in browning by inclusion of o-diphenol, PPO, and fruit weight data with the treatment contrasts in the regression equation over treatment contrasts alone. Other possible contributing factors in explanation of browning may be ascorbic acid content of the ripe fruit (Weaver and Charley, 1974; Douglas et al., 1977) and type of o-diphenol (Luh and Phithakpol, 1972).

The factors yielding decreased browning of "Redhaven" peaches were growth regulator treatment, treatment 46 days after bloom, decreases in PPO and o-diphenol content of the fruit, and increases in fruit weight. These factors accounted for 81% of the variation in browning. The reason for decreased browning with late but not early treatment application is unknown, but may be related to the stage of fruit development. Environmental factors may also have been important. The spring of 1975 was particularly cold and wet and a light drizzle of rain fell within hours of the 21-day application. Whether or not the treatments were washed off the trees by the rain was unknown, but they were not reapplied. The breakdown of ethephon to ethylene as well as uptake of chemicals from a spray application have been seen to be temperature dependent (Lougheed and Franklin, 1972; Leopold and Kriedemann, 1975).

Thin Layer Chromatography

Knapp et al. (1970) reported slight qualitative differences in the phenolic compounds of "Early Amber" peaches that had been sprayed with GA or ethephon and had decreased enzymatic browning. Ethylene

has been found to induce the de novo synthesis of phenolic compounds not normally present in carrot roots as well as increase the levels of pre-existing phenols, particularly isochlorogenic acid (Sarkar and Ton Phan, 1974). In addition, it has been shown that certain phenolic compounds such as ferulic acid and coumaric acid inhibit PPO (Walker and Wilson, 1975), while others such as phloroglucinol and resorcinol are competitive inhibitors of PPO but paradoxically are able to increase the rate of browning by reacting with the quinones produced by the enzymatic oxidation of o-diphenolic substrates (Wong et al., 1971b). To determine whether treatment with GA and ethephon had altered the qualitative distribution of "Redhaven" peach phenolic compounds or induced the synthesis of new phenolic compounds, extracts of peach tissue were separated by two-dimentional chromatography on cellulose thin layers. The spots were revealed by ultra-violet light with and without ammonia (Seikel, 1962) and by spraying separate plates with different location reagents (Table XI). Those compounds seen to be PPO substrates were tentatively identified by their mobilities and behavior with the location reagents.

The first plate of each treatment was sprayed with Folin-Ciocalteau reagent. The hydroxyl groups of the phenolic compounds reduce the reagent to a blue color (Ribereau-Gayon, 1972) yielding light-blue to dark-blue spots on a light blue background. Twenty compounds were seen to react with this reagent (Fig. 2). Figures 2-6 were drawn from a representative plate of each reagent.

TABLE XI. COLOR CHARACTERISTICS[a] AND R_f VALUES OF POLYPHENOLIC COMPOUNDS EXTRACTED FROM "REDHAVEN" PEACHES[bc].

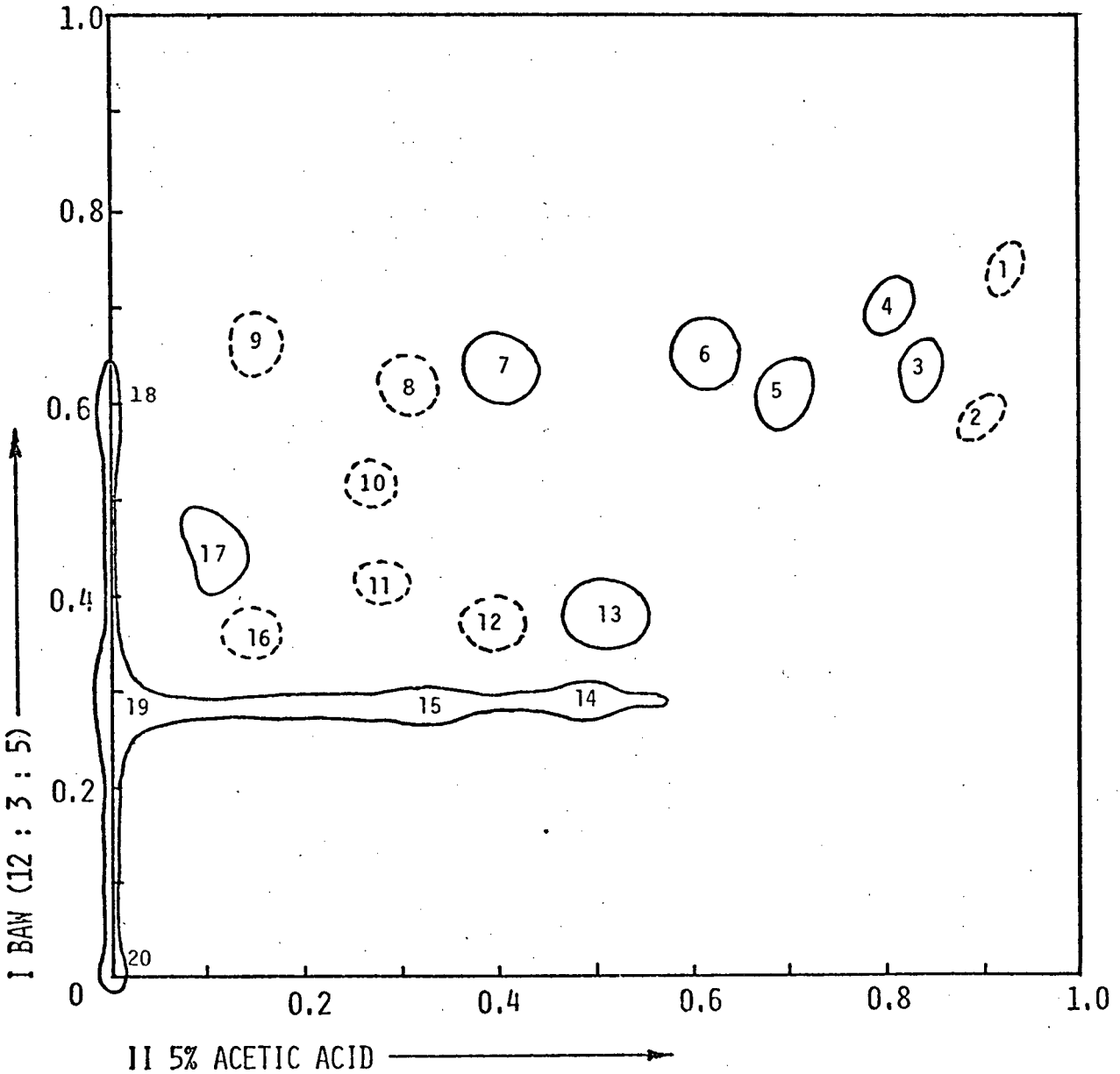
Spot No.	R_f BAW	R_f HOAc	Location Reagent Folin	DPNA	Vanillin	Molybdate	PPO
1	0.71	0.91	fB	slT	C	C	C
2	0.57	0.89	fB	slT	C	C	C
3	0.61	0.83	B	T	C	lY	fY
4	0.67	0.79	B	T	C	lY	fY
5	0.59	0.67	B	T	C	Y	Y
6	0.65	0.60	B	T	C	Y	Y
7	0.62	0.37	B	OT	OP	lY	YBr
8	0.61	0.27	fB	fYBr	C	fY	C
9	0.66	0.15	fB	fYBr	C	fY	C
10	0.53	0.24	fB	fYBr	C	C	C
11	0.43	0.26	fB	fYBr	fP	C	C
12	0.38	0.37	lB	YBr	lP	fY	C
13	0.38	0.47	B	YBr	OP	lY	Y
14	0.30	0.46	B	YBr	fP	C	C
15	0.30	0.30	B	YBr	fP	B	C
16	0.36	0.15	fB	fYBr	fP	C	C
17	0.44	0.10	B	YBr	P	C	C
18	0.58	0.00	B	fYBr	C	C	C
19	0.30	0.00	B	YBr	P	YT	fYBr
20	0.00	0.00	B	YBr	P	Yt	fYBr
21	0.53	0.29	C	fYBr	fP	C	C

a. B=Blue, Br=Brown, C=Colorless, O=Orange, P=Pink, T=Tan, Y=Yellow, f=faint, l=light, sl=slight

b. All spots were colorless under visible light except spot 17 which appeared pink.

c. Mobilities of all spots are average values.

Figure 2. Peach polyphenols visualized with Folin-Cioucalteau Reagent.



Broken lines indicate lighter intensity.

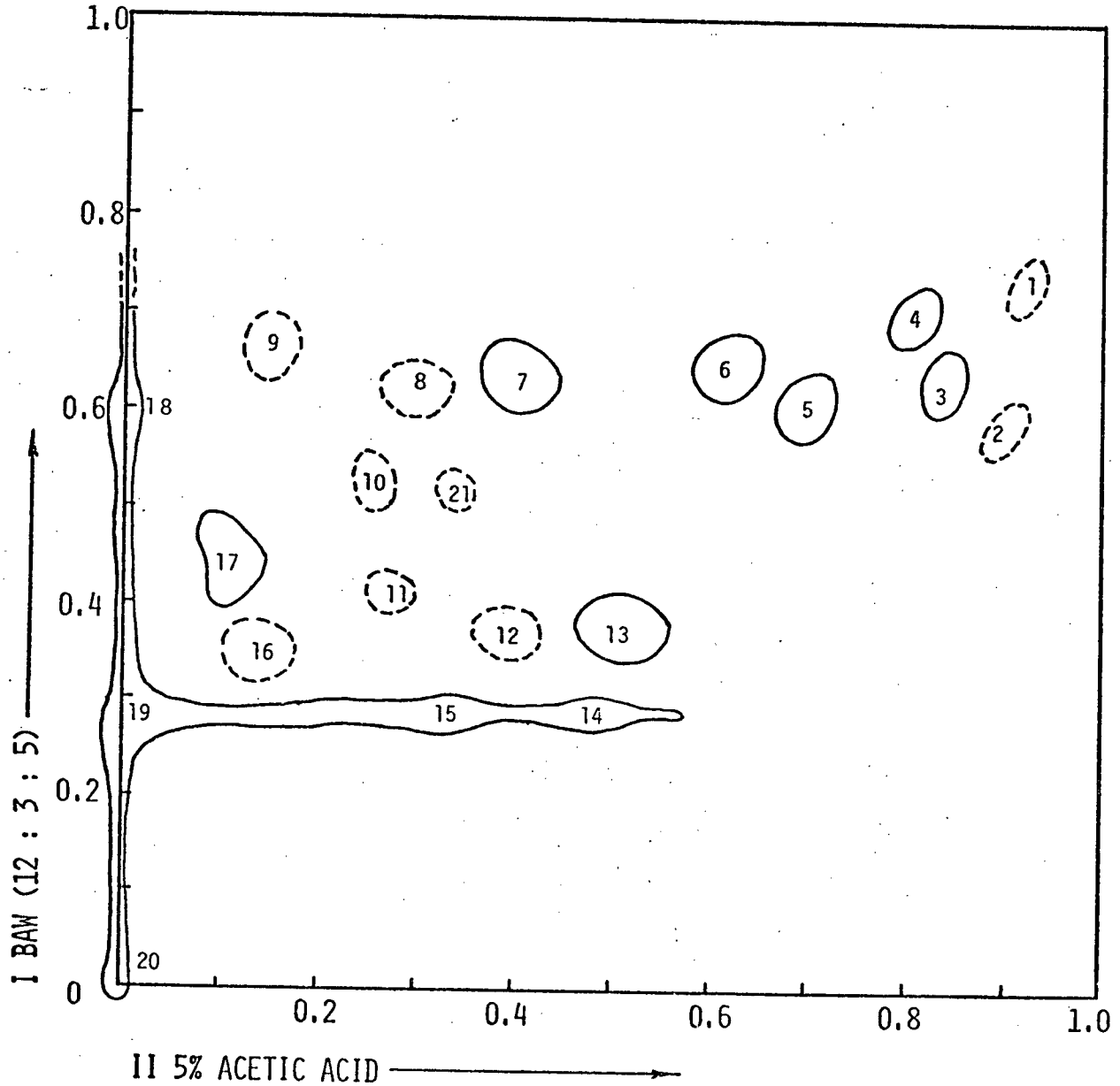
A second plate was sprayed with DPNA reagent which undergoes a coupling reaction with phenolic compounds giving azo dyes (Ribereau-Gayon, 1972) the colors ranging from tan to orange-brown depending on the nature of the phenol. DPNA rea gent revealed the 20 spots seen with the Folin-Ciocalteau reagent, plus a 21-st spot which was very light orange-brown (Fig. 3). The absence of this spot on the plate sprayed with Folin-Ciocalteau reagent may be due to the light blue background color obscuring the spot or differing sensitivity of the compound to the reagents.

A third plate was sprayed with vanillin-HCl reagent which reacts with the "A" ring of catechins and leucoanthocyanidins (Ribereau-Gayon, 1972) yielding spots which are pink to orange-pink in color. Eleven of the spots reacted with this reagent including spot 21, revealed with DPNA reagent (Fig. 4).

To visualize potential browning substrates, a fourth plate was sprayed with 5% Na molybdate which gives a yellow color with o-dihydroxy and vicinal trihydroxy phenolic compounds. Six spots reacted strongly with this reagent and 7 spots were lighter in color (Fig. 5).

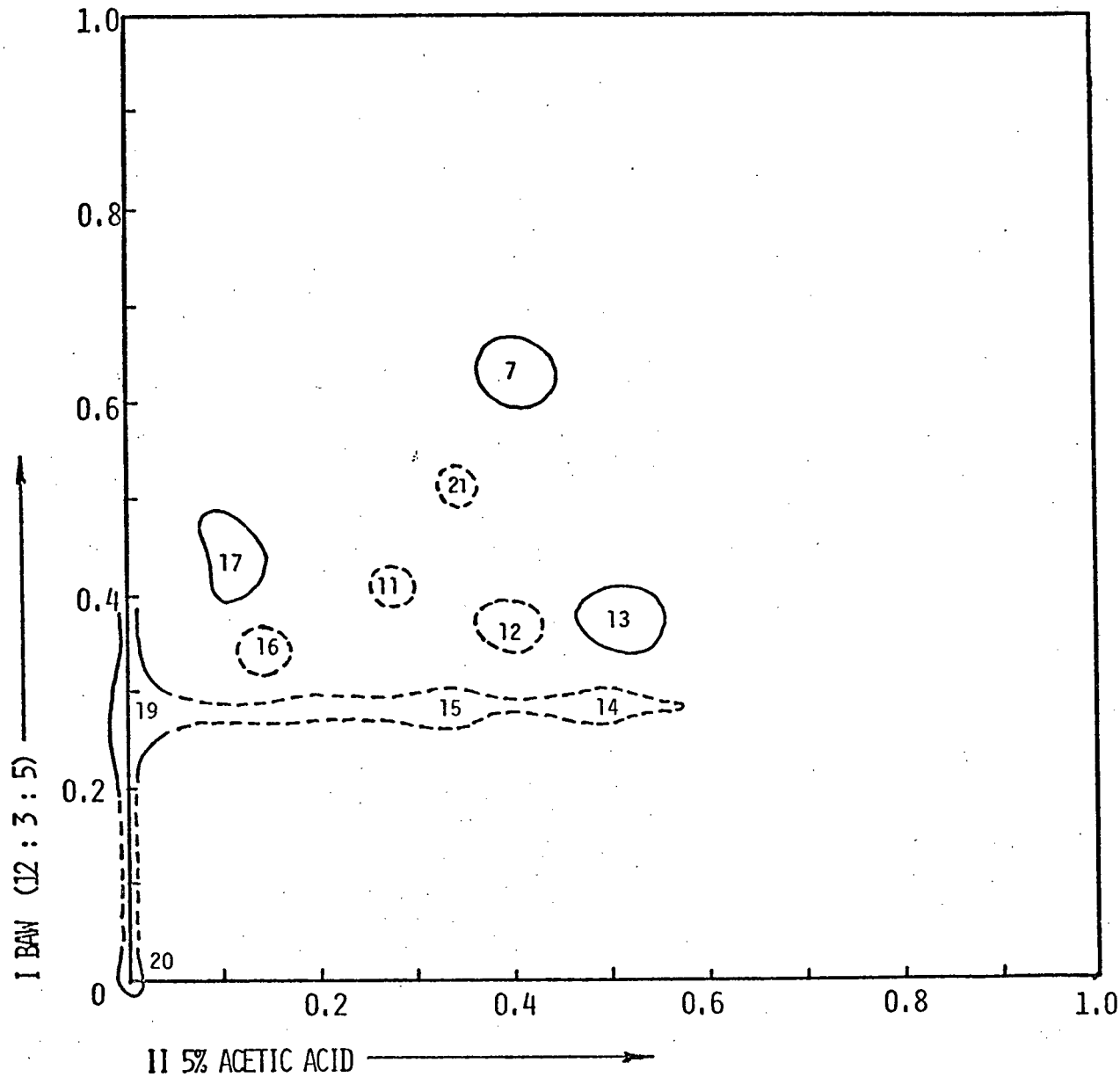
A fifth plate sprayed with crude PPO prepared from control fruit showed that 8 o-diphenolic compounds were oxidized by the enzyme, giving spots ranging in color from very light yellow-brown to intense orange-brown (Fig. 6). The light spots seen with Na molybdate reagent but not seen to be oxidized by PPO were either poor browning substrates or too low in concentration to show a detectable reaction with PPO. No differences were observed in the qualitative distribution of phenolic compounds due to growth regulator treatment.

Figure 3. Peach polyphenols visualized with diazotized p-nitroaniline reagent.



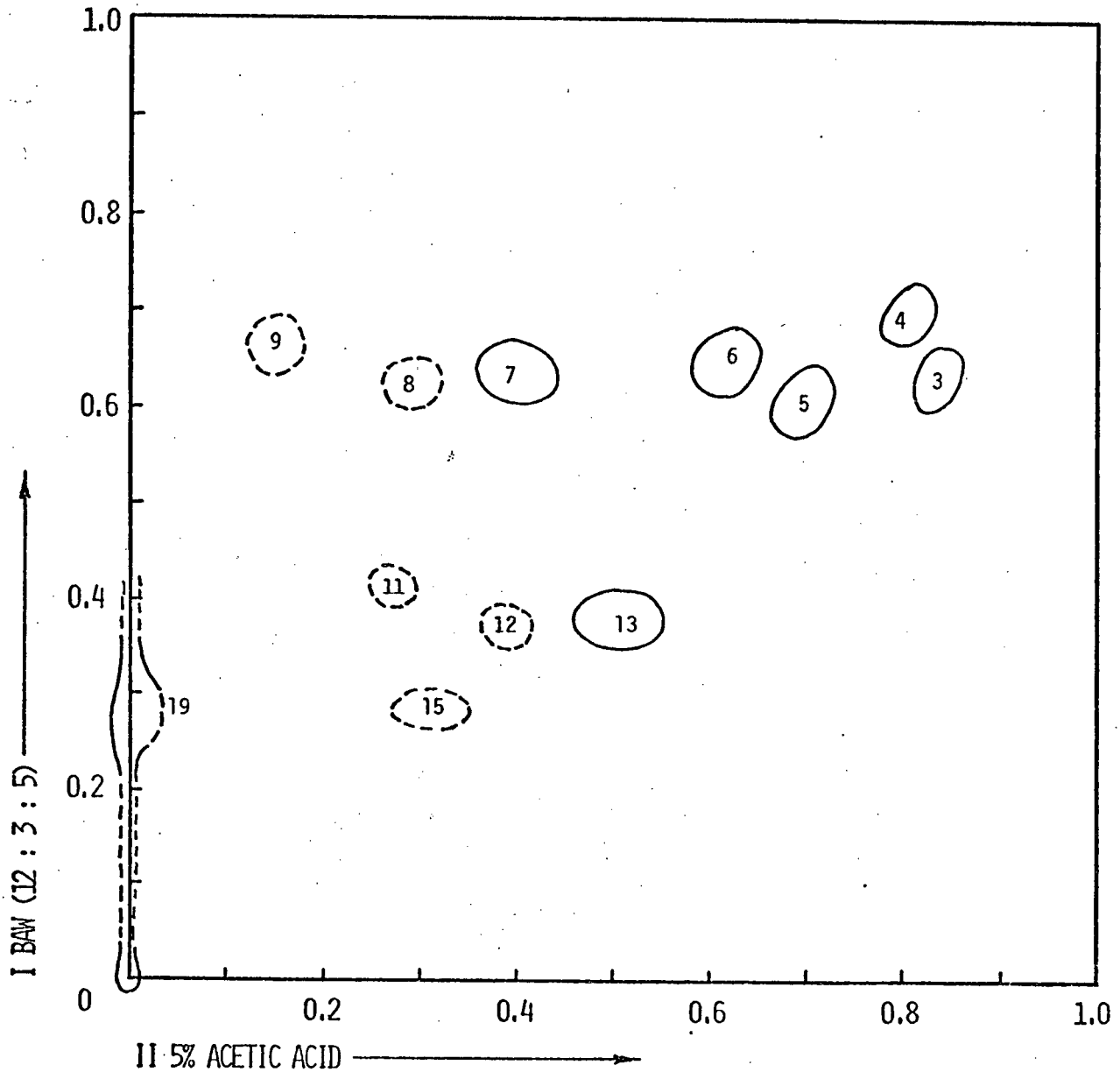
Broken lines indicate lighter intensity.

Figure 4. Peach polyphenols visualized with Vanillin-HCl reagent.



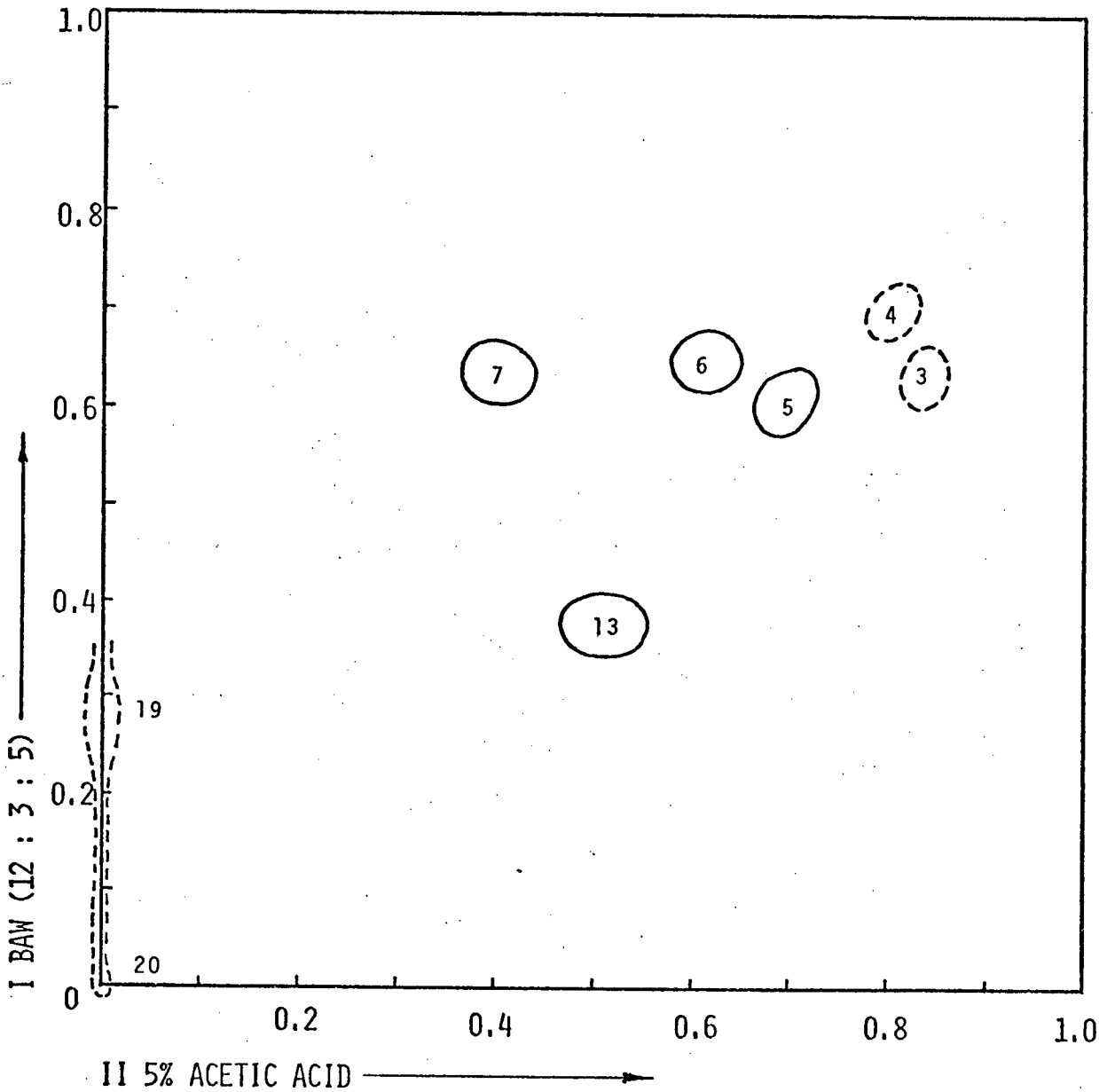
Broken lines indicate lighter intensity.

Figure 5. Peach polyphenols visualized with Na molybdate reagent.



Broken lines indicate lighter intensity.

Figure 6. Peach polyphenols visualized with polyphenoloxidase.



Broken lines indicate lighter intensity.

No attempt was made to conclusively identify each of the phenolic compounds separated, as the primary objective was to determine whether growth regulator treatment qualitatively affected the distribution of peach phenolics, but a tentative identification was made of the PPO reactive compounds.

Spots 3, 4, 5 and 6 were identified as chlorogenic acid isomers. They displayed a strong blue fluorescence under UV light which changed to a blue-green fluorescence after fuming with ammonia vapor (Schaller and von Elbe, 1970). They did not react with vanillin-HCl reagent and gave a tan color with DPNA reagent. Spots 4 and 6 are probably the cis and trans isomers respectively of chlorogenic acid as they had similar mobilities to the authentic compounds (Table XII). Spots 3 and 5 are possibly the cis and trans isomers respectively of neochlorogenic acid which have R_f values similar to chlorogenic acid in weak acid systems but lower R_f values in butanol systems than chlorogenic acid (Schaller and von Elbe, 1970). The cis isomers of chlorogenic acid and neochlorogenic acid have higher R_f values than the trans isomers in dilute acid systems on cellulose (Williams, 1955; Roberts, 1956). The trans isomers are the more stable (Walker, 1975) and showed greater intensity of reaction with the location reagents. Chlorogenic acid is known to be a good PPO substrate and has been shown to be present in both freestone (Craft, 1961) and clingstone (Luh et al., 1967) peaches. Neochlorogenic acid has been identified in peaches by Corse (1953).

TABLE XII. R_f VALUES OF AUTHENTIC POLYPHENOLIC COMPOUNDS.

Compound	R_f	
	BAW	5% HOAc
Caffeic Acid	0.78	0.24
Chlorogenic Acid - cis	0.67	0.81
- trans	0.67	0.60
l-Epicatechin	0.58	0.34

Spot 7 was tentatively identified as catechin. It was colorless under UV light before fuming with ammonia but turned dark after fuming. It reacted with DPNA reagent giving the orange-tan color characteristic of catechin and also the characteristic orange-pink to reddish-pink color with vanillin-HCl reagent (Swain and Hillis, 1959; Luh et al., 1967). Catechin has R_f values slightly greater than epicatechin in both BAW and acetic acid systems on cellulose (El-Sayed and Luh, 1965) as does spot 7 (Tables 13 and 14). Siegelman (1955) showed catechin to be a major browning substrate in pear, and it has been identified as a browning substrate in peach (Craft, 1961; Luh et al., 1967).

Spots 13, 19 and 20 resemble leucoanthocyanidins in mobility (Craft, 1961; Luh et al., 1967). They were colorless under UV light with and without ammonia vapor and appeared orange-pink and pink when sprayed with vanillin-HCl reagent, a characteristic of leucoanthocyanidins (Luh et al., 1967). These compounds have been shown to be browning substrates in peaches (Craft, 1961; Luh et al., 1967; Fel'dman and Kostinskaya, 1970) Caffeic acid was not detected, which is in agreement with the findings of Craft (1961) for "Elberta" peaches, although it was present in "Halford" peaches (Luh et al., 1967).

Only one spot was seen under visible light, spot 17, which was pink and thought to be anthocyanin. Hsia et al. (1965) reported that the major anthocyanin in peaches is cyanidin-3-monoglucoside.

The principal polyphenolic compounds in "Redhaven" peaches of the present study oxidized by PPO are tentatively identified as chlorogenic acid isomers, a compound with the characteristics of catechin, and

leucoanthocyanidins. This is in agreement with the results of Craft (1961), Luh et al. (1967) and Fel'dman and Kostinskaya (1970).

The color and intensity of the spots produced with PPO on the chromatograms do not necessarily reflect the true importance of each compound in the browning reaction of the whole fruit. The final color of enzymatic browning is largely the result of non-enzymatic secondary reactions by the quinones after initial oxidation (Mathew and Parbia, 1971) under conditions which are not duplicated on the chromatograms. Substrate specificity of PPO is also important. Fel'dman and Kostinskaya (1970) reported that the amount of oxidizable polyphenols in peach varied with the cultivar and type of polyphenol. Browning resulted in a decrease in catechins of 70-100%, leucoanthocyanidins 33-87% and chlorogenic acids 32-40%, depending on cultivar. Craft (1961) found that 75% of the total phenolic compounds and 80% of the leucoanthocyanins in "Elberta" peaches were no longer detected after browning and presumably oxidized. No attempt was made to quantitate individual o-diphenols in the present study, either before or after oxidation.

No qualitative changes in peach polyphenols with maturity has been reported except the appearance of anthocyanin with ripening (Craft, 1961; Van Blaricom and Senn, 1967). Ethylene has been shown to stimulate anthocyanin biosynthesis (Craker, 1975) possibly due to stimulation of the enzyme phenylalanine ammonia lyase (PAL) (Camm and Towers, 1973), thought to be a controlling enzyme in the shikimic acid pathway of phenol biosynthesis (Walker, 1975). GA has also been shown to stimulate PAL activity (Camm and Towers, 1973) but Proebsting et al. (1973) found that

GA treatment decreased cherry anthocyanins. Aoki et al. (1971) found PAL to be present only in the red, anthocyanin containing portions, of mature peaches. Buchanan et al. (1969) noted that ethephon treated peaches had more red color than non-treated or GA treated fruit. Anthocyanin is a poor browning substrate but can take part in coupled oxidations with o-quinones (Mathew and Parbia, 1971) being decolorized in the process. The reaction is similar to the coupled oxidation of ascorbic acid, used in controlling browning (Eskin et al., 1971). There have been no reports on the effect of anthocyanin concentration on rate of enzymatic browning however.

Polyphenoloxidase Isozymes

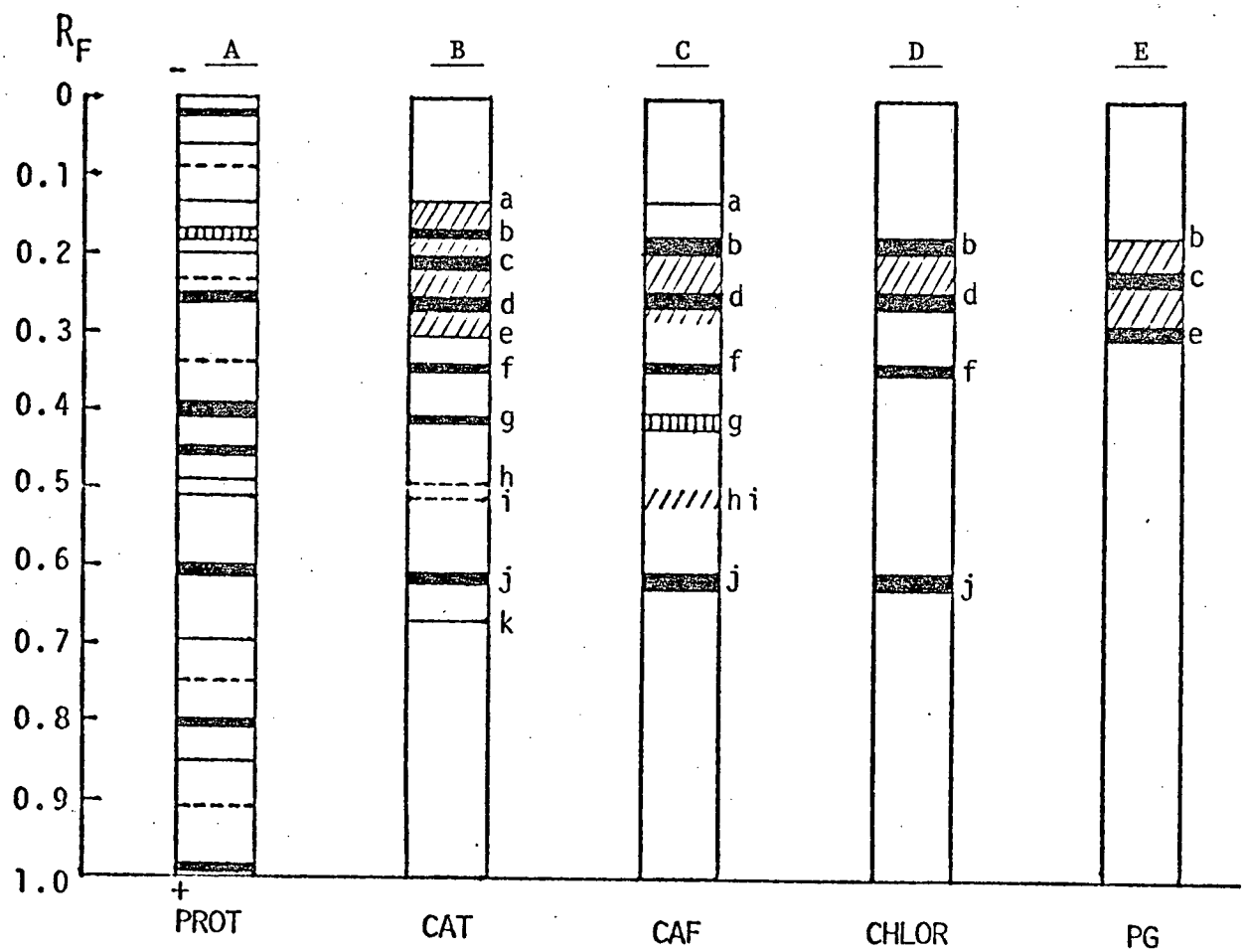
The effects of plant growth regulators on isozyme formation are many and varied. No reports have been found on their effects on PPO isozymes in particular, but there has been numerous mention made of alterations in peroxidase (PRO) isozymes. Although PRO contains an iron porphyrin as its prosthetic group and PPO contains copper, their isozymes have sometimes been found to be closely associated (Sheen and Calvert, 1969; Srivastava and van Huystee, 1973). The nature of the association is unknown. Galston et al. (1968) reported induction of a PRO isozyme in tobacco tissue culture by IAA. Imaseki et al. (1968) found that ethylene stimulated several PRO isozymes but not others, indicating that ethylene may preferentially affect the synthesis of particular isozymes. Bireka et al. (1976) however, reported no changes in the qualitative isozyme spectrum of PRO from sweet potato tissue treated with ethylene. Lee (1971) found that GA caused increases in three IAA oxidase isozymes in tobacco callus culture, but the effectiveness of GA was dependent on IAA and kinetin. IAA oxidase activity has often been attributed to peroxidases (Shinshi and Noguchi, 1975). Applications of GA to dwarf corn and pea produced no qualitative change in PRO isozyme patterns but quantitatively increased the level of certain isozymes and decreased that of others (Lee, 1972). Inhibition of PRO activity in sugar cane stem tissue by GA produced no change in the isozyme banding pattern (Glasziou et al., 1968).

It was decided to examine the PPO isozymes in treated and untreated "Redhaven" peaches to determine whether treatment with GA and ethephon affects isozyme number and/or substrate specificity.

Preliminary attempts at separating crude PPO preparation by electrophoresis were unsatisfactory due to low protein concentration as well as poor resolution and artifacts probably caused by high pectin content (Frenkel et al., 1969). Removal of pectic materials by precipitation with calcium chloride and subsequent concentration by ultrafiltration allowed satisfactory separation. Results are shown in Figure 7.

Figure 7A shows the banding pattern of the gels when stained for protein with coomassie blue. Visual observations and densitometric scans of the gels showed very similar banding patterns from control and treated fruit. Incubation of gels in catechol, the simplest o-diphenol, revealed up to 11 brown bands after 1.5 hr (Fig. 7B). Bands b, c, d, and j were visible after approximately 5 min. The region from a to e had a dark brown background, as indicated by shading. All bands except band k were visible on gels from each treatment. Band k was only visible on gels from peaches treated 21 days after bloom with both GA and ethephon (75 and 150 ppm) and appeared very slowly. Comparison of the mobilities of the bands on the catechol gels with those on the protein gels showed good agreement. The intensity of staining is not the same, however, as the bands on the catechol gels result from the enzymatic oxidation of a substrate while those on the protein gels resulted from direct staining with coomassie blue.

Figure 7. Reactions of polyphenoloxidase isozymes with o-diphenolic substrates.



PROT = protein, CAT = catechol, CAF = caffeic acid, CHLOR = chlorogenic acid, PG = pyrogallol

Using caffeic acid as substrate revealed a banding pattern similar to that with catechol (Fig. 7C). Bands b, d, and j formed within 5 min and the remainder appeared over the course of the 1.5 hr incubation. Immediately noticeable is the disappearance of bands c, e, and k. The background in the region from b to d extended slightly below band d suggesting the presence of another isozyme, but no discrete band was visible. Band g was more diffuse with caffeic acid than with catechol and bands h and i, seen as discrete bands on the catechol gels appeared as one light band with caffeic acid. Band j was more intense with caffeic acid than catechol. None of the growth regulator treatments affected the banding pattern with caffeic acid.

Chlorogenic acid, a natural browning substrate in peaches, proved to be unsatisfactory for staining PPO isozymes as the bands formed were very water soluble and quickly diffused, making it difficult to detect minor bands. A similar effect was noted by Van Loon (1971). A green background quickly formed thus increasing the difficulty. Bands b, d, f, and j were visible with chlorogenic acid (Fig. 7D).

Pyrogallol, a vicinal-trihydroxyphenol, was oxidized by only 3 isozymes (Fig. 7E). The mobilities of the bands were similar to bands b, c and e of the catechol gels, but the intensities differed. PPO from different growth regulator treatments showed identical banding patterns with pyrogallol except ethephon (150 ppm, 46-day treatment), in which band b wasn't apparent.

Electrophoresis of different amounts of PPO preparation as well as incubation of the gels in differing substrate concentrations at different pH's may give different results (Kahn, 1976; Constantinides and Bedford, 1967). Method of PPO extraction has also been shown to influence

isozyme pattern (Benjamin and Montgomery, 1973; Kahn, 1977).

The lack of knowledge of PPO function in the cell makes it difficult to determine the functions of the PPO isozymes. A common regulatory feature of branched biosynthetic pathways is the presence of isozymes with differing susceptibilities to end-product control. Constantinides and Bedford (1967) found that PPO isozymes from mushroom had differing susceptibilities to high substrate concentration, suggesting a kind of defence mechanism against product inhibition. In addition, Wong et al. (1971a) noted differences in sensitivities of peach PPO isozymes to heat and chemical inhibitors. It has been suggested that multiple forms of an enzyme are needed to catalyze the same reaction but under different metabolic conditions, cellular locations, and stages of differentiation in order to maximize biological capacity (Markert, 1974). On the other hand, some isozymes may be merely evolutionary accidents with no pressure of natural selection favoring or opposing their existence (Moss and Butterworth, 1974). The differing substrate specificities of peach PPO isozymes may therefore represent alterations in structure which modify the substrate specificities without impairing physiological effectiveness, or they may indicate specific metabolic roles for the isozymes in the cell. Markert (1974) suggests that the differences in charge distribution over the surface of the enzyme molecules, which makes electrophoretic separation possible, probably affects the topographic location of the molecule within the cell. This view is interesting as PPO has been shown to exist in forms soluble as well as bound to mitochondria and chloroplasts (Harel et al., 1965; Sato and Hasegawa, 1976).

The significance of the appearance of band k with catechol in those peaches treated 21 days post-bloom, and the disappearance of band b with pyrogallol in peaches treated with 150 ppm ethephon 46 days post-bloom is presently unknown. As band k was faint and formed slowly with catechol only, it probably isn't important in the overall browning reaction. The disappearance of band b, however, may indicate a modification of the activity of this isozyme with certain substrates, as it was seen to oxidize catechol and caffeic acid quite readily. If specific activity of this isozyme with pyrogallol was decreased, a greater amount of PPO preparation added to the gel may cause this band to appear. Band b on the other gels stained with pyrogallol were quite light. It would be necessary to isolate each isozyme and determine their separate kinetic properties with naturally occurring substrates to gain a better understanding of their importance in browning.

PPO Substrate Specificity

The relative activity of crude PPO preparations from control, GA (100 ppm), and ethephon (75 and 150 ppm) treated peaches (46 days after bloom) with various phenolic compounds is shown in Table XIII.

It is apparent that treatment with GA and ethephon 46 days after bloom resulted in alterations in the relative activities of the crude PPO preparations with o-diphenolic substrates. The significance of these alterations is not clear; rate and amount of browning may be affected, but it would be necessary to use naturally occurring peach o-diphenols as substrates to gain a better understanding.

Although caffeic acid was oxidized by many isozymes, the relative PPO activities with this substrate are low. Pyrogallol, on the other hand, was only oxidized by 3 isozymes yet relative activity with this substrate is high. The disappearance of isozyme b with pyrogallol may be related to the low activity of PPO from ethephon (150 ppm) treated peaches toward this substrate.

None of the PPO preparations had activity with p-cresol or quinol, even after the addition of a small amount of catechol (Whitaker, 1972). Luh and Phithakpol (1972) found PRO from "Halford" peaches to be active with o-diphenols only, but Reyes and Luh (1960) and Harel et al. (1970) found slight PPO activity with quinol. Harel and Mayer (1970) attributed this activity to a single isozyme.

TABLE XIII. RELATIVE ACTIVITY OF PPO FROM TREATED AND UNTREATED "REDHAVEN" PEACHES WITH PHENOLIC COMPOUNDS AT pH 6.3.

			PPO Source			
					Ethephon	
Substrate	Configuration	Concentration (mM)	Control	GA (late)	75ppm (late)	150ppm (late)
Catechol	o-diphenol	9.3	100	100	100	100
Pyrogallol	o-diphenol	9.3	121	124	102	94
Chlorogenic acid	o-diphenol	4.2	59	31	33	64
Caffeic acid	o-diphenol	9.3	17	14	13	18
p-Cresol	monophenol	9.3	0	0	0	0
Quinol	p-diphenol	9.3	0	0	0	0

PPO pH Optima

The effect of pH on activity of the PPO preparations just discussed is shown in Figure 8. Two pH optima were seen with PPO from each source; pH 4.4 and 6.2 for PPO from control and GA treated peaches, and pH 4.4 and 6.6 for PPO from ethephon treated peaches (both 75 and 150 ppm). The reason for the shift in pH optimum (from pH 6.2 to 6.6) with ethephon treatment is unknown. Jen and Kahler (1974) reported a shift in pH optima of "Redhaven" peach PPO with ripening from a single optimum at pH 6.2 to double optima at pH 6.0 and 6.5. Although the peaches in the present study were harvested at the same maturity, the effect of ethephon in advancing peach maturity may have also accelerated ripening after harvest over control and GA treated fruit.

The decrease in relative activity at pH 4.4 in the treated peaches may indicate a lower PPO activity with natural substrates at the pH of the peach slurries, leading to decreased browning. No reports have been found of peach PPO with a pH optimum near 4.4.

Effect of Substrate Concentration

The effect of varying catechol concentration on activity of a crude PPO preparation from control peaches was studied. The Michaelis constant (Km) was determined by least squares treatment of the straight line obtained by plotting 1/substrate conc., versus $1/v_o$ (Lineweaver and

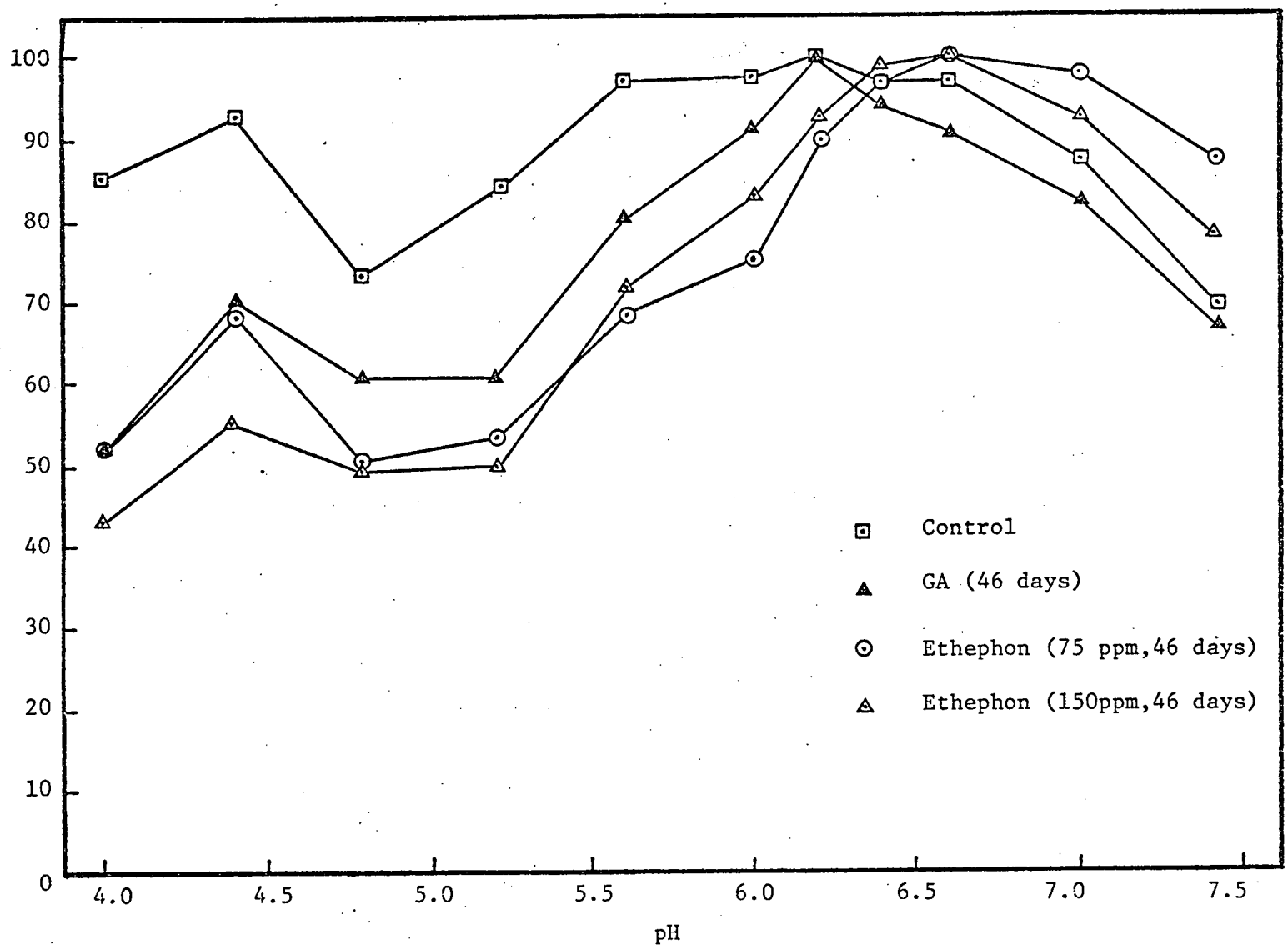


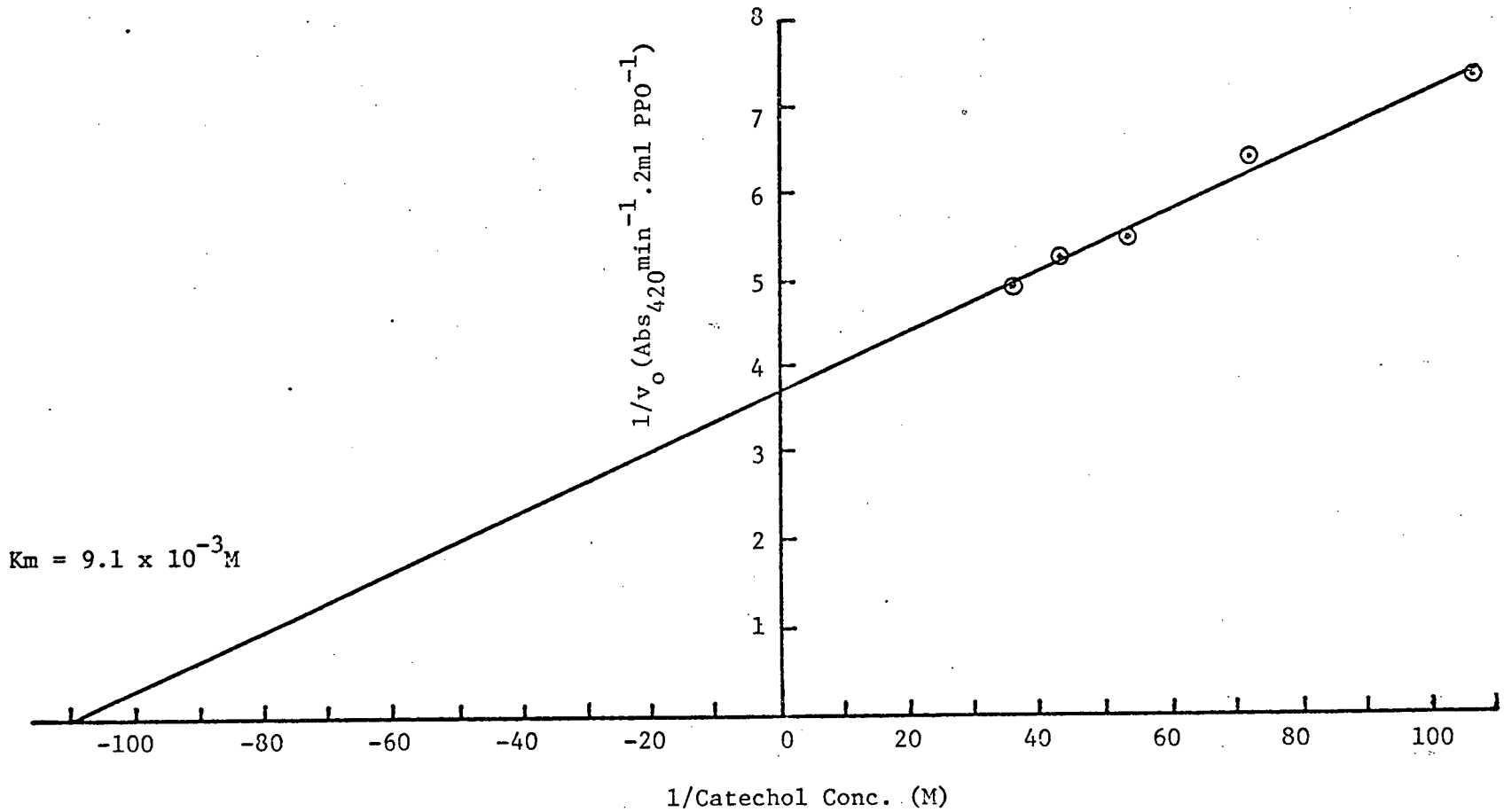
Figure 8. Effect of pH on Polyphenoloxidase Activity from Untreated and Treated (46-day application) "Redhaven" Peaches.

Burke, 1934) (Figure 9), and was found to be 9.1×10^{-3}M (catechol) at pH 6.3, and 25°C. The Km value is a measure of the affinity of the enzyme for the substrate and represents the substrate concentration when v_o is half of the maximum velocity of the enzyme. Smaller Km values represent greater affinity for the substrate.

The Km value is one of the characteristics of an enzyme; similar Km values under similar conditions indicate similar enzyme characteristics (Jen and Kahler, 1974). Km values for peach PPO from different sources have been reported to be: 15 mM catechol for "Halford" peaches (Luh and Phithakpol, 1972), 29 mM catechol for "Redhaven" peaches (Jen and Kahler, 1974), and 120 mM for "Elberta" peaches (Reyes and Luh, 1960). Wong et al. (1971a) reported that peach PPO isozymes have differing Km values.

The differing characteristics of "Redhaven" peach PPO of the present study compared to that of Jen and Kahler (1974) may indicate differences in area, growing conditions and rootstalk.

Figure 9. Double Reciprocal Plot of Crude Polyphenoloxidase from Untreated "Redhaven" Peaches.



SUMMARY AND CONCLUSIONS

Redhaven peaches treated with gibberellic acid (100 ppm) or ethephon (75 or 150 ppm), 21 or 46 days after full bloom, were evaluated for enzymatic browning in the ripe fruit. Treated fruit had less browning than untreated fruit, and fruit treated 46 days after bloom had less browning than fruit treated 21 days after bloom. Fruit pH and fresh weight were affected by treatment, but not o-diphenol content or PPO activity. Stepwise multiple regression revealed that 81% of the variation in browning was explained by differences in treatment, treatment application time, o-diphenol content, PPO activity, and fresh weight. Unlike previous reports, the reduction in browning observed in the present study could not be attributed to any single factor.

Qualitative analysis of "Redhaven" peach polyphenol compounds by 2-dimentional thin layer chromatography showed the presence of 21 spots on the TLC plates, eight of which were oxidized by a crude PPO preparation from control peaches. These were tentatively identified as 4 chlorogenic acid isomers, a compound with properties similar to catechin, and 3 leucoanthocyanidin-like compounds. There were no differences observed between treatments in qualitative distribution of phenolic compounds, ruling out the possibility of a treatment induced phenolic PPO inhibitor or the disappearance of a PPO substrate in low browning peaches. Possible quantitative changes in amount of each PPO substrate were not investigated, nor the importance of each type of PPO substrate in the browning reaction.

Analysis of partially purified peach PPO preparations by polyacrylamide disc-gel electrophoresis showed the presence of up to 11 isozymes with activity toward catechol. The isozymes had differing substrate specificities and were present in differing amounts. Treatment 21 days after bloom with both GA and ethephon (75 and 150 ppm) seemed to induce the appearance of a minor catechol reactive isozyme. This isozyme had no activity with the other substrates tested. Treatment with 150 ppm ethephon 46 days after bloom appeared to decrease the substrate specificity of one isozyme. The importance of these changes on the degree of enzymatic browning were not determined.

Crude PPO preparations from control fruit and fruits treated 46 days after bloom with GA and ethephon (75 and 150 ppm) were analyzed for substrate specificities. Enzymatic activity was seen with o-diphenolic compounds only; no activity was seen with either a monophenol or a p-diphenol. Activity toward o-diphenols relative to catechol showed slight variations with treatment which may indicate alterations in reactivity with naturally occurring PPO substrates in peach.

The same PPO preparations exhibited two pH optima in phosphate-citrate buffer. PPO from ethephon treated fruit had pH optima of 6.6 and 4.4 while that from GA treated fruit and control fruit had pH optima of 6.2 and 4.4. PPO from GA and ethephon treated fruits had lower amounts of total activity at pH 4.4 than control fruit. As pH 4.4 is closer to the fruit pH than pH 6.3, at which PPO activity was measured,

decreased PPO activity at this pH optimum may be reflected in reduced enzymatic browning in the peach tissue.

LITERATURE CITED

Abeles, F.B. 1972. Biosynthesis and mechanism of action of ethylene. Ann. Rev. Plant Physiol. 23:259.

Anderson, J.W. 1968. Extraction of enzymes and subcellular organelles from plant tissues. Phytochem. 7:1973.

Aoki, S., Araki, C., Kaneko, K. and Katayama, O. 1971. Occurrence of L-Phenylalanine ammonia lyase activity in peach fruit. Agr. Biol. Chem. 35:784.

Benjamin, N.D. and Montgomery, M.W. 1973. Polyphenol oxidase of Royal Anne cherries: purification and characterization. J. Food Sci. 38:799.

Bireka, H., Catalfamo, J. and Urban, P. 1976. Isoperoxidases in sweet potato plants in relation to mechanical injury and ethylene. Plant Physiol. 57:74.

Boswell, J.G. 1963. Plant polyphenol oxidases and their relation to other oxidase systems in plants. In "Enzyme Chemistry of Phenolic Compounds". Ed. Pridham, J.B., p.25. Permagon Press, New York.

Bright, H.J., Wood, B.J.B., Ingraham, L.L. 1963. Copper, tyrosinase and the kinetic stability of oxygen. Ann. N.Y. Acad. Sci. 100:965.

Buchanan, D.W., Hall, C.B., Biggs, R.H. and Knapp, F.W. 1969. Influence of Alar, Ethrel and gibberellic acid on browning of peaches. Hort. Science 4:302.

Byers, R.E. and Emerson, F.H. 1973. Effect of SADH and ethephon on peach fruit growth and maturation. Hort. Science 8:48.

Byers, R.E., Dostal, H.C. and Emerson, F.H. 1969. Regulation of fruit growth with 2-chloroethylphosphonic acid. Bioscience 19:903.

Camm, E.L. and Towers, G.H.N. 1973. Phenylalanine Ammonia Lyase. Phytochem. 12:961.

Chalmers, D.J. and van den Ende, B. 1975. A reappraisal of the growth and development of peach fruit. Aust. J. Plant Physiol. 2:263.

Chalmers, D.J., van den Ende, B. and Jerie, P.H. 1976. The effect of (2-chloroethyl) phosphonic acid on the sink strength of developing peach (Prunus Persica L.) fruit. Planta 131:203.

Cohen, J. 1968. Multiple regression as a general data-analytic system. Psych. Bull. 70:426.

Conn, E.E. 1964. Enzymology of phenolic biosynthesis. In "Biochemistry of Phenolic Compounds," Ed. Harborne, J.B., Ch. 10. Academic Press, New York.

Constantinides, S.M. and Bedford, C.L. 1967. Multiple forms of phenoloxidase. J. Food Sci. 32:446.

Corgan, J.N. and Widmoyer, F.B. 1971. The effects of gibberellic acid on flower differentiation, date of bloom, and flower hardiness of peach. J. Am. Soc. Hort. Sci. 96:54.

Corse, J. 1953. A new isomer of chlorogenic acid from peaches. Nature 172:771.

Craft, C.C. 1961. Polyphenolic compounds in Elberta peaches during storage and ripening. J.Am.Soc.Hort.Sci. 78:119.

Craker, L.E. 1975. Effect of ethylene and metabolic inhibitors on anthocyanin biosynthesis. Phytochem. 14:151.

Davis, B.J. 1964. Disc electrophoresis. II Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404.

Douglas, M.A., Vanderstoep, J. and Paulson, A.T. 1977. Effect of gibberellic acid and ethephon on ascorbic acid content and ascorbic acid oxidase activity of Redhaven peaches. Can.Inst.Food Sci. Technol.J. 10:233.

Duckworth, H. and Coleman, J.E. 1970. Physicochemical and kinetic properties of mushroom tyrosinase. J. Biol. Chem. 245:1611.

Edgerton, L.J. 1966. Some effects of gibberellin and growth retardants on bud development and cold hardiness of peach. Proc.Am.Soc. Hort.Sci. 88:197.

El-Sayed, A.S. and Luh, B.S. 1965. Polyphenolic compounds in apricots. J. Food Sci. 30:1017.

Eskin, N.A.M., Henderson, H.M. and Townsend, R.J. 1971. "Biochemistry of Foods". Academic Press, New York.

Fel'dman, A.L. and Kostinskaya, L.I. 1970. Peach polyphenols and their role in color changes in the fruit. Prikl. Biochim. i Mikrobiol. 6:442.

Frenkel, C., Klein, I. and Dilley, D.R. 1969. Methods for the study of ripening and protein synthesis in intact pome fruits. Phytochem. 8:945

Gajzago, I., Vamos-Vigyazo, L. and Nadudveri-Markus, V. 1976. Investigations into the enzymic browning of apricot cultivars. Acta. Alimentaria 6:95.

Galston, A.W., Vance, S. and Siegel, B.Z. 1968. The induction and repression of peroxidase isozymes by 3-IAA. In "Biochemistry and Physiology of Plant Growth Substances," Ed. Wightman, F. and Setterfield, G., p.455. Runge Press, Ottawa, Canada.

Glaszion, K.T., Gaylor, K.R. and Waldon, J.C. 1968. Effects of auxin and gibberellic acid on the regulation of enzyme synthesis in sugar-cane stem tissue. In "Biochemistry and Physiology of Plant Growth Substances," Ed. Wightman, F. and Setterfield, G., p.433. Runge Press, Ottawa, Canada.

Goldstein, J.L. and Swain, T. 1963. Changes in tannins in ripening fruits. Phytochem. 2:371.

Grice, M.R., Brown, H.D. and Burrell, R.C. 1952. Varietal characteristics influence browning of frozen peaches. Food Eng. p.131.

Guadagni, D.G., Sorber, D.G. and Wilber, J.S. 1949. Enzymatic oxidation of phenolic compounds in frozen peaches. Food Tech. 3:359.

Gujarati, D. 1970. Use of dummy variables in testing for equality between sets of coefficients in linear regressions: a generalization. Amer. Stat. December, p.18.

Haight, G.P. Jr. and Paragamian, V. 1960. Color complexes of catechol with molybdate. Analytical Chem. 32:642.

Harel, E. and Mayer, A.M. 1970. The use of a fungal pectate lyase in the purification of laccase from peaches. Phytochem. 9:2447.

Harel, E., Mayer, A.M. and Lerner, H.R. 1970. Changes in the levels of catechol oxidase and laccase in developing peaches. J.Sci. Fd. Agric. 21:542.

Harel, E., Mayer, A.M. and Shain, Y. 1965. Purification and multiplicity of catechol oxidase from apple chloroplasts. Phytochem. 4:738.

Hess, D. 1975. "Plant Physiology" Springer-Verlag, New York.

Hsia, C.L., Luh, B.S. and Chichester, C.O. 1965. Anthocyanin in freestone peaches. J. Food Sci. 30:5

Imaseki, H., Uchiyama, M. and Uritani, I. 1968. Effect of ethylene on the inductive increase in metabolic activities in sliced potato roots. Agr. Biol. Chem. 32:387.

Jackson, D.I. 1968. Gibberellin and the growth of peach and apricot fruits. Aust. J. Biol. Sciences 21:209.

Jen, J.J. and Kahler, K.R. 1974. Characterization of polyphenol oxidase in peaches grown in the southeast. Hort.Science 9:590.

Jerie, P.H. and Taylor, B.K. 1971. Influence of foliar sprays of growth regulating materials on the vegetative growth of one-year-old peach trees. Hort.Res. 11:136.

Jones, R. 1973. Gibberellins: their physiological role. Ann. Rev. Plant Physiol. 24:571.

Kahn, V. 1975. Polyphenol oxidase activity and browning of three avocado varieties. J. Sci. Fd. Agric. 26:1319.

Kahn, V. 1976. Polyphenol oxidase isozymes in avocado. Phytochem. 15:267.

Kahn, V. 1977. Latency properties of polyphenol oxidase in two avocado cultivars differing in their rate of browning. J. Sci. Fd. Agric. 28:233.

Knapp, F.W., Hall, C.B., Buchanan, W.D. and Biggs, R.H. 1970. Reduction in polyphenoloxidase activity in peaches sprayed with Alar, Ethrel, or gibberellic acid. Phytochem. 9:1453.

Krebs, K.G., Heusser, D. and Wimmer, H. 1969. Spray reagents. In "Thin-layer Chromatography", ed. Stahl, E., p. 854. Springer-Verlag, New York.

Lang, A. 1970. Gibberellins: structure and metabolism. Ann. Rev. Plant Physiol. 21:537.

Lanzarini, G., Pifferi, P.G. and Zamorani, A. 1972. Specificity of an o-diphenol oxidase from prunus avium fruits. Phytochem. 11:89.

Lavee, S. and Martin, G.C. 1974. Ethephon {1,2-^{14}C(2-Chloroethyl) phosphonic acid} in Peach Fruits. I. Penetration and Persistence. J. Am. Soc. Hort. Sci. 99:97.

Lavee, S. and Martin, G.C. 1975. Ethephon { 1,2-^{14}C(2-Chloroethyl) phosphonic acid}in peach (Prunus persica L.) fruits. III. Stability and persistence. J. Am. Soc. Hort. Sci. 100:28.

Le, C. and Tenisci, T. 1977. UBC TRP Triangular Regression Package, Computing Centre, the University of British Columbia, Vancouver, B.C.

Lee, T.T. 1971. Increase of IAA oxidase by GA in tobacco callus cultures. Can. J. Bot. 49:687.

Lee, T.T. 1972. Interaction of cytokinin, auxin, and gibberellin on peroxidase isoenzymes in tobacco tissues cultured in vitro. Can. J. Bot. 50:2471.

Leopold, A.C. and Kriedemann, P.E. 1975. "Plant Growth and Development", McGraw-Hill, Toronto.

Lerner, H.R. and Mayer, A.M. 1976. Reaction mechanism of grape catechol oxidase: a kinetic study. Phytochem. 15:57.

Li, J.C.R. 1964a. "Statistical Inference I". Edwards Brothers Inc., Ann Arbor, Mich.

Li, J.C.R. 1964b. "Statistical Inference II". Edwards Brothers Inc., Ann Arbor, Mich.

Li, K.C., Boggess, T.S. Jr. and Heaton, E.K. 1972. Relationship of sensory ratings with tannin components of canned peaches. J.Food Sci.37:177.

Lineweaver, H. and Burke, D. 1934. The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56:658.

Looney, N.E. 1972. Effects of succinic acid 2,2-dimethylhydrazide, 2-chloroethylphosphonic acid, and Ethylene on respiration, ethylene production, and ripening of "Redhaven" peaches. Can. J. Plant Sci. 52:73.

Looney, N.E., McGlasson, W.B. and Coombe, B.G. 1974. Control of fruit ripening in peach, Prunus persica: action of Succinic acid -2, 2-dimethylhydrazide and (2-chloroethyl) phosphonic acid. Aust. J. Plant Physiol. 1:77.

Loughheed, E.C. and Franklin, E.W. 1972. Effects of temperature on ethylene evolution from ethephon. Can.J. Plant Sci. 52:769.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.

Luh, B.S. and Phithakpol, B. 1972. Characteristics of polyphenoloxidase related to browning in cling peaches. J. Food Sci. 37:264.

Luh, B.S., Hsu, E.T. and Stachowicz, K. 1967. Polyphenolic compounds in canned cling peaches. J. Food Sci. 22:807.

Mapson, L.W., Swain, T. and Tomalin, A.W. 1963. Influence of variety, cultural conditions and temperature of storage on enzymic browning of potato tubers. J. Sci. Fd. Agric. 14:673.

Markert, C.L. 1974. Biology of isozymes. In: "Isozymes: I Molecular Structure," Ed. Markert, C.L. p.11. Academic Press, New York.

Mason, H.S. 1957. Mechanisms of oxygen metabolism. Adv. Enz. 19:131.

Mathew, A.G. and Parbia, H.A.B. 1971. Food browning as a polyphenol reaction. Adv. Food Res. 19:75.

Moss, D.W. and Butterworth, P.J. 1974. "Enzymology and Medicine", p.28, Copp Clark Pub. Co., Toronto, Ontario.

Nakabayashi, T. and Uhai, N. 1963. Browning of peaches by polyphenolase. Japanese J. Food Tech. 10:211.

Nitsch, J.P. 1970. Hormonal factors in growth and development. In "The Biochemistry of Fruits and Their Products". Vol. I. Ed. Hulme, A.C., Ch. 15, Academic Press, New York.

Ogawa, Y. 1965. Changes in the content of gibberellin - like substances in the seed of Prunus persica Bot. Mag. 78:412.

Paulson, A. 1973. Effect of foliar sprays of Ethrel and gibberellic acid on enzymatic browning of Fairhaven and Redhaven peaches. Unpublished undergraduate thesis, Dept. of Food Science, U.B.C.

Ponting, J.D. 1960. Control of enzymatic browning of fruits. In "Food Enzymes", ed. Schultz, H.W. Ch. 9 Avi Publishing Company, Westport, Conn.

Ponting, J.D. and Joslyn, M.A. 1948. Ascorbic acid oxidation and browning in apple tissue extracts. Arch. Biochem. 19:47.

Porritt, B. 1974. Effect of storage treatments and foliar sprays of ethephon and gibberellic acid on Redhaven peaches for processed refrigerated peach slices. Unpublished undergraduate thesis, Dept. of Food Science, U.B.C.

Potty, V.H. 1969. Determination of proteins in the presence of phenols and pectins. Analytical Bioc. 29:535.

Proebsting, E.L. Jr. and Mills, H.H. 1966. Effect of gibberellic acid and other growth regulators on quality of Early Italian prunes (Prunus domestica L.) Proc. Am. Soc. Hort. Sci. 89:135.

Proebsting, E.L. Jr. and Mills, H.H. 1969. Effects of 2-chloroethane phosphonic acid and its interaction with gibberellic acid on quality of Early Italian prunes. J. Am. Soc. Hort. Sci. 94: 443.

Proebsting, E.L. Jr., Carter, G.H. and Mills, H.H. 1973. Quality improvement in canned Rainier cherries (P. avium L.) with gibberellic acid. J. Am. Soc. Hort. Sci. 98:334.

Reeve, R.M. 1959. Histological and histochemical changes in developing and ripening peaches. I The catechol tannins. Am. J. Bot. 46:210.

Reyes, P. and Luh, B.S. 1960. Characteristics of browning enzymes in Fay Elberta freestone peaches. Food Tech. 14:570.

Ribereau-Gayon, P. 1972. "Plant Phenolics", Hafner Pub. Co., New York.

Rivas, N. and Whitaker, J.R. 1973. Purification and some properties of two polyphenoloxidases from Bartlett pears. Plant Physiol. 52:501.

Roberts, E.A.H. 1956. The chlorogenic acids of tea and mate. Chemistry and Industry. p.985.

Rom, R. and Scott, K. 1971. The effect of 2-chloroethylphosphonic acid (ethephon) on maturation of a processing peach. Hort. Science 6:134.

Salisbury, F.B. and Ross, C. 1969. "Plant Physiology". p.398, Wadsworth Pub. Co., Belmont, Cal.

Sal'kova, E.G., Zuyagintseva, Y.V., Kuliev, A.A. and Akhundov, R.M. 1977. (Effect of Ethrel on biochemical processes during fruit ripening). Prikl. Biokhim. i Mikrobiol. 13:97 (Abstract).

Sarkar, S.K. and Ton Phan, C. 1974. Effect of ethylene on the qualitative and quantitative composition of the phenol content of carrot roots. Physiol. Plant. 30:72.

Sato, M. and Hasegawa, M. 1976. The latency of spinach chloroplast phenolase. Phytochem. 15:61.

Schaller, D. and von Elbe, J. 1970. Polyphenols in Montmorency cherries. J. Food Sci. 35:762.

Siegelman, H.W. 1955. Detection and identification of polyphenoloxidase in apple and pear skins. Arch. Biochem. Biophys. 56:97.

Seikel, M.K. 1962. Chromatographic methods of separation, isolation, and identification of flavanoid compounds. In "The Chemistry of Flavanoid Compounds", p. 34, Ed. Geissman, T.A. Macmillan Pub. Co., New York.

Sheen, S.J. and Calvert, J. 1969. Studies on polyphenol content, activities and isozymes of polyphenol oxidase and peroxidase during air-curing in three tobacco types. Plant Physiol. 44:199.

Shinshi, H. and Noguchi, M. 1975. Relationships between peroxidase, IAA Oxidase and Polyphenol Oxidase. Phytochem. 14:1255.

Srinivasan, C., Pappiah, C.M. and Doraipandian, A. 1973. Effect of gibberellic acid on ascorbic acid, sugar content and oxidative enzyme activity of West Indian Cherry (Malpighia punicifola) fruit. J. Exp. Biol. 11:469.

Srivastava, O.P. and van Huystee, R.B. 1973. Evidence for close association of peroxidase, polyphenol oxidase, and IAA oxidase of peanut suspension culture medium. Can. J. Bot. 51:2207.

Stembridge, G.E. and Raff, J.W. 1973. Ethephon and peach fruit development. Hort. Science 8:500.

Stuart, N.W. and Cathey, H.M. 1961. Applied aspects of the gibberellins. Ann. Rev. Plant Physiol. 12:369.

Swain, T. and Hillis, W.E. 1959. The phenolic constituents of Prunus domestica. I. The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10:63.

Taneja, S.R. and Sachar, R.C. 1974. Separate monophenolase and o-diphenolase enzymes in Triticum Aestivum. Phytochem. 13:1367.

Van Blaricom, L.O. and Senn, T.L. 1967. Anthocyanidin pigments in freestone peaches grown in the Southeast. Proc. Am. Soc. Hort. Sci. 90:541.

Van Buren, J. 1970. Phenolics.In "The biochemistry of Fruits and Their Products". Vol. I. Ed. Hulme, A.C., Ch. 11, Academic Press, New York.

Van Loon, L.C. 1971. Tobacco polyphenoloxidases: a specific staining method indicating non-identity with peroxidases. Phytochem. 10:503.

Walker, J.R.L. 1962. Studies on the enzymic browning of apple fruit. N.Z.J.Sci. 5:316.

Walker, J.R.L. 1975. "The Biology of Plant Phenolics". Edward Arnold (Publishers) Ltd. London.

Walker, J.R.L. and Wilson, E. 1975. Studies on the enzymatic browning of applies. Inhibition of apple o-diphenol oxidase by phenolic acid. J. Sci. Food Agric. 26:1825.

Weaver, C. and Charley, H. 1974. Enzymatic browning of ripening bananas. J. Food Sci. 39:1200.

Whitaker, J.R. 1972. "Principles of Enzymology for the Food Sciences." p. 577. Marcel Dekker, Inc., New York.

Williams, A.H. 1955. Paper chromatography of cinnamic acid derivatives. Chemistry and Industry, p.120.

Wong, T.C., Luh, B.S. and Whitaker, J.R. 1971a. Isolation and characterization of polyphenol oxidase isozymes of clingstone peach. Plant Physiol. 48:19.

Wong, T.C., Luh, B.S. and Whitaker, J.R. 1971b. Effect of phloroglucinol and resorcinol on the clingstone peach polyphenol oxidase - catalyzed oxidation of 4-methyl catechol. Plant Physiol. 48:24.

Yang, S.F. 1969. Ethylene evolution from 2-chloroethylphosphonic acid. Plant Physiol. 44:1203.