ENZYMATIC SYSTEMS AND SUBSTRATES INVOLVED IN FREESTONE PEACH BROWNING

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Kamal El-Din Hussien Motawi

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ABSTRACT

ENZYMATIC SYSTEMS AND SUBSTRATES INVOLVED IN FREESTONE PEACH BROWNING

by Kamal El-Din Hussien Motawi

The browning of peaches during preparation for processing and frozen storage is a serious and costly problem. The process is considered to be enzymatic in nature and due to the oxidation of phenolic compounds by oxidizing enzymes in the presence of molecular oxygen.

This study was conducted to investigate the relationships between the enzyme system and the substrates responsible for bringing about brown discoloration in freestone peaches. The enzyme, polyphenol oxidase (PPO), was prepared by acetone precipitation and purified by ammonium sulfate fractionation and Sephadex G-100. The enzyme solution was not stable at pH 8.0 at all the temperatures used, from 22° to -31.7° C. The enzyme was not very stable at room temperature, 22° C., losing 93.3% of its activity in 6 days. The frozen solutions stored for 12 weeks at -17.8° C. and -31.7° C. showed a loss in activity of 20 and 16.6%, respectively. The enzyme showed an optimum pH of 6.4 with citrate-phosphate buffer and

catechol as substrate and an optimum temperature of 43° C. The enzyme from the eight varieties studied showed the same substrate specificity. Elberta peaches had the highest enzyme activity while Redskin showed the lowest level of activity.

The phenolic constituents of peach extract in order of prominence were leucoanthocyanins, 35.6 to 39.4%, chlorogenic acid, 22.8 to 25.9%, flavonols, 18.8 to 19.4% and catechin, 12.0 to 14.8%. Richhaven peaches had the highest total phenolic content, 173.2 mg./100 g. and Sunhaven the lowest, 85.4 mg./100 g. Catechin exhibited the highest reactivity with the enzyme followed by chlorogenic acid, leucoanthocyanins, and flavonols. The degree of discoloration of peaches during preparation for processing, frozen storage or on thawing is primarily attributed to the original activity of the enzyme in the varieties that have from moderate to high total phenolic content. Paper chromatographic and spectrophotometric techniques were used for isolation and identification of the phenolic compounds in peach extract.

ENZYMATIC SYSTEMS AND SUBSTRATES INVOLVED IN FREESTONE PEACH BROWNING

By

Kamal El-Din Hussien Motawi

A THESIS

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DEDICATION

To my Mother and my Wife

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The author is deeply indebted to Dr. C. L. Bedford for his assistance in conducting this study and his counsel in the preparation of the manuscript. The author wishes to express his sincere appreciation for the assistance given him by Dr. P. Markakis who provided laboratory facilities and contributed valuable discussions; and to Dr. R. C. Nicholas for his encouragement throughout this study.

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TABLE OF CONTENTS

TABLE OF CONTENTS--(Continued)

LIST OF TABLES

LIST OF TABLES--(Continued)

LIST OF FIGURES

LIST OF FIGURES--(Continued)

INTRODUCTION

The enzymatic discoloration of plant tissues following cell damage is a serious and long standing problem. Many fruits undergo rapid change in color after mechanical or physiological injury during harvesting and storage. Such color change in fruit products is accentuated during preparation for processing by canning, dehydration or freezing and continues during freezing storage and subsequent defrosting of frozen fruits. The natural color of the product may be destroyed or masked by formation of dark brown pigments. This browning is generally accompanied by change in color, flavor and nutritive value which greatly detract from the quality of the finished product.

The problem of browning in peaches during processing and frozen storage has been the subject of numerous articles and reports. The process is considered to be enzymatic in nature and due to oxidation of the so called catechol tannins by oxidizing enzymes in the presence of molecular oxygen.

The purpose of this study is to investigate the relationship between the enzyme system and the substrates responsible for bringing about brown discoloration in frozen freestone peaches.

REVIEW OF LITERATURE

Enzyme catalyzed oxidative browning was recognized by Kastle (1910). Lindet (1895) concluded that the changes in color occuring in fresh cider are due to oxidation of tannin by a laccase-like enzyme contained in the tissues of apples.

Most of the early research on enzymatic oxidation of fruit products has been directed to qualitative characterization of the oxidizing enzyme present and methods of inactivation or control of the enzyme involved in browning (Basset and Thompson, 1911; Onslow, 1920; Overholser and Cruess, 1923; Cruess and Fong, 1926, 1929a, 1929b, Joslyn, 1941; and Guadagni, Sorber and Wilbur, 1949).

Quantitative methods in studying plant oxidases were first used in the United States by Bunzell (1912a, 1912b, 1916a, 1916b, 1916c). Nelson and his coworkers investigated the tyrosinase of the common edible and wild mushroom during the period of 1938-1941 and made many contributions to quantitative techniques reviewed by Nelson and Dawson (1944).

Quantitative investigations of enzymatic oxidation of fruit products were carried out by Samisch, 1935a and 1937; Hussien and Cruess, 1940; Jimenez, 1947; Cruess and Sugihara, 1948; Ponting

and Joslyn, 1948; Cruess, 1948; El-Tabey and Cruess, 1949 and Tauber, 1949.

There are many different enzymes having the ability to catalyze the oxidation of phenols by molecular oxygen.

Several theories have been proposed for the nature and course of enzymatic browning differing considerably in nomenclature as well as in mechanism. It was believed that plant tissues which darken on injury contain a substance called oxygenase which in the presence of air undergoes autooxidation, yielding a peroxide. This peroxide, activated by the enzyme peroxidase present in most plants, then brings about the oxidation of the natural phenolic substances. Kastle and Lovenhart (1901) and Overholser and Cruess (1923) ascribed discoloration to such a system.

As early as 1920 Onslow showed that peaches contain both oxidizing enzymes and certain phenolic compounds, the latter being converted to brown-black substances of unknown constitution during the oxidation. She modified the existing concept of oxygenase by considering it as an enzyme which in the presence of air catalyzed the oxidation of o-dihydric phenols yielding as one of the oxidation products a peroxide or hydrogen peroxide. The latter, in the presence of peroxidase was activated and oxidized a suitable chromogen. She believed that this secondary oxidation involved mono-, di-, and poly-hydroxyphenolic compounds, including tannins which after oxidation transferred into the characteristic colored pigments.

Onslow (1931) systematically investigated the oxidizing enzymes present in higher plants and segregated them into two groups, those which contain oxygenase and catechol compounds and those in which oxygenase and catechol compounds were absent, the peroxidase plants. The first group of plants discolors rapidly on injury and includes apple, apricot, banana, cherry, fig, grape, peach, pear, and strawberry. The second group of plants which does not discolor on injury includes citrus fruit (lemon, orange, lime, and grapefruit), red currants, melon, pineapple and tomato.

Szent-Gyorgyi (1925) succeeded in showing that darkening of plant tissues could take place in the absence of peroxidase.

Balls and Hale (1935) reported that pigment formation in injured apple tissue was accelerated by the addition of horseradish peroxidase and inhibited by the addition of direct inhibitors of peroxidase. They concluded that the darkening of freshly cut surfaces of apples is a reaction catalyzed by peroxidase and that the formation of hydrogen peroxide from molecular oxygen by a respiratory enzyme is a necessary preliminary step.

Raper and co-workers (1932-1938) investigated the intermediate reactions involved in the conversion of tyrosine into melanin by tyrosinase without the intervention of peroxidase.

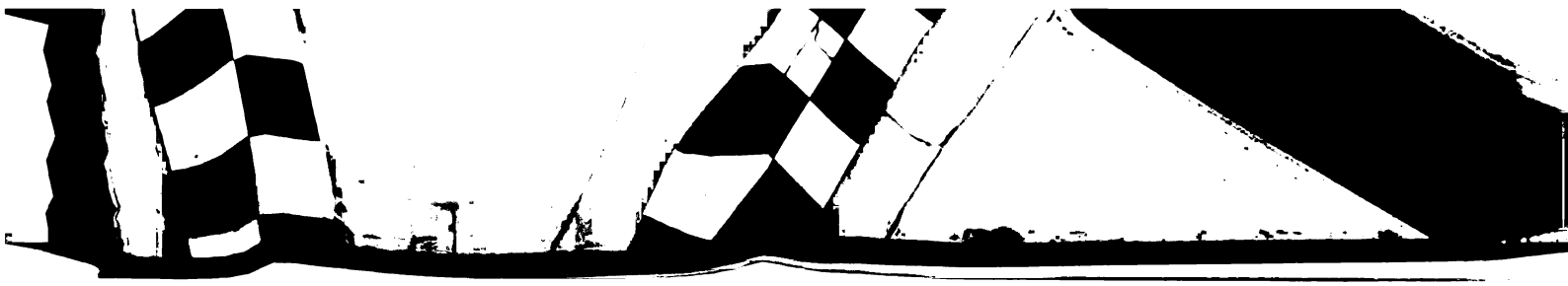
Shortly afterward, with the improvement of methods of enzyme separation and assaying techniques, the existence of copper-containing oxidases catalyzing the oxidation of phenols by molecular

oxygen, as distinct from the iron porphyrin peroxidases, catalyzing the oxidation of phenols by hydrogen peroxide was established through the classical work of Keilin and Mann (1938) and Parkinson and Nelson (1940).

Nelson and Dawson (1944) used the term tyrosinase for the preparation they obtained from mushrooms and found that it had the ability to catalyze the oxidation of monophenols in addition to catalyzing the oxidation of polyphenols. The ratio of rate of oxidation of monophenol oxidation to that of polyphenol oxidation, however, varied considerably in preparations from different mushroom sources.

Keilin and Mann (1938) purified polyphenol oxidase from mushroom and found it did not oxidize monophenols appreciably.

Cruess and Sugihara (1948) used the term oxidase for the mixture of polyphenol oxidases and peroxidases in extracts of crude enzyme preparations from fruit tissues. The phenolase in apricot extract prepared by extraction of frozen ground tissue by Samisch and Cruess (1934) was able to catalyze the oxidation of catechol and pyrogallol and not that of phenol, resorcinol, quinol, phloroglucinol or tyrosine. Samisch (1927) also reported that the extracts of avocado behaved the same as that of apicot and Jimenez (1947) reported similar results with guava extract. Cruess and Sugihara (1948) extracted olive tissue with cold acetone and found that it catalyzed only the oxidation of o-dihydroxyphenols.



Kertesz (1933) showed qualitatively that the oxidizing enzymes of Sunbeam peaches were similar to those of other peach varieties. He concluded that the low concentration of catechol tannins in this variety was responsible for the failure of the sliced fruit to darken.

Adams and Nelson (1938) reported that the phenol oxidizing enzyme in aqueous extract of sweet potato possesses the ability to catalyze the aerobic oxidation of o-dihydric phenols but cannot bring about the oxidation of monohydric phenols such as p-cresol and tyrosine. As a result the enzyme has been called catecholase by Graubard (1939) and orthophenolase by Somner and Somner (1943).

Eiger and Dawson (1949) found that purified sweet potato phenolase oxidizes only polyphenols with the ortho-dihydroxy grouping, although sweet potatoe slices will oxidize p-cresol also.

El-Tabey and Cruess (1949) also reported that apricot oxidase containing phenolase and peroxidase catalyzed the oxidation of catechol, protocathuic acid, caffeic acid, and digallic acid.

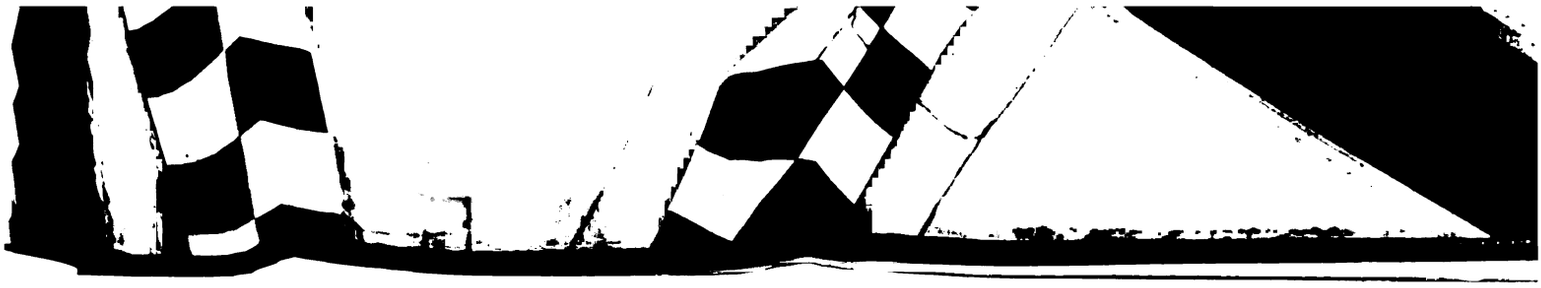
Ponting and Joslyn (1948) prepared polyphenolase from apples mainly by acetone precipitation. Freezing in dry ice and acetone caused no loss, but subsequent storage at -34.6° C. caused a loss of 80% of the activity in three months. Freeze-drying the enzyme solution rendered the enzyme completely insoluble. The loss in solution at 0° C. was about as low as in the frozen storage. They reported

an optimum temperature of 40° C. and optimum pH of 7.0. It was also noted that polyphenolase is quite sensitive to heat. Catechol, resorcinol, p-cresol, quiacol and phenol were tested as substrates. Only catechol was oxidized by the enzyme.

Studying the characteristics of browning enzymes in Fay Elberta freestone peaches, Reyes and Luh (1960) reported an optimum pH of 5.9 to 6.3 for the polyphenolase with citrate-phosphate buffer and catechol as substrate. Michaelis constant of 0.12 M catechol was also reported. They found that peach peroxidase and polyphenolase act primarily on the ortho-dihydroxy configuration. The para-dihydroxy structure was also oxidized by both enzymes but at a slower rate. Neither enzyme exhibited significant activity with mono-hydroxy and meta-dihydroxy compounds as substrates.

Palmer (1963) isolated a polyphenoloxidase from banana and showed that it catalyzed the oxidation of o-dihydric phenols, but not that of monophenols such as tyrosine, tyramine, ortho- and para-cresol. He noted also that addition of catechol or dopamine in an attempt to eliminate any induction period had no effect on the oxidation of tyrosinase.

Most of the investigations of the natural substrates in fruits have been qualitative in nature and largely based on the technique introduced by Onslow (1931) for the domonstration of the presence of catechol compounds. Flavones and flavonols have long been suspected



of being involved in discoloration. Nagi (1921) reported that certain anthocyanins were completely decolorized by the action of oxidizing enzymes which also caused certain flavones, flavonols and their glucosides to yield characteristic oxidation colors. He showed that the color of aqueous extract of many plant tissues rich in flavones changes to brown or reddish-brown when treated with fresh plant juices containing oxidizing enzymes. He reported also that quercetin and quercitrin were oxidized by plant oxidases into deep colored pigments which rapidly changed to brown.

Sando (1924) isolated and identified quercetin from the peels of the McIntosh apple and postulated that it may be the chromogen involved in storage scald.

Cruess and Alsberg (1934) made an extensive study on the bitter principle of the olive, oleuropein. This substance was later found by Cruess and Sugihara (1948) to be a substrate for polyphenolase, proved to be a glucoside of caffeic acid, the latter being esterified with a phenol. Only the caffeic acid portion of this compound contains the ortho-dihydroxy grouping necessary for polyphenolase oxidation. They reported that natural substrate should not be called a tannin, since when purified it was not precipitated by gelatin or absorbed by hide powder, reactions typical of tannins. Thus the statement commonly made, that the darkening of fruit is caused by oxidation of tannins, does not apply in the case of the only well investigated fruit and it probably does not apply to any other fruit.

Blake and Davidson (1941) classified a number of peach varieties on the basis of the color reaction between 4 ml. of peach juice and 3 drops of 6% $FeCl_3$ solution. They estimated the tannins by the color and thickness of the discolored layer formed by adding the $FeCl_3$ to the peach juice. On this basis they found that varieties high in acidity as well as in tannin were objectionably astringent and more susceptible to browning.

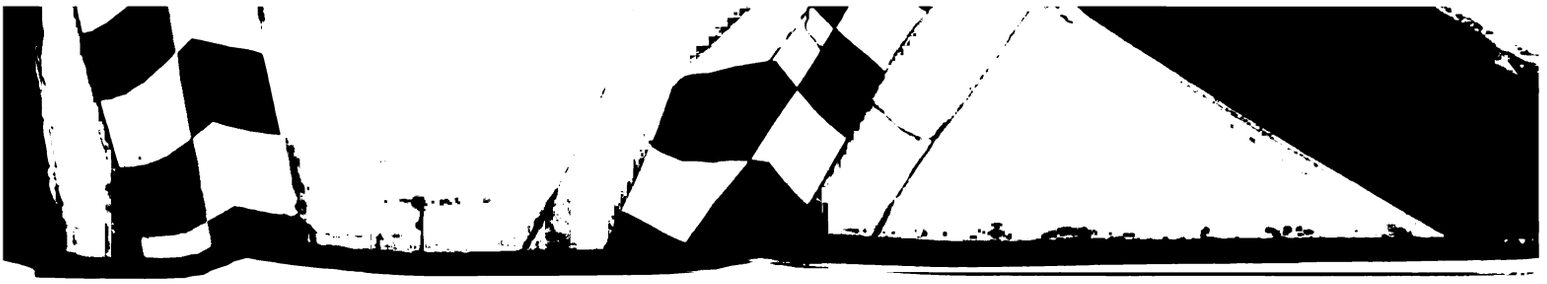
Guadagni et al. (1949) studied enzymatic oxidation of phenolic compounds in frozen peaches. They found fairly good correlation between the oxidation of phenolic compounds and the changes in the optical density of aqueous extracts of peach tissue using Folin-Denis reagent. It was found that only a fraction of the total phenolic substances in peaches is of the oxidizable type. The susceptibility of enzymatic browning in several peach varieties was found to be directly related to the amount of oxidizable tannin present. Initial browning tendency, however, was also governed by the original activity of the enzymes.

Johnson et al. (1950) reported using synthetic anion exchange resin for the isolation of peach tannins and obtained the latter in relatively pure form by this method. They used paper chromatography and ultraviolet absorption techniques in the characterization and identification of the phenolic substances in the isolated material. The isolated tannins contained D-catechin, a reddish colored substance,

probably polymeric derivitives of catechin, chlorogenic acid and anthocyanin pigment.

Guadagni and Nimmo (1952) studied the effect of growing area on tannin and its relation to astringency in frozen Elberta peaches. Peaches representing three areas of different climatic conditions were analyzed for tannin content and subjectively compared for difference in astringency. They found the area having the warmest and clearest weather produced peaches of lowest tannin content while the cooler, cloudy area produced fruit of highest tannin content. The area having climate considered to be intermediate between these two extremes produced peaches with intermediate tannin content. The ability to detect differences in astringency due to the tannins found in these peaches appears to depend on the magnitude of the total amount present as well as the magnitude of the differences between samples. It was concluded that tannins appear to be correlated fairly well with relative astringency determined by organoleptic means.

Bate-Smith (1954), in a study on astringency in foods, paid considerable attention to astringency in peaches. In peaches, as in many other fruits, a certain degree of astringency is desirable, otherwise the fruit is insipid, but the astringency must not be excessive. Some varieties, in particular one named "Sunbeam," are well known for the complete absence of tannin from their flesh, but they



have the advantage of not becoming brown by phenolase action during processing.

Siegelman (1954) reported on a modified paper chromatographic method for detection and identification of polyphenoloxidase substrates in apple and pear skins. The apple polyphenoloxidase enzyme was used as a chromatographic spray for detecting the possible endogenous substrates of the browning reaction. In the tests described, the browning reaction of chlorogenic acids on the chromatograms after it was sprayed with PPO was not nearly as intense as that of catechin. Three additional unidentified compounds were found which darkened after treatment with PPO, but none corresponds to the already known catechins. He emphasized that a browning reaction on the chromatogram is not conclusive evidence of the participation by that substance in the browning reaction.

Swain and Hillis (1959) critically examined and made some modifications of existing methods of quantitative analysis of anthocyanins, leucoanthocyanins, flavones and total phenols in plant tissue extract. They concluded that all the procedures for their estimation are necessarily empirical. For total phenol determination the methods based on the use of oxidizing agents are the most useful. The more modified method of Folin and Denis has proved to be more convenient and consistent. All the phenolic compounds tested showed an almost linear relationship between optical density

and concentration, although the slopes of the curves were slightly different.

Corse (1953) isolated a new isomer of chlorogenic acid, "neochlorogenic acid," from Elberta and Halford peaches by Counter-Current distribution. The ultraviolet absorption spectrum is typical of the other isomers, but the rotation, melting point, and the crystalline nature were different.

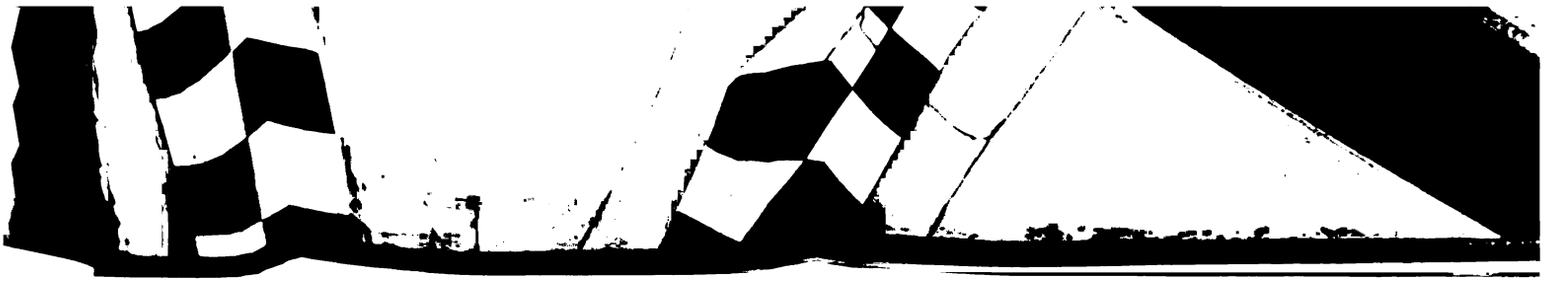
The polyphenolic compounds in Elberta peaches during storage and ripening were studied by Craft (1961). He found that the principal polyphenols separated from ethanolic extracts of immature peaches were in order of prominence on the paper chromatograms; leucoanthocyanins, chlorogenic acids, catechin, and flavonols. There was no qualitative change in this polyphenolic pattern during ripening, and the relative proportions of the individual components remained fairly constant. Total phenolic compounds were estimated by the Folin-Denis reagent method and leuco-anthocyanin by hydrolysis to anthocyanidin.

A long term study of the discoloration of canned peaches was carried out by Claypool (1962). He reported that the most pronounced change in maturing peaches is the appearance of anthocyanins and a decrease in leucoanthocyanins. Other compounds of phenolic origin which have been tentatively identified on chromatograms are L-epicatechin, D-catechin and four isomers of chlorogenic acids.

Present information shows a negative correlation between discoloration and the total tannins present in the fruit even though these compounds are susceptible to enzymatic browning. He concluded that two factors, advanced maturity and nutritional status of the trees, contribute to susceptibility to discoloration of canned freestone peaches.

Luh et al. (1962) reported that discoloration of canned freestone peaches seems to be related to the chelation of anthocyanin with tin lining to form a stannous-anthocyanin complex which is blue to purple in color. The formation of such a complex causes the discoloration of the syrup and the peach tissue. During storage the anthocyanin fades away, while brown colored oxidation and polymerization products are formed.

Hsia et al. (1964) isolated leucoanthocyanidin from immature Elberta peaches by Counter-Current extraction. Cleavage with hydrochloric acid yielded cyanidin chloride and catechin. Alkali degradation of the resultant cyanidin chloride to protocatechuic acid and phloroglucinol further substantiates the cyanidin structure. They reported also that the peach leucocyan does not have a glycoside linkage, which was proven by a negative reaction with the glucostat enzymatic system, after leucocyan was hydrolyzed with dilute hydrochloric acid. They confirmed the presence of catechin and certain chlorogenic acids and their isomers in Elberta peaches. Traces of closely related but unidentified companion leucocyanidin compounds were found on chromatograms.



Hsia et al. (1965) studied anthocyanin from Elberta freestone peaches. The pigment was characterized by its absorption in the visible and infrared regions. Acid hydrolysis of the pigment yielded glucose and cyanidin, present in equal amounts on a molar basis. No intermediate glycoside was found when the pigment was hydrolyzed under mild conditions, indicating a monoglucoside structure. Alkali degradation of the aglycone yielded phyloroglucinol and protocatechuic acid, showing the presence of cyanidin in the pigment. The absorption peak of the pigment in a 0.01% methanolic HCl solution shifted from 525 to 568 mμ when aluminum chloride was added to form a chelate, indicating the presence of ortho-phenolic groups in the molecule. The peach anthocyanin is identified as a 3-monoglucoside of cyanidin.

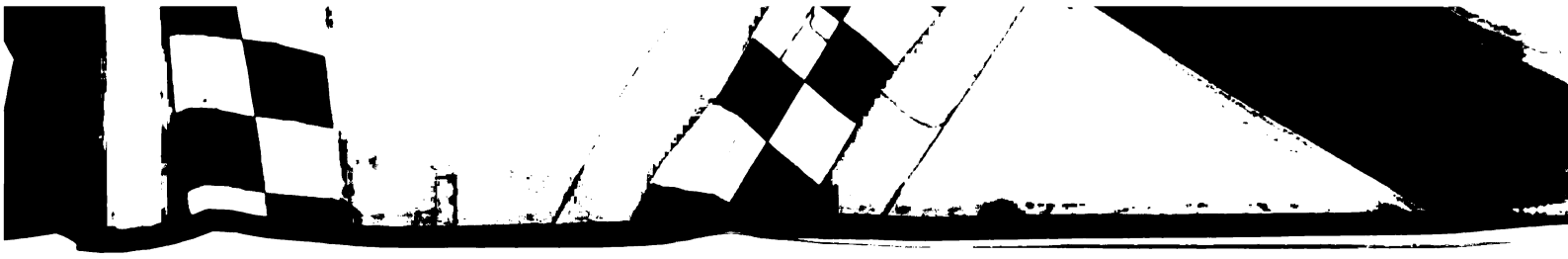
Griffith (1959) found that the principle browning substrate in edible banana is 3, 4-dihydroxyphenylethylamine. It was oxidized rapidly with banana PPO resulting in dark brown color. He reported also that the enzyme failed to oxidize the leucoanthocyanin present in banana.

Patil et al. (1956) reported that crude extracts of potato tubers contain three electrophoretically different polyphenol oxidases. One fraction had high cerolase activity and the other two showed catecholase activity, though they oxidized chlorogenic acid faster than catechol.

Bauchilloux et al. (1963) showed multiple forms of mushroom tyrosinase in homogenous state by a process involving preparative electrophoresis and chromatography on hydroxylapatite. Four enzymes, α, β, γ, and δ tyrosinase were obtained, the last three essentially pure. Although these enzymes possessed partially different activities toward mono- and o-diphenols, the three homogenous ones had very similar amino acid composition. Both cuprous and cupric copper were present in each enzyme.

Joslyn and Goldstein (1964) studied the changes in phenolic content of persimmons during ripening and processing with relation to astringency. They observed changes in astringency with changes in concentration of phenolic substances extractable with methanol and aqueous methanolic solution. Oxidation was found to be responsible for loss in astringency on air drying of sliced persimmons or high speed blending. Pureeing and freezing of astringent tissue resulted in loss of astringency and decrease in phenolics even in the presence of added ascorbic acid or sulfite. The leucoanthocyanin isolated from methanolic extracts of astringent tissue contained both leucodelphinidin and leucocyanidin, apparently being present together in the molecule.

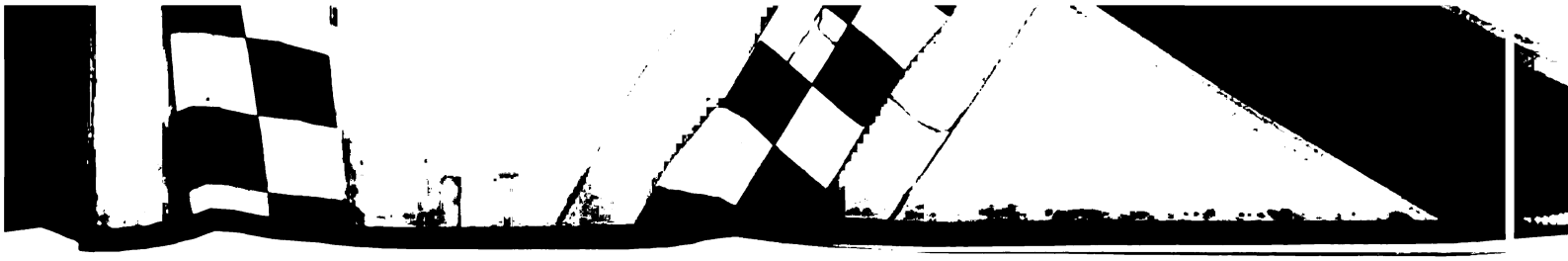
A widely held view of the function of polyphenol oxidase in plants is that it functions as the terminal oxidase in respiration. This was proposed by Boswell and Whiting (1938). They added



catechol to respiring potato slices and found a sharp increase in the rate of oxygen uptake followed by a gradual decrease to 33% of that with potato slices alone. Further addition of catechol caused no response, indicating that the polyphenol oxidase was inactive. Robinson and Nelson (1944) and Nelson (1945) reported that the enzyme tyrosinase is involved in plant respiration. They concluded that the 3-, 4-dihydroxyphenylalanine is the hydrogen carrier functioning adjacent to the terminal oxidase tyrosinase in a respiration chain present in sweet and common potato tubers.

Lamb and Sreerangachar (1940b) noted that a particular tea bush has leaves which did not undergo the typical polyphenolase catalyzed fermentation when crushed. They showed that this was due to the absence of polyphenolase; fermentation proceeded when an enzyme preparation from other tea leaves was added. Thus respiration is not necessarily related to polyphenolase in tea leaves.

In view of the contradicting reports about the role of polyphenol oxidase in plant respiration, it can only be stated that the role of the enzyme in plants remains unknown at present.

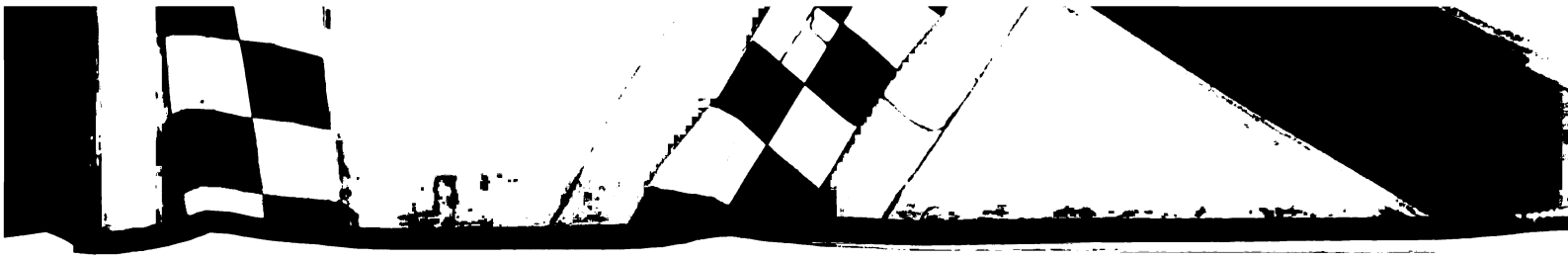


MATERIALS AND METHODS

Eight freestone peach varieties were obtained during the 1964 and 1965 seasons from orchards in the South Haven area. They were harvested at the firm ripe stage of maturity. The peaches were halved, pitted, packed in polyethylene bags under partial vacuum, sealed and stored at -17.7° C., 0° F. In preparation for analysis, 20 peaches of each variety were cut into thin slices and ground with a meat grinder at -17.7° C., 0° F. The ground frozen material was thoroughly mixed and stored at -31.6° C., -25° F. in polyethylene bags until used.

Enzyme Extraction

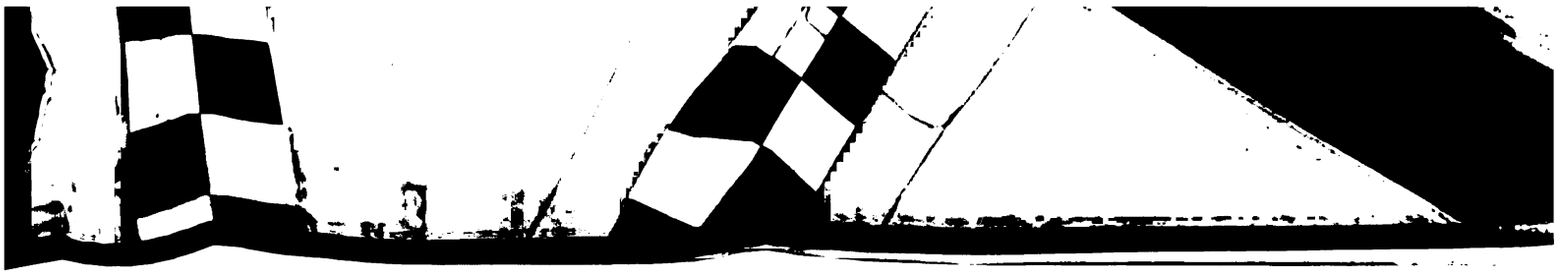
The crude enzyme solution was prepared by blending the peach tissue with cold acetone. Although initial studies indicated that the acetone extraction procedure resulted in about 15% more enzyme inactivation than the extraction with citrate-phosphate buffer, pH 7.0, acetone had the advantage of eliminating most of the phenolic substances that resulted in browning in the buffer extract. All extractions were made in a cold room at 1.1° C., 34° F.



One hundred grams of frozen peach tissue were blended for 5 minutes in a Waring blender with 200 ml. of -31.6° C., -25° F. acetone and centrifuged at 2200 rpm for 20 minutes. The supernatant liquid was discraded and the precipitate was reblended with 200 ml. 1.1° C., 34° F. distilled water for 3 minutes and centrifuged at 2200 rpm for 20 minutes. The supernatant liquid was removed, filtered, and 2.5 volumes of -23.3° C., -10°F. acetone were added. The mixture was allowed to stand for 5 minutes, then centrifuged at 2200 rpm for 15 minutes, and the supernatant liquid was discarded. The precipitated enzyme was dissolved in 30 ml. of 0.1 M citrate -0.2 M phosphate buffer pH 7.0, centrifuged at 10,000 rpm for 20 minutes to remove debris, and the supernatant liquid containing the crude polyphenol oxidase enzyme was filtered. The enzyme solution was pale, yellow and clear and had little or no peroxidase activity. Several preparations were pooled for further purification.

Fractionation by Ammonium Sulfate

Five-hundred ml. of crude enzyme solution were fractionated with ammonium sulfate. Sufficient solid ammonium sulfate was added to the enzyme solution with continuous mechanical stirring to make it 40% saturation. After 30 minutes the protein precipitate was removed by centrifugation at 10,000 rpm for 20 minutes and dissolved in cold citrate-phosphate buffer, pH 7.0 Successive additions of ammonium



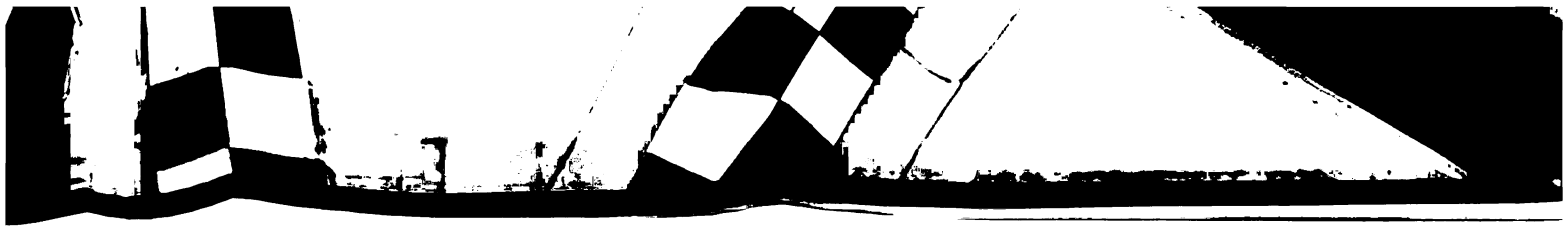
sulfate, collections of precipitate, and dissolving in cold buffer were carried out in the same way for 50, 60, 70, 80, and 90% saturation. The resulting enzyme solutions were desalted by passing over a column of Sephadex G-25, concentrated by dialysis against sucrose powder, and assayed for protein content and enzyme activity.

Enzyme Purification

The fraction with the highest specific enzyme activity from the ammonium sulfate fractionation was used for further purification. Sephadex G-100 was prepared by soaking in excess citrate-phosphate buffer, pH 7.0, for three days with occasional stirring. It was packed in a glass column (1.5 cm. X 85 cm.) to a height of 70 cm., equilibrated and washed with buffer for 72 hours at 34 degrees F. Two ml. of concentrated enzyme solution, having a protein content of 3.71 mg./ml. and a specific activity of 1600 units per mg., was charged gently at the top of the gel bed. It was eluted with citrate-phosphate buffer pH 7.0. Three ml. fractions were collected and assayed for protein content and polyphenol oxidase activity. The flow rate of the column was 13 ml. per hour for the buffer and 8 ml. per hour for the enzyme solution.

Protein Determination

The spectrophotometric method of Warburg and Christian (1942) was used and the protein content calculated by the Kalckar



(1947) formula:

1.45 absorbance at 280 mμ - 0.74 absorbance at 260 mμ

= mg. protein/ml.

Assay Procedure

The enzyme activity was determined by a modification of the tyrosinase assay method of Fox and Burnett (1958). The reactions were carried out in small, thin-walled test tubes (1 cm. x 7 cm.) in a 30° C., 86° F. water bath. The reaction mixture contained 1 ml. 0.03 M catechol (final concentration of 0.01 M substrate), 1.0 ml. 0.1 M citrate - 0.2 M phosphate buffer, 0.8 ml. distilled water and 0.2 ml. enzyme solution. The reaction was stopped by the addition of 0.2 ml. 0.001 M potassium cyanide. In all cases a blank of all reagents with inactive enzymes (potassium cyanide added to the enzyme) was used. Within one or two minutes, the tube contents were transferred to the 1 cm. light path spectrophotometer cells and absorbance at 420 mμ was determined.

One unit of enzyme activity represented the amount of enzyme that causes an increase of 0.001 in absorbance at 420 mμ per minute under the assay conditions.

Effect of pH on Enzyme Activity

The rate of catechol oxidation was determined over the pH range of 4.0 to 10 at 0.5 intervals at 30° C. A blank determination

was made at each pH to compensate for any autooxidation. The reaction mixture was the same as described in the assay procedure.

Effect of Temperature on Enzyme Activity

The enzyme activity was determined at temperatures from 15° to 70° C. at 5° intervals, using catechol as the substrate. The reactions were carried out in small, thin-walled (1 cm. x 7 cm.) test tubes in a thermostatically controlled water bath. The enzyme was added after the tube contents had attained the bath temperature. The reaction was stopped after 2 minutes with 0.2 ml. of 0.001 M potassium cyanide.

Enzyme Stability

Aliquots of the enzyme solution were adjusted to pH values from pH 4.0 to 10 at 0.5 intervals with citrate-phosphate buffer. The final volume at each pH was 60 ml., with protein content of 0.42 mg./ml. and specific activity of 750 units/mg. 15 ml. aliquots at each pH were transferred to rubber stoppered tubes and stored at -31.6°, -17.7°, 1.1° and 21° C., -25°, 0°, 34°, and 70° F. Enzyme activity determination was made at regular intervals for three months. To determine activity of these solutions they were thawed at 1.1° C., 34° F. and then refrozen. Some solutions were also stored at -31.6° and -17.7° C., -25° and 0° F. for three months

without thawing to compare with the other solutions for the effect of repeated thawing and freezing on enzyme activity.

Substrate Specificity

Monophenols as 0-, m-, and p-cresol, 2, 4-dimethylphenol, phenol, tyrosine, hydroquinone, resorcinol, orcinol, catechol, caffeic acid, D, L-dopa, dopamine and pyrogallol and polyphenols as chlorogenic acid, catechin, quercetin, quercitrin and rutin were used to determine their suitability as substrates for purified peach polyphenol oxidase. In the cases where no oxidation was immediately apparent, the reaction mixture was held for 2 hours to insure the absence of a long induction period. In addition a further study was made using larger amounts of enzyme and maximum substrate concentration.

The Michaelis constants and maximum velocities were calculated for all enzymatic reactions, plotting 1/activity against 1/substrate concentration (Lineweaver and Burk, 1934).

Extraction of Phenolic Compounds

One-hundred g. ground frozen peach tissue were dropped into 300 ml. of boiling 95% ethanol and boiled for 5 minutes to inactivate the enzyme systems. The mixture was transferred to a Waring blender and sufficient 95% ethanol added to obtain a final concentration of 70% ethanol. It was blended for 5 minutes, centrifuged 15 minutes at 2000 rpm and the supernatant decanted off. The

precipitate was suspended in 200 ml. of 95% ethanol, blended for 5 minutes, filtered and the filtrate pooled with previous extract. The ethanolic extract was concentrated under reduced pressure at 40° C. to about 50 ml. This concentrate was washed three times with 100 ml. petroleum ether to remove chlorophylls and carotenes and then extracted with 500 ml. of ethyl acetate. The ethyl acetate extract was concentrated under reduced pressure in a flash evaporator at 40° C. This extract was used for identification of the phenolic compounds by chromatography and spectrophotometric methods.

Total Phenolic Determination

Total phenolic concentration was determined by the Folin-Denis procedure (1915) as modified by Swain and Hillis (1959). A standard curve was prepared for catechin (Figure 1) and the results are reported as catechin equivalent. A suitable aliquot of the solution was diluted with distilled water to 8.5 ml. in a test tube. Five-tenths ml. Folin-Denis reagent was added and the contents were thoroughly mixed. Exactly three minutes later, 1.0 ml. of saturated sodium carbonate sulution was added and well mixed. After one hour the optical density was determined by a Beckman DU spectrophotometer at 675 mμ using a blank of water and reagents only. The solutions were filtered before optical density determination.

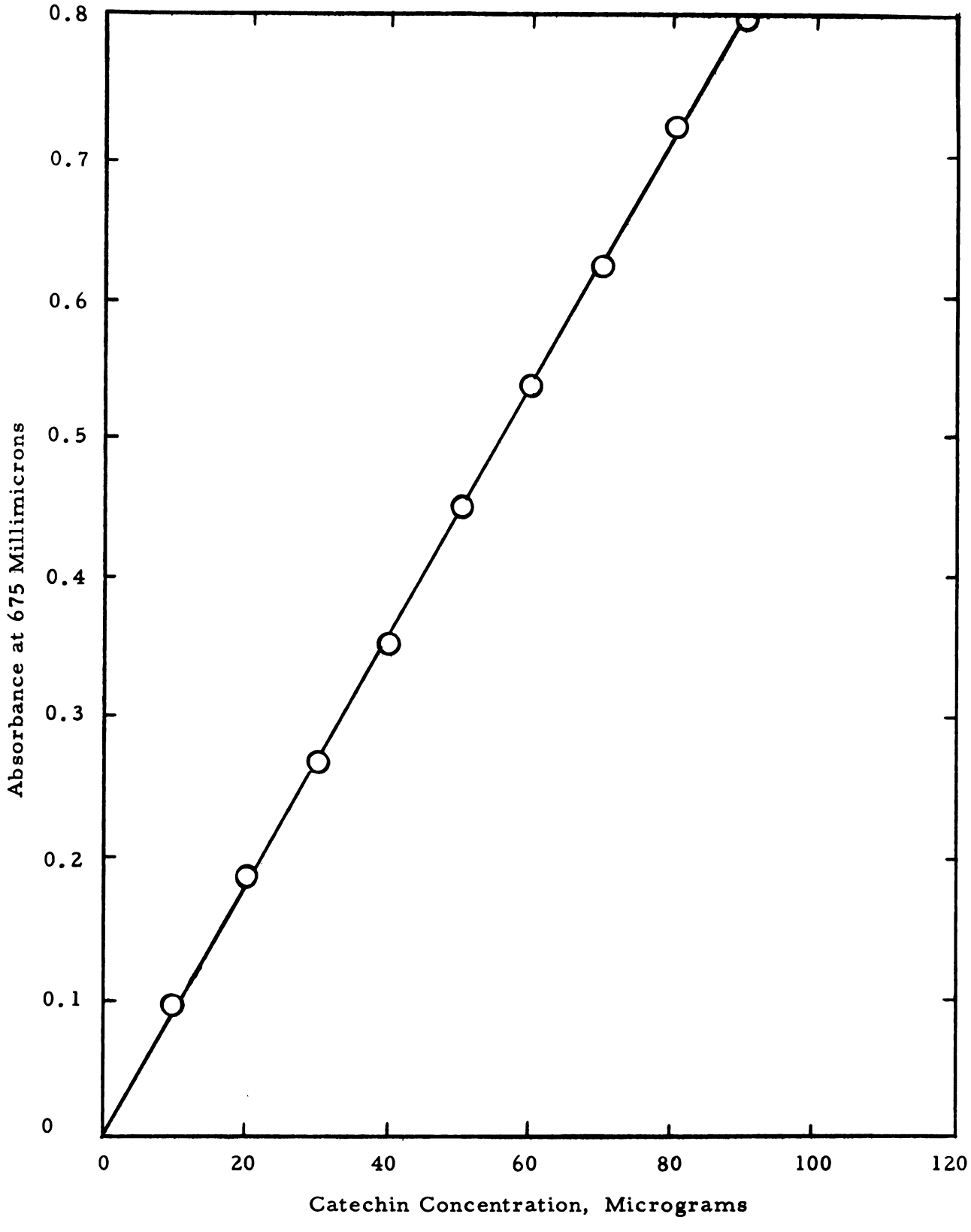
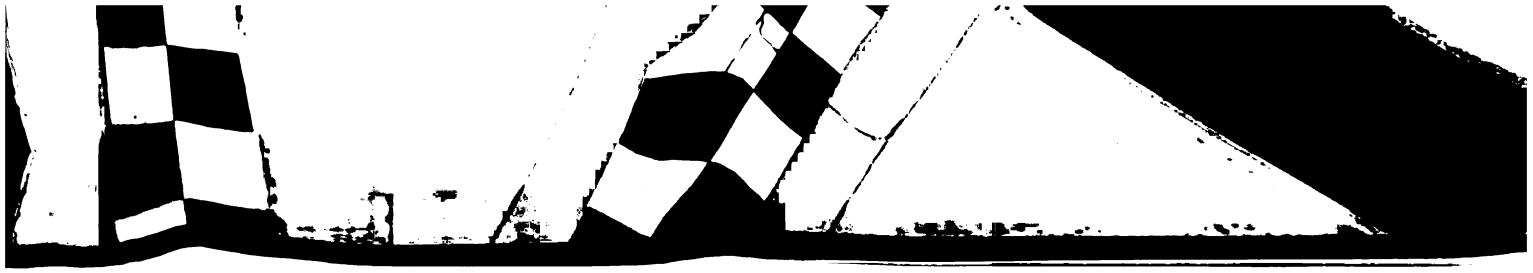


Figure 1. -- Folin-Denis pigment formation by catechin in a standard curve for phenolic determination.

Phenolic Oxidation

Enzymatic oxidation of phenolic substrates in peaches was achieved by blending peach tissue with citrate-phosphate buffer, pH 6.4, in a Waring blender.

Four 100 g. samples of ground frozen peach tissue were used for this experiment. One sample was extracted for total phenolic content as previously described and served as a control. The second sample was dropped into 100 ml. of boiling citrate-phosphate buffer, pH 6.4, and boiled for 5 minutes to inactivate enzymes. It was then transferred into a Waring blender and blended for an hour. Sufficient boiling 95% ethanol was added to obtain a final ethanol concentration of 70%. It was blended at high speed for 5 minutes, centrifuged at 2000 rpm for 20 minutes and the supernatant (ethanolic extract) was collected and concentrated under reduced pressure at 40° C. The third and fourth samples were blended with 100 ml. of citrate-phosphate buffer, pH 6.4, in a Waring blender for 30 and 60 minutes respectively. The blending speed was regulated by a variable transformer to keep the temperature in the range of 25.5° to 26.6° C., 78° to 80° F. and to obtain adequate areation of the mixture for enzymatic oxidation of the oxidizable phenolics by the endogenous enzyme in peaches. Total phenolic extraction was made on the blends as above. The ethanolic extracts were concentrated to identical volumes of 50 ml. These extracts were used for spectrophotometric analysis.



In addition, 50 g. samples of ground frozen peach tissue of four varieties (Elberta, Richhaven, Sunhaven, Redskin) were blended with 50 ml. of citrate-phosphate buffer, pH 6.4, for 0, 15, 30, 45, 60, 75, and 90 minutes respectively. Blending was carried out on these samples as previously described. Total phenolic determination was made on the ethanolic extracts from different samples at different oxidation times. The ethanolic concentrates were washed with petroleum ether and extracted with ethyl acetate. The ethyl acetate extracts were concentrated under reduced pressure at 40° C. to identical volumes of 55 ml. These concentrates were used later for paper chromatography.

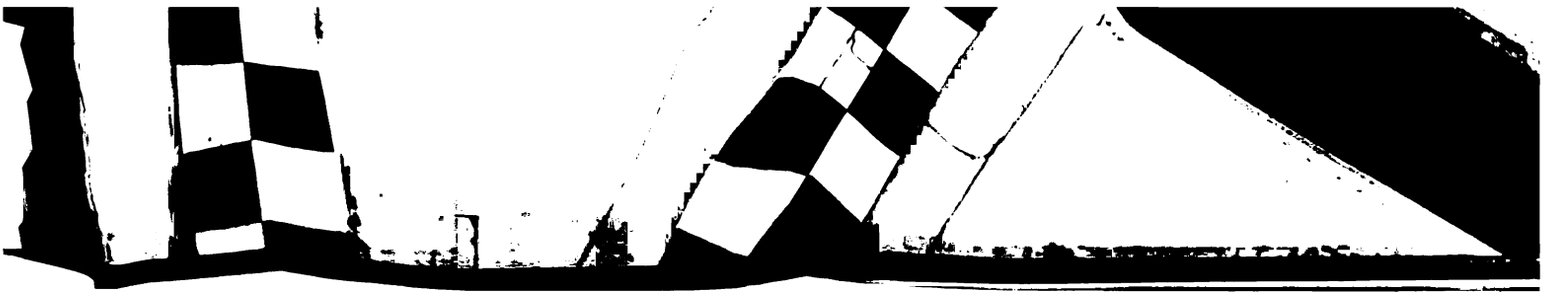
Spectrophotometric Analysis

Beckman DU and Bausch and Lomb 505 spectrophotometers were used in this study. All phenolic substances exhibit intense absorption in the ultraviolet region. The ultraviolet absorption of the ethanolic extracts of the control and the third and fourth samples of the oxidation study were determined and compared. The ultraviolet absorption was determined on 0.2 ml. aliquots of the extracts diluted to 10 ml. with 95% ethanol using ethanol as a blank. Spectrophotometry was also used in isolation and purification of the phenolic substances from peach extract along with paper chromatography.

Paper Chromatography

Paper chromatography was used for detection, isolation and identification of polyphenol oxidase substrates in peach extracts. The ethyl acetate extracts were chromatographed using the ascending method. The chromatograms were developed using the upper phase of n-butanol-acetic acid-water (4:1:5, V/V) for 20 hours at 20° - 22° C. They were then dried in an air current at 20° C. for 2 hours.

For the isolation and identification of the peach phenolics, one cm. wide streaks (about 15 ml. ethyl acetate concentrate) were placed on Whatman No. 3 chromatographic paper, 22" x 18" and developed. The chromatograms were examined under UV light at 366 mμ before and after exposure to ammonia vapor, the fluorescent areas marked and their R_f values determined. Small side strips were cut from the paper and were treated with a freshly prepared mixture of 0.3% $FeCl_3$ - 0.3% $K_3Fe(CN)_6$ for color development. These were then matched with the center portion of the paper, and the bands or combination of bands were cut out. These were then eluted with about 300 ml. 95% ethanol at 20° C. for 24 hours. The elutes were concentrated under reduced pressure at 40° C. and representative aliquots were examined spectrophotometerically to determine their maximum absorbance in the ultraviolet region. The concentrates of the different fractions were also rechromatographed on Whatman No. 1 chromatographic 6" x 22" strips. Those

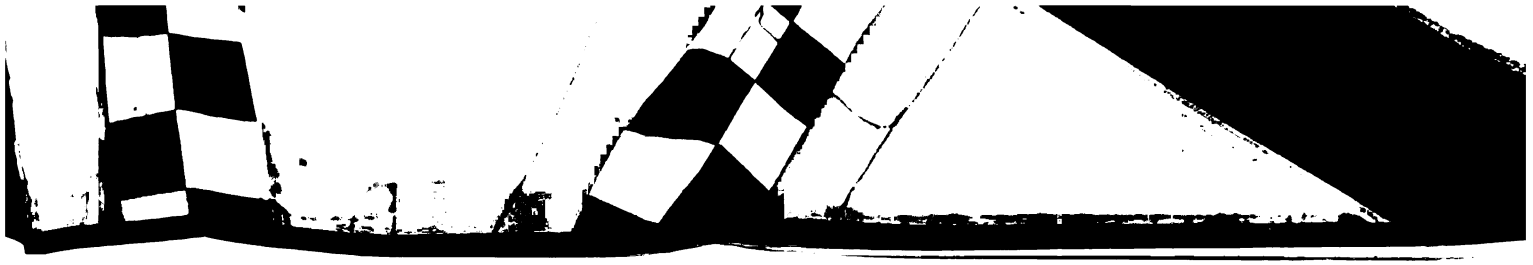


having R_f values of 0 to 0.4 were developed with BAW for 20 hours at 20° C. and those with R_f values of 0.4 to 0.7 with 5% acetic acid for five hours at 20° C. Representative chromatograms were sprayed with 0.5N NaOH, 0.6 N HCL in propanol, Folin-Denis reagent followed by 10% Na_2CO_3, diozotized sulfanilic acid (DSA), 3% paratoluene sulfonic acid (PTSA), mixture of 0.3% $FeCl_3$ and 0.3% $K_3Fe(CN)_6$, saturated sulution of 3, 5-dinitrosalsylic acid in 50% ethanol containing 2% NaOH (DNSA), and 5% $AlCl_3$ for identification and comparison with known compounds. From other representative chromatograms individual bands were cut out and eluted with 95% ethanol for 24 hours. The elutes were concentrated and their absorbance determined and compared with those of known compounds.

For detection of the enzymatically oxidizable substances in peach extracts, aliquots of non-oxidized and oxidized (blended for 2 hours) peach extracts were applied on No. 1 Whatman chromatographic strips 6" x 22", developed with BAW for 20 hours and dried. They were examined under UV light at 366 mμ before and after exposure to ammonia vapor. Some were sprayed with a mixture of 0.3% $FeCl_3$ and 0.3% $K_3Fe(CN)_6$ for color development and the R_f values of the different bands determined. Representative strips were sprayed with partly purified peach polyphenol oxidase after a light spray with citrate-phosphate buffer, pH 6.4, and kept in a humid chamber for 18 hours at 20° - 22° C. and examined for color development as indicative of enzymatic oxidation.

To investigate the activity of peach polyphenol oxidase toward the different components of peach extract, 2 ml. aliquots of ethyl acetate extract with total phenolic content of 2.2 mg. were applied on No. 1 chromatographic paper, 6" x 22", developed with BAW for 20 hours and dried. Successive strips of each papergram were cut out according to R_f values. The strips were eluted with 95% ethanol for 24 hours. The elutes were concentrated under reduced pressure at 40° C. to identical volumes of 10 ml. Total phenolic determination was made on the elutes from the different strips. The enzyme activity toward the elutes was determined spectrophotometerically as the increase in absorbance at 420 mμ. The reaction mixture contained 2 ml. elute, 1 ml. 0.1 M citrate -0.2 M phosphate buffer, pH 6.4 and 0.2 ml. enzyme was added with good mixing. The reaction was carried out for 30 minutes at 30° C.

The progress of the enzymatic oxidation of four peach varieties by their endogenous polyphenol oxidase was followed quantitatively. Aliquots of 2 ml. of ethyl acetate extracts of the different samples at different oxidation times (from the phenolic oxidation study) were chromatographed on No. 1 chromatographic papers, 6" x 22", developed with BAW for 20 hours and dried. Representative chromatograms were examined under UV light at 366 mμ and sprayed with different reagents for color reactions. Three sets of



chromatograms representing samples were cut out into strips according to R_f values. The strips were eluted with 95% ethanol for 24 hours at 20° C. and the elutes concentrated under reduced pressure at 40° C. to identical volumes of 10 ml. Total phenolic determination was carried out on the different elutes using an aliquot of 2 ml.

RESULTS AND DISCUSSION

Maximum enzyme activity was obtained in the fraction precipitated by 60% ammonium sulfate. The specific activity was double that of the original solution. No enzyme activity was found in the fractions precipitated below 40% or above 80% saturation (Table 1) and about 35% of the original enzyme activity was lost in the fractionation process.

Table 1.--Fractionation of the enzyme solution with ammonium sulfate

Salt conc. g./ml.	Volume ml.	Protein Content mg./ml.	Enzyme Activity units/ml.	Specific Activity units/mg.
0.4	50	1.50	-	-
0.5	50	1.10	400	440
0.6	50	0.51	820	1607
0.7	50	0.43	310	720
0.8	50	0.20	60	300
0.9	50	-	-	-

The pattern of protein separation and enzyme activity as fractionated on a Sephadex G-100 column is shown (Figure 2). Most of the inactive protein emerged first from the column. The first

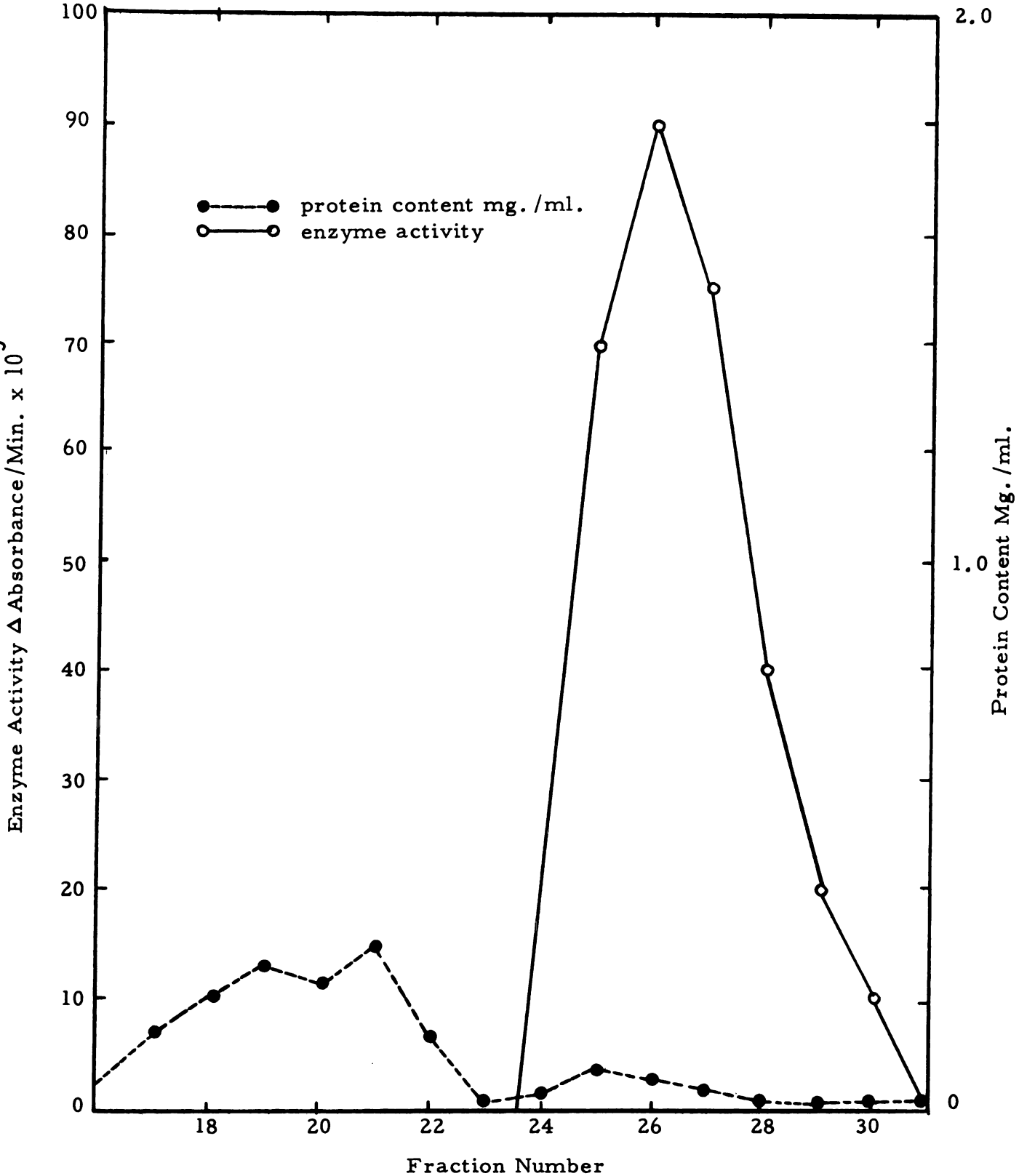


Figure 2. -- The chromatography of partly purified peach polyphenol oxidase on Sephadex G-100 column (1.5 x 70 cm.).

enzyme activity appeared in fraction 24 and continued till fraction 30. The highest enzyme activity was found in fraction 27 with a specific activity of 8000 units/mg. This was about a tenfold purification. The recovery of enzyme activity from the Sephadex column was 75%. No further separation was obtained by passing fraction 27 through the Sephadex column a second time and both protein and enzyme activity emerged as one peak.

The enzyme extracts from the eight freestone peach varieties showed the same pattern of protein separation and enzyme activity in the fractions. Elberta peaches were found to have the highest enzyme activity and Redskin the lowest level of activity, about 56% of that of the Elberta. The other varieties ranged between these two (Table 2).

The enzyme extracts from all varieties showed the same substrate specificity. The activity of the crude enzyme preparations from all varieties toward catechol is shown (Figure 3).

Effect of pH and Temperature on Enzyme Activity

The rate of catechol oxidation (increase in absorbance at 420 mμ) for a given amount of enzyme was found to be markedly dependent on the pH of the system (Figure 4). The enzyme is only 5.5% as active at pH 4.0 as it is at pH 6.5. It is worth noting that while the enzyme is most stable at pH 8.0, it is most active at pH 6.4.

Table 2. --Comparison of enzyme activity of the different varieties of peaches.

Variety	1964 Season		1965 Season	
	Enzyme Activity Δabsorbance at 420 mμ X 10^3/min**	No. of Enzyme units/100 gm.*	Enzyme Activity Δabsorbance at 42°mμ X 10^3/min**	No. of Enzyme units/100 gm.*
Elberta	78	17,820	66	16,640
Richhaven	57	14,210	53	13,200
Kalehaven	51	12,730	50	12,240
Sunhaven	44	11,290	48	11,880
Fairhaven	43	10,970	46	11,660
Redhaven	40	10,000	44	10,650
Halehaven	38	9,360	42	10,400
Redskin	36	9,000	38	9,180

*Enzyme unit: The amount of enzyme that will cause a change in absorbance of .001 per minute under the standard assay conditions.

**The reaction mixture contained 0.2 ml. enzyme solution, 1 ml. of 0.003M catechol, 1 ml. of 0.01 M citrate-0.02 phosphate buffer at pH 6.4, and 0.8 ml. distilled water.

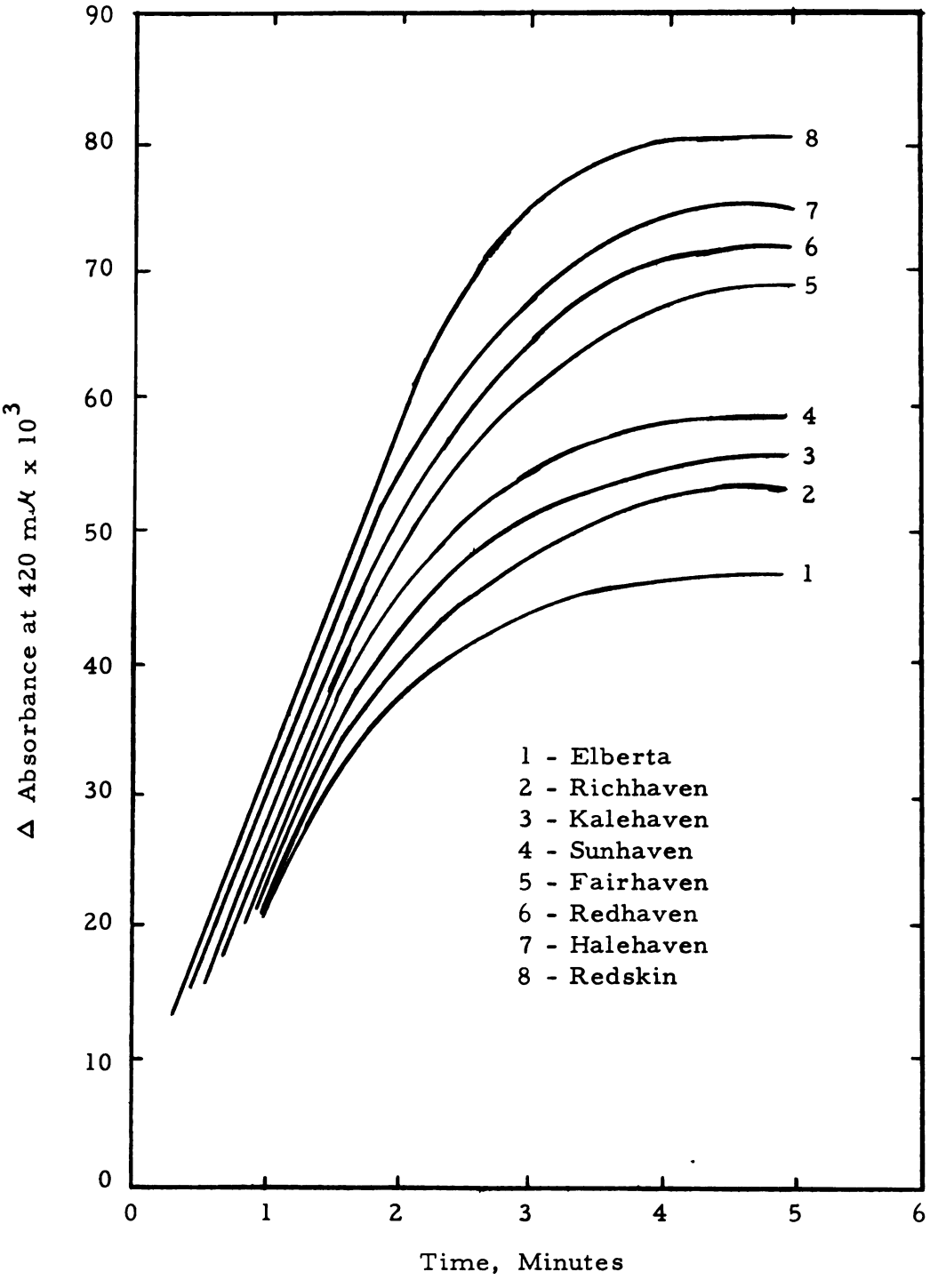


Figure 3. -- Enzyme activity of the crude enzyme preparations (0.001 M catechol, 0.1 ml. enzyme and citrate-phosphate buffer, pH 6.4).

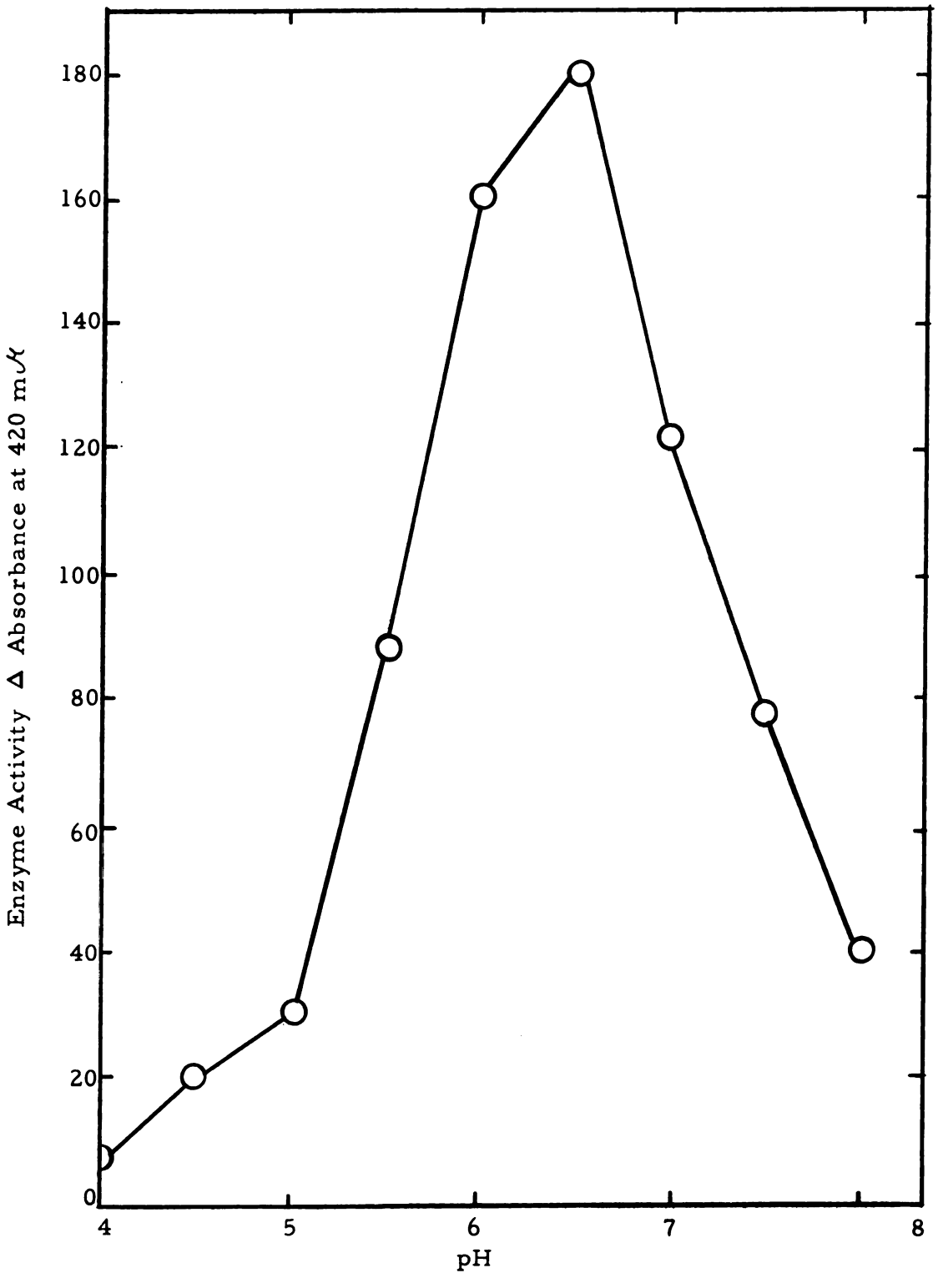


Figure 4.--Influence of pH on peach PPO activity.

The maximum activity of the peach PPO in citrate-phosphate buffer was found to be in the pH range of 6.3 to 6.6 with optimum activity at pH6.4. Reyes and Luh (1960) reported an optimum pH range of 5.9 to 6.3 for Elberta PPO using a crude peach extract containing peroxidase and PPO.

Peach PPO showed optimum activity at 42° to 44° C. (Figure 5). The activity at 70°C. was about 13% of that at 44° C. and no activity was detected at higher temperatures. Ponting et al. (1948) reported an optimum temperature of 40° C. for PPO from apples.

Stability of the Enzyme

The enzyme was most stable at pH 8.0 at all of the temperatures studied. At room temperature, 20° C. (68° F.) there was no decrease in enzyme activity during the first 24 hours and only a 2.6% decrease occurred after 48 hours. Activity decreased rapidly thereafter and there was a 93.3% loss after 6 days storage. The samples stored at 1.1° C., 34° F. showed a continual loss in activity during storage and at the end of 12 weeks the loss was 83.3% (Figure 6). The frozen samples stored at -17.8° C., 0° F. and -31.7° C., -25° F. for 12 weeks showed a loss in activity of 20 and 16.6% respectively. The samples that were thawed and refrozen periodically during the twelve weeks had a continual loss in activity, and at the end of 12 weeks, the activity losses were 66.6 and 60% respectively (Table 3).

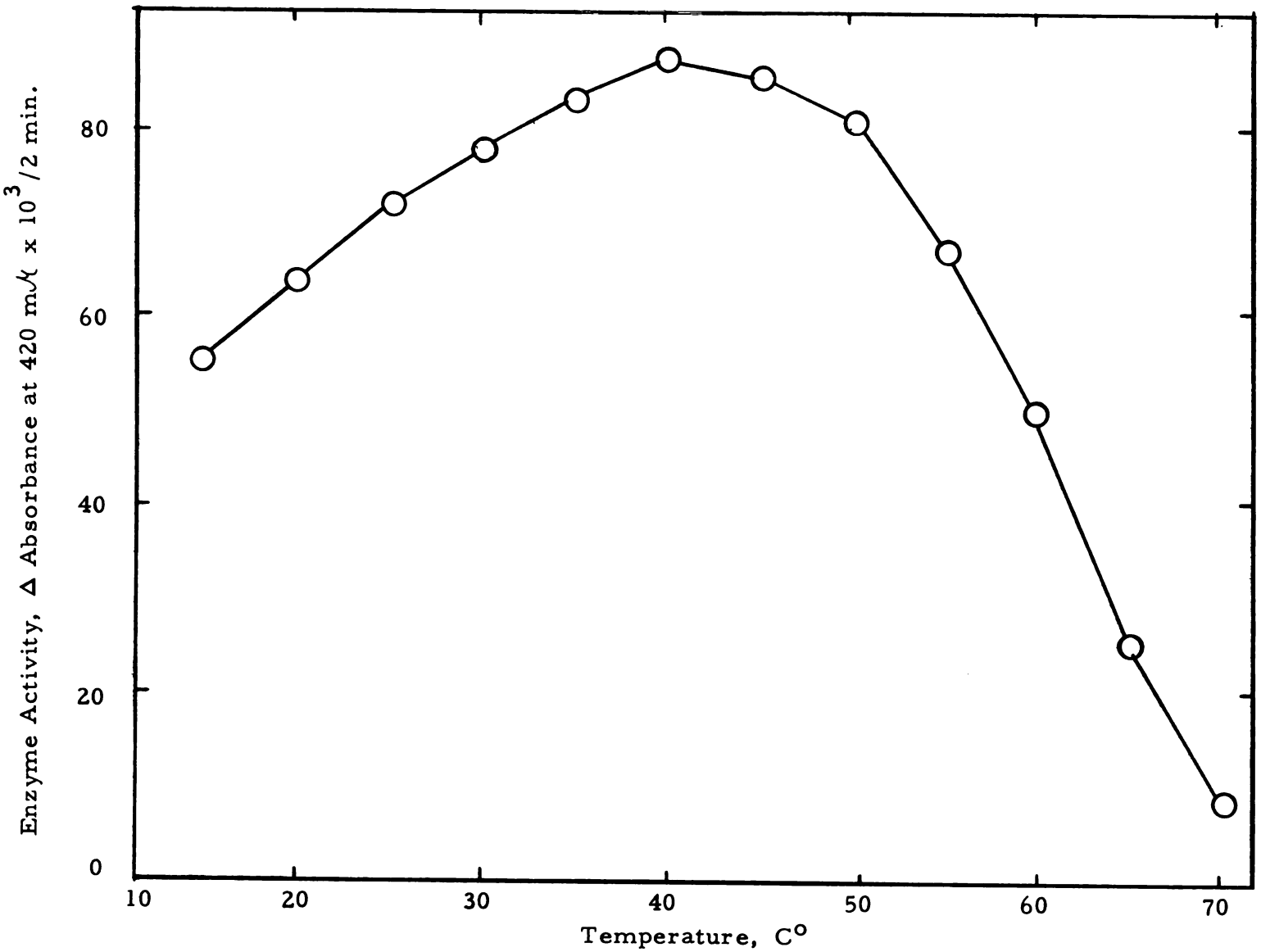


Figure 5. -- Influence on temperature on peach PPO activity.

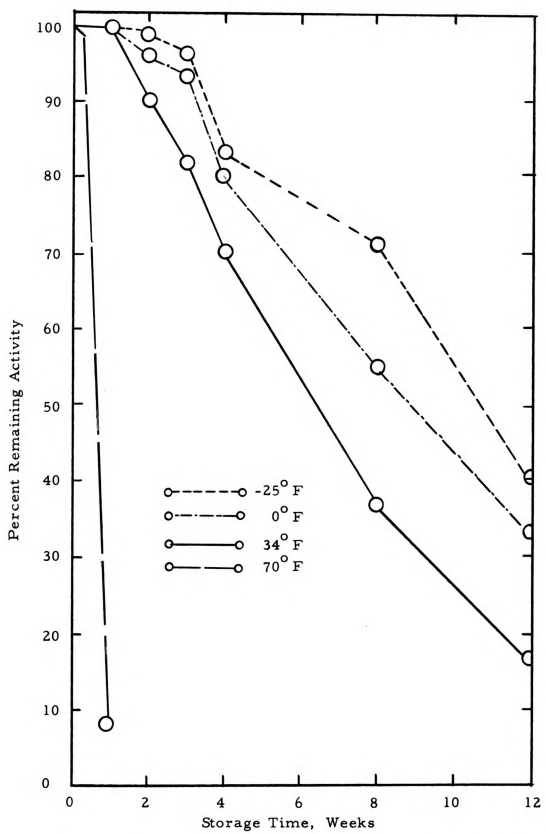


Figure 6.--Stability of crude peach PPO at different storage temperatures, and pH 8.0.

Table 3. -- Effect of storage on enzyme activity at 12 weeks.*

Temperature °F.	Treatment	pH 4	5	6	7	8	9	10	Percent Loss at pH 8.0
		Enzyme Activity**							
34		5	5	14	32	62	21	5	58.66
0	Continuous freezing	15	20	41	72	120	40	17	20.00
0	Frozen & thawed	5	5	10	26	50	17	5	66.60
-25	Continuous freezing	20	23	46	78	125	47	21	16.66
-25	Frozen & thawed	5	5	15	31	60	17	5	60.00

* The original activity of the enzyme solution was 150.

** Standard assay procedure.

Substrate Specificity

Monophenols with the ortho-dihydroxy configuration such as catechol, caffeic acid, D, L-dopa, L-arterenol, pyrogallol, and dopamine acted as substrates for the enzyme, though varying in effectiveness. Other monophenols such as hydroquinone, resorcinol, orcinol, o-, m-, and p-cresol and L-tyrosine were not oxidized. Catechin and chlorogenic acid, polyphenolic compounds containing the ortho-dihydroxy grouping in their molecules were natural substrates for the enzyme and were present in peaches. Other polyphenols, as quercetin, quercitrin and rutin, did not serve as substrates, although they have the ortho-dihydroxy groupings. Apparently the side groupings on the phenolic ring adjacent to the one carrying the ortho-dihydroxy configuration interfered or prevented the enzyme from getting its active sites into the right orientation for attacking the molecule.

Michaelis constants and maximum velocities for the enzyme reactions with the substrates in decreasing reactivity rates are given (Table 4). These values were calculated by the method of Lineweaver and Burk (1934). A reaction curve for catechol is shown (Figure 7).

It was also noted that the enzyme became inactivated as it catalyzed the oxidation of its substrates. All of the data reported were obtained during the first two minutes before any significant inactivation took place. Inactivation when the enzyme reacted with catechol was

noted after about five minutes. This inactivation as the reaction proceeds is referred to as reaction inactivation, Ingraham (1954).

Table 4.--Michaelis constant and maximum velocities for purified peach polyphenol oxidase.*

Substrate	Km, M	V max, M
Caffeic acid	8.0×10^{-4}	2.63×10^{-1}
Catechin	1.0×10^{-3}	3.58×10^{-1}
D, L-arternol	1.2×10^{-3}	2.42×10^{-1}
Chlorogenic acid	2.2×10^{-3}	5.26×10^{-2}
Pyrogallol	2.9×10^{-3}	6.66×10^{-2}
Catechol	9.0×10^{-3}	3.33×10^{-1}
Dopamine	8.0×10^{-3}	1.96×10^{-1}
D, L-dopa	2.5×10^{-2}	2.00×10^{-1}

*Calculated by the method given by Lineweaver and Burk (1934).

Total Phenolic Compounds

Richhaven variety had the highest phenolic content, 173.2 mg./100 g. and Sunhaven the lowest phenolic content, 85.4 mg./100 g. (Table 5). No linear relationship was found between the total phenolic content and the amount enzymatically oxidized. Guadagni et al. (1949) reported the existence of a straight-line relationship between total and enzymatically oxidizable tannins in the range of 32 to 194 mg. per 100 grams of peaches. However there was a direct relationship between the per cent of phenolics oxidized and enzyme activity of the peach varieties (Table 6).

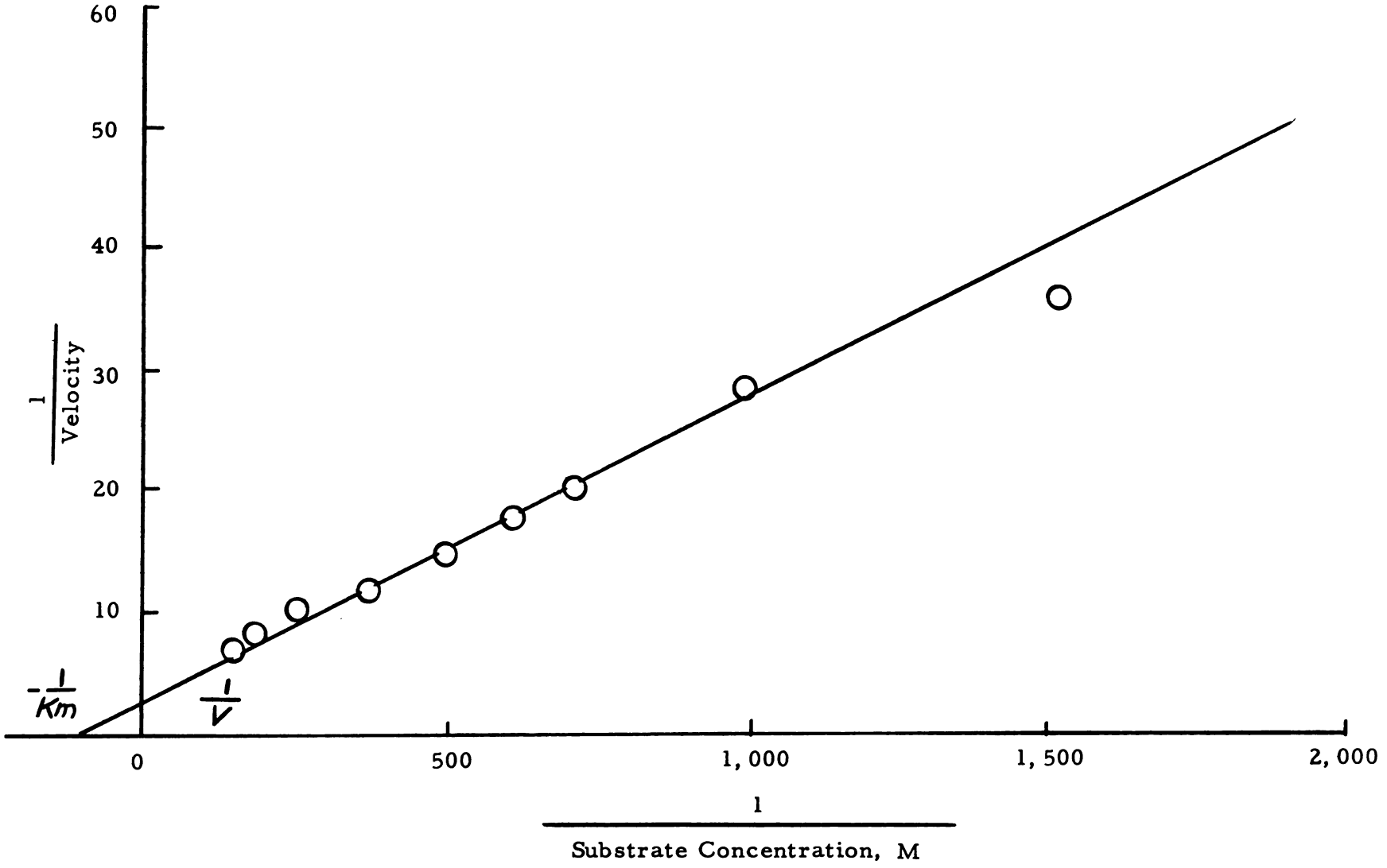


Figure 7. -- A reaction curve of peach PPO with catechol by using the Lineweaver and Burk procedure (1934).

Table 5.--Total extractable phenolics of the different varieties of peaches for the 1964 and 1965 seasons.

Variety	1964 Total Phenolics mg./100 g.*	1965 Total Phenolics mg./100 g.
Richhaven	141.9	173.2
Kalehaven	138.6	160.0
Halehaven	125.4	135.2
Fairhaven	104.0	122.0
Elberta	99.8	126.0
Redskin	78.2	94.4
Redhaven	75.4	86.6
Sunhaven	62.4	85.4

*Calculated as mg. of catechin per 100 g. peach tissue.

Table 6.--Oxidation of extractable phenolics of 100 g. of peach tissue from different varieties, blended with 100 mg. of distilled water for 15 minutes.

Variety	Number Enzyme units/100 g.	Total Phenolics		Percent Oxidation
		Before Oxidation	Before Oxidation	
Elberta	16,640	126.0	52.42	58.4
Richhaven	13,200	173.2	80.36	53.6
Kalehaven	12,240	160.0	77.60	51.5
Sunhaven	11,880	85.4	36.70	48.7
Fairhaven	11,660	122.2	65.23	46.7
Redhaven	10,650	86.6	41.40	45.6
Halehaven	10,400	135.2	90.60	44.6
Redskin	9,180	94.4	51.00	43.4

Detection of Enzyme Substrates in Peach Extract

One dimensional paper chromatograms of the peach extract with BAW showed the presence of 8 bands when sprayed with $FeCl_3$ - $K_3Fe(CN)_6$, (Figure 8). Maximum blue color developed in bands 1, 2, and 7 (Table 7). Under ultraviolet light, bands 5 and 6 emitted a blue fluorescence which turned green when exposed to ammonia vapor. Band 7 emitted pale blue fluorescence under UV light and ammonia vapor while band 8 appeared as a dark shade. When the chromatograms were sprayed with partly purified peach PPO, the bands showed brown to pale yellow color development in the following order; 1, 2, 7, 6, 5, and 8. Bands 3 and 4 occasionally showed a very pale yellow color.

Chromatograms of the oxidized peach extract (blended with buffer, pH 6.4 for 2 hours to allow oxidation with endogenous enzyme) showed less blue color development in bands 1, 2, 3, and 4 and no color development or fluorescence in the areas corresponding to bands 5, 6, 7, and 8. No color development was observed on the chromatograms of the oxidized peach extract when sprayed with enzyme. This showed that not all of the substances represented by the first 4 bands were completely oxidized by the endogenous peach PPO while those of the latter 4 bands were completely oxidized. These results indicated the presence of 3 major and at least two minor compounds capable of being oxidized by the peach PPO with the development of yellow to

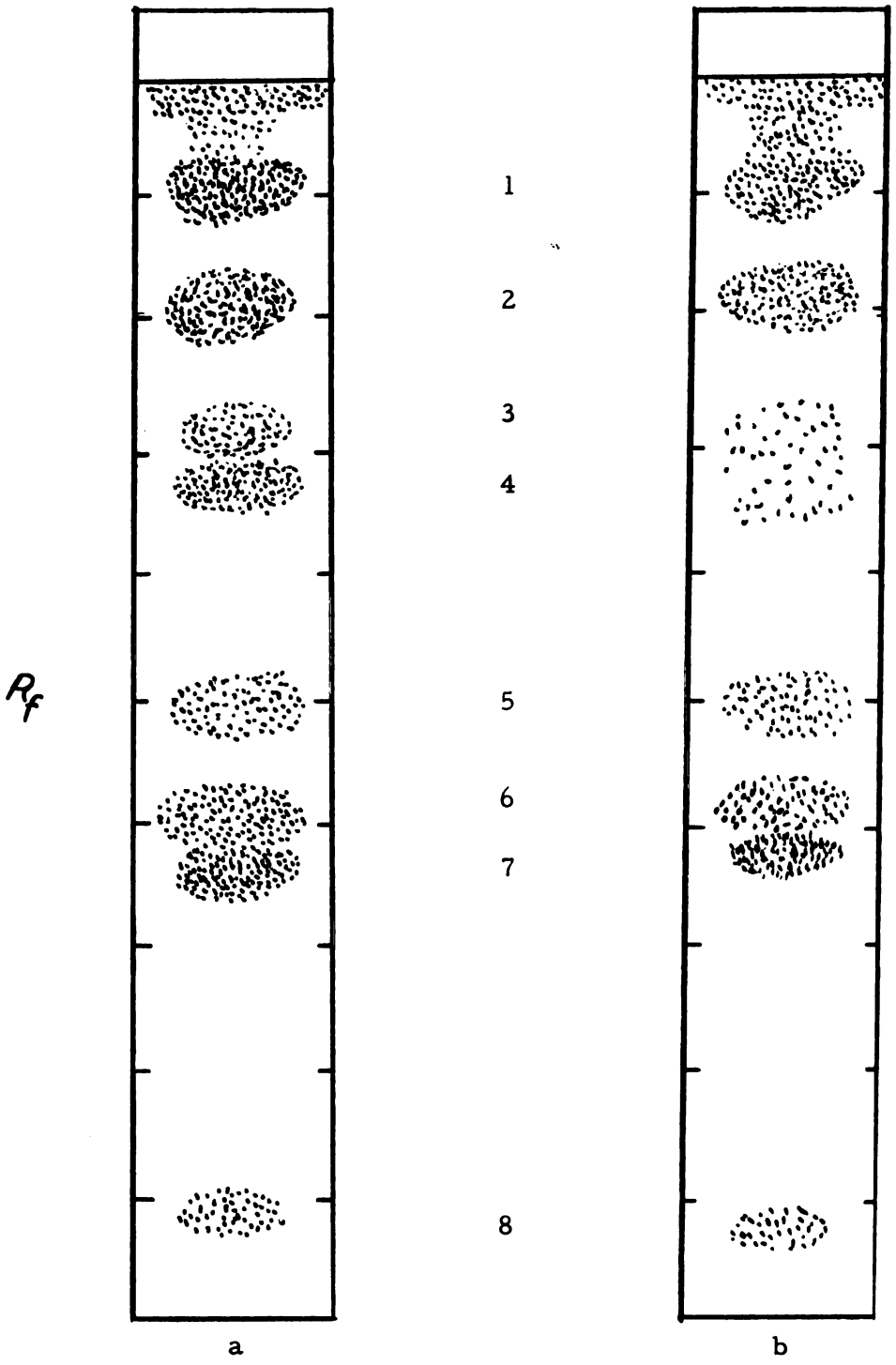


Figure 8. -- Paper chromatograms of Elberta peach extract

a - Treated with $FeCl_3$-$K_3Fe(CN)_6$

b - Sprayed with partly purified peach PPO

brown color. Siegelman (1954) stated that a browning reaction on the chromatogram after spraying with PPO was not conclusive evidence of the participation by this substance in the browning reaction, although he gave no supporting evidence.

Table 7.--Description of the phenolic component of Elberta peach extract as separated on one dimensional paper chromatograms.

Band Number	R_f Value	Treatment	
		$FeCl_3$-$K_3Fe(CN)_6$	Peach Enzyme
1	.11	very dark blue	brown
2	.19	very dark blue	brown
3	.28	light blue	-
4	.32	light blue	-
5	.51	dark blue	yellow
6	.58	dark blue	yellow
7	.63	very dark blue	brown
8	.93	light blue	pale yellow

Total Phenolic Oxidation by Endogenous Enzymes

For total phenolic extraction, boiling the peach tissue in 95% ethanol or citrate-phosphate buffer, pH 6.4, were found equally effective in preventing enzymatic oxidation of the phenolic compounds. There also was no autooxidation of the phenolic compounds when the boiled samples were blended in a Waring blender for 60 minutes (Table 8). The samples blended with unheated buffer for 30 and 60

minutes, respectively, showed extreme browning and 73% oxidation of the phenolics that can be attributed to enzyme activity.

Table 8.--Effect of blending heated and unheated samples of peach tissue on total phenolic content.

Treatment	Total Phenolics mg./100 g. peach tissue
1. Extracted with 95% ethanol non-oxidized, unheated	126.70
2. Extracted with 95% ethanol after being boiled in buffer and blended for an hour	126.10
3. Extracted with 95% ethanol after being blended with buffer for an hour	34.00

The ultraviolet absorbance spectra of the phenolic extracts of the samples are shown (Figure 9). The control sample showed two absorption maxima at 280 mμ and 324-328 mμ. The spectra of the samples, blended for 30 and 60 minutes respectively, showed no maximum absorption at 326 mμ, and therefore the substances originally present were apparently completely enzymatically oxidized. There was also a very marked decrease in absorption at 280 mμ indicating that these substances were also oxidized, but their oxidation was not as complete as those at 326 mμ. This indicated that either they oxidized at a slower rate or that not all of the substances were oxidized. Since the amount of oxidation at 60 minutes was not much greater than

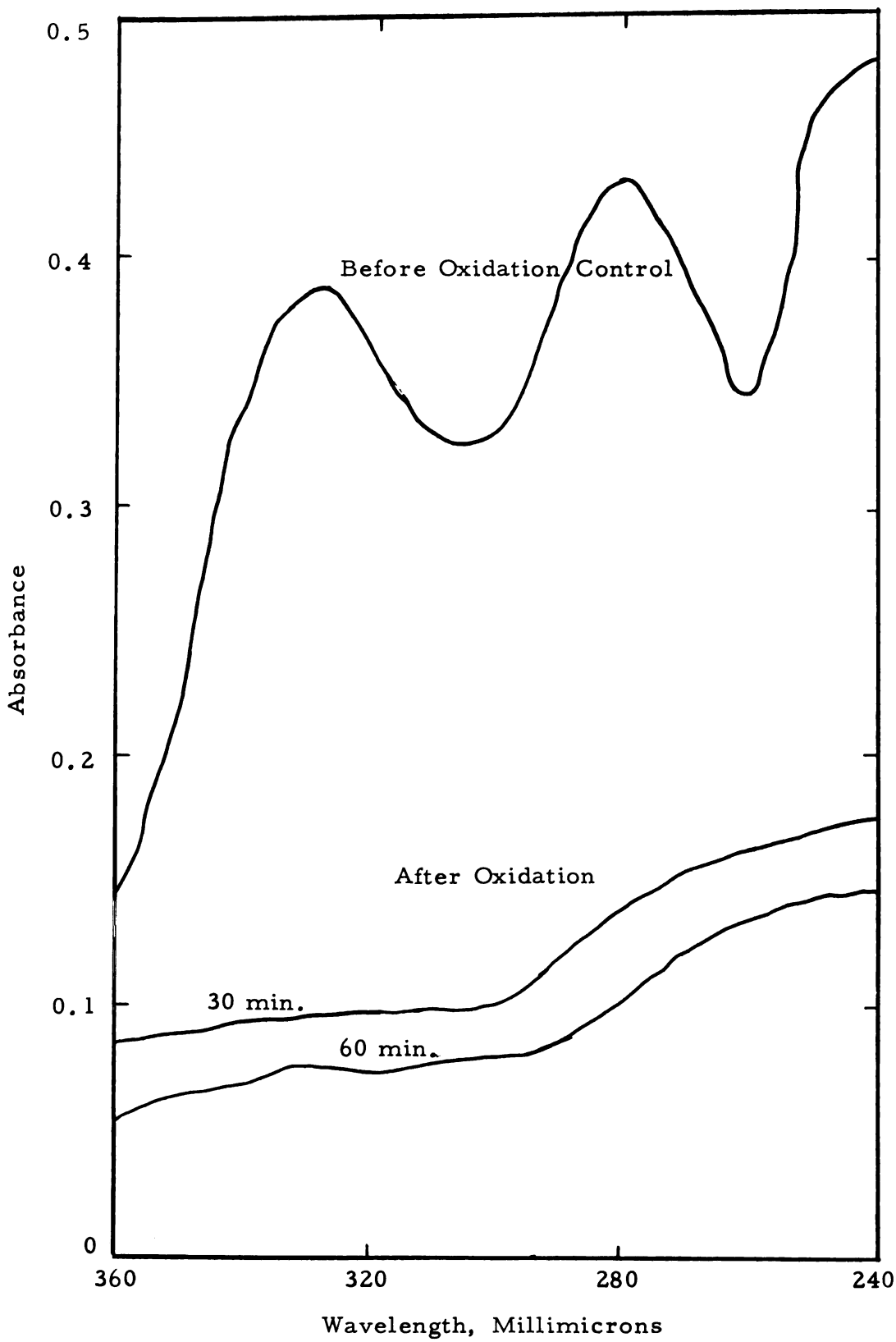


Figure 9. -- Ultraviolet spectra of peach phenolic extract before and after oxidation with endogenous PPO.

that for 30 minutes, it is apparent that most of the oxidizable substances were oxidized fairly rapidly during the initial period of blending.

Further studies on the rate of phenolic oxidation by the endogenous enzymes showed that maximum oxidation occurred during the first 15 minutes (Table 9). It would appear that the enzyme became less active or the remaining oxidizable phenolic substances had become limiting factors. The total amount of oxidation after 90 munutes was not significantly greater than that at 60 minutes. Guadagni et al. (1949), using a similar method of oxidation, reported that the amount of tannins oxidized in 24 hours was not appreciably greater than the amount oxidized in one hour.

Isolation and Identification of Peach Phenolics

Paper chromatograms of the ethyl acetate extract of peaches developed with BAW showed the presence of 8 spots on treatment with $FeCl_3$ - $K_3Fe(CN)_6$. Their R_f values were 0.12, 0.20, 0.28, 0.33, 0.51, 0.60, 0.63 and 0.93 respectively. These will be referred to as spots 1 to 8 in order of increasing R_f values. The color reactions of spots 1 and 2 with the different spray reagents used were typical of those reported for leucoanthocyanins and those of spots 3 and 4 for flavonols, Bate-Smith (1948 and 1954), Harborne (1961)

Table 9. -- Phenolic oxidation of peach tissue of different varieties by endogenous enzymes.

Oxidation Time in Minutes	Variety							
	Elberta		Richhaven		Sunhaven		Redskin	
	Total Phenolics mg./50 g.	Percent Oxidized	Total Phenolics mg./50 g.	Percent Oxidized	Total Phenolics mg./50 g.	Percent Oxidized	Total Phenolics mg./50 g.	Percent Oxidized
0	62.7	0	86.6	0	42.8	0	49.6	0
15	27.5	56.1	42.3	51.2	22.2	48.2	27.8	44.0
30	20.6	65.5	32.2	62.8	20.1	53.3	23.7	52.2
45	18.8	70.4	27.3	68.6	18.2	57.6	23.2	55.4
60	15.5	75.3	25.8	70.0	14.9	65.1	21.4	56.2
75	14.6	76.8	24.6	71.6	13.3	67.9	20.8	59.6
90	13.9	77.9	24.1	72.2	13.2	63.0	20.2	60.0

and Craft (1961), (Table 10). The combined elute of the 4 spots had a maximum absorption peak at 280 mμ. The elutes of the individual spots showed a broad absorption maxima between 275 and 283 mμ, (Figure 10). These absorption maxima have been reported for leucoanthocyanins by Hsia et al. (1964). When the elutes from spots 3 and 4 were treated with concentrated sulfuric acid they developed a yellow color that fluoresced at 366 mμ. On addition of aqueous NaOH, a dark yellow color developed that turned brown on standing. Further effort to obtain identification as to the type of flavonol was not successful.

Spots 5 and 6 gave color reactions similar to those obtained for chlorogenic acid, and that of 7 was identical to those shown by d-catechin, (Table 10). When the elutes were rechromatographed with chlorogenic acid and d-catechin, the same R_f values were obtained, using both BAW and 5% acetic acid as solvents. The spectra of the elutes of spots 5 and 6 were identical to that of pure chlorogenic acid (N. B. Co.) (Figure 11) and that of 7 to commercial d-catechin (N. B. Co.) (Figure 12).

The eighth spot appeared as a dark area under ultraviolet light. It developed a weak blue color when sprayed with $FeCl_3$ - $K_3Fe(CN)_6$, and a yellow color when sprayed with 5% $AlCl_3$ in ethanol that faded out on standing. The elute in 95% ethanol was pale yellow in color with a greenish tan fluorescence under ultraviolet light.

Table 10.--Color reaction of spots 1 to 8 with different spray reagents and the absorbance of their elutes.

Band Number	R_f Value		UV Fluo		Color Reactions						Absorption Maximum	Identity
	BAW 20 hours	5% HAc 5 hours	-	NH_3	PTSA	NaOH	DSA	Pro-HCl	Vanilin HCl	DNSA	EeOH mμ	
1	0.12	-	-	-	pink	-	yellow	dark pink	dark pink	-	-	leucoantho-cyanin
2	0.20	-	-	-	pink	-	yellow	dark pink	dark pink	-	-	leucoantho-cyanin
3	0.28	-	dark	yellow	yellow	yellow	-	-	-	-	-	flavonol
4	0.33	-	dark	yellow	yellow	yellow	-	-	-	-	-	flavonol
5	0.51	0.62	blue	green	-	yellow	-	-	-	yellow fades on standing	328	chlorogenic acid
6	0.60	0.71	blue	green	-	yellow	-	-	-	yellow fades on standing	328	chlorogenic acid
7	0.63	0.52	-	pale blue	light brown	-	yellow	-	red	brown	280	catechin
8	0.93	-	dark	dark	-	-	-	-	-	-	348	flavonol

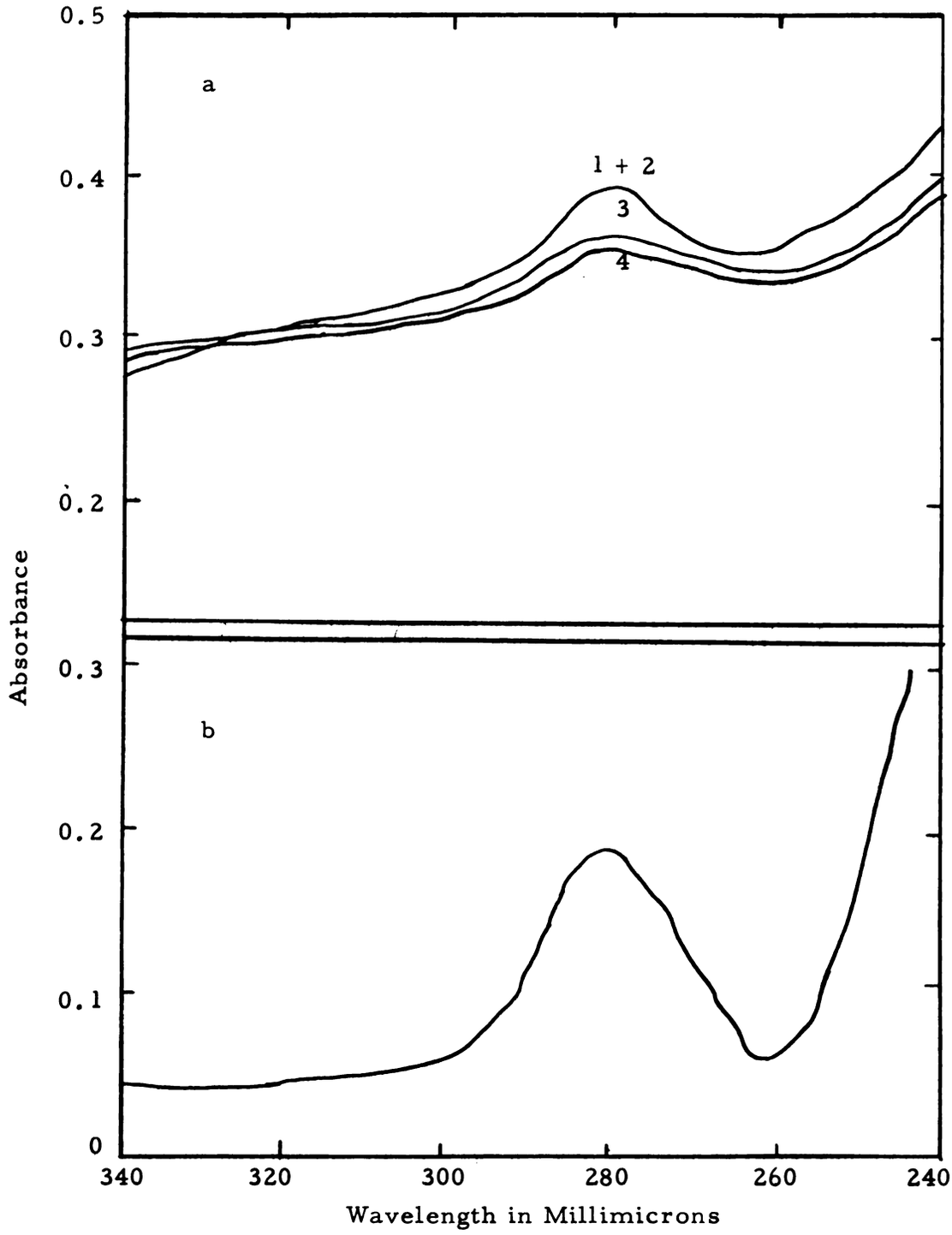


Figure 10. -- Ultraviolet spectra of a-the combined elute spots, 1, 2, 3 and 4; and b-the individual spots.

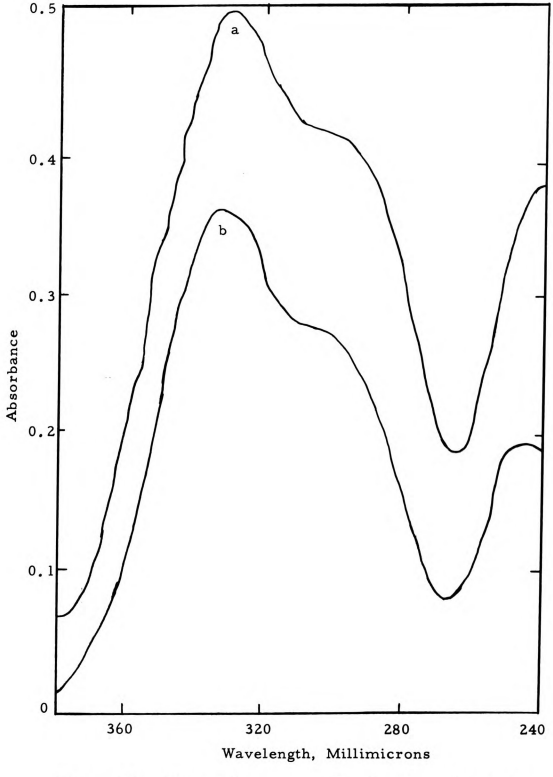


Figure 11. -- Ultraviolet spectra of a-the elutes from spots 5 and 6; and b-pure chlorogenic acid.

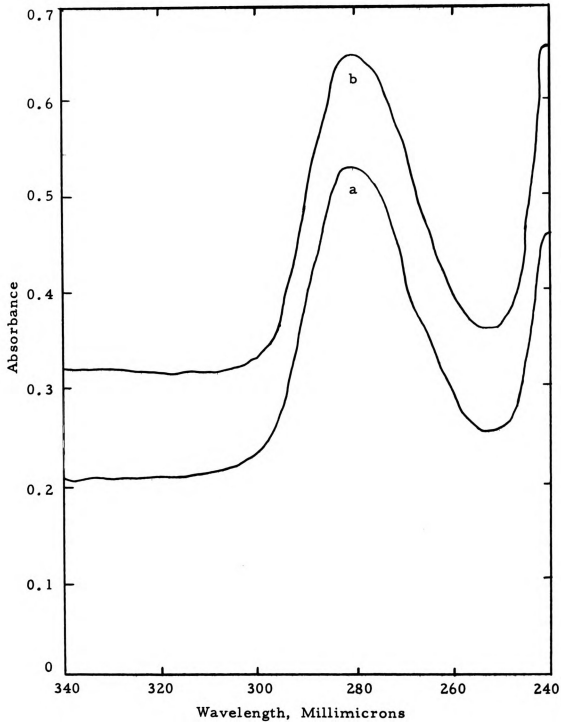


Figure 12. -- Ultraviolet spectra of a-the elute of spot 7; and b-commercial catechin.

It became yellow in aqueous NaOH and turned brown on standing. Addition of concentrated sulfuric acid gave an intense yellow color that showed a white with yellowish tan fluorescence under ultraviolet light. It had a maximum absorption at 348 mμ (Figure 13). The addition of a few drops of $AlCl_3$ or NaAc to the elute did not cause any shift in its maximum absorption. On the basis of the color reactions, it appeared to be a flavonol, but which flavonol could not be determined.

The ethyl acetate peach extract was also chromatographed using two dimensional chromatography. BAW was used as the first solvent and 5% acetic acid as the second solvent. Examination of the chromatograms under ultraviolet light and by spraying with different reagents as given previously showed the presence of 10 spots (Figure 14). These were identified the same as previously and their R_f values recorded. The two additional spots 5a and 6a, on the basis of their R_f values of .51/.76 and .62/.72, respectively, were trans and cis chlorogenic acids (Hsia et al. 1964). Spots 5 and 6 are evidently the trans and cis isomers of closely related compounds such as isomeric chlorogenic acids since their spectral properties were the same in the ultraviolet region.

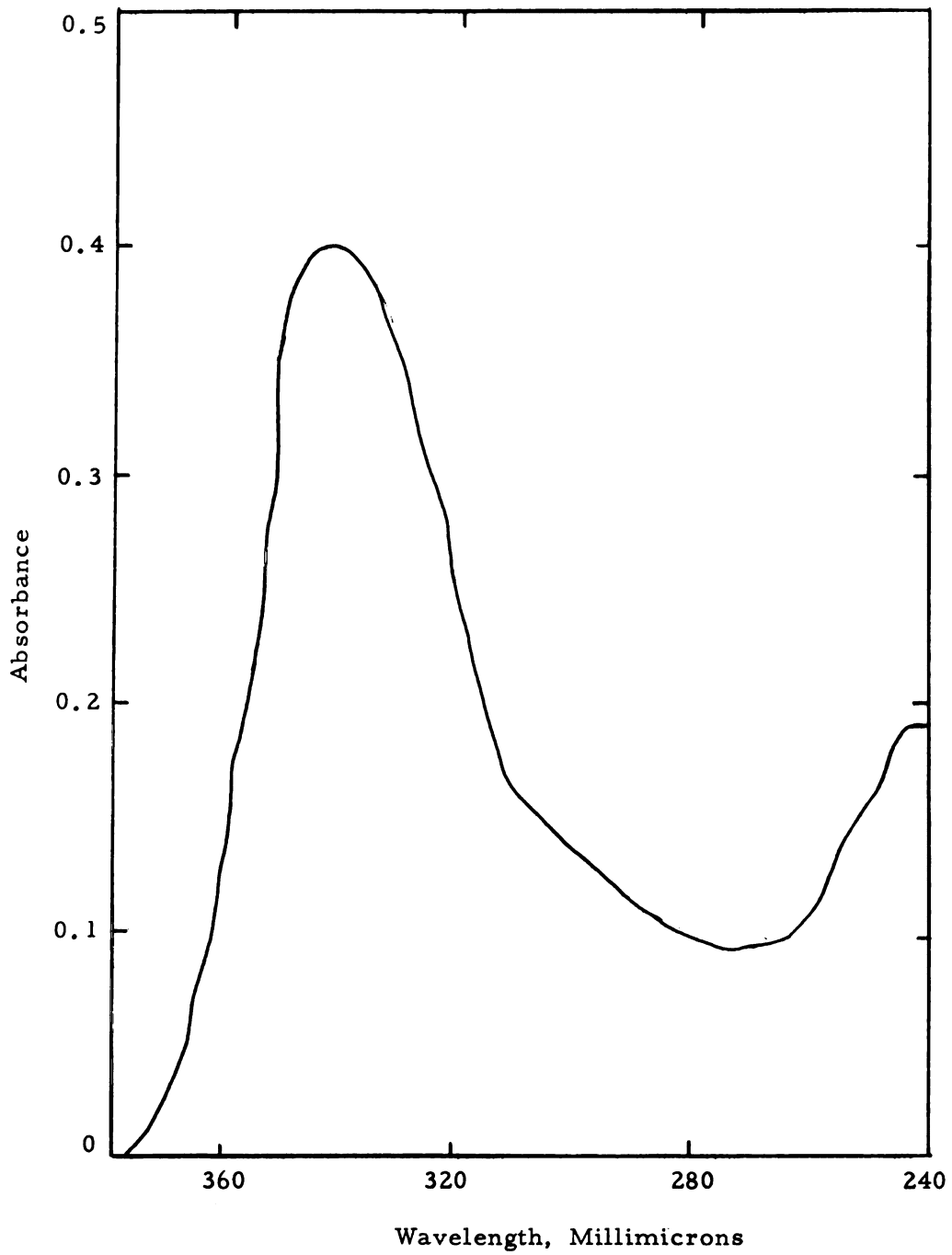


Figure 13.--Ultraviolet spectrum of the eulte of spot 8.

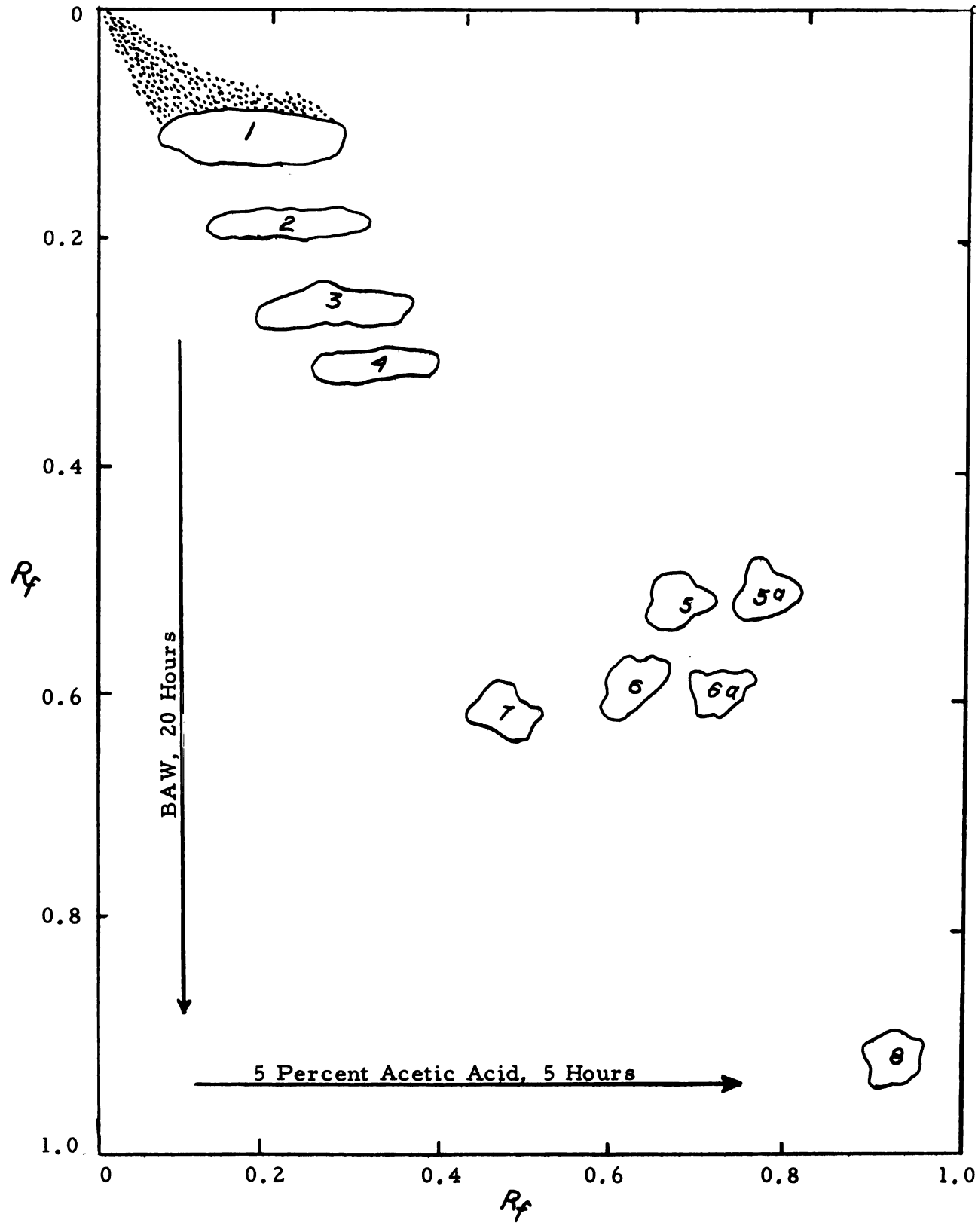


Figure 14. -- Two-dimensional paper chromatography of peach ethyl acetate extract.

Enzymatic Oxidation of the Phenolic Components

The concentration of the phenolic substances in Elberta peach extract as eluted from one-dimensional paper chromatograms is shown (Figure 15). The leucoanthocyanins constituted 38.50%, chlorogenic acid 24.54%, flavonol 19.37% and catechin 13.43% of the total phenolics. Similar results for the proportions of these phenolic constituents were obtained for other peach varieties (Table 11). The elutes were incubated with purified enzyme for 30 minutes at 30° C. and pH 6.4, and the absorbance at 420 mμ determined. It was found that the greatest change in absorbance occurred with chlorogenic acid as the substrate, followed by leucoanthocyanins, catechin and flavonols. It was also noted that the flavonol with R_f 0.93, although small in quantity, resulted in a relatively large change in absorbance at 420 mμ (Figure 16).

Table 11. -- Concentration of the identified phenolic components in four peach varieties.

Variety	Phenolic Components			
	% Leucoanthocyanins	% Chlorogenic Acids	% Flavonols	% Catechin
Richhaven	35.6	25.9	18.9	14.8
Elberta	38.5	24.6	19.4	13.4
Redskin	38.8	22.8	18.8	14.2
Sunhaven	39.4	24.1	19.3	12.0

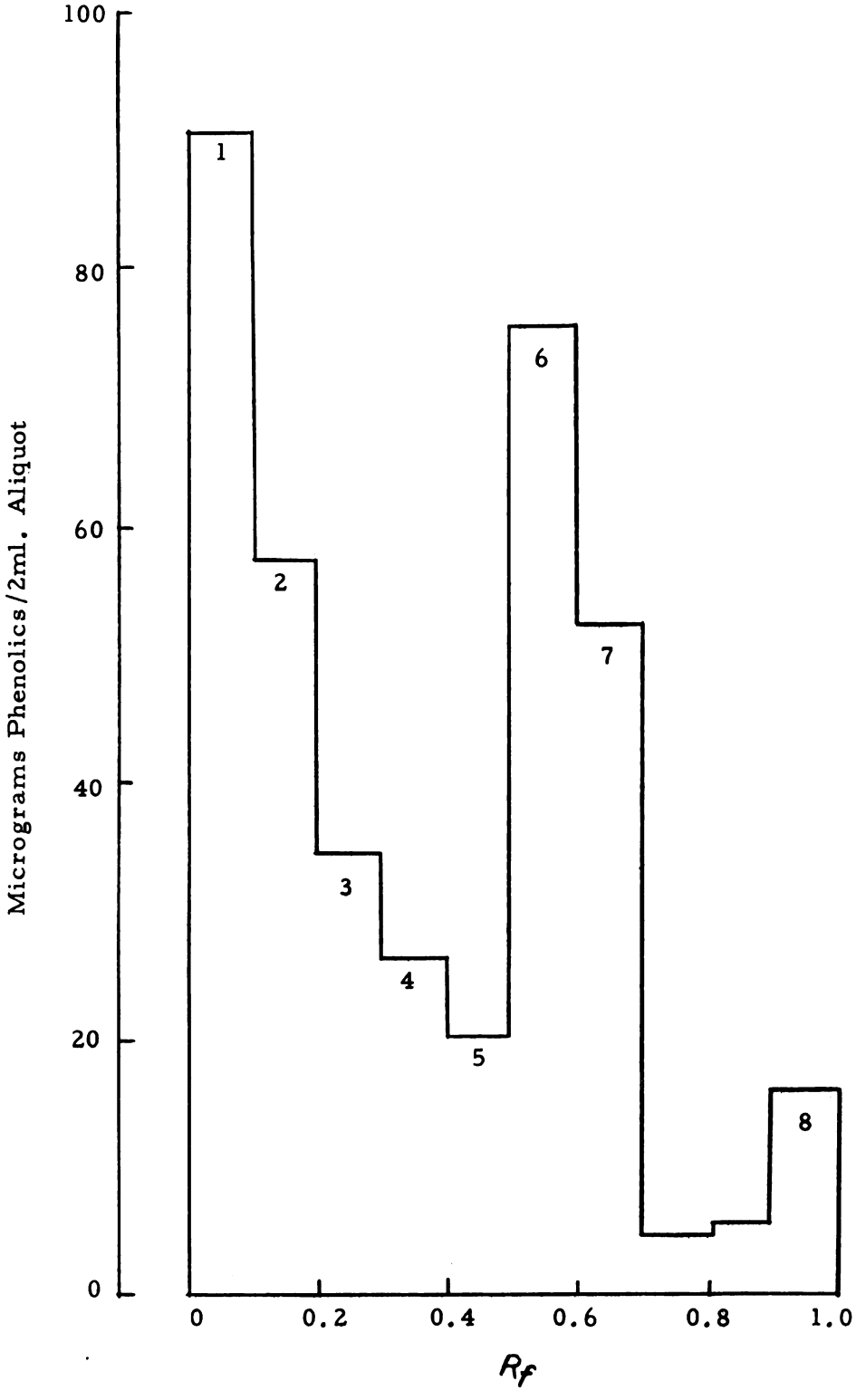


Figure 15. -- Phenolic components of Elberta peach extract as separated on one-dimentional paper chromatograms. 1 and 2 leucoanthocyanins, 3 and 4 flavonols, 5 and 6 chlorogenic acid, 7 catechin and 8 flavonol.

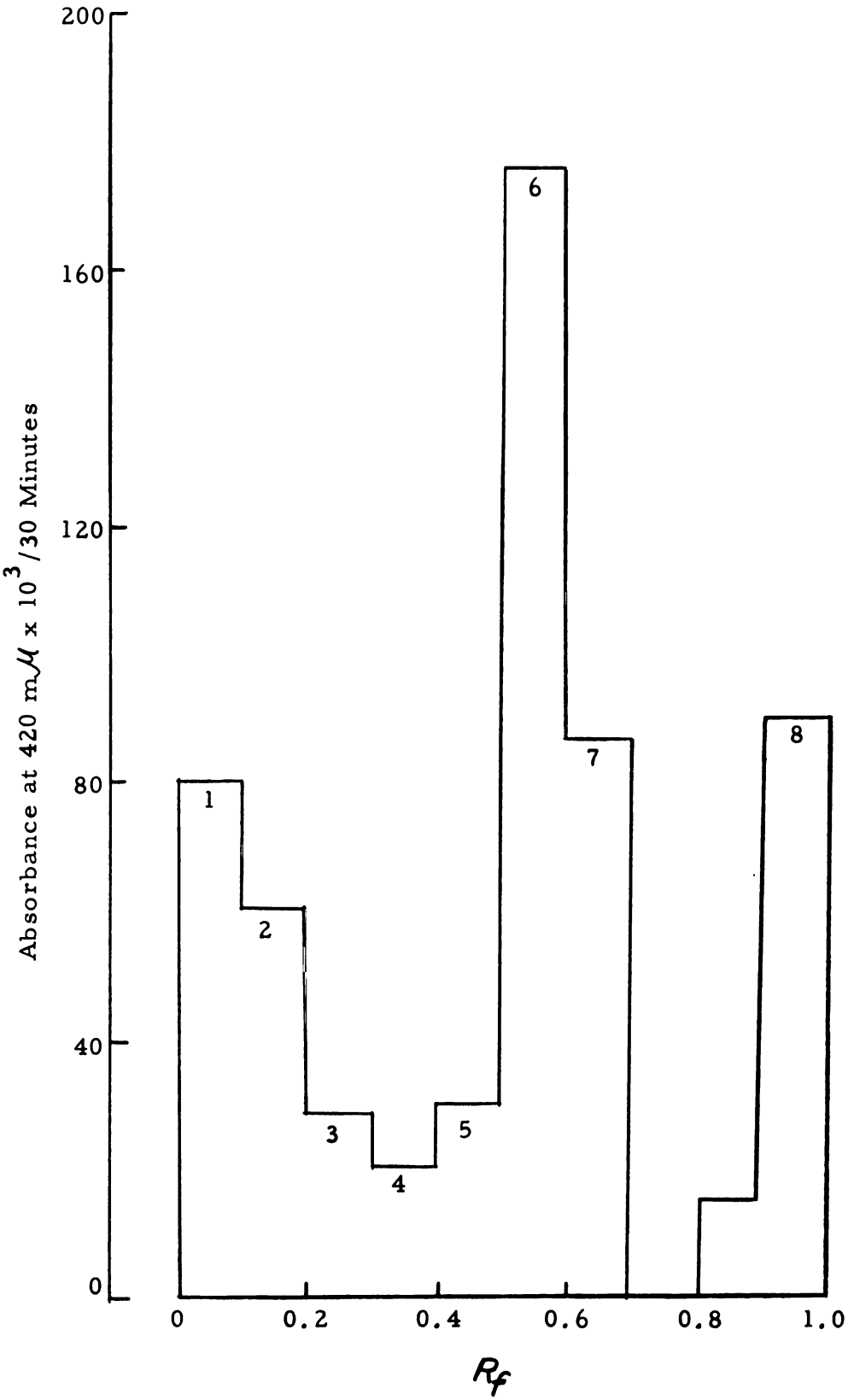


Figure 16. -- Peach polyphenol oxidase activity toward the different components of Elberta peach extract.

When the chromatograms were sprayed with partly purified enzyme, the development of brown to pale yellow color did not follow the same pattern as the change in absorbance at 420 mμ . Leucoanthocyanins developed maximum brown color followed by catechin, chlorogenic acid and flavonols (Figure 8).

The progress of oxidation of the phenolic components by endogenous enzyme in Elberta, Richhaven, Sunhaven and Redskin peaches is shown (Tables 12 to 15). It was found that 73.16% of the leucoanthocyanins in Elberta peaches was oxidized in 45 minutes with no further oxidation up to 90 minutes. Oxidation of flavonols also stopped after 45 minutes with 58.34% oxidized. Chlorogenic acid and catechin were completely oxidized in 45 minutes and the flavonol (R_f 0.93) was completely oxidized in 15 minutes. The results obtained for the varieties Richhaven, Sunhaven and Redskin were similar to those for Elberta for the leucoanthocyanin and flavonols. Chlorogenic acid was not completely oxidized in 90 minutes. The remaining chlorogenic acid was oxidized on addition of more enzyme. Catechin reqired 75 minutes for complete oxidation.

These results indicated that only a fraction of leucoanthocyanins and flavonols were enzymatically oxidizable. Chlorogenic acid and catechin can be completely oxidized by the enzyme when the latter is present in high concentration (as in Elberta peaches). Their rates of oxidation were higher than those of leucoanthocyanins and flavonols.

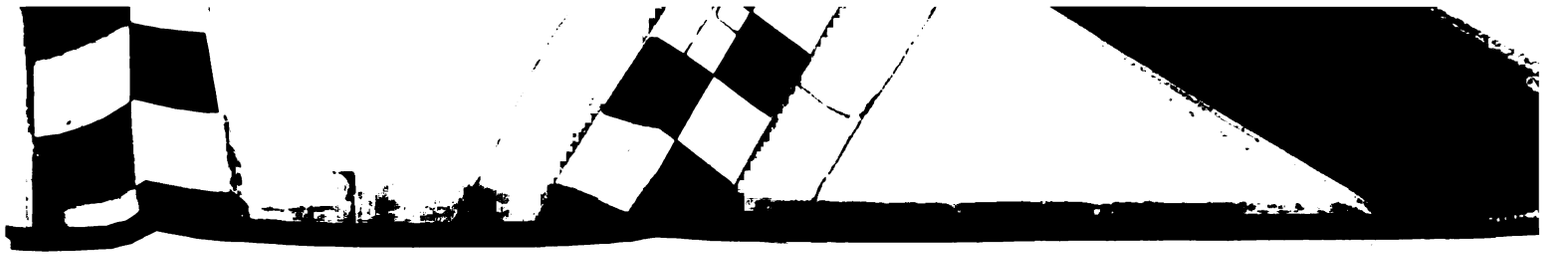


Table 12. -- Progress of oxidation of the different phenolic components of Elberta peaches by endogenous enzyme.

Identity	R_f	Oxidation Time, Minutes						
		0	15	30	45	60	75	90
		Total Phenolics Remaining after Oxidation, micg.						
Leucoanthocyanins	0,1	92	45	35	22	22	22	22
	0.2	57	26	20	18	18	18	18
Flavonol	0.3	34	20	16	13	13	13	13
	0.4	26	16	14	12	12	12	12
Chlorogenic Acids	0.5	20	6	-	-	-	-	-
	0.6	75	40	15	-	-	-	-
Catechin	0.7	52	22	10	-	-	-	-
Unknown	0.8	6	6	6	6	6	6	6
	0.9	10	4	-	-	-	-	-
Flavonol	1.0	15	5	-	-	-	-	-

Table 13. -- Progress of oxidation of the phenolic components of Richhaven peaches by endogenous enzyme.

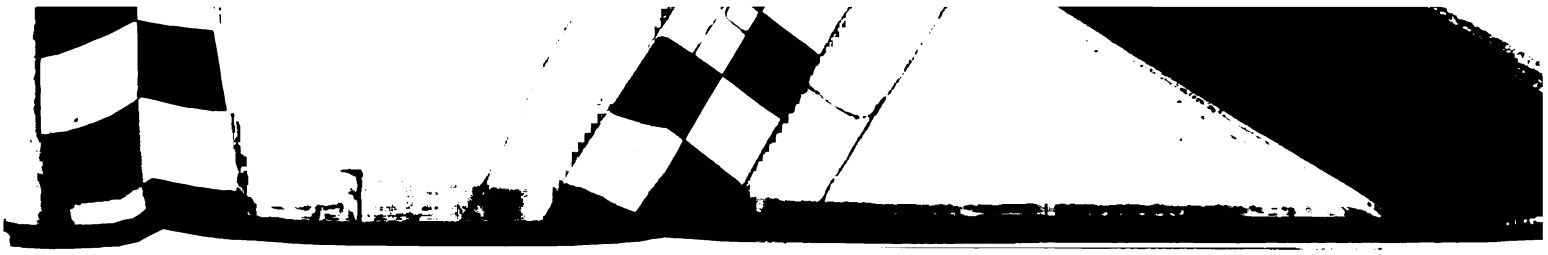
Identity	R_f	Oxidation Time, Minutes						
		0	15	30	45	60	75	90
		Total Phenolics Remaining after Oxidation, micg.						
Leucoanthocyanins	0.1	112	72	46	38	36	36	36
	0.2	80	50	34	28	26	24	24
Flavonol	0.3	44	28	20	16	16	18	18
	0.4	38	22	18	18	16	16	16
Chlorogenic Acids	0.5	32	12	-	-	-	-	-
	0.6	108	53	36	24	20	16	14
Catechin	0.7	80	42	30	16	8	-	-
Unknown	0.8	10	10	12	13	12	12	14
	0.9	16	6	-	-	-	-	-
Flavonol	1.0	20	10	-	-	-	-	-

Table 14. -- Progress of oxidation of the phenolic components of Sunhaven peaches by endogenous enzyme.

Identity	R_f	Oxidation Time, Minutes						
		0	15	30	45	60	75	90
		Total Phenolics Remaining after Oxidation, micg.						
Leucoanthocyanin	0.1	64	38	30	26	24	22	22
	0.2	44	20	16	14	14	14	14
Flavonol	0.3	25	16	14	12	12	12	12
	0.4	18	12	8	8	8	8	8
Chlorogenic Acids	0.5	14	5	-	-	-	-	-
	0.6	52	30	24	20	15	12	8
Catechin	0.7	33	18	12	10	10	6	-
Unknown	0.8	6	8	6	9	6	10	6
	0.9	8	-	-	-	-	-	-
Flavonol	1.0	10	5	-	-	-	-	-

Table 15. -- Progress of oxidation of the phenolic components of Redskin peaches with endogenous enzyme.

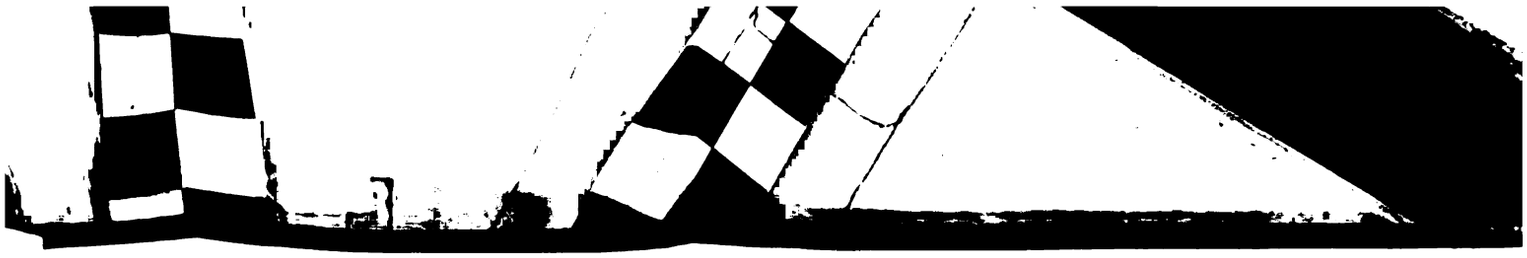
Identity	R_f	Oxidation Time, Minutes						
		0	15	30	45	60	75	90
		Total Phenolics Remaining after Oxidation, micg.						
Leucoanthocyanins	0.1	74	58	45	34	28	26	26
	0.2	52	38	32	28	24	20	20
Flavonols	0.3	29	18	16	14	14	14	14
	0.4	20	16	12	10	10	10	10
Chlorogenic Acids	0.5	14	8	-	-	-	-	-
	0.6	60	38	30	24	19	14	12
Catechin	0.7	46	32	26	18	12	6	-
Unknown	0.8	8	8	9	8	10	8	8
	0.9	10	4	-	-	-	-	-
Flavonol	1.0	12	4	-	-	-	-	-



CONCLUSIONS

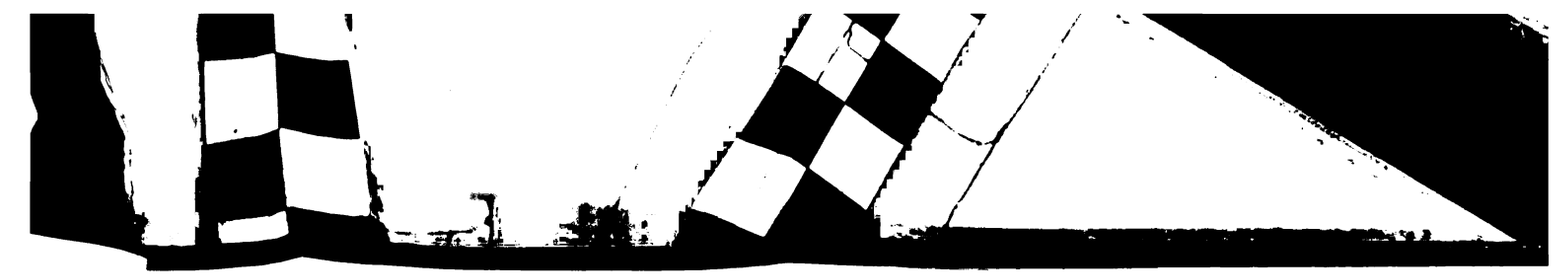
Polyphenol oxidase activity differs considerably among the peach varieties investigated. Elberta showed the highest level of enzyme activity and Redskin the lowest. The enzymes of all eight varieties had the same substrate specificity. The enzyme solution was not very stable at room temperature, 22.2° C., 72° F., losing 93.3% of its activity in 6 days. The enzyme was more stable at low temperatures losing only 20 and 16.6% activity in 12 weeks at -17.8° C., 0° F., and -31.7° C., -25° F., respectively. The enzyme was most active at pH 6.4 while most stable at pH 8.0.

The phenolic constituents of peach extract in order of prominence were leucoanthocyanins, 35.6 to 39.4%, chlorogenic acid, 22.8 to 25.9%, catechin, 12.0 to 14.8% and flavonols, 18.8 to 19.4%. Catechin exhibited the highest reactivity with the enzyme followed by chlorogenic acid, leucoanthocyanins and flavonols. However, their contributions to discoloration in enzymatic browning of peaches did not follow the same pattern as reactivity to the enzyme. Leucoanthocyanins contributed most of the browning followed by catechin, chlorogenic acid and flavonols. The degree of discoloration of frozen peaches during storage or on thawing is primarily attributed to the original



activity of the enzyme in the varieties that have from moderate to high total phenolic content. In such cases the enzyme could cause discoloration to a profound extent before the substrate became a limiting factor, which seems to be the case in all eight varieties investigated in this study. However, in some varieties distinguished for low total phenolic content, the substrate could be the limiting factor for browning since there is not much for the enzyme to do without the substrate. Elberta peaches showed a higher rate of browning (phenolic oxidation by peach PPO) than the other varieties. This was due to the higher enzyme concentration in Elberta peach tissue as compared with these varieties.

Spectrophotometric and chromatographic methods were used to identify and to determine the concentration of the different phenolic substances in peach extract. More work is still needed to establish the identity of the flavonols in peaches since this work identified them only as a group through their color reactions with spray reagents.



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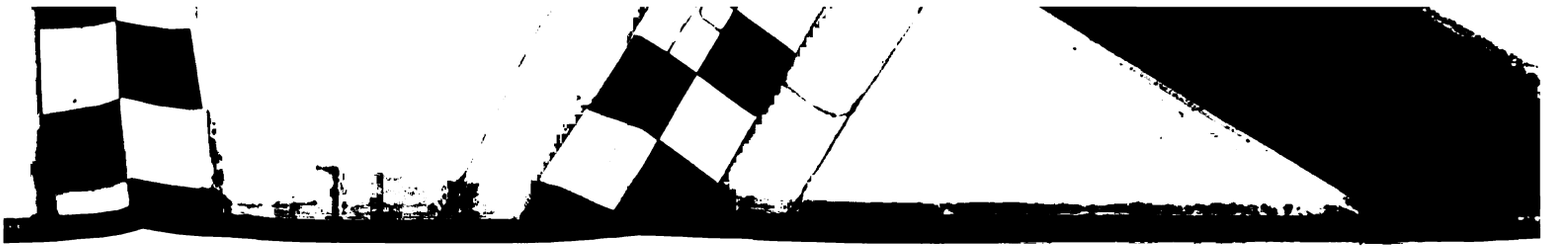

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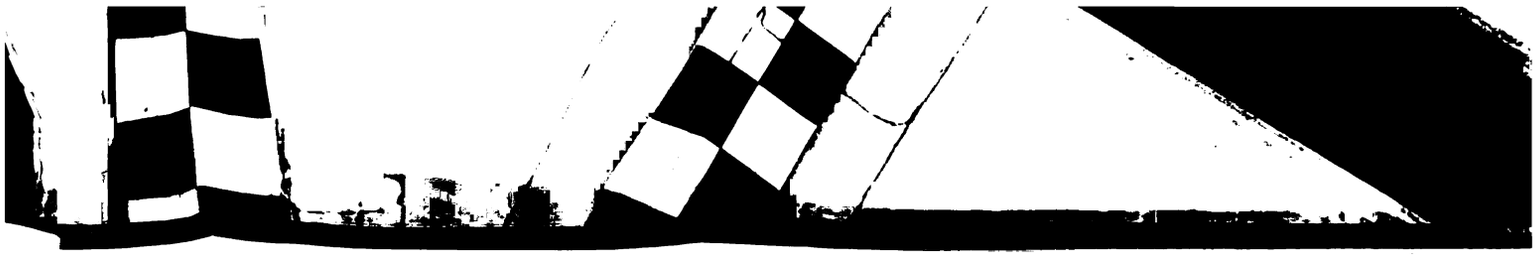

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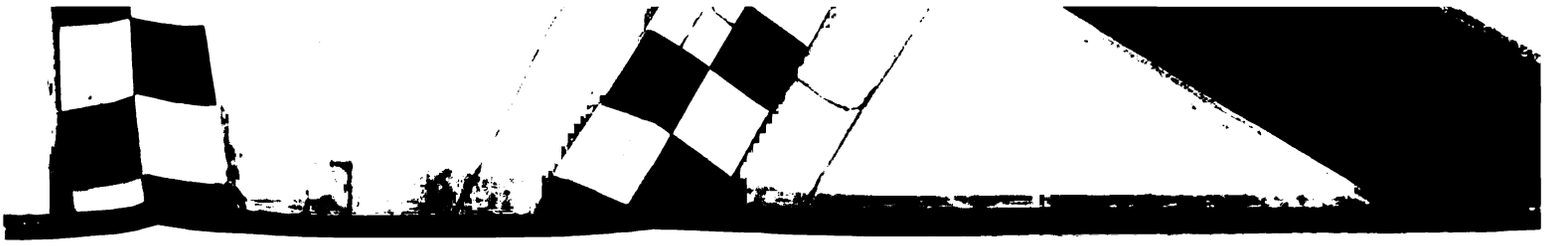

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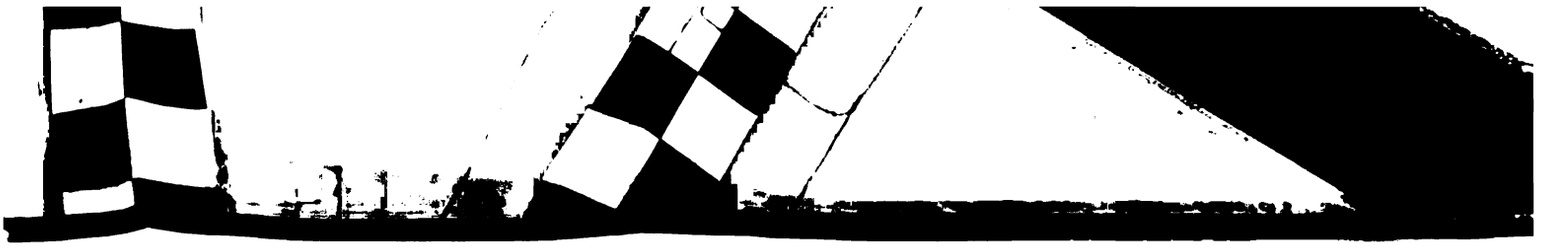

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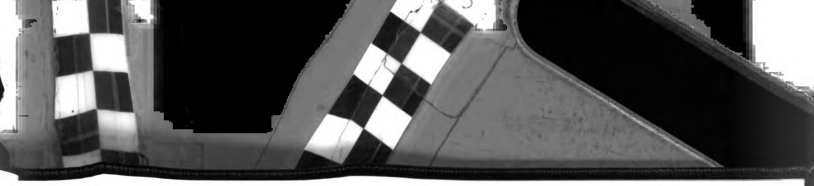

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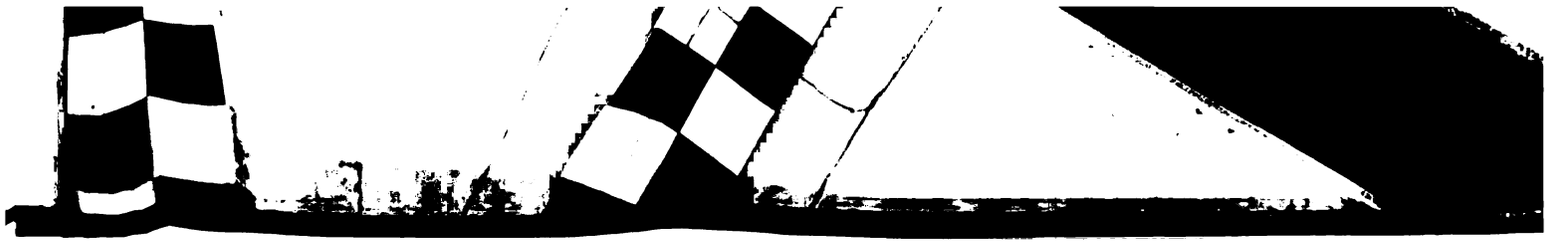

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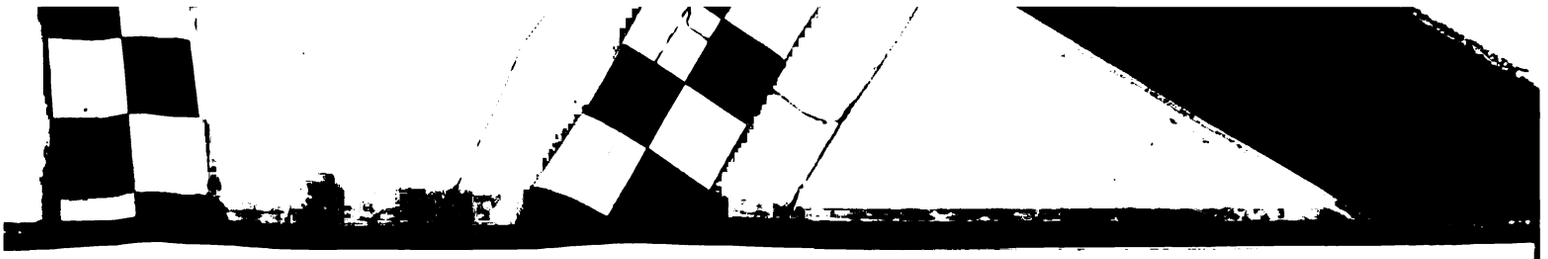

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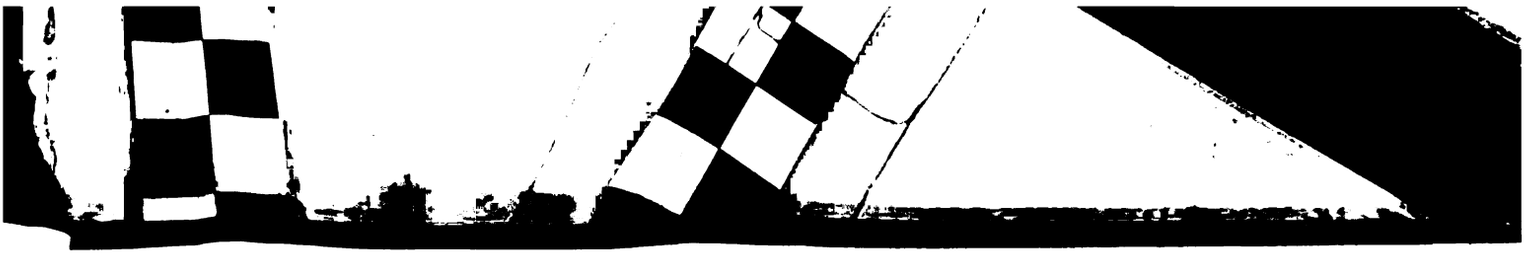

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