AN ABSTRACT OF THESIS OF

YIH HERMON CHANG for the degree of MASTER OF SCIENCE

in Food Science and Technology presented on January 11,1979

Title: CHEMICAL ANALYSIS OF ACID PRECIPITATED COLLOID OF RIPE BARTLETT PEAR JUICE

Abstract approved: D.V. Beavers

With acidification, the clarification-filtration difficulties of the 1975 ripe Bartlett pear juice were generally overcome. Chemical analyses were conducted to determine the composition of the acid precipitated colloid in an attempt to determine the reason that the 1975 juice was so difficult to clarify and filter.

Samples of the acid precipitated colloid were obtained from the clarification process of the 1975, 1976 and 1977 juices. The process includes acidification with citric acid and depectinization with pectinase enzyme. After conducting the tests with different washing media, the precipitate was washed with the selected medium (citrate buffer), air dried, powdered, freeze dried and stored in a desiccator.

A wide variation of copper, iron, magnesium, calcium, sodium and potassium was detected in all the precipitate samples. High calcium content was noted in all samples. This might be part of the reason for the clarification-filtration difficulties of pear juice. No pectin or starch was detected in any of the precipitate samples. Fructose,

galactose and glucose were found in the precipitates. Lipids were found in the precipitates, linoleic, palmitic and oleic acids were the major fatty acids. Amino acid analyses and the determination of total phenolics and crude protein were also conducted. The percent compositions of the precipitates are summarized below:

	1975 precipitate	1976 precipitate	1977 precipitate
Protein	30.8 ± 0.5	36.6 ± 0.7	35.6 ± 0.7
Mineral	1.6	1.4	1.8
Phenolics	38.1 ± 0.5	33.9 ± 0.4	29.1 ± 0.4
Sugars	7.8	8.9	7.4
Lipid	26.5 ± 0.4	21.3 ± 0.5	20.4 ± 0.4
Total	105	102	94

According to the above analytical results, acid precipitated colloid of the ripe Bartlett pear juice is a protein-phenolic-lipid complex containing lesser quantities of sugars and metal ions.

Amino acid analyses were also conducted on the cloudy and clarified ripe Bartlett pear juice. Protein contents of the 1975, 1976 and 1977 cloudy pear juice were 0.07%, 0.10% and 0.18%, respectively. The percentages of free amino acid in total amino acid of the 1975, 1976 and 1977 juices were 47.3%, 36.8% and 32.8%, respectively.

The juice of 1975 season had the lowest protein content (0.07%), and the colloidal precipitate from this juice showed the lowest crude protein content (30.8%). In addition, the 1975 precipitate had the highest phenolic and lipid contents. High phenolic and lipid contents plus low protein content may be the cause of the clarification-

filtration difficulties of the 1975 juice.

Chemical Analysis of Acid Precipitated Colloid of Ripe Bartlett Pear Juice

by

Yih Hermon Chang

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed January 1979

Commencement June 1979

APPROVED:

Associate Professor of Food Science and Technology
in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented January 11, 1979

Typed by researcher for Yih Hermon Chang

ACKNOWLEDGEMENT

I wish to express my most sincere gratitude and appreciation to my major professor, Mr. Darrell V. Beavers for his guidance and valuable assistance during the course of this study and the preparation of this thesis.

My sincere appreciation is also extended to my minor professor, Dr. Robert R. Becker of the Biochemistry Department for running the amino acid analyses for me, and for his advice and help.

I would like to extend my sincere thanks to Dr. Daryl G. Richardson of the Horticulture Department for his valuable suggestion of running lipid analyses. His assistance in helping with the analytical method and providing his valuable unpublished data is likewise greatly appreciated.

I am grateful to Dr. Morris W. Montgomery for serving on my graduate committee and showing great concern for me. His valuable suggestions and assistance during the course of this investigation are also greatly appreciated.

I would like to acknowledge the assistance of Dr. Frank W. Adams of the Agricultural Chemistry Department who helped with the mineral analyses, and Mr. Bob Parker's assistance in running the fatty acid analyses.

TABLE OF CONTENTS

Table of Contents -- continued

LIST OF FIGURES

LIST OF TABLES

CHEMICAL ANALYSIS OF ACID PRECIPITATED COLLOID OF RIPE BARTLETT PEAR JUICE

INTRODUCTION

Some juices, for example orange juice, are consumed in a naturally cloudy state as clarification would impair the appearance and flavor of the juice. The juices of apples and berries have traditionally been consumed as filtered-clear juices and the problem has been to effectively clarify these juices and to maintain this brilliantly clear condition throughout the storage life of the juice.

A freshly pressed juice contains various amounts of fine cellular debris with pectic substances, proteins, phenolics and other components. The system is initially stabilized either by the electrical charges and the hydration of the particles or by the presence of soluble pectin.

Although the colloidal systems in juices are inherently unstable, natural clarification is usually slow and it is necessary to accelerate the process if a clear juice is required. Direct centrifugation or filtration is sometimes possible but often is uneconomical as the presence of pectin renders the juice viscous which results in slow filtration. Therefore, juice is usually depectinized by pectinase enzymes before filtration.

During the 1975 processing season, the pear juice expressed in an industrial pilot plant in California was extremely difficult to clarify and filter. Since the end of 1975, research on the problems of

clarification and filtration has been conducted here at the Department of Food Science and Technology of Oregon State University. The major portion of the work has been with ripe pear juice, because it presented the greatest problems. However, knowledge gained with the ripe pear juice can be applied to other juices. At the end of 1976, it was found that the most important factor in the clarification-filtration of the ripe pear and other juices is acidification of the juice and pH control. With acidification, pear and many other juices can be easily filtered; whereas, without acidification, ripe pear juice can not be filtered satisfactorily. Acidification process lowers the pH of the pear juice from ca. 4.2-4.6 to 3.5. The pH change probably reduces the electrical charges of the suspended particles which causes a destabilization of the collidal system. This destabilization promotes the clarification of the juices.

Different clarification behavior among the pear juices of 1975, 1976 and 1977 seasons has been observed: Without acidification, the 1975 juice was almost impossible to clarify and filter, the 1976 juice was easier to clarify, and the 1977 juice was the easiest one to clarify. When the juices were acidified, they were very easy to clarify and filter; but they still followed the same sequence: The 1975 juice was the most difficult and the 1977 juice was the easiest to clarify. Therefore, chemical analysis was conducted to determine the composition of the acid precipitated colloid of the 1975, 1976 and 1977 juices in an attempt to determine the cause of the clarification and filtration problems with the 1975 juice.

REVIEW OF LITERATURE

Chemical Composition of Juice Precipitates

Available literature contains little information on the formation and composition of the precipitate of pear juice. As early as 1908, Kelhofer reported on the chemical nature of the sediment formed in the fermented pear juice. He concluded that the sediment contained protein, pectin and oxidized tannins.

The literature on the subject of the formation of nonbiological hazes and storage deposits in clarified apple juice has been reviewed by Esselen (1945), Marshall (1951) and more recently by Heatherbell (1976a, 1976b, 1976c). Sedimentation of the clarified apple juice has been of two types: The first arising from the precipitation of partly degraded pectin and the second associated with the formation of products containing degraded tannin. The simultaneous formation of both types of deposit has been an added complication (Kieser et al., 1957). In general, the formation of nonbiological hazes and sediments in clarified apple juice mainly arises from incompletely degraded pectins and from complexes of phenolics, proteins and metal ions (Heatherbell, 1976a).

In the report of Neubert and Veldhuis (1944), they concluded that the sediment of clarified apple juice was probably a phlobaphene, a substance derived by heating condensed tannins with diluted acid. Qualitative chemical tests showed the absence of nitrogen, sulfur, halides and phosphates. Qualitative tests showed the presence of iron and

copper, but no calcium. Yamasaki (1964) showed that the precipitate of apple juice was a protein-carbohydrate complex containing 36% protein. He postulated that the protein-carbohydrate complex was surrounded by the negatively charged protective colloids, such as pectin. The work of Zitko and Rosik (1962b) indicated that polyphenols were involved also. Johnson et al. (1968) investigated the sediment formed in the clarified apple juice and showed that the sediment was a polymeric phenolic-protein complex.

Letzig and Nurnberger (1963) investigated the nature of the sediment from "cloudy pressed apple juice" and concluded that the cloudy substances were a heterogenous complex consisting of protein, polyphenolic matter, fragments of cell wall, nucleus and other ingredients.

Krug (1969) and Krebs (1971) have reported that solubilized starch and starch tannin complexes may also be involved in the sediment formation of the clarified apple juice. Amerine et al. (1967) described the clouding of cider due to the presence of excess copper or iron. Monties and Barret (1965) described a reversible chill haze present in both apple juice and cider, containing 70% condensed tannin , 20% protein and 5% reducing sugars. More recently, in Heatherbell's (1976a) research, the hazes and sediments formed in apple wine and clarified apple juice were found to consist mainly of incompletely degraded starch polymers. Small amounts of protein and phenolic material were present in some instances.

Pectic Substances

Pectic substances is a group designation for those complex, colloidal carbohydrate derivatives containing a large proportion of anhydrogalacturonic acid units (Kertesz, 1951). Pectic substances are found in the tissue of all higher plants they are mainly deposited in the middle lamella and the primary cell wall, where they act as intercellular cement. Parenchymous and meristematic tissues are particularly rich in pectic substances (Krop, 1974). The structure of the pectic substances is only partially understood. Protopectin, the giant water insoluble parent pectic substance, is believed to consist of high molecular weight soluble pectin and enmeshed mechanically and chemically with the cell wall substances, such as hemicellulose and cellulose (Keegstra et al., 1973). The backbone of pectic substances, with a tendency to coiling, consists of 1,4 linked d-galacturonic acid units. The carboxyl groups of the galacturonic acid are partly esterified with methanol, and in some cases, the hydroxyl groups are partially acetylated (Deuel and Stutz, 1958). On hydrolysis most pectin preparations, even after extensive purification, yield not only galacturonic acid but also neutral sugars. Therefore, a model pectin molecule might consist of a main chain of galacturonan, containing blocks of rhamnose rich regions, with mainly arabinose, galactose and xylose in the side chains (Krop, 1974; Pilnik and Voragen, 1970).

Pectic substances are characterized by:

1. Degree of polymerization (DP). Values for the molecular

weight of pectic preparation given in the literature (Kertesz, 1951) vary between 30,000 and 300,000, consequently the DP varies between 160 and 1,600.

2. Degree of esterification (DE). This represents the number of esterified carboxyl groups. When all the carboxyl groups in pure polygalacturonic acids are esterified, the degree of esterification is 100% and the methoxyl content is 16.32% (Doesburg, 1965). In fruit pectins, usually 80% of the carboxyl groups are esterified (McCready and McComb, 1954). Apple pectin is highly esterified, and has a DE of ca. 90% (Endo, 1965b). In pears, DE remained constant at approximately 70% before harvest but dropped post harvest to 50% (Raunhardt and Neukom, 1964).

3. Degree of esterification with acetic acid. Pectic substances of some plants (especially sugar-beet and pear) are partially esterified with acetic acid at C_2 and C_3.

4. Content of non-galacturonide materials and the distribution of these along the main chain (Krop, 1974).

Pectins form a colloidal solution in water, which is a viscous "solution". The viscosity depends on the molecular weight and is influenced by degree of esterification, pH and electrolyte concentration (Deuel, 1943; Deuel and Stutz, 1958). Pectins can be precipitated with proteins (Doesburg, 1965) and with polyvalent cations (Deuel and Stutz, 1958). Acids hydrolyze the ester and the glycosidic linkages. At low temperatures saponification prevails and at high temperatures depolymerization (Doesburg, 1965). Alkali also acts on the ester groups which can be split off at low temperatures without depolymeri-

zation (Pilnik and Voragen, 1970). At higher temperatures, β-elimi-native cleavage of glycosidic linkages becomes dominant (Krop, 1974).

The predominating colloids present in fruit juices, according to Kertesz (1936) are pectic substances. Pectic substances may act as a stabilizing protective colloid in the juice system (Yamasaki et al., 1967). The formation of flocculent precipitates of apple juice seems to be centered about the disintegration of the pectin molecules (Kertesz, 1930; Fabian and Marshall, 1935; Forgac, 1945). Endo (1965d) also reported that the hydrolysis of pectic substances in apple juice was necessary for clarification.

Pectic Enzymes

Pectic substances can be attacked by two main groups of enzymes: saponifying enzymes (pectin esterases) and depolymerizing enzymes. Although "protopectinase" has been mentioned as a distinct enzyme which solubilizes protopectin, most workers believe that such an enzyme does not exist (Doesburg, 1965).

Saponifying enzymes: Pectin esterase (PE) or pectin methyl esterase (EC 3.1.1.11) is almost completely specific in hydrolyzing the methylesters of pectinic acids. PE attacks only ester groups next to a free carboxyl group and then continues to act along the molecule. Through the saponification, the pectic substances may become increasingly negatively charged (Lineweaver and Ballou, 1945).

Depolymerizing enzymes: Pectic depolymerizing enzymes can be classified according to three criteria namely: (1) hydrolytic or

transeliminative splitting of the glycosidic bonds (Figure 1), (2) random or terminal mechanism of attack (endo- or exo- enzyme, respectively) and (3) preference for pectic acid or pectin as substrate. Thus theoretically eight groups of the depolymerizing enzymes can be obtained (Krop, 1974). The scheme of the classification of the depolymerizing pectic enzymes, according to Koller (1966), is shown in Table 1.

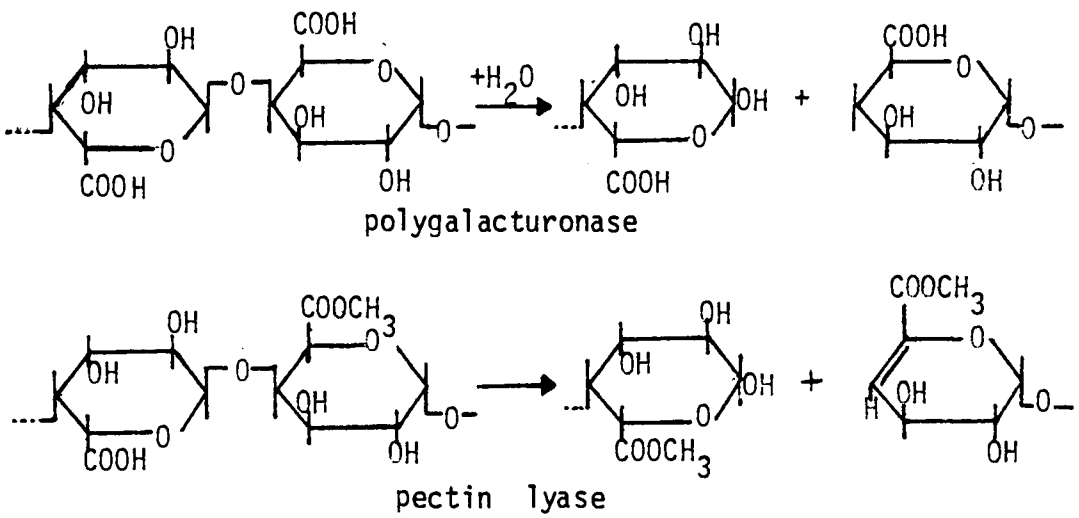


Figure 1. Splitting mechanism of the 1,4 glycosidic bond. Hydrolytic splitting of a pectic acid chain by polygalacturonase and transeliminative splitting of a pectin chain by pectin lyase.

Pectin lyases (PL) are very specific for methoxyl groups and pectic acid lyases (PAL) (Table 1) for free carboxyl groups. Breakdown products from PL action will show a methylated unsaturated galacturonide unit at the nonreducing end and those from PAL action will show oligomers with a nonmethylated unsaturated galacturonide unit at the nonreducing end.

Table 1. Schematic classification of depolymerizing pectic enzymes (Koller, 1966)

Pectic enzymes acting mainly on pectin	
Polymethylgalacturonase (PMG)	Pectin lyases (PL)
1. endo PMG (3.2.1.41)	3. endo PL (4.2.2.3)
2. exo PMG	4. exo PL
Pectic enzymes acting mainly on pectic acid	
Polygalacturonases (PG)	Pectic acid lyases (PAL)
5. endo PG (3.2.1.15)	7. endo PAL (4.2.2.1)
6. exo PG (3.2.1.40)	8. exo PAL (4.2.2.2)

Endo-polygalacturonases (PG) are the most widely distributed and most frequently occurring pectin depolymerases in nature. Most fungi produce PG adaptively together with other pectic enzymes such as PE, exo-PG and PL. The preferred substrate is pectic acid but also pectin is attacked at a lower rate and to a lower hydrolysis limit. The studies of Endo (1965a, 1965b) and Yamasaki et al. (1966,1967) showed clearly that the clarification of apple juice can be obtained by the use of a mixture of endo-PG and PE alone without the presence of other activities, such as cellulase, hemicellulase, protease or amylase, frequently associated with pectic enzymes.

Clarification Mechanisms of Apple and Orange Juices

Although this study is concerned with the acid precipitated colloid prepared from the clarification process of the pear juice; it is helpful to consider the clarification mechanisms of other juices.

Electrostatic Charge Neutralization Mechanism

Yamasaki et al. (1964) studied the resuspension of ultracentrifuged precipitate of apple juice in aqueous media and proposed a mechanism of enzymic clarification: The suspended materials are a protein-carbohydrate complex, surrounded by negatively charged protective colloids, such as pectin. By the total or partial degradation of the protective colloid with pectic enzymes, the positive charge of the protein complex would be exposed and flocculation occurs due to the electrostatic attraction between particles with positive (exposed protein complex) and negative (protective colloid) charges. The suspended particles were shown to be negatively charged at pH 3.5. No flocculation occurs above pH 4.75. This pH value probably being above the isoelectric point of the protein.

Three Stages of Clarification Process

Endo (1965a, 1965d) demonstrated that clarification of apple juice could be accomplished by the joint action of a purified endo-PG and a purified PE. Soluble pectin acts as a protective colloid and that the partial hydrolysis of this pectin permits insoluble and finely divided particles to flocculate. Therefore, hydrolysis of pectic

substances in apple juice seemed to be indispensable for clarification. He distinguished three stages in the clarification process: (1) solubilization of insoluble pectin bound to suspended particles; (2) decrease in viscosity by hydrolysis of soluble pectin, and (3) flocculation of the suspended particles. It was shown that endo-PG and PE were involved in the first and second stages but not in the third stage. The third stage occurred extremely early in the glycosidic hydrolysis of the pectin.

Cloud Loss Mechanism of Orange Juice

Krop (1974) studied the mechanism of the cloud loss phenomena in orange juice by investigating many factors for the cloud stability. He found that the cloud loss of orange juice could be ascribed directly to the action of PE. Clarification did not occur if the formation of high polymer calcium pectate was prevented either by degrading the juice pectin by a purified PL before the substantial action of the PE, or by the enzymic hydrolysis (by a yeast PG) of the low methoxyl pectin formed. This mechanism was confirmed when the addition of pectic acid clarified the orange juice artificially.

Plant Phenolics

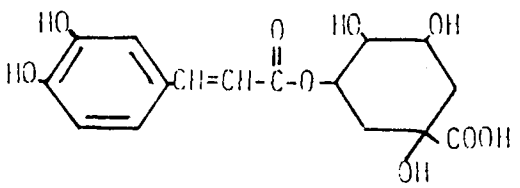
Plant phenolics embrace an extremely heterogenous chemical compounds having an aromatic ring with one or more hydroxyl groups and other substituents. It has been suggested that phenolics were originally byproducts of the metabolism of aromatic amino acids (Neish, 1964) . In many cases the structures are still unknown.

Fifteen major groupings of plant phenolics have been classified by Harborne and Simmonds (1964). However, most of them can be divided into two groups: (1) the flavonoids, including condensed tannins (C_6-C_3-C_6structures), (2) the phenylpropanoid compounds, including the hydrolyzable tannins (C_6-C_1 and C_6-C_3 structures and their derivatives). Generally, compounds of the first type contain only phenolic hydroxyl groups as reactive centers. Compounds of the second type commonly contain also carboxyl groups (Loomis and Battaile, 1966).

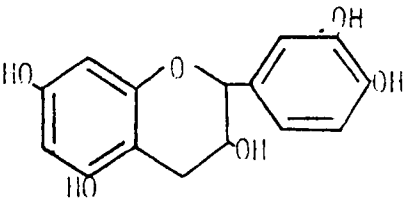
Sioud and Luh (1966) identified the presence of chlorogenic acid, (+)-catechin, (-)-epicatechin, leucoanthocyanidins, and caffeic acid in the ripe Bartlett pear puree. The predominant polyphenolics were chlorogenic acid, (-)-epicatechin and leucoanthocyanidins. The structures of these pear phenolics were shown in Figure 2.

Chlorogenic acid is the most important cinnamic acid derivative found in fruits, and the amount is large enough in apple to permit its isolation and characterization (Hulme, 1953). It is well established that chlorogenic acid is the main phenolic substrate involved in the enzymic browning reaction by polyphenoloxidase (EC 1.10.3.1) in pears (Weurman and Swain, 1953; Hulme, 1958; Walker, 1964).

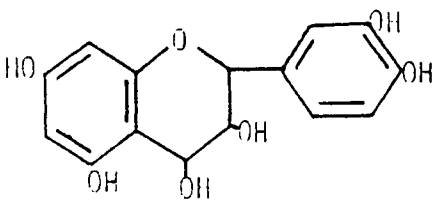
(+)-Catechin and (-)-epicatechin are the most common forms of the flavans. They often appear together in fruits. In pears (Pyrus communis), (+)-catechin and (-)-epicatechin are present only on the dessert types such as the Bartlett variety (Nortje, 1966) but are not in the perry pears (Williams, 1957). It was reported that (+)-catechin and (-)-epicatechin are the principal substrates for enzymic browing in the skins of Bartlett pears when the tissue was damaged (Sioud and



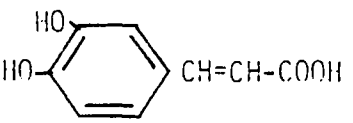
Chlorogenic acid



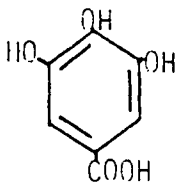
Catechin or Epicatechin



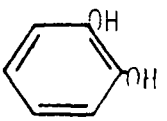
Flavan-3,4-diol
(Leucoanthocyanidins)



Caffeic acid



Gallic acid



Catechol

Figure 2. Structures of phenolic compounds.

Luh, 1965; Siegelman, 1955).

Leucoanthocyanidin exists in all fruits. It is a type of condensed flavan which yields anthocyanidin when heated with dilute acids. The flavans have a pronounced tendency to condense with themselves, the flavan-3,4-diols undergoing self-condensation in hot water or cold acids at a faster rate than catechins. When fruits are heated the flavan condensation causes the loss of low molecular weight flavans. Flavan-3,4-diols are suspected to be precursors of the leucoanthocyanidins (Van Buren, 1970).

Tate et al. (1964) reported that pear polyphenoloxidase was active only on polyphenols having an ortho-diphenolic configuration; chlorogenic acid, caffeic acid and catechin were shown to be phenolic substrates for polyphenoloxidases.

Tannins

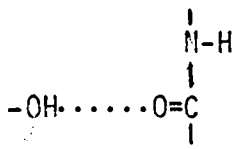
"Tannins" is used loosely to designate a heterogenous group of plant phenolics which are able to combine with animal skin protein and convert the raw hide into leather.

Tannins are high molecular weight compounds (M.W. 500-5,000). They are classified into two types of compounds: The first type are the condensed tannins. They are mostly polymers of catechins and/or flavan-3,4-diols (leucoanthocyanidins). The second type are the hydrolyzable tannins. They are generally derived from gallic acid. (Figure 2) and contain a central core of glucose (Walker, 1975; Loomis and Battaile, 1966).

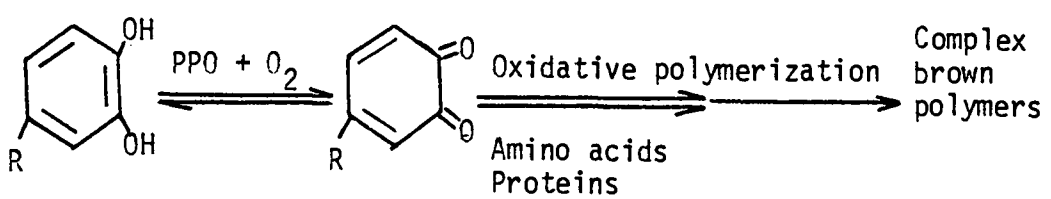
Phenolic and Protein Interactions

Phenolics and proteins frequently occur in associated insoluble complexes in juices and wines (Heatherbell, 1976). Much of our knowledge of these reactions comes from works in the field of leather research (Van Buren and Robinson, 1969). Phenolics combine with protein reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensations. Their reactions with proteins may be divided into four principal classes (Loomis, 1974):

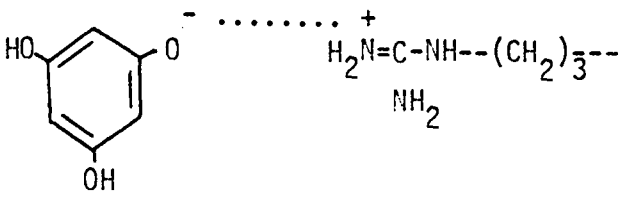
(1) Hydrogen bonding. The hydroxyl groups of the phenolics form very strong hydrogen bonds with the carbonyl groups of the protein peptide bonds (Gustavson, 1954). The reaction is shown below:



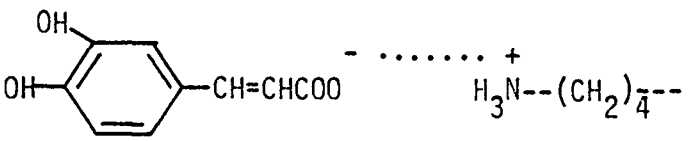
(2) Covalent reactions. Quinones, the oxidation products of phenolics, are highly reactive and tend to polymerize. They condense readily with protein by covalent bondings. This is the basic reaction of the enzymic browning occurred in fruits. The oxidation of a range of o-dihydroxy-phenols to the corresponding o-quinones is carried out by polyphenol oxidase (PPO, EC 1.10.3.1). The generalized reaction scheme is shown below (Walker, 1977):



(3) Ionic interactions. At high pH's the dissociated hydroxyl groups of phenolics may interact with the basic amino acid residues of proteins. At neutral pH and below, the phenylpropanoid phenolics may interact with proteins through their negatively charged carboxyl groups. Two examples of the interactions are shown below:



phloroglucinol arginine



caffeic acid lysine

(4) Hydrophobic interactions. The hydrophobic interactions occur between the aromatic rings of phenolics and the hydrophobic regions of proteins, i.e. the regions which are rich in phenylalanine, valine, leucine etc.

Monomeric phenolic substances such as gallic acid and catechol (Figure 2) do not interact with the protein (Hoff and Singleton, 1977). Heatherbell (1976b) also indicated that simpler phenolics such as chlorogenic acid have little affinity for proteins but under the influence of heat and oxygen can condense to form typical tannins which may bind proteins.

Total Phenolic Determination

The quantitative estimation of the total phenolics in biological extracts can be accomplished in a number of different ways. It was found that methods based on the use of oxidizing agents are the most useful (Swain and Hillis, 1959). The modified method of Folin and Denis has proved to be more convenient than the method of Lowenthal, and is considered the method of choice for estimating total phenolic content in complex plant materials (Swain and Hillis, 1959; Swain and Goldstein, 1964). The A.O.A.C. official procedures include the Folin-Denis colorimetric method for distilled alcoholic beverages and wines.

Folin and Denis (1912) proposed a colorimetric method based on the reduction of phosphomolybdic-phosphotungstic acid reagent in alkaline solution as a general phenol reagent. Folin and Ciocalteu (1927) subsequently modified the reagent to increase its sensitivity by increasing the molybdate content and added lithium sulfate to prevent precipitation. It was further modified for wine by Singleton and Rossi (1965).

Singleton and Rossi (1965) indicated that direct application of the official A.O.A.C. method or descriptions in other reference works often leads to difficulties such as the formation of a troublesome precipitate and deviation from the Beer-Lambert law. In their modification, gallic acid was adopted as the standard instead of tannic acid. Tannic acid is glucose-pentagalloyl gallate and contains ten potential gallic acid moieties. Since the purity of gallic acid is easier to obtain and to demonstrate, gallic acid was adopted as the standard.

MATERIALS AND MATHODS

Source of Pear Juice

The ripe Bartlett pear juices from 1975, 1976 and 1977 seasons were pressed from pears obtained from Modesto, California, ripened at 70°F to a pressure test of 3-5 lbs, and hammer-milled in a Fitzpatrick mill at a blade tip speed of 36 mph with a 3/4 inch round holed screen. Pear pulp was heated to 130°F and held for one hour without addition of enzyme. One percent of fluffed paper and one percent of rice hulls were added and the pulp was pressed in a rack and cloth hydraulic press at 100 p.s.i. on the pressed surface. The frozen juice samples were stored at -10°F until used.

Preparation of Acid Precipitated Colloid

Pretreatment of Juice and Acidification

The juice samples were removed from freezing storage, thawed, an aliquot of the juice was taken and was centrifuged in a International centrifuge (University Model UV) at 2,000 rpm (1,120g) for 10 min to remove cellular materials and paper fibers. The supernatant juice was decanted off and the pH of the juice was adjusted with 50% (w/v) citric acid to 3.5.

Enzymic Clarification

Fifty ml juice samples of the acidified juice were measured into

50 ml graduated cylinders, Rohm and Haas Pectinol 59-L pectinase enzyme was diluted to 10% (v/v) and one ml was pipetted into the juice. After mixing each sample (inverting the cylinder seven times), the juice was held for two hours in a water bath at 120°F. Water level in the water bath was 1/2 inch above the upper level of the juice in the graduated cylinder.

Isolation of the Precipitate

After two hours of the holding period, the acid precipitated colloid was isolated by centrifuging for 10 min in a Servall centrifuge at 15,000 rpm (27,000g).

Washing Test of the Precipitate

Washing tests were conducted to select the proper washing medium for the precipitates. Two tenths gm of the precipitate samples were washed five times by resuspending them in 10 ml of four different washing media. The washing media tested were distilled water, 80% (v/v) ethanol, 95% (v/v) ethanol and 0.2M citrate buffer (pH 3.5). After each washing, the precipitate was centrifuged at 15,000 rpm (27,000g) for 10 min. The supernatants were decanted off. After each washing period the absorbance at 280 nm was taken by using Perkin Elmer Spectrophotometer Model 550.

Washing of the Precipitate

After the washing medium was selected, each 1.5 gm portion of the colloidal precipitate was washed three times by resuspending it in

25 ml of 0.2M citrate buffer adjusted to pH 3.5 with citric acid and sodium citrate and centrifuging as indicated above. The supernatants were decanted off.

Freeze Drying and Moisture Determination

The buffer-washed precipitate was air dried by spreading it on watch glasses and when dry was ground into a fine powder with a mortar and pestle. The powdered samples were freeze-dried at 20-30 microns vacuum at a plate temperature of 38°C for 48 hr in a Hull Corporation freeze dryer. The samples were stored in screw-capped bottles in a silica gel desiccator at ambient temperature until used.

The moisture content was determined by the vacuum oven method at 26"Hg at 70±1°C for 16 hr according to the official methods of A.O.A.C. (1975a).

Amino Acid Analysis

Total Amino Acid Analysis

Amino acid content of the pretreated cloudy juice samples, clarified juice samples and precipitate samples from the 1975, 1976 and 1977 juices were determined according to the method of Spackman *et at.* (1958). Analyses were conducted in an Automatic Amino Acid Analyzer, Beckman Model 120B, modified to use a single column. The samples were hydrolyzed with 6N HCl at 110°C for 20 hr prior to analyses.

Free Amino Acid Analysis of Pretreated juice

Pretreated juice samples from the three years were deproteinized by mixing 5 ml of the sample with 20 ml of a 30 gm/l aqueous sulfosalicylic acid solution, and then the precipitate was removed by centrifuging in a Servall centrifuge as indicated before. An aliquot of the supernatant was mixed with an equal volume of citrate buffer (0.2M, pH 2.2) and the amino acid analysis was carried out as indicated above, with the exception that the samples were not hydrolyzed prior to the analysis (Gerritsen and Niederwieser, 1974).

Crude Protein Determination of Precipitate

The official method of the A.O.A.C. (1975b) was used for the determination of nitrogen content (micro-Kjeldahl, sections 42.021, 47.022, 47.023). The crude protein content was claculated as percent N x 6.25.

Mineral Analysis

One hundred mg samples of the precipitates from the 1975, 1976 and 1977 juices were digested overnight with 2 ml of 70% $HClO_4$ and 5 ml of concentrated HNO_3. After the digestion, the residues were washed into 25 ml volumetric flasks with 0.1N HCl.The solutions were analyzed for copper, iron, magnesium and calcium by using a Jarrel-Ash 82-500 Atomic Absorption/Flame Spectrophotometer. Sodium and Potassium were analyzed by using a Perkin-Elmer Coleman 51-Ca Flame Emission Photometer.

Phenolic Analysis

Alkali Fusion Test

The basis of the alkali fusion test was a modification of the potassium hydroxide fusion of Johnson et al. (1968). Potassium hydroxide (1.5 gm) was added to 25 mg samples of precipitate and the mixture was heated on a hot plate at temperatures of 415°C, 390°C, 370°C and 315°C for various lengths of time from 50 seconds to 15 minutes. The cooled mass was dissolved in 5 ml of water and then acidified with concentrated HCl. The resulting solution was extracted with two 20 ml portions of diethyl ether. The combined ether extracts were dried under a reduced pressure in a rotary evaporator. The residue was dissolved in 10 ml of 80% (v/v) ethanol and the total phenolics was determined by the method of Singleton and Rossi (1965).

Total Phenolic Determination

Twenty five mg of the precipitate samples were alkali fused at 370°C for 5 min as described before. The acidified extract was brought up to the volume in a 50 ml volumetric flask with distilled water. A 2 ml aliquot was mixed with 10 ml of a 1/10 aqueous dilution of Folin-Ciocalteu reagent (Fisher Scientific Company, 2N solution). After 2 min, 8 ml of water containing 0.6 gm of anhydrous Na_2CO_3 (75gm/l) was added and mixed. After 2 hr, the absorbance was determined at 765 nm by using the Beckman DB spectrophotometer. The total phenolic content was calculated in gallic acid equivalents by comparing with a standard curve.

The degree of destruction of phenolics during the severe alkali fusion process was estimated by determining the percent recovery of tannic acid. A 25 mg sample of tannic acid (Sigma Chemical Company) was alkali fused at 370°C for 5 min and the total phenolics was determined. The percent recovery of tannic acid after alkali fusion was calculated as:

$$\text{Percent recovery of tannic acid} = \frac{\text{Total phenolics (mg) determined after alkali fusion}}{\text{Weight (mg) of tannic acid sample (calculated on a moisture free basis)}} \times 100$$

Pectin and Sugar Analysis

Hydrolysis Test for Pectin Determination

Polygalacturonic acid was used as a substitute for the precipitate to find the best hydrolysis conditions for pectin determination. Twenty mg of polygalacturonic acid (Sigma Chemical Company, Grade 3, Approx. 98%) were hydrolyzed under different conditions as follows: (1) with 5 ml of 2N HCl at 100°C for 3 hr. (2) with 5 ml of 6N HCl at 100°C, 110°C and 120°C for various lengths of time, from 2.5 min to 18 min. The extent of the hydrolysis was measured by gas chromatography on the basis of galacturonic acid produced from the polygalacturonic acid.

Acid Hydrolysis of the Precipitate

Samples (200 mg) of the pear precipitate were hydrolyzed under two different conditions: (1) with 20 ml of 2N HCl at 100°C for 3 hr and (2) with 20 ml of 6N HCl at 100°C for 14 min. The hydrolysate samples

were adjusted with sodium hydroxide solution to a pH of 3.0.

Isolation of Acids from Sugars by Ion Exchange

The method used for the separation and determination of the acids and sugars of the precipitate was a modification of the procedures of Akhavan (1977). Two chromatographic columns were plugged with glass wool. Approximately 9 ml Dowex 1-X8 anion exchange resin (200-400 mesh, acetate form, Bio-Rad Chemical Co.) and 6 ml Dowex 50W-X4 cation exchange resin (200-400 mesh, hydrogen form, Bio-Rad Chemical Co.) were placed in the two columns respectively and the resin beds covered with glass wool pads.

Fifty ml 0.1N acetic acid was added to 1-X8 column and allowed to elute. The 50W-X4 column was then placed above the 1-X8 column and the precipitate hydrolysate sample was poured into the connected column. When all the sample had been eluted, the column was washed with deionized water until 200 ml of the effluent containing sugars was collected.

After the upper 50W-X4 column was removed, 50 ml of 10N formic acid was poured through the 1-X8 column to displace the acid. Care was taken to ascertain that no liquid remained above the resin bed prior to the addition of formic acid, since this would effectively dilute the acid. The column was then washed with deionized water until 100 ml of effluent containing acids was collected.

Gas Chromatographic Equipment and Conditions

A dual-column Varian Aerograph Model 200 gas chromatography (GC) unit with hygrogen flame ionization dectors was used for GC analysis of the acids and sugars. Two 10' X 2 mm i.d. glass columns were packed: one with 3% SE-30 (for acid analysis) and the other with 5% SE-52 (for sugar analysis) on 80/100 mesh Chromosorb W (HP). GC operating conditions common to both columns were: injector temperature (190°C), and detector temperature (250°C) and nitrogen carrier gas flow rate (25 ml/min). The SE-30 column was programmed from 100°C to 250°C at 6°C/min. The SE-52 column was operated isothermally at 165°C for 14 min, then programmed at 12°C/min to 250°C.

Pectin and Sugar Determination by Gas Chromatography

For pectin determination, the 100 ml acid effluent collected was placed in a 500 ml round bottom flask and evaporated to complete dryness on a rotary evaporator. The residue was taken up by thoroughly washing the wall of the flask with 3 ml deionized water. One ml aliquots were transferred to 3 ml vials with Teflon-lined screw caps and 100 µl of a 1% (w/v) tartaric acid solution (internal standard for acid) were added to the vials.

For sugar determination, one ml aliquots of the sugar effluent were transferred to 3 ml vials and 100 µl of a 0.2% (w/v) rhamnose solution (internal standard for sugars) were added to the vials.

The acid and sugar vials were taken to dryness on a rotary evaporator. The vials were stored under vacuum over P_2O_5 until silylated.

Three hundred µl of "Tri-Sil" silylation reagent (Pierce Chemical Co.) was added to each vial using a micropipet (Centaur). The acid vials were shaken for 5 min, heated at 50°C for 30 min and finally centrifuged for 5 min. The sugar vials were shaken for 5 min, heated at 70°C for 20 min, then shaken again for 15 min and finally centrifuged for 5 min. Two µl of the supernatant samples were injected directly into the appropriate column of the gas chromatograph and retention times and areas were obtained.

Lipid Analysis

Total lipid extraction and determination

The method used for the extraction and determination of total lipids of the pear precipiate was a modification of the method of Folch et al. (1957).

Ten ml of 2:1 chloroform-methanol (CM) mixture (v/v) was added to a 0.5 gm sample of pear precipitate, and was homogenized with a mortar and pestle. The homogenate was filtered through a Whatman No.5 filter paper. The solid residue was rehomogenized with 10 ml of 2:1 CM mixture. The combined filtrate was mixed thoroughly with 0.2 of its volume of 0.9% NaCl solution in a separatory funnel and was acidified with one drop of 20% HCl solution. The mixture was shaken thoroughly and allowed to separate into two layers. The lower layer was then eluted into a 100 ml round bottom flask. The upper layer was further mixed with 10 ml of chloroform and allowed to settle. The lower layer was again eluted and combined with the previous lower layer portion in

the round bottom flask.

The combined lower layer was concentrated to dryness on a rotary evaporator. The residue was taken up with 2:1 CM mixture and transferred into a tared vial. The vial was placed under a stream of nitrogen to evaporate the solvent, and then stored under vaccum for one hr in a desiccator, and weighed. The percent of total lipids was calculated as follows:

$$\text{Percent of total lipids} = \frac{\text{weight of lipid residue}}{\text{weight of precipitate sample (calculated on a moisture free basis)}} \times 100$$

Analysis of the component fatty acids

The fatty acids were released from the lipid extracts of the pear precipitate by saponification with 4% methanolic H_2SO_4 for 1 hr at 85 °C. After transesterification, the methyl esters were extracted with hexane and separated by gas chromatography. Gas chromatography was carried out in a Varian (1200) instrument equipped with a flame ionization detector and a 15% ethylene glycol succinate column at 185°C.

RESULTS AND DISCUSSION

Washing the Precipitate

The freshly isolated acid precipitated colloid from the Servall centrifuge contains occluded pear juice. In order to determine the composition of the precipitate, it was necessary to wash the precipitate to remove the occluded pear juice.

Due to the possibility that some of the precipitate components might be soluble in the washing medium and thus be removed, the selection of the washing medium was carefully considered. Prolamines (a group of wheat proteins) are soluble in aqueous alcohol, and were classified according to this characteristic (Anglemier and Montgomery, 1976). Scott et al. (1965) also mentioned that the commonly recognized nitrogenous components, amino acids and phospholipids, may be removed as water-, alcohol- or hexane soluble materials.

Neubert and Veldhuis (1944) washed the sediment of clarified apple juice with water; Johnson et al. (1968) washed the same kind of material without mentioning the washing medium in their report; Heatherbell (1976a) washed the centrifugal precipitates of wine sediments by resuspending it in chilled 80% (v/v) ethanol.

Washing studies were conducted with distilled water, 80% ethanol, 95% ethanol and citrate buffer. The first two media were tried because they were mentioned in the literature as cited above. The reason for testing 95% ethanol was to determine if there was any difference between ethanol of different concentrations. The citrate buffer (pH 3.5)

was tried, because the juice is a natural buffer system and citric acid is a common juice acid. Another reason for using citrate buffer is because the precipitate was gathered from the clarification process using citric acid to lower the pH to 3.5.

The results are shown as curves in Figure 3. According to these washing curves, water appeared to be the poorest washing medium. Its washing behavior was irregular (zig-zag curve) and apparently it dissolved a considerable amount of material each time from the precipitate, therefore the possibility of using water as washing medium was excluded.

Comparing the ethanols (80% and 95%) and citrate buffer, they all seemed quite efficient in removing the entrapped juice, since the washing curves all showed a drastic change in absorbance (280 nm) after the first washing. The high absorbance of the alcohol washings (both 80% and 95%) indicated the possibility that the ethanols not only removed the entrapped juice but also dissolved some alcohol soluble components of the precipitate.

Since the citrate buffer was able to remove the entrapped juice as efficiently as the ethanols and dissolved the least amount of the precipitate components, the citrate buffer was chosen as the washing medium of the precipitate. Three washing were adopted as standard procedure, since after the third washing, further washings made little changes in absorbance at 280 nm.

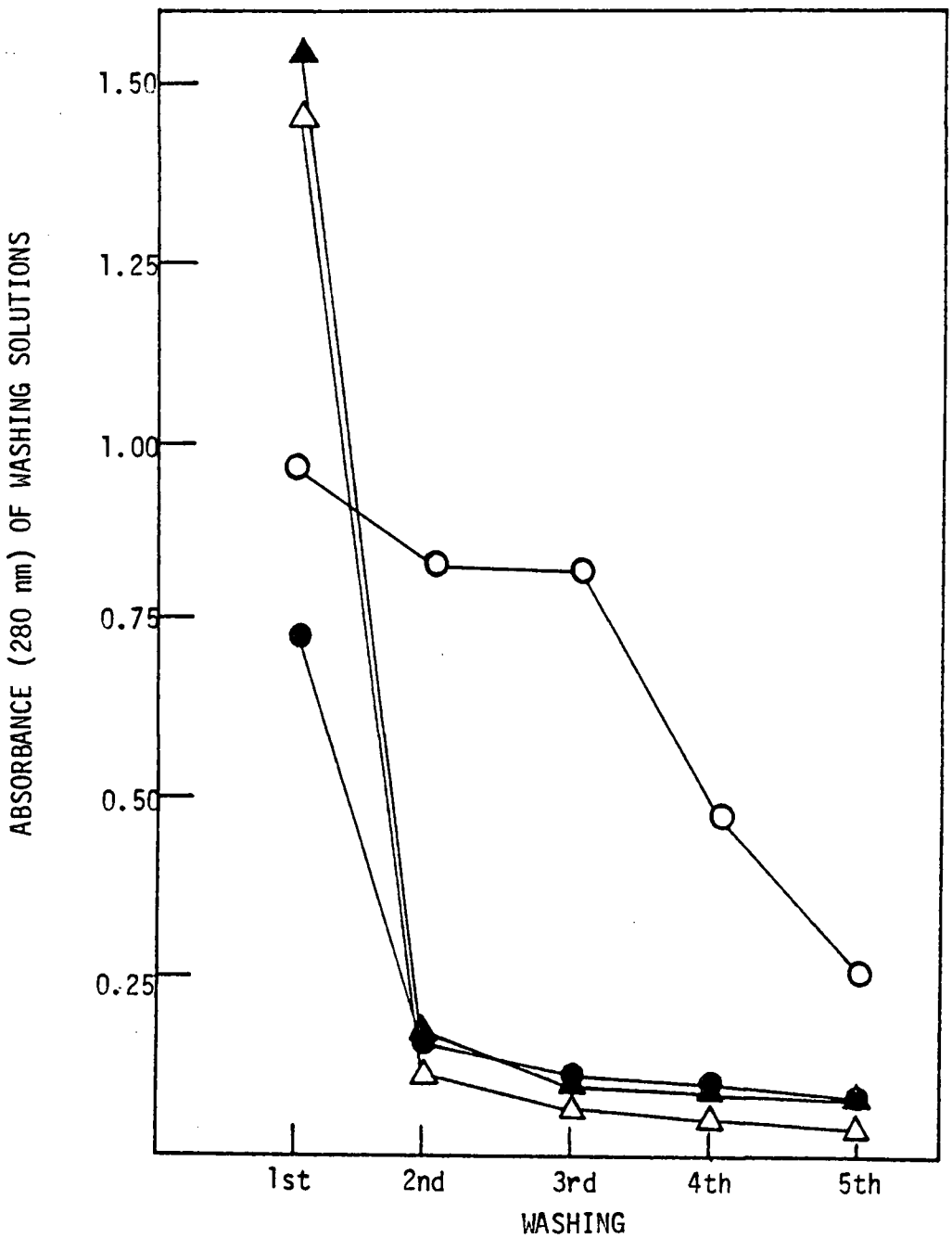


Figure 3. Washing tests of acid precipitated colloid of ripe Bartpear juice. Citrate buffer, ●——●; 95% ethanol, ▲——▲; 80% ethanol, △——△; distilled water, ○——○.

Amino Acid Analysis and Crude Protein Determination of Precipitate

The amino acid compositions of the precipitates are shown in Table 2. It shows high amounts of leucine, aspartic acid and glutamic acid, in which aspartic and glutamic acids were partly produced as a result of the hydrolysis of asparagine and glutamine.

The lack of methonine is also shown. This may be explained by the low methionine content of the pear juice samples (Table 4). Heatherbell (1976a) demonstrated the presence of 19 amino acids in the hydrolyzed extracts of apple juice and apple wine sediments. Methionine was also absent in his samples. The amino acid composition of the clarified apple juice sediment reported by Johnson et al. (1968) also listed methionine as the lowest amino acid. Tryptophan is not included because it was destroyed during acid hydrolysis of the precipitate samples.

The estimated protein content (by adding up the weights of individual amino acids and ammonia presented in Table 2) of 1975, 1976 and 1977 precipitate is 31%, 26% and 37%, respectively. Table 3 shows the crude protein contents (calculated as percent N x 6.25) of 1975, 1976 and 1977 precipitate as 30.8%, 36.6% and 35.6%, respectively. The estimated protein contents and the crude protein contents match fairly closely, except for the 1976 determination. The 1975 precipitate has the lowest crude protein content (30.8%). This may be due to the lowest protein content (0,07%) of the 1975 juice (see Table 6).

Yamasaki (1964) showed that the precipitate of apple juice contained 36% protein; Monties and Barret (1965) reported 20% protein in the haze of apple juice and cider. The results of the protein content

in this study also fall into a simillar range.

Table 2. Amino acid composition of acid precipitated colloid of ripe Bartlett pear juice (calculated on moisture free basis).

Amino acid (AA)	1975 precipitate	1976 precipitate	1977 precipitate
	(gm AA/100 gm precipitate)		
Lysine	2.15	1.98	2.77
Histidine	0.90	0.86	1.17
Arginine	1.86	1.53	2.03
Aspartic acid	3.55	3.06	4.36
Threonine	1.56	1.32	1.92
Serine	1.66	1.42	1.97
Glutamic acid	3.89	3.29	4.78
Prolin	1.54	1.35	1.96
Glycine	1.47	1.26	1.85
Alanine	1.66	1.38	2.00
Half cystine	0.02	0.04	0.04
Valine	1.89	1.63	2.36
Methionine	--	--	0.06
Isoleucine	1.66	1.42	2.03
Leucine	2.87	2.42	3.48
Tyrosine	1.47	1.26	1.62
Phenyl alanine	1.86	1.55	2.25
(Ammonia)	0.64	0.58	0.76
Total	30.6	26.4	37.4

Table 3. Crude protein content of acid precipitated colloid of ripe Bartlett pear juice (calculated on moisture-free basis).

Year	Nitrogen (percent)	Protein* (percent)
1975	4.93	30.8 ± 0.5
1976	5.86	36.6 ± 0.7
1977	5.69	35.6 ± 0.7

* Calculated as %N x 6.25.

Amino Acid Analysis of the Cloudy Ripe Bartlett Pear Juice

The presence of methionine, which was absent in the precipitate, was shown in all the analyses of the cloudy juices (Table 4), although the amount is very low compared to other amino acids. It is also shown that the majority of the methionine exists as free amino acid. The total amino acid analyses show the extremely high amount of aspartic acid and ammonia in all the samples. In free amino acid analyses, threonine and serine are apparently predominant amino acids in all the samples; but this is due to the combination of asparagine and/or glutamine. Ulrich and Thaler (1955, 1957) examined the amino acids of ripe Williams (Bartlett) and Passe Crasanne pears, they also found chiefly aspartic acid and asparagine.

The percentages of the free amino acid in total amino acid of the juices were calculated and shown in Table 5. They were 47.3%, 36.8% and 32.8% for the 1975, 1976 and 1977 juices, respectively. It demonstrated that a very large amount of the juice "protein" existed as free amino acids, especially in the juice of 1975 season.

Table 4. Amino acid composition of the cloudy ripe Bartlett pear juice (mg AA/100 ml juice).

Amino acid (AA)	1975 juice		1976 juice		1977 juice	
	Total AA	Free AA	Total AA	Free AA	Total AA	Free AA
Lysine	4.51	1.91	4.01	0.78	5.27	1.34
Histidine	1.97	0.72	1.16	0.54	2.08	0.52
Arginine	1.98	0.67	2.07	0.55	3.50	0.68
Aspartic acid	59.6	4.47	96.2	--	189	--
Threonine	3.41	1.30	3.20	18.8*	5.21	33.3*
Serine	5.64	30.8	4.90	24.5*	7.17	36.2*
Glutamic acid	9.20	1.37	8.90	3.40	13.8	5.22
Proline	14.1	12.7	5.94	4.40	7.60	4.76
Glycine	3.00	0.46	2.63	0.16	4.15	0.27
Alanine	4.37	1.94	3.12	0.73	4.97	1.52
Half cystine	--	--	--	tr	--	tr
Valine	4.60	2.22	3.58	1.21	5.43	1.73
Methionine	0.69	0.43	0.40	0.44	0.89	0.55
Isoleucine	5.76	1.93	3.25	1.40	4.61	1.62
Leucine	5.49	2.03	3.91	0.42	6.01	0.97
Tyrosine	1.68	0.66	1.49	0.22	2.28	0.81
Phenyl alanine	5.27	2.46	3.74	1.70	6.76	4.42
(Ammonia)	9.04	0.08	11.9	0.40	19.1	0.72
Total	140	66.2	162	59.6	288	94.6

* Asparagine and/or glutamine cochromatographed.

Table 5. Amino acid and protein content of the cloudy ripe Bartlett pear juice (percent).

	1975 juice	1976 juice	1977 juice
% Free AA in total AA[1]	47.3	36.8	32.8
Total AA content[2]	0.13	0.15	0.27
Free AA content[3]	0.06	0.05	0.09
Protein content[4]	0.07	0.10	0.18

1. Percent free AA in total AA = wt. of free AA (per ml juice)/ wt. of total AA (per ml juice).
2. Total AA content = wt. (gm) of total AA (per ml juice)/ specific gravity of juice (see Table 6).
3. Free AA content = wt. (gm) of free AA (per ml juice)/ specific gravity of juice.
4. Protein content = total AA content - free AA content.

Table 6. Degree Brix and specific gravity of ripe Bartlett pear juice.

Year	Degree Brix	Specific gravity at 20/20°*
1975	12.8	1.05168
1976	13.7	1.05548
1977	13.0	1.05252

* From methods of A.O.A.C. (1975), page 990.

Since the 1975 juice has the highest percentage (47.3%) of free amino acid in total amino acid and has the lowest total amino acid content (o.13%), it therefore has the lowest protein content (0.07%). Table 5 also shows that the protein content of 1976 juice (0.10%) is higher than that of 1975 juice, and the 1977 juice (0.18%) again, is higher than the 1976 juice. Therefore, lack of protein in 1975 juice may be one of the possible cause of the clarification difficulty. The higher protein content of the 1976 and 1977 juices may be one of the reasons that they were easier to clarify than the 1975 juice.

Lack of protein may influence the proper formation (growing) of the protein-phenolic complexes. The small-sized complexes thus formed could still exist as suspended colloids and may not be removed as precipitates.Protein may also serve as a positive charge carrier in the juice. The flocculation due to the positively charged protein and the negatively charged suspended particles might be decreased, if there were an insufficient amount of protein.

Amino Acid Analysis of The Clarified Ripe Bartlett Pear Juice

The results of Table 7 show that at least about 80% of the total amino acid composition was retained in the juice after the clarifition process. In the clarified juice, it still shows high amounts of aspartic acid and ammonia. The content of glutamic acid and proline are also relatively high. From the nutritional standpoint, except for tryptophan, (which would be destroyed by HCl hydrolysis if it was present) the clarified ripe Bartlett pear juice contained all the essential amino acids, i.e. lysine, phenyl alanine, threonine, valine, methionine, leucine and isoleucine.

Table 7. Amino acid composition of the clarified ripe Bartlett pear juice (mg AA/100 ml juice).

Amino acid	1975 juice	1976 juice	1977 juice
Lysine	2.70	2.28	3.17
Histidine	1.56	2.34	1.37
Arginine	0.87	1.15	1.50
Aspartic acid	60.1	94.8	187
Threonine	2.12	2.26	3.52
Serine	4.30	3.42	6.23
Glutamic acid	5.60	6.15	10.2
Proline	13.1	4.60	6.32
Glycine	1.63	1.42	2.40
Alanine	2.90	1.93	3.44
Half cystine	tr	tr	--
Valine	2.98	2.15	3.41
Methionine	0.07	0.20	0.31
Isoleucine	2.40	1.96	2.89
Leucine	2.72	1.72	3.21
Tyrosine	0.97	tr	1.37
Phenyl alanine	4.06	1.68	5.47
(Ammonia)	8.77	1.17	21.0
Total	117	129	263
Total AA of clarified juice/total AA of cloudy juice	83.6%	79.6%	91.3%

Mineral Analysis

Table 8 shows the mineral content of the pear precipitate. A wide range of metals, notably calcium and copper, were found in the precipitate. Compared to the analytical results of clarified apple juice sediment for metal ions by Johnson et al. (1968), the pear juice precipitate is extremely low in copper and iron. Calcium content appears to be in the same order of magnitude.

Table 8. Mineral content of the acid precipitated colloid of ripe Bartlett pear juice (calculated on moisture-free basis).

Year	Ca %	Na %	Cu ppm	Fe ppm	Mg ppm	K ppm
1975	0.11	1.48	35	154	141	361
1976	0.06	1.26	73	123	40	201
1977	0.23	1.51	68	209	83	295

Neubert et al. (1944) reported a negative qualitative test for calcium of clarified apple juice sediment and eliminated the possibility of the sediment being calcium pectate. If pectin is demethoxylated to any great extent, calcium, magnesium and other divalent ions in the juice will cross link adjacent pectin chains at the carboxyl groups. Depectinization inhibition will occur and the juice clarification rates will be impared. If these divalent ions cross linking the pectins were removed, then the pectinase enzymes could attack the pectins more easily and improve the clarification and filtration

rates. By using the chelating agents, such as EDTA (disodium salt of ethylenediaminetetraacetic acid), sodium hexametaphosphate and sodium phytate; this concept was substantiated (Beavers and Youtz, 1976). In the pear precipitates, except for sodium (due to the sodium citrate in the washing buffer), the amount of calcium is the highest among the six metal ions analyzed. It was reported that calcium ion strongly inhibited the depectinization and clarification of apple juice (Endo, 1965c). Since pear pectin (DE 50%) (Raunhardt and Neukom, 1964) has more free carboxyl groups available for calcium cross linking than apple pectin (DE ca. 90%) (Endo, 1965b), the inhibition of depectinization and clarification due to the calcium ion should be more profound in the pear juice. Therefore, high calcium content might be part of the reasons for the clarification and filtration difficulties of the pear juice. Comparing the calcium contents of 1975, 1976 and 1977 precipitates (0.11%, 0.06% and 0.23%, respectively), no conclusions can be made concerning the clarification difficulties of the 1975 juice.

Magnesium and potassium contents appear moderately higher and copper content moderately lower in the 1975 precipitate. Metal ions, in particular copper have been shown to catalyze the degradation of juice phenolics, particularly of leucoanthocyanins and epicatechin, leading to their precipitation as oxidized polymerized phenolics and as phenolic-protein-metal ion complexes (Kieser et al., 1957). In addition, polyphenol oxidase (EC 1.10.3.1), which may cause the oxidation of the phenolics (o-dihydroxyphenols), contains bivalent copper ion as its prothetic group (Kubowitz, 1938). Therefore, low copper

content might influence the proper oxidation, condensation and precipitation of the phenolics, thus might contribute some difficulties to the juice clarification.

Alkali Fusion Test

Many phenolics are readily oxidized to quinones (nonenzymatically or by phenol oxidase), polymerize rapidly, and form covalent bonds with protein rapidly (Loomis and Battaile, 1965). Van Buren et al. (1976) also reported that tannins (polyphenols) naturally occurring in the juice are oxidized and polymerized during the enzymatic treatment of apple juice. Therefore, the polyphenols in the precipitates most likely existed as oxidized polymerized complexes covalently bonded with proteins. On the basis of the above consideration, neither insoluble polyvinylpyrolidone (PVP), 6-8M urea, dilute alkali, nor aqueous organic solvents which are capable of hydrogen bonding, such as alcohols or acetone (Loomis and Battaile, 1965) can serve as a satisfactory phenolic stripping agent for the pear precipitate.

Potassium hydroxide fusion method was widely adopted for the tannin (polyphenols) investigations of the juice sediments (Muller and Zellner, 1953; Neubert and Veldhuis, 1944; Johnson et al., 1968; Heatherbell, 1976a). The preliminary test of the potassium hydroxide fusion method according to Johnson et al. (1968) i.e. at ca. 300°C for 5 min, showed only a trace amount (less than 0.4%) of the phenolics in the precipitate samples. Therefore, different fusion temperatures and different lengths of reaction time were tested. The

results are shown in Figure 4. The fusion curves show clearly that higher temperatures are necessary to obtain phenolics. Although the maximum phenolic content was obtained by alkali fusion at 415°C, after a critical point of time (one min), the phenolics tend to be destroyed very rapidly. Since the destruction at 370°C is much less drastic, alkali fusion at 370°C for 5 min was adopted as the analytical method to determine the phenolic content of the precipitate.

Total Phenolic Determination

The term "total phenolics" embraces a large array of chemical compounds (Walker, 1975). Since individual compounds vary widely in their chemical and physical properties, all the procedures for the estimation of total phenolics are necessarily empirical (Swain and Hillis, 1959). The basis of the total phenolic determination method used in this study is on the color forming reaction of the Folin-Ciocalteu reagent with monohydric phenols, polyphenols, flavonoids, tannins and other readily oxidized substances such as ascorbic acid (Singleton and Rossi, 1965).

The bonded phenolics in the precipitates were liberated by alkali fusion. Since the phenolics were subjected to the severe potassium hydroxide fusion, part of the phenolics were destroyed during the fusion process. Therefore, tannic acid was used as a reference to determine the degree of destruction of the phenolics during the fusion process. It was found that 23.8% of the tannic acid was recovered from the alkali fusion process. Therefore, a correction factor of 4.20 was

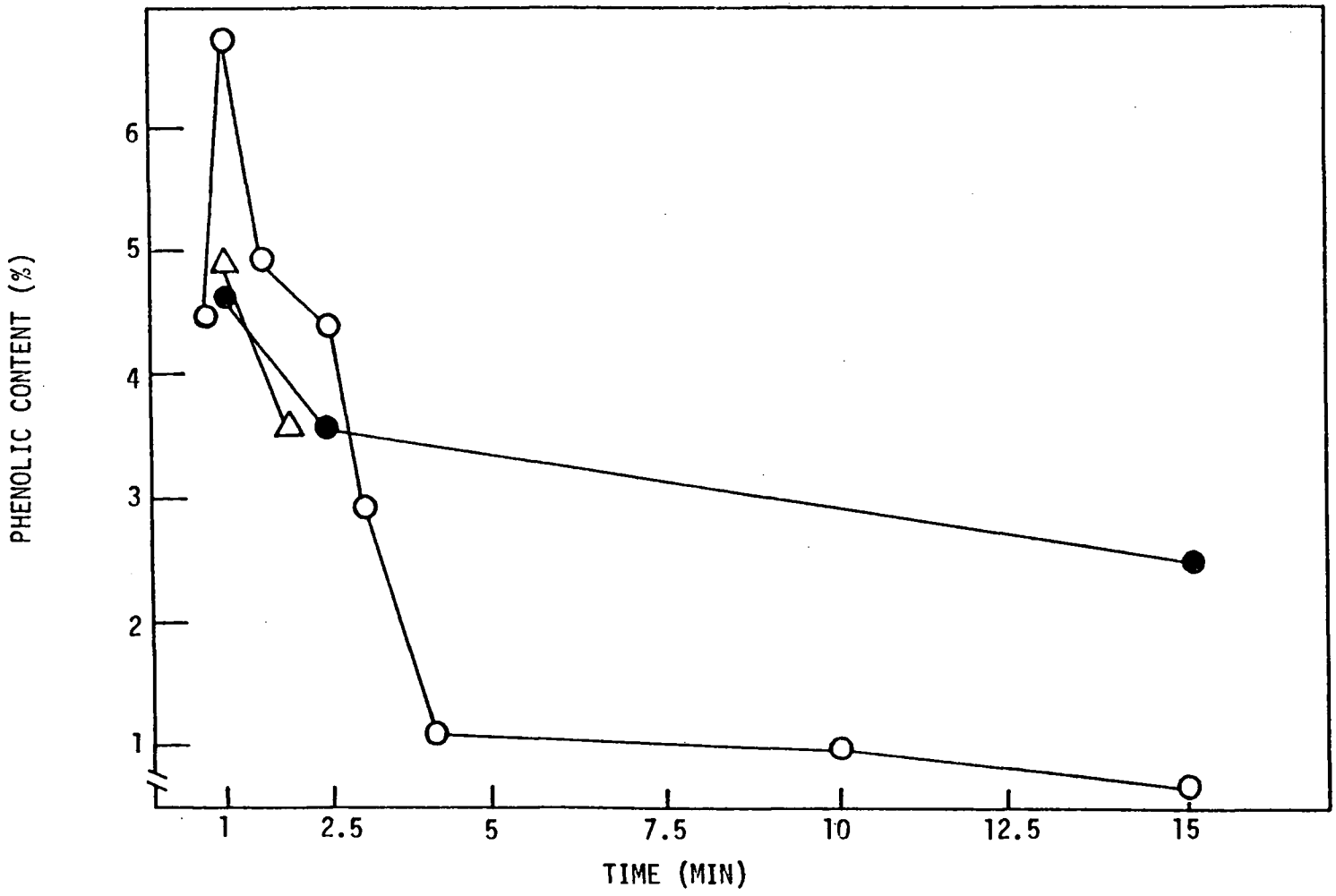


Figure 4. Potassium hydroxide fusion test of the acid precipitated colloid of ripe Bartlett pear juice. Sample at 415°C, O—O; at 390°C, Δ—Δ; at 370°C, ●—●.

used to calculate the total phenolic contents of the precipitates after the amounts of the phenolics recovered from the fusion were determined.

The results of the total phenolic contents of the precipitates are shown in Table 9. It shows that the total phenolic content of the 1975 sample (38.1%) is higher than that of the 1976 sample (33.9%) and the 1977 sample has the lowest (29.1%) total phenolic content. Phenolics are often reported as one of the major components of the sediments in the fermented pear juice and clarified apple juice (Kelhofer, 1908; Neubert and Veldhuis, 1944; Kieser et al., 1957), the large amount of the phenolics found in the pear precipitate (Table 9) was expected.

Table 9. Total phenolics of the acid precipitated colloid of ripe Bartlett pear juice (calculated on moisture free basis).

Year	Total phenolics (percent)
1975	38.1 ± 0.5
1976	33.9 ± 0.4
1977	29.1 ± 0.4

Plant phenolics play an important role in the juice clarification process. This may be shown by the tannin (polyphenols)-gelatin fining of fruit juice. Tannin molecules are negatively charged (Ratushnyi, 1958), which is caused by the dissociation of the carboxyl groups of the tannin molecules (Zitko and Rosik, 1962a). The fining process was generally considered to be based on the electrostatic reaction between

the positively charged gelatin molecule and the negatively charged tannins of the juice. This reaction produces a gelatin-tannin complex precipitate which carries down much of the suspended particles and thus clarifies the juice (Rüdiger and Mayr, 1929; Endo, 1965c). When tannin-gelatin complexes are formed, the positively charged centers in the gelatin molecules are often spacially blocked by tannin molecules. Therefore, the amount of tannin bonded in the complex and the pH of the juice, which determine the dissociation degree of the tannin carboxyl groups, greatly influence the electrical charge of the tannin-gelatin complex. Rüdiger and Mayr (1929) showed that at optimal gelatin addition, the complexes are electrically neutral.

Since the phenolics may spacially block the positively charged centers of the protein molecules and have the potential to convey negative charges to the protein-phenolic complexes, the relative amount (ratio) of the phenolics and proteins existing in the pear precipitate may greatly influence the electrical charge of the protein-phenolic complexes. The higher the phenolic/protein ratio is, the more negatively charged the complexes would be. The ratio of these two ingredients may possibily explain the clarification difficulties of the 1975 juice, and may also explain why the juices of 1976 and 1977 were easier to clarify. Table 9 shows that the total phenolic content of the 1975 precipitate was the highest (38.1%) among the three. It was also shown that (Table 3) the 1975 precipitate has the lowest crude protein content (30.8%). Therefore, the ratio of these two ingredients of the 1975 precipitate was the highest (1.24) among the three.

The high phenolic/protein ratio of the 1975 precipitate may cause the protein-phenolic complexes to become more negatively charged. These complexes would not readily aggregate and precipitate due to the greater repulsive forces between each other and the greater stabilizing effect of the negatively charged pectin molecules. Therefore, the juice of the 1975 season was the most difficult to clarify. Since the phenolic/protein ratios of the 1976 and 1977 precipitates (0.93 and 0.82, respectively) are lower than that of the 1975 precipitate (1.24), these precipitates are less negatively charged, and may aggregate and precipitate more readily. Therefore, the juice of the 1976 and 1977 seasons were more easily to clarify.

According to Beavers and Youtz (1976), the acidification and pH control greatly improve the clarification-filtration of the pear juice. The acidification of the pear juice, by lowering the pH to 3.5, caused the protein molecules to become more positively charged, and the phenolics and pectins less negatively charged (by supressing the dissociation of the carboxyl groups). Therefore, the protein-phenolic complexes will neutralize and flocculate more easily.

Hydrolysis Test for Pectin Determination

The purpose of this test was to find suitable hydrolysis conditions which could be used for the analysis of pectin in precipitate samples. When the precipitate was subjected to the acid hydrolysis, the methyl ester of its pectin component (if pectin were present) would be removed to yield polygalacturonic acid, and the glycosidic linkages would be depolymerized to yield galacturonic acid (Aurand and Woods, 1973). Therefore, polygalacturonic acid was chosen as a substitute for the precipitate samples for the acid hydrolysis tests.

The galacturonic acid recovery obtained by using the hydrolysis method of Heatherbell (1976a) (2N HCl at 100°C for 3 hr) was rather low (20%). The results of other hydrolysis conditions are shown in Figure 5. In the treatments of using 6N HCl at 110°C and 120°C, degradation of galacturonic acid occurred rapidly after a short period of time (3.5 min and 2.5 min, respectively). Under such conditions, time was very critical and the reaction time was very short. Therefore, it has the disadvantages of demanding very accurate timing and the short reaction time probably will not be sufficient for adequate hydrolysis. Using 6N HCl at 100°C (boiling water bath) for 14 min gave the most satisfactory results (30% recovery of the galacturonic acid). The reaction time (14 min) is longer for more adequate hydrolysis of the precipitate and the timing is not so critical. The GC chromatogram of the hydrolysate of the polygalacturonic acid is shown in Figure 6. The group of galacturonic acid peaks in Figure 6 may be identified by comparing with the standard GC chromatogram (Figure 7)

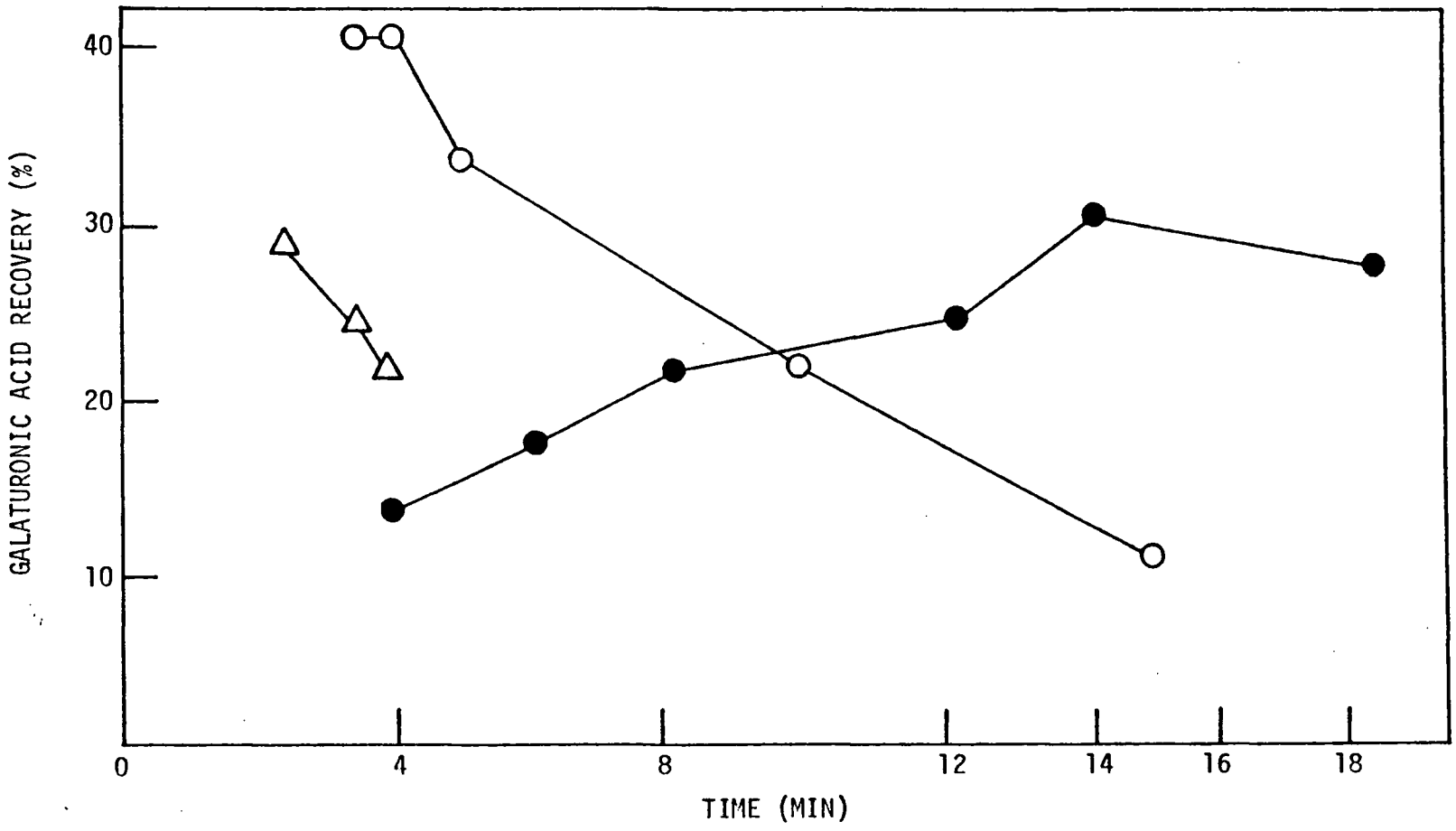


Figure 5. Galacturonic acid recovery from the hydrolysis of polygalacturonic acid under different reaction conditions. With 6N HCl at 100°C, ●——●; with 6N HCl at 110°C, ○——○; with 6N HCl at 120°C, △——△.

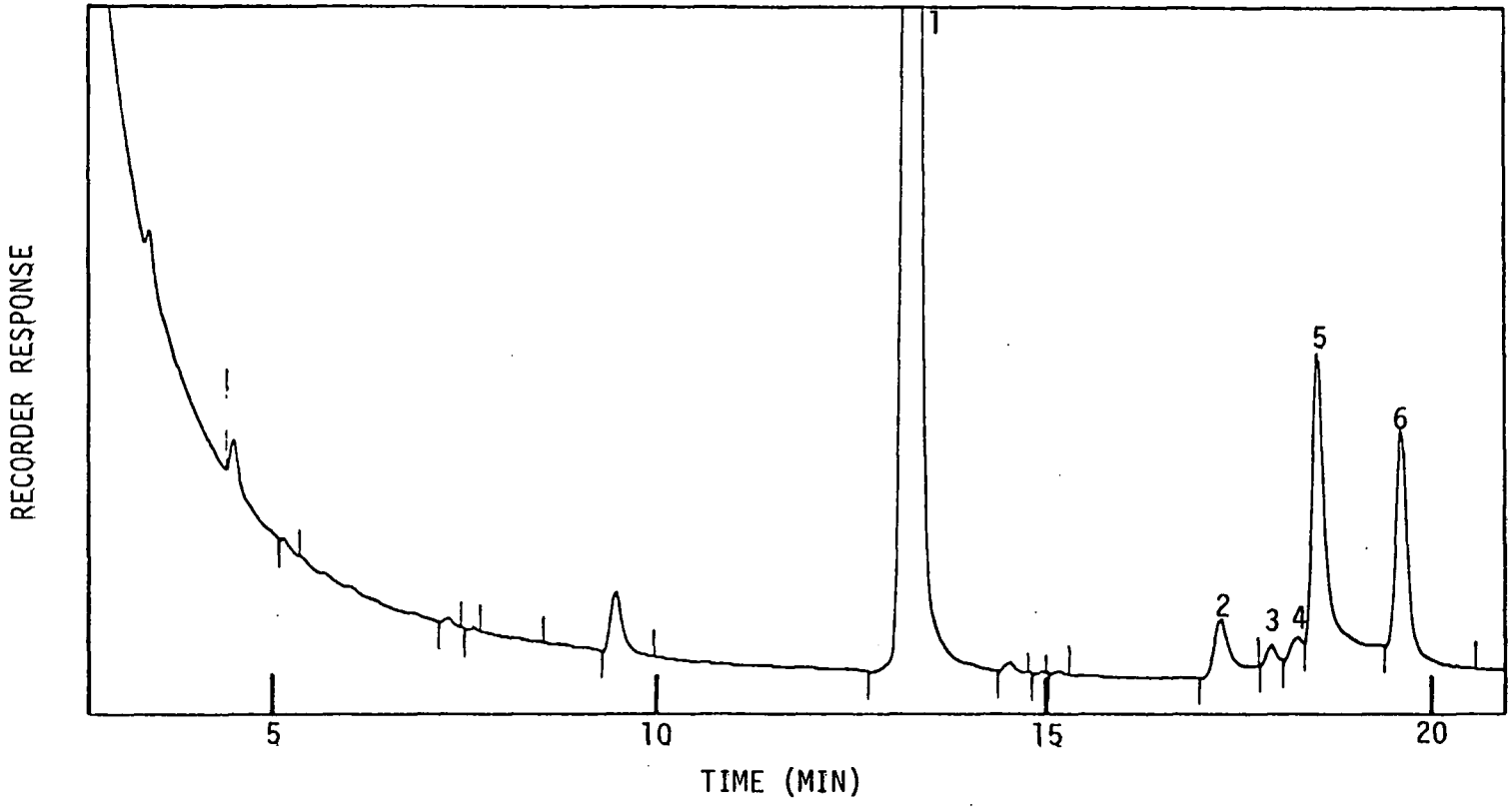


Figure 6. GC chromatogram of TMS-derivatives of polygalacturonic acid hydrolysate. Peak 2 through 6: galacturonic acid (see Figure 7). Peak 1: tartaric acid (internal standard).

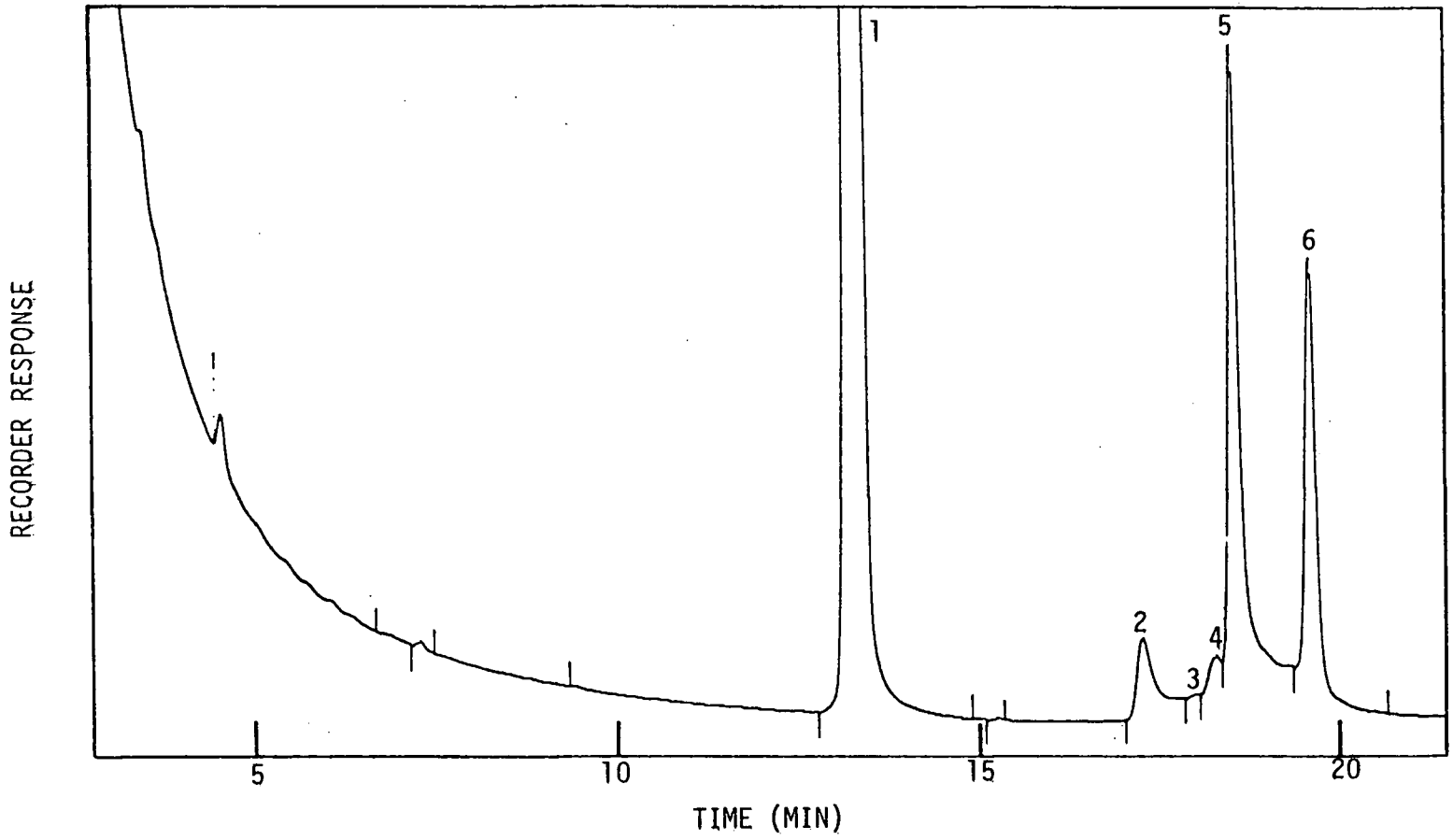


Figure 7. GC chromatogram of TMS-galacturonic acid (peak 2 through 6). Peak 1: tartaric acid (internal standard).

of the pure galacturonic acid.

Pectin Determination

One of the most widely used colorimetric method of determining pectic substances is Dische's (1947) sulfuric acid-carbazole reaction as applied by McCready and McComb (1952). This method is cumbersome, especially in handling the concentrated sulfuric acid in the spectrophotometric cell, a large amount of sample is also needed. Heatherbell (1974, 1976a) applied GC method to determine galacturonic acid (of its trimethylsilyl derivative) in the hydrolyzed sediment of apple juice and apple wine. This GC method was sensitive, specific (for galacturonic acid) and accurate. The reported GC results for total acids were comparable with those obtained using standard A.O.A.C. (Association of Official Analytical Chemists) procedures. Therefore, GC was chosen as the method for pectin determination, after the precipitate samples were hydrolyzed and acids separated.

Both Heatherbell's (1976a) hydrolysis conditions (2N HCl at 100°C for 3 hr) and the hydrolysis conditions of 6N HCl at 100°C for 14 min were used prior to the application of the GC method for pectin determination. At first, a small sample size (20 mg, as described by Heatherbell, 1976a) was tried. The results showed the absence of galacturonic acid. Therefore, the sample size was increased to 200 mg. All the results (chromatograms) failed to demonstrate the presence of galacturonic acid. No pectin was detected in any of the

precipitate samples. No other significant peak was found in the chromatogram. A typical chromatogram of the acids of the precipitate hydrolysate for pectin determination is shown in Figure 8. The presence of the citric acid peak is due to the citrate washing buffer.

Kieser et al. (1957) reported that the sedimentation in bottled pasteurized apple juice has been of two types: the first arising from the precipitation of partly degraded pectin and the second associated with the formation of products containing degraded tannin. Due to the absence of pectin, apparently the pear juice precipitate was of the second type. Pectic substances were reported to be present in small amount or absent in the storage deposits of the bottled apple juice (Kieser et al., 1957). The pectin contents of the deposits may be accounted for by incomplete depectinization of the juices.

Zitko and Rosik (1962b) studied the reactions in a pectin-tannin-gelatin system and reported that pectin-tannin-gelatin complexes only arise when the value of the weight ratio between tannin and gelatin is less than 2, if it is greater than 2, only tannin-gelatin complexes arise. Therefore, the absence of pectin in the pear precipitate may be due to the high weight ratio between phenolics and proteins in the pear juice. High phenolic/protein ratio may cause the positively charged centers of the protein molecules to be blocked by the phenolics. This may render the protein-phenolic complexes more negatively charged. Since the major driving force for the interactions between pectin and protein molecules are electrostatic forces (Imeson et al., 1977), the negatively charged protein-phenolic complexes would then not be able to flocculate with the negatively

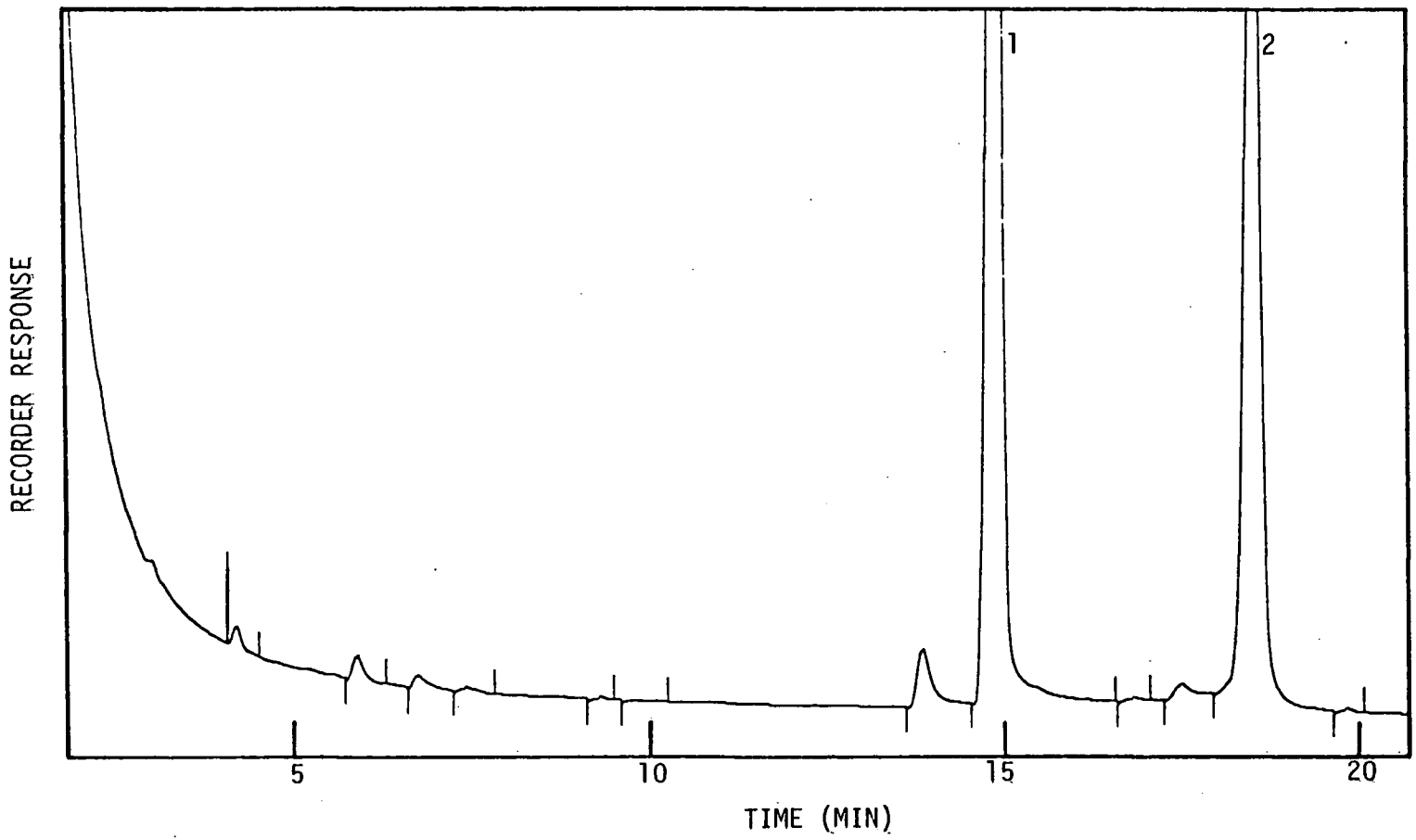


Figure 8. Typical GC chromatogram of the acids from the pear precipitate hydrolysate for pectin determination. Note the absence of galacturonic acid peaks. Peak 1: tartaric acid (internal standard). Peak 2: citric acid.

charged pectin. Therefore, we would not expect to find pectin in the precipitated complexes.

Furthermore, the clarification theory of Endo (1965d) may help to explain the absence of pectin in the pear precipitate. In his theory the flocculation of suspended particles is due to the decrease of the juice viscosity (pectin hydrolysis). When the soluble pectin (served as a protective colloid) was hydrolyzed, it lost its stabilizing function, allowing other particles to flocculate and precipitate.

Sugar Analysis

Table 10 shows the sugar contents of the pear precipitate samples. Fructose, galactose and glucose were found in all the precipitate samples. Typical GC chromatogram of these sugars was shown in Figure 9.

Table 10. Sugar content of the acid precipitated colloid of ripe Bartlett pear juice (calculated on moisture-free basis).

Year	Sugars (percent)			
	Fructose	Galactose	Glucose	Total
1975	3.4	2.9	1.5	7.8
1976	5.1	2.9	0.9	8.9
1977	4.3	2.2	0.9	7.4

Sugars are the main storage material in pears (Hulme, 1970). Fructose, glucose, sorbitol and sucrose were found as the four major sugars in Bartlett pear (Kline et al., 1970; Akhavan, 1976). Sorbitol may be metabolized to form fructose (Kidd et al., 1940; Lewis et al., 1967). Sucrose may produce fructose and glucose during acid hydrolysis

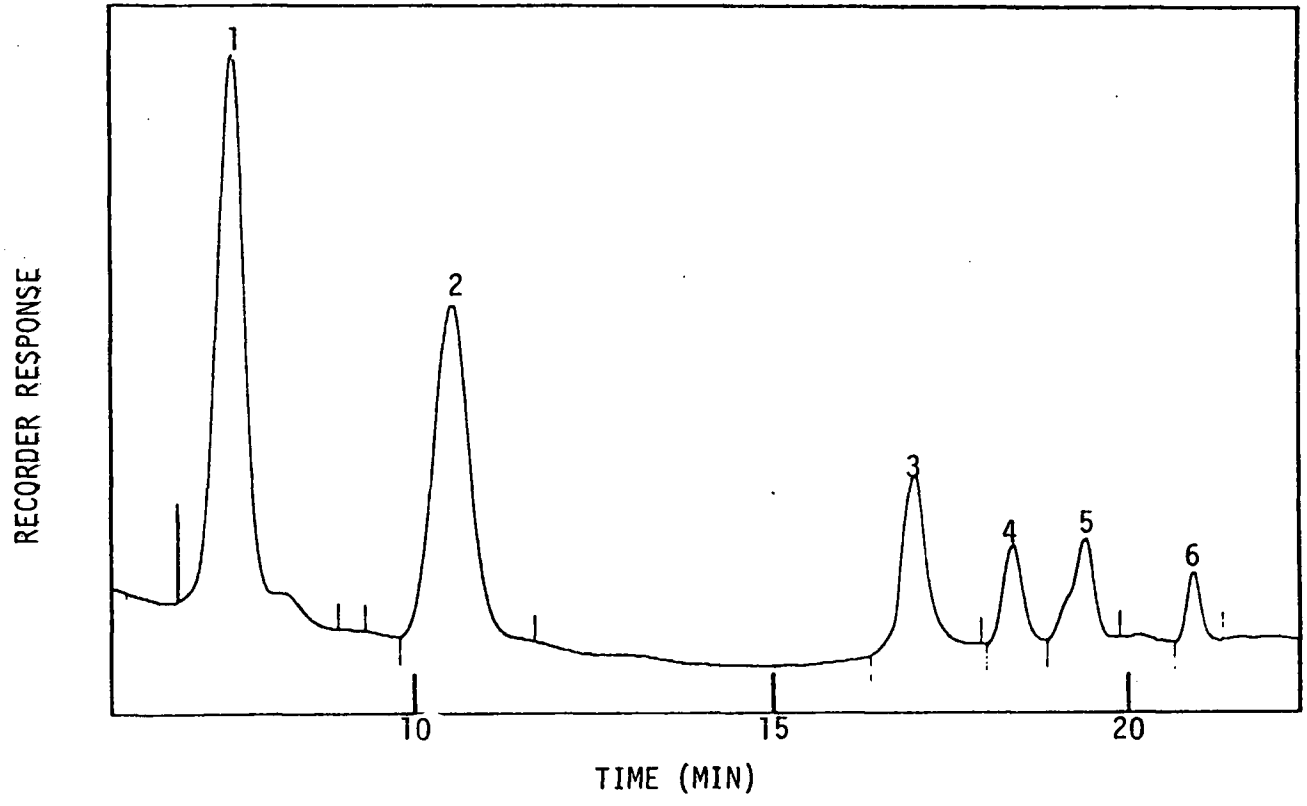


Figure 9. Typical GC chromatogram of the sugars from the pear precipitate hydrolysate. 1 and 2, αβ rhamnose (internal standard); 3, α+β fructose; 4, α galactose; 5, α glucose + β galactose (co-chromatographed); 6, β glucose.

(Roberts et al., 1970).

Most flavonoid aglycones and many other phenolic acids exist within the plant cell linked to sugars (e.g. galactose, glucose etc.) as glycosides (Walker, 1975). Therefore, part of the sugars in the precipitate may be liberated from the phenolics during acid hydrolysis. Some of the sugars (e.g. galactose) may also come from glycolipids (Mazliak and Catesson, 1968; Hulme, 1970).

No detailed starch analysis was conducted. However the iodine-iodide test of the precipitate samples indicated the absence of the starch.

Lipid Analysis

The results of total lipid determination of the pear precipitates are shown in table 11. The 1975 precipitate had the highest lipid content (26.5%) and the lipid contents of 1976 and 1977 precipitates were basically the same (21.3% and 20.4%, respectively).

Table 11. Total lipid content of the acid precipitated colloid of ripe Bartlett pear juice (calculated on moisture-free basis).

Year	Total lipids (percent)
1975	26.5 ± 0.4
1976	21.3 ± 0.5
1977	20.4 ± 0.4

The fatty acid composition of the pear precipitate lipids are shown in Table 12. The major component of the fatty acids is linoleic acid (18:2 n-6) which accounted for 52.8%, 49.7% and 43.5% of the

total fatty acids of 1975, 1976 and 1977 sample, respectively. Palmitic acid (16:0) and oleic acid (18:1) were present in a relatively high amount (19.7% & 8.2%, 23.2% & 11.0% and 23.8% & 20.1% for 1975, 1976 and 1977 sample, respectively). Linoleic acid is the major fatty acid in plant lipids. Oleic and palmitic acids are the most abundant fatty acids in both plants and animals (Gurr and James, 1971). In fact, all the fruit lipids contain oleic and palmitic acid (Hulme, 1970). Except for palmitoleic acid (16:1), all the fatty acids found in pear precipitate are "major fatty acids" which are responsible for a large proportion of fatty acids present in most plant lipids (Hitchcock and Nichols, 1971).

Table 12. Fatty acid composition of the total lipid extract of acid precipitated colloid of ripe Bartlett pear juice.

Fatty acid	1975	1976 (percent)	1977
12:0, lauric	tr	tr	tr
14:0, myristic	0.6	tr	tr
14:1, myristoleic	tr	tr	tr
16:0, palmitic	19.7	23.2	23.8
16:1, palmitoleic	0.8	tr	tr
18:0, stearic	3.1	3.8	3.6
18:1, oleic	8.2	11.0	20.1
18:2(n-6), linoleic	52.8	49.7	43.5
18:3(n-3), linolenic	4.6	4.1	3.3
unidentified*	10.2	8.3	5.6

*probably oxidized materials.

According to the available literature, lipid has never been reported as a component of the sediments of the pear or apple juices.

The presence of linoleic acid and palmitic acid as the major fatty acid components of the pear precipitate lipids corresponded with the fatty acid composition of the total lipid extract from pear fruit. In pear fruit lipids, linoleic acid and palmitic acid contents were 52.0% and 23.0%, respectively (Richardson, 1978). Due to this similarity in fatty acid composition, it is possible that most of the lipids in the pear precipitate may be from the insoluble discrete lipid particles suspended in the pear juice. After the juices were clarified, these lipid particles were centrifuged down together with other precipitated complexes.

It was found that 35% of the total lipid extract from Bartlett pear are phospholipids (Richardson, 1978). Thibaudin et al. (1968) also reported that the main phospholipids of plant lipoprotein membranes are characterized by a high linoleic acid content. Phospholipids may form microscopic inclusions (spherosomes) in the cytoplasms of many fruit cells (Sorokin, 1967). They are also the major lipids of the cytoplasmic or mitochondrial membranes (Hulme, 1970). Therefore, these facts lead to the speculation that phospholipids may be one of the major components of the pear precipitate lipids.

Lipids may form hydrophobic associations through their hydrocarbon chains with the hydrophobic regions of proteins and phenolics. Polar lipids, such as phospolipids and glycolipids, may also form the hydrogen bondings or electrostatic associations through their polar "heads" (polarizable or ionizable chemical groups) with proteins and phenolics.

Through the interactions of lipids with protein-phenolic complexes, lipids may also possibly block the positively charged centers of the protein molecules. Furthermore, free fatty acids and phospholipids, such as phosphotidylethanolamine, phosphotidylserine and phosphatidic acid, may even convey more negative charges to the protein-phenolic complexes. Therefore, this may enhance the clarification difficulties. Since the 1975 precipitate has the highest total lipid content (26.5%), this may also be one of the possible reasons for the clarification difficulties of the 1975 juice.

SUMMARY AND CONCLUSIONS

The chemical analyses of the acid precipitated colloid of 1975, 1976 and 1977 ripe Bartlett pear juice are summarized below:

1. The estimated protein contents (by total amino acid analysis) were 31%,26% and 37%, respectively. The crude protein contents (by micro-Kjeldahl method) were 30.8%, 36.6% and 35.6%, respectively.

2. The total phenolic contents were 38.1%, 33.9% and 29.1%, respectively.

3. A wide variation of copper, iron, magnesium, calcium, sodium and potassium was detected in all the samples.

4. No pectin was detected in any of the samples.

5. Fructose, galactose and glucose were found in the precipi tates. Sugar contents were 7.8%, 8.9% and 7.4%, respectively.

6. Total lipid contents were 26.5%, 21.3% and 20.4%, respectively. Linoleic, palmitic and oleic acids were the major components of the fatty acids.

Therefore, according to the analytical results, acid precipitated colloid of the ripe Bartlett pear juice is mainly a protein-phenolic-lipid complex containing sugars and metal ions.

The protein contents of 1975, 1976 and 1977 cloudy pear juices were 0.07%, 0.10% and 0.18%, respectively. The percentages of free amino acid in total amino acid of the 1975, 1976 and 1977 juices were 47.3%, 36.8% and 32.8%, respectively.

The juice of the 1975 season had the lowest protein content (0.07%), and its colloidal precipitate also showed the lowest crude protein content (30.8%). In addition, the 1975 precipitate had the highest phenolic and lipid contents. High phenolic and lipid contents plus low protein content may be the cause of the clarification-filtration difficulties of the 1975 juice.

High calcium content was also noted in all precipitate samples, this might be part of the reason for the clarification-filtration difficulties of the pear juice.

BIBLOGRAPHY

Akhavan, I. 1977. Variation of sugars and acid during ripening of pears and in the production and storage of pear concentrate. Ph. D. thesis, Oregon State University, Corvallis, Ore.

Amerine, M. A., Berg, H. W. and Cruess, W. V. 1967. Fruit Wines. In The technology of Wine Making. p. 531. Avi Publishing Co., Westport, Conn.

Anglemier, A. F. and Montgomery, M. W. 1976. Amino acid, peptides, and proteins. In Principles of Food Science. (Fennema, O. R. ed.) Part I. Food Chemistry. p. 244. Marcel Dekker Inc., N.Y.

A.O.A.C. 1975a. Official Methods of Analysis. 12th ed. p. 394, 22. 013. Assoc. of Official Analytical Chemists, Washington, D.C.

_____. 1975b. Official Methods of Analysis. 12th ed. p. 927, 47.021, 47.022, 47.023. Assoc. of Official Analytical Chemists, Washington, D.C.

Aurand, L. W. and Woods, A. E. 1973. Food glycosides. In Food Chemistry. p. 332. Avi Publishing Co., Westport, Conn.

Beavers, D. V. and Youtz, J. A. 1976. Investigating juice clarification and filtration procedure for juices made from fruit wastes and surplus fruits. Research Report Number 2 to Tri Valley Growers. Oregon Agric. Exp. Stn. Corvallis, Ore.

Deuel, H. 1943. Oxydativer abbau von pektin in wässeriger lösung. Helv. Chim. Acta. 26:2002. Quoted in Pilnik, W. and Voragen, A. G. J. 1970. The Biochemistry of Fruits and their Products. (Hulme, A. C. ed.) Vol. 1, p. 61. Academic Press, N.Y.

Deuel, H. and Stutz, E. 1958. Pectic Substances and pectic enzymes. Adv. Enzymol. 20:341.

Dische, Z. 1947. A new specific color reaction for hexuronic acids. J. Biol. Chem. 167:187

Doesberg, J. J. 1950. A modification of the carbazole reaction of hexuronic acids for the study of polyuronides. J. Biol. Chem. 183:489.

Doesberg, J. J. 1965a. Nomenclature and Structure of pectic substances. In Pectic substances in Fresh and Preserved Fruits and vegetables. p. 10. Institute for Research on Storage and Processing of Horticultural Produce. Wageningen, The Netherlands.

_____. 1965b. Pectic enzymes. In Pectic substances in Fresh and Preserved Fruits and Vegetables. p. 74. Institute for Research on Storage and Processing of Horticultural Produce. Wageningen, The Netherlands.

Endo, A. 1965a. Studies on pectolytic enzymes of molds. Part XIII. Clarification of apple juice by the joint action of purified pectolytic enzymes. Agric. Biol. Chem. 29:129.

_____. 1965b. Studies on pectolytic enzymes of molds. Part XIV. Properties of pectin in apple juice. Agric. Biol. Chem. 29:137

_____. 1965c. Studies on pectolytic enzymes of molds. Part XV. Effect of pH and some chemical agents on the clarification of apple juice. Agric. Biol. Chem. 29:222.

_____. 1965d. Studies on pectolytic enzymes of molds. Part XVI. Mechanism of enzymatic clarification of apple juice. Agric. Biol. Chem. 29:229.

Esselen, W. B. 1945. Methods of clarification and blends of Massachusetts apples for apple juice. Fruit Product J. 24:165.

Fabian, F. W. and Marshall, R. E. 1935. How to make, clarify and preserve cider. Mich. Agric. Expt. Stn. Circ. 98:1. Quoted in Forgacs, I., Ruth, W. A. and Tanner, F. W. 1945. Examination of canned apple juice. J. Fd. Sci. 10:227.

Fergus, M. C. and Francis, F. J. 1976. Pigments. In Principles of Food Science. (Fennema, O. R. ed.) Part I. Food Chemistry. p. 413. Marcel Dekker Inc., N.Y.

Folch, J., Lees, M. and Stanley, G. H. S. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 73:627.

Folin, O. and Ciocalteu, V. 1927. On tyrosine and tryptophan determination in proteins. J. Biol. Chem. 73:627.

Folin, O. and Denis, W. 1912. On phosphotungstic-phosphomolybdic compounds as color reagents. J. Biol. Chem. 12:239.

Forgacs, I., Ruth, W. A. and Tanner, F. W. 1945. Examination of canned apple juice. J. Fd. Sci. 10:227.

Gerritsen, Th. and Niederwieser. 1974. Amino acids. In Clinical Biochemistry. (Curtius, H. Ch. and Roth, M. ed.) Vol. 2, p. 1093. Walter de Gruyter, N.Y.

Gurr, M. I. and James, A. T. 1971. Fatty acids. In Lipid Biochemistry. p. 25. Cornell University Press, Ithaca, N.Y.

Gustavson, K. H. 1954. Interaction of vegetable tannin with polyamides as proof of the dominant function of the peptide bond of collagen for its binding of tannins. J. Polymer Sci. 12:317.

Harborne, J. B. and Simmonds, N. W. 1964. The natural distribution of the phenolic aglycones. In Biochemistry of Phenolic Compounds. (Harborne, J. B. ed.) p. 77. Academic Press, N.Y.

Heatherbell, D. A. 1974. Rapid current analysis of fruit sugars and acids by gas-liquid chromatography. J. Sci. Fd. Agric. 25:1095.

_____. 1976a. Haze and sediment formation from starch degradation products in apple wine and clarified apple juice. Confructa 21:36.

_____. 1976b. Haze and sediment formation in clarified apple juice and apple wine. I: The role of pectin and starch. Fd. Tech. in New Zealand, May.

_____. 1976c. Haze and sediment formation in clarified apple juice and apple wine. II: The role of polyvalent cations, polyphenolics and proteins. Fd. Tech. in New Zealand, June.

Hitchcock, C. and Nichols, B. W. 1971. Structure and distribution of plant fatty acids. In Plant Lipid Biochemistry. p. 2. Academic Press, N.Y.

Hoff, J. E. and Singleton, K. I. 1977. A method for determination of tannins in foods by means of immobilized protein. J. Fd. Sci. 42:1566.

Hulme, A. C. 1953. The isolation of chlorogenic acid from the apple fruit. Biochem. J. 53:337.

_____. 1958. Some aspects of the biochemistry of apple and pear fruits. Advances in Food Research. 8:351.

Imeson, A. P., Ledward, D. A. and Mitchell, J. R. 1977. On the nature of the interaction between some anionic polysaccharides and proteins. J. Sci. Fd. Agric. 28:661.

Johnson, G., Donnelly, B. J. and Johnson, D. K. 1968. The chemical nature and precursors of clarified apple juice sediment. J. Fd. Sci. 33:254.

Keegstra, K., Talmadge, K. W., Bauer, W. D. and Albersheim, P. 1973. The structure of plant cell wall. III. A model of the walls of suspension-cultured sycamore cell based on the interconnections of the macromolecular components. Pl. Physiol.51:188.

Kelhofer, Von W. 1908. Beitrage zur kenntnis des bringerbstoffs und seiner beränderungen bei der ŏbstweinbereitung. Landw. Jahrb. Schweiz. 22:343. Quoted in Johnson, G., Donnelly, B. J. and Johnson, D. K. 1968. The chemical nature and precursors of clarified apple juice sediment. J. Fd. Sci. 33:254.

Kertesz, Z. I. 1930. A new method of enzymatic clarification of unfermented apple juice. Bull. 589. Geneva Agr. Expt. Stn., Geneva, N.Y.

_____. 1936. Pectic enzymes. Ergeb. Enzymforsch. 5:233. Quoted in Forgacs, I., Ruth, W. A. and Tanner, F. W. 1945. Examination of canned apple juices. J. Fd. Sci. 10:227.

_____. 1951. Definition and nomemclature. In The Pectic Substances. p. 6. Interscience Publisher, N.Y.

Kidd, F., West, C., Griffith, D. G. and Potter, N. A. 1940. An investigation of the changes in chemical composition and respiration during the ripening and storage of conference pears. Ann. Bot. N. S. 4:1.

Kieser, M. E., Pollard, A. and Timberlake, C. F. 1957. Metallic components of fruit juices. I. Copper as a factor affecting sedimentation in bottled apple juices. J. Sci. Fd. Agric. 8:151.

Kline, D. A., Fernandez-Flores, E. and Johnson, A. R. 1970. Quantitative determination of sugars in fruits by GLC separation of TMS derivatives. J. Ass. Off. Analyt. Chem. 53:1198.

Koller, A. 1966. Diss. no. 3774, E.T.H. Zürich. Quoted in Pilnik, W. and Voragen, A. G. J. 1970. The Biochemistry of Fruits and their Products. (Hulme, A. C. ed.) Vol. 1. p. 74. Academic Press, N.Y.

Krebs, J. 1971. Die apfelstärke und ihr einfluss auf die saftklärung. Flüssieges Obst. 38:137. Quoted in Heatherbell, D. A. 1976. Haze and sediment formation from starch degradation products in apple wine and clarified apple juice. Confructa 21:36.

Krop, J. J. P. 1974. The mechanism of cloud loss phenomena in orange juice. Ph.D. thesis. Agricultural University in Wageningen, The Netherlands.

Krug, K. 1969. Probleme bei der herstellung von apfelsaft und apfelsaftkonzentraten (II). Flüssiges Obst. 36:333. Quoted in Heatherbell, D. A. 1976. Haze and sediment formation from starch degradation products in apple wine and clarified apple juice. Confructa 21:36.

Kubowitz, F. 1938. Cleavage and resynthesis of polyphenol oxidase and of hemocyanin. Biochemistry Z. 299:32.

Letzig, E. and Nurnberger. 1963. Chemical composition of the cloudy precipitates from press-dregs apple juice. (in German). Quoted in Johnson, G., Donnelly, B. J. and Johnson, D. K. 1968. The chemical nature and precursors of clarified apple juice sediment. J. Fd. Sci. 33:254.

Lineweaver, H. and Ballou, G. A. 1945. Effect of cations on activity of alfalfa pectin-esterase. Archs. Biochem. 6:373.

Lewis, D. H. and Smith, D. C. 1967. Sugar alcohols (polyols) in fungi and tree plants. New Physiologist 66:143.

Loomis, W. D. and Battaile, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5:423.

Loomis, W. D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods in Enzymology 31:528.

Marshall, C. R. 1951. Oxidation in apple juice. J. Sci. Fd. Agric. 2:321.

Mazliak, P. and Catesson, A-M. 1968. Metabolism of the lipids in the external parenchyma of apple skin. II. Development of lipogenesis during the growth of fruits; relation to plant morphology. (in Chem. Abstr. 1968, 84147p).

McCready, R. M. and Gee, M. 1960. Determination of pectic substances by paper chromatography. J. Agric. Fd. Chem. 8:510.

McCready, R. M. and McComb, E. A. 1952. Extraction and determination of total pectic materials in fruits. Anal. Chem. 24:1986.

_____. 1954. Pectic substituents in ripe and unripe fruit. J. Fd. Sci. 19:530.

Monties, B. and Barret, A. 1965. Chemical Studies on reversible chill haze in apple juice (in French). Ann. Technol. Agric. 14: 167. Quoted in Heatherbell, D. A. 1976. Haze and sediment formation from starch degradation products in apple wine and clarified apple juice. Confructa 21:36.

Müller, E. and Zellner, J. 1935. Zur kenntnis der phlobaphene. Biochem. Z. 277:383. Quoted in Neubert, A. M. and Veldhuis, M. K. 1944. Clouding and sedimentation in clarified apple juice. Fruit Product J. and Amer. Fd. Manufacturer July:324.

Neish, A. C. 1964. Major pathways of biosynthesis of phenols. In Biochemistry of Phenolic compounds. (Harborne, J. B. ed.) p. 295. Academic Press, N.Y.

Neubert, A. M. and Veldhuis, M. K. 1944. Clouding and sedimentation in clarified apple juice. Fruit Product J. and Amer. Fd. Manufacturer July:324.

Nortje, B. K. 1966. Some catechins and proanthocyanindins in the core of Bartlett pears. J. Fd. Sci. 31:733.

Pilnik, W. and Voragen, A. G. J. 1970. Pectic substances and other uronides. In The Biochemistry of Fruits and their Products. (Hulme, A. C. ed.) Vol. I. p. 55. Academic Press, N.Y.

Ratushnyi, G. D. 1958. The physicochemical mechanism of wine clarification. Trudy Krasnodarsk. Inst. Pisc. Prom. 18:65. Quoted in Zitko, V. and Rosik, J. 1962. A contribution to the theory of the tannin-gelatin clarification of fruit juices (in German). Nahrung 6:560.

Raunhardt, O. and Neukom, H. 1964. Modification of the apple and pear pectins during the ripening process (in German). Mitt. Geb. Lebensm. Hyg. 55:446. Quoted in Pilnik, W. and Voragen, A. G. J. 1970. The Biochemistry of Fruits and their Products. (Hulme, A. C. ed.) Vol. 1. p. 65. Academic Press, N.Y.

Richardson, D. G. 1978. Unpublished data. Dept. of Horticulture, Oregon State University, Corvallis, Ore.

Roberts, J. D., Stewart, R. and Caserio, M. C. 1970. Carbohydrates. In Organic Chemistry. p. 409. W.A.Benjamin Inc., Menlo Park, CA.

Rüdiger, M. and Mayr, E. 1929. Wine clarification. Kolloid. Z. 47: 141. Quoted in Zitko, V. and Rosik, J. 1962. A contribution to the theory of the tannin-gelatin clarification of fruit juices (in German). Nahrung 6:560.

Schanderl, S. H. 1970. Tannins and related phenolics. In Methods in Food Analysis. (Joslyn, M. A. ed.) p. 709. Academic Press, N.Y.

Scott, W. C., Kew, T. j. and Veldhuis, M. K. 1965. Composition of orange juice cloud. J. Fd. Sci. 30:833.

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

Singleton, V. L. and Rossi, J. A. Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic reagents. Am. J. Enol. Vitcult. 16:144.

Sioud, F. B. and Luh, B. S. 1966. Polyphenolic compounds in pear purees. Fd. Tech. 20:534.

Smit, C. J. B., Joslyn, M. A. and Lukton, A. 1955. Determination of tannins and related polyphenols in foods. Analyt. Chem. 27:1159.

Sorokin, H. P. 1967. The spherosomes and the reserve fat in plant cells. Am. J. Bot. 54:1008.

Spackman, D. H., Stein, W. H. and Moore, S. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30:1190.

Swain, T. and Goldstein, J. L. 1964. The quantitative analysis of phenolic compounds. In Methods in Polyphenol Chemistry. (Pridham, J. B. ed.) p. 134. Macmillan Co., N.Y.

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

Tate, J. N., Luh, B. S. and York, G. K. 1964. Polyphenoloxidase in Bartlett pears. J. Fd. Sci. 29:829.

Thibaudin, A., Mazliak, P. and Catesson, A. M. 1968. Biosynthesis of fatty acids by isolated microsomes from apple juice, Pirus malus, parenchyma (in French). C. R. Acad. Sci., Paris. Ser. D. 266: 784. Quoted in Mazliak, P. 1970. The Biochemistry of Fruits and their Products. (Hulme, A. C. ed.) p.223. Academic Press, N.Y.

Van Buren, J. P. 1970. Fruit phenolics. In The Biochemistry of Fruits and their Products. (Hulme, A. C. ed.) Vol. 1. p. 271. Academic Press, N.Y.

Van Buren, J. P., deVos, L. and Pilnik, W. 1976. Polyphenols in golden delicious apple juice in relation to method of preparation. J. Agr. Fd. Chem. 24:448.

Van Buren, J. P. and Robinson, W. B. 1969. Formation of complexes between protein and tannic acid. J. Agr. Fd. Chem. 17:772.

Walker, J. R. L. 1964. The polyphenoloxidase of pear fruit. Aust. J. Biol. Sci. 17:575.

_____. 1975. Plant phenolics. In The Biology of Plant Phenolics. p. 5. Edward Arnold Ltd., London.

_____. 1977. Enzymic browning in foods. It's chemistry and control. Fd. Tech. in New Zealand March:19.

Weurman, C. and Swain, T. 1953. Chlorogenic acid and the enzymic browning of apples and pears. Nature 172:678.

Williams, A. H. 1957. The simpler phenolic substances of plants. J. Sci. Fd. Agr. 8:385.

Yamasaki, M., Yasui, T. and Arima, K. 1964. Pectic enzymes in the clarification of apple juice. Part I. Study on the clarification in a simplified model. Agr. Biol. Chem. 28:779.

_____. 1966. Studies on pectic enzymes of microorganisms. Part III. Endo-polygalacturonase of Aspergillus saitoi. Agr. Biol. Chem. 30:1119.

Yamasaki, M., Kato, S., Chu, S. and Arima, K. 1967. Pectic enzymes in the clarification of apple juice. Part II. The mechanism of clarification. Agr. Biol. Chem. 31:552.

Zitko, V. and Rosik, J. 1962a. Composition of pectin-gelatin complexes (in German). Chem. zvesti. 16:474. Quoted in Zitko, V. and Rosik, J. 1962. A contribution to the theory of the tannin-gelatin clarification of fruit juices (in German). Nahrung 6:560.

_____. 1962b. A contribution to the theory of the tannin-gelatin clarification of fruit juices (in German). Nahrung 6: 560.