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AN INVESTIGATION OF THE KINETICS AND INHIBITION OF PECTINESTERASE

By

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ABSTRACT

AN INVESTIGATION OF THE KINETICS AND INHIBITION OF PECTINESTERASE

By
Anna Tamnai C. Maghasi

Methanol in fruit brandy is regulated by the FDA because of its toxic properties. To reduce the amount of methanol in fruit brandy, it is necessary to understand the source and mode of its generation. Pectinesterase enzyme is responsible for the deesterification of fruit pectin and generates methanol as one of the major products. The kinetics of pectinesterase and its inactivation by heat were studied. At 70°C, Pectinesterase was found to be inactivated significantly with an inactivation rate constant of $2.8 \times 10^{-3} s^{-1}$.

Various inhibitors were tested for their effect on the activity of pectinesterase. Tannic acid, polyacrylic acid, polymaleimide, citric acid and ethylenediamminetetraacetic acid (EDTA) were tested as potential inhibitors. Polyacrylic acid was found to reduce the activity of pectinesterase by nearly 45%. EDTA also significantly reduced the amount of methanol generated by pectinesterase. It is postulated that they act by providing excess carboxylate functionality thereby shifting the equilibrium of the deesterification reaction.

Dedicated to my Parents

For their wisdom and guidance

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1. INTRODUCTION

1.1 Michigan Fruit Brandy Industry

The Michigan fruit industry has benefited greatly from advances made in agricultural techniques. Consumption of the major fruits such as apples, plums and cherries has remained the same despite the increase in production. In some cases such as pears, consumption has even decreased. The improved productivity has led to surpluses for most of these major fruits. Some fruit also has defects in either appearance or size that reduce its market value significantly. These surpluses have created a need to develop new ways to utilize the excess fruit. An alternative widely practiced in Europe is the production of fruit brandy. In the United States, this technology is virtually non-existent and the development of such technology will greatly benefit the fruit industry as a whole.

Many fruit and vegetable juices contain some amount of methanol. The methanol content of fresh juices is dependent upon the method used to extract the juice, the type of fruit and the stage of harvesting (Kazeniac, 1970). Lund (1981) showed that the average methanol content of fresh orange juice was 34 mg/liter, while fresh grapefruit juice averaged 27 mg/liter in the same study. Processed juices have been reported to contain much less methanol than their fresh counterparts. For example, Kazeniac (1970) found that blended tomatoes had a methanol content of between 64 and 138 mg/liter depending upon the ripeness of the tomatoes. Methanol is also found in alcoholic

beverages, especially those derived from fruit. Table 1.1 shows a summary of the average methanol content of some beverages (Monte, 1984).

The pectin in fruits and vegetables is usually esterified by methyl groups. The naturally occurring presence of the enzyme pectinesterase in the fruit is responsible for deesterifying the pectin to release methanol. The activity of the enzyme gradually increases as fruits ripen and tissue is damaged. Fruits at different stages of maturity will contain methanol, but the activity of the pectinesterase enzyme is rather low until after harvesting when the fruit tissue drastically softens.

Table 1.1 Methanol content of Fruit Juice and Beverages[1]

Juice/ Beverage	Methanol (mg/liter)
Orange	34
Grapefruit	27
Pear Wine	188
Cherry Wine	276

As noted in table 1.1, methanol is present in many fruit juices. This poses a problem in the fruit brandy industry. The methanol levels that are low in fruit juices become highly significant when fruit mash is distilled. During distillation, separation of alcohols from the fruit mash always results in the concentration of methanol in the product.

Use of Michigan excess fruits for brandy production therefore calls for the need to address the methanol content of the fruit brandies. In order to develop quality products,

[1] **Data obtained from Monte, W.C. (1984).**

the amount of methanol needs to be closely monitored. Methanol contributes to good attributes in the fruit brandy, but there are health concerns associated with methanol toxicity. The aim of this project is therefore to quantitate the methanol content of Michigan fruit brandies and establish ways in which this can be moderated. The main approach used in this project is to target the pectinesterase enzyme that is responsible for the generation of methanol and find ways to inhibit its activity.

1.2 Regulation of methanol

The United States Environmental Protection Agency recommends a minimum acute toxicity concentration of methanol in drinking water at 3.9 parts per million (Cleland and Kingsbury, 1977). The EPA has also set the permissible exposure limit in air as 200 parts per million for an 8-hour time weighted average.

In the production of fruit brandy, methanol has been widely regulated in Europe (Tanner and Brunner, 1982). In Germany, maximum methanol content in fruit brandies varies from fruit to fruit. Cherry brandy methanol content is regulated at 400 mg/100 mL absolute alcohol, while the maximum methanol content in brandy from Bartlett pears is 790 mg/100 mL absolute alcohol. In Italy the maximum methanol content in any fruit brandy is 800 mg/100 mL absolute alcohol. In Austria the maximum allowed content of methanol in spirits is 1000 mg/100 mL absolute alcohol.

The United States Food and Drug Administration (FDA) regulates the amount of methanol that is allowed in fruit brandy and the current limit is 0.35 % v/v for both local and imported fruit brandy. This corresponds to 700 mg/100 mL absolute alcohol.

1.3 Objectives

The objectives of this study are to determine the amount of methanol in fruit brandy and find possible means of minimizing it. The approach used in this work is to study the activity of pectinesterase, which catalyzes the formation of methanol and use various substances to inhibit its activity. A variety of potential inhibitors are tested for their effect on pectinesterase activity.

2. LITERATURE REVIEW

2.1 Source and Nature of Pectin

Pectic substances are carbohydrate derivatives that are widely distributed in plant tissues, where they occupy intracellular spaces. They contribute to the adhesion between cells and overall mechanical strength of the cell wall. Cell walls contain approximately 60% water and 40% biopolymers. Pectins make up 20-35% of the polymers (Jarvis, 1982). Pectins are usually classified by the procedures that are used to extract them from cell walls. The three major classifications are: water-soluble, chelator-soluble and protopectin that are dissolved with alkali. Either one or several of these pectin types may exist in a given fruit tissue (Sajjaanatakul *et al.*, 1989).

Pectin is a polysaccharide, whose main component is D-galacturonic acid, joined by means of α-(1$\rightarrow$4) glycosidic linkages. Figure 2.1 shows the structure of pectin. The galacturonic acid molecule has a carboxyl group on C5 that may be esterified with methyl alcohol. The degree of esterification of this carboxyl group is an important factor in characterizing pectin and has a bearing on the firmness and extent of cohesion of plant tissues.

There are a wide range of degrees of esterification, depending on the species, tissue and maturity (Van Buren, 1991). In general, the degree of esterification in plant tissues can be up to 90%.

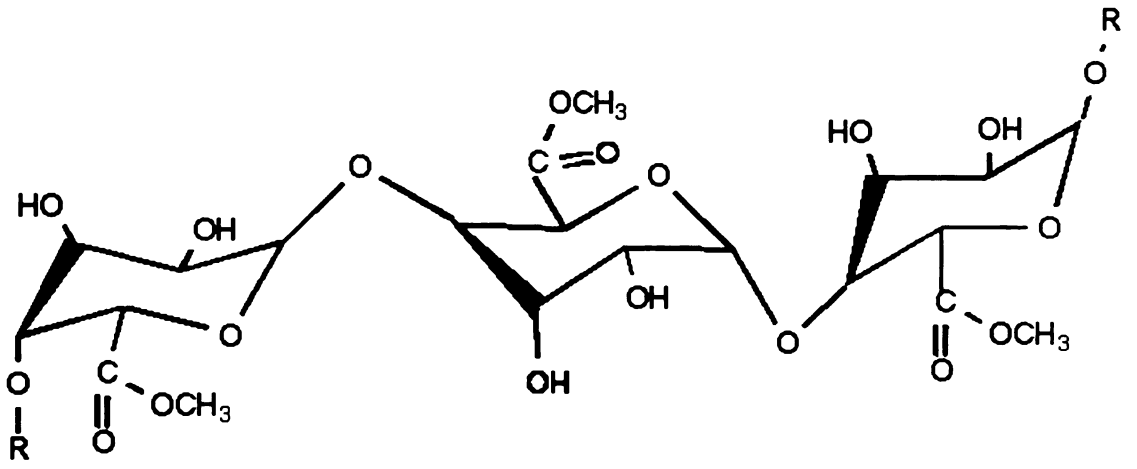


Figure 2.1 The structure of fruit pectin.

2.2 Pectic Enzymes

The chemistry of pectic substances has been extensively reviewed by Kertesz (1951), Dewel and Stutz (1958), and Joslyn and Dewel (1963). Enzymes that catalyze the formation of pectin from its water insoluble precursor protopectin, are called protopectinases. The enzymes that catalyze the degradation of pectic substances are called "pectic enzymes."

Depolymerization of pectin generally occurs during fruit ripening. These enzymes also play a significant role in changes that occur after the harvest of fruits. Pectic enzymes have been known as the cause of cloud loss in citrus juice in the food processing industry (Pilnik and Voragen, 1993).

There are reports in the literature of various types of pectic enzymes. Some of these are: pectate lyase, polygalacuturonase and pectinesterase (Wong, 1995). Of these, polygalacturonase and pectinesterase have been widely studied for various reasons. Pectate lyases degrade pectin by the mechanism of β-elimination (Rexova-Benkova and

Markovic, 1976). The degradation occurs either in a random (endo) or terminal (exo) fashion. When endo-pectate lyase acts on pectin, the product of the degradation is a 4,5-unsaturated galacturonosyl residue. Exo-pectate lyase is considered relatively rare compared to endo-pectate lyase. It attacks from the reducing end of the chain and the only product of degradation is unsaturated digalacturonic acid.

Polygalacturonase is widely studied because its activity is usually associated with the softening of fruit. The enzyme catalyzes the hydrolytic cleavage of the O-glycosyl bond of α-D- (1→4) polygalacturonate (Burns, 1991). The pattern of degradation is either random (endo-polygalacturonase) or terminal (exo-polygalacturonase). Endo-polygalacturonase usually prefers substrates with a low degree of esterification (Archer, 1979), typically less than 20%. Exo-polygalacturonase generally acts on deesterified pectin (Pressey and Avants, 1977). Pressey (1986) found that within short reaction times, random cleavage of polygalacturonate resulted in large decreases in viscosity. Very little change in viscosity was observed in the case of terminal cleavage. Table 2.1 shows the main classifications of pectic enzymes.

Table 2.1 Classification of pectic enzymes

Enzyme	Mode of action
Polygalacturonase (PG)	Cleaves α-(1→4) glycosidic bonds
Pectate Lyase (PEL)	Cleaves non-esterified galacturonate units via β-elimination
Pectinesterase (PE)	Hydrolyzes the methyl ester groups of pectin.

This study will focus on the activity of pectinesterase enzyme, which is responsible for the generation of methanol from pectin. A literature survey of reports on pectinesterase and the theories involved in the mode of action of pectinesterase are discussed in the next section.

2.3 Pectinesterase

Pectinesterases have been detected in a variety of plants. Most of the fruits have been reported to have more than one isozyme of pectinesterase. Two pectinesterase isozymes, each having a molecular weight of 30,000 Da, were detected in banana pulp (Hultin *et al*, 1966) and they both exhibited an optimum activity at pH 7.5. Orange pectinesterase is a well-studied enzyme. Two isozymes have been reported, each having a molecular weight of 36,200 Da. Unlike the banana pectinesterase, these isozymes have different optimal pH. Structural studies have shown that the enzyme is a glycoprotein formed by a single low molecular weight polypeptide (Markovic and Jorvall, 1986; Pressey and Woods, 1992; Glover and Brady, 1994). Generally, the isoelectric points for pectinesterase isozymes are between 7 and 11 (Alonso *et al*). This enzyme is also produced by a number of micro-organisms, including bacteria yeast and mold (Fogarty and Kelly, 1983).

Pectinesterase catalyzes the hydrolysis of the methyl esters of pectin, generating methanol. It removes the methoxyl group by a nucleophilic attack on the ester, resulting in an acyl-enzyme intermediate. This is then followed by deacylation, where the intermediate is hydrolyzed to regenerate the enzyme and a carboxylic acid (Wong, 1995). The schematic of this mechanism is shown in Figure 2.2.

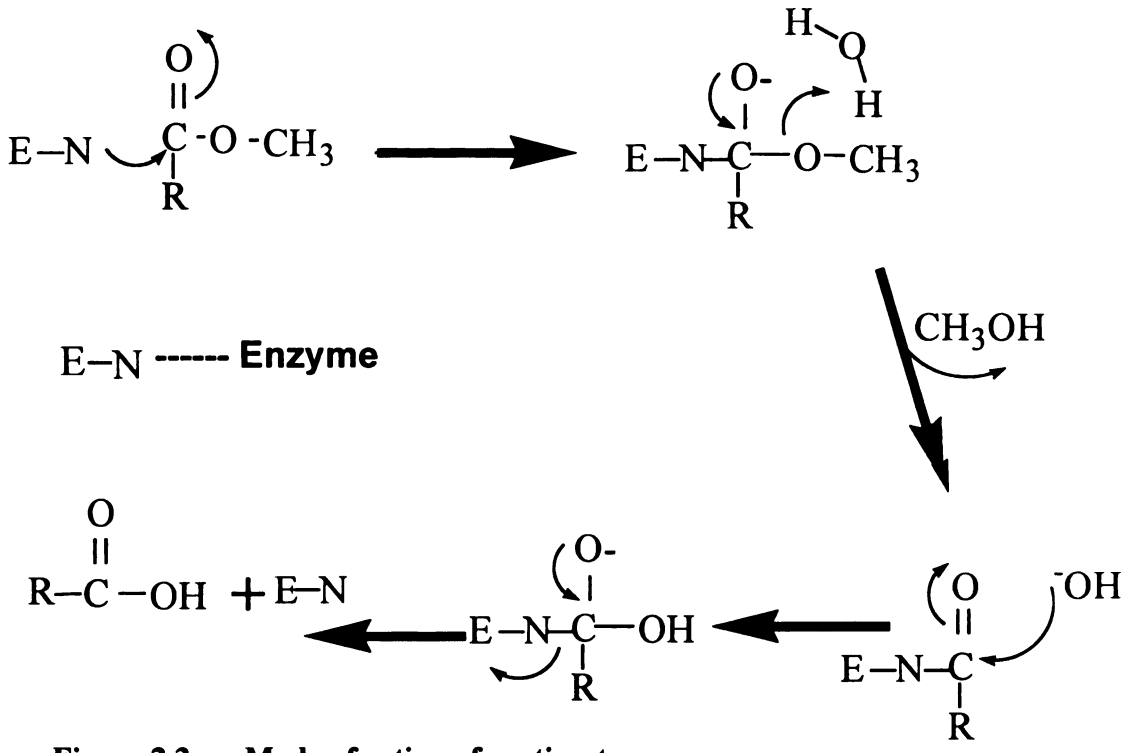


Figure 2.2. Mode of action of pectinesterase.

The effect of pH on pectinesterase has also been studied. Sun and Wicker (1996) used the fluorescent probe 8-anilinonaphthalene-1-sulfonate (ANS) to determine the surface hydrophobicity of the enzyme. ANS is non-fluorescent in water and other polar environments, but highly fluorescent in non-polar environments or when bound to hydrophobic sites on proteins. Changes in protein structure that result in an increase in hydrophobicity can be estimated by the change in fluorescence of ANS. If pH causes a change in the hydrophobic sites of protein, the structural change is detected by ANS fluorescence. Based on ANS fluorescence, pectinesterase molecules were found to be more hydrophilic at neutral and alkaline pH than at acidic pH values.

Current studies of pectinesterase activity indicate that the degree of activity can be enhanced by the presence of cations. The stimulatory effect of NaCl on pectinesterase

varies considerably. Fayyaz and coworkers (1995) found that the stimulatory effect of NaCl was quite high for NaCl concentrations between 0.1 and 0.15 M. The enzyme had approximately the same activity between 0.15 and 0.3 M, but as the concentration of NaCl increased the activity of the enzyme declined. Labib and El-Ashwah (1995) reported a similar effect on mango pectinesterase by NaCl. The maximum pectinesterase activity was at 0.1 M, and the activity decreased gradually as the concentration was increased to 0.4 M. Calcium chloride has also been shown to have a similar effect on the activity of pectinesterase. It is worth noting that the cations increased the activity of pectinesterase, but were not a requirement for its activity.

2.4 Enzyme Kinetics

Enzymes are proteins by nature, and they have highly specialized biological activity. They retain their biological capacity and function in a very narrow range of temperature and pH. Exposure of an enzyme to extremes of temperature or pH results in denaturation of the enzyme and loss of its ability to catalyze a chemical reaction. The polypeptide backbone of the protein molecule is not broken, but the polypeptide chain is unfolded and the molecule loses its structure. Local structure is key to the functioning of an enzyme.

Enzymatic activity is generally proportional to the enzyme concentration. During the early stages of the enzymatic reaction, the relationship between enzyme concentration and the rate of reaction is linear. This linear relationship has been reported (Mangos and Haas, 1997) for orange pectinesterase.

During the early stages of an enzymatic reaction and for very short time intervals, the substrate can be assumed to be constant (initial rate assumption). This is especially true in cases where the substrate is present in excess. Beyond the initial phase, the reaction slows down with time. Most enzymatic reactions follow the kinetics of a first-order reaction described by the equation,

$$d[S]/dt = -k[S] \qquad (1)$$

where

$d[S]/dt$ = rate of substrate consumption

$[S]$ = substrate concentration at any given time during the reaction

k = reaction rate constant

The concentration of substrate remaining at any given time, may be expressed in terms of the initial substrate concentration and product formed in view of the mass balance relationship:

$$[S] = [S]_i - [M]_t \qquad (2)$$

where,

$[S]_i$= Initial substrate concentration

$[M]_t$ = Concentration of product at any given time

Enzyme activity is affected by a variety of factors. Two major factors are temperature and pH. As the temperature is increased, two simultaneous reactions occur. First, the enzyme activity increases, as observed in many chemical reactions, and secondly, the enzyme stability decreases due to thermal denaturation (Laidler, 1954). The net result of these two effects is a bell-shaped curve.

In the food processing industry, inactivation of enzymes by heat is a very common procedure. Vegetables and fruits are heat treated before canning or freezing. Cloudy juices, such as orange and tomato juice retain their cloudiness by inactivating the pectic enzymes by heat. The inactivation of an enzyme can be described by first order kinetics. After integration and substituting equation (2), equation (1) can be written as:

$$\ln ([S]_i - [M]_t)/ [S]_i = -kt \qquad (3)$$

where,

$[S]_i$= Initial substrate concentration

$[M]_t$ = Concentration of product at any given time

t = heating time

k = inactivation rate constant

A plot of equation (3) is linear and the slope represents the inactivation rate constant, k. The constant k is highly pH and temperature dependent. Various reports have demonstrated that thermal inactivation rates of pectinesterase in citrus juices increased at lower pH values (Rouse and Atkins, 1952; Atkins and Rouse, 1954).

The rates of chemical reactions generally vary with temperature according to Arrhenius law:

$$k = A\, e^{(-Ea/RT)} \qquad (4)$$

where

k = reaction rate constant,

A = Arrhenius constant

E_a = Activation energy

T = Absolute temperature

To measure the inactivation rate constant, a small range of temperature is chosen, at which inactivation is taking place. A plot of the overall temperature effect on the enzyme activity will assist in determining the temperature at which inactivation occurs.

Fayyaz *et al* (1995) studied the activity of papaya pectinesterase. They found that papaya pectinesterase showed an optimum reaction temperature at 65°C and declines after 70°C. They calculated the activation energy of papaya pectinesterase to be 5690 cal mol^{-1} from 20 to 60°C. Similar results have been reported for orange pectinesterase by Korner *et al* (1980), who determined a value of 5740 cal mol^{-1}. Another study by Lee and Wiley (1970) found an activation energy of 5800 cal mol^{-1} for apple pectinesterase.

2.5 Current methods of measuring pectinesterase activity

Several methods have been used to monitor the activity of pectinesterase. The analytical methods can be categorized into three, depending on changes that are used to quantify the activity of pectinesterase. The activity may be measured by: 1) the disappearance of the substrate; 2) the formation of certain products from the substrate; 3) changes in physical properties of the substrate mixture. In this study the second method was used, whereby the amount of methanol produced was used as a measure of the activity of pectinesterase (PE). Other methods that are generally spectrophotometric in nature have been extensively reported.

Wood and Siddiqui (1971) developed a titrimetric method to measure the amount of methanol generated from pectin. Pectin is hydrolyzed using sodium hydroxide to generate methanol. The methanol is then oxidized to formaldehyde with potassium

permanganate, followed by condensation with 2,4-pentanedione to yield the colored product 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine. The absorbance of this colored product is monitored by UV-VIS. The permanganate oxidation method requires the reduction of unreacted permanganate with sodium arsenite.

In an attempt to improve on the technique of Wood and Siddiqui, an enzymatic method was proposed by Klavons and Bennett (1986) using alcohol oxidase to oxidize methanol liberated upon hydrolysis of pectin. The use of alcohol oxidase eliminated the reduction step with sodium arsenite and offers a two-fold increase in sensitivity. The specifity of alcohol oxidase to oxidize lower primary alcohols to aldehydes was also utilized.

Mangos and Haas (1997) have developed an alternate procedure that also utilizes alcohol oxidase (AOD) to oxidize methanol to formaldehyde, but subsequently employs the use of peroxidase and hydrogen peroxide. When methanol is oxidized it produces formaldehyde and hydrogen peroxide. They quantitated hydrogen peroxide with peroxidase (POD) and the chromogen 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The reaction scheme is shown below:

$$\text{Pectin-COOCH}_3 + \text{H}_2\text{O} \xrightarrow{\text{NaOH}} \text{Pectin-COOH} + \text{CH}_3\text{OH}$$

$$\text{CH}_3\text{OH} + \text{O}_2 \xrightarrow{\text{AOD}} \text{HCHO} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + 2\ \text{ABTS(H)} \xrightarrow{\text{POD}} 2\ \text{H}_2\text{O} + 2\ \text{ABTS}^{\bullet+}$$

The generation of ABTS radical cation, which absorbs strongly at 420 nm, is proportional to the amount of pectinesterase in the reaction.

In the fruit brandy industry, presence of methanol is accompanied by relatively large amounts of ethanol. The techniques discussed above are not selective for methanol

over ethanol. In fact, ethanol has been reported to interfere with the analysis of methanol (Wood and Siddiqui, 1971). Measurement of the activity of pectinesterase in the brandy industry requires a technique that is particularly selective toward methanol in the presence of large amounts of ethanol.

Gas chromatography is a fast and accurate method to measure alcohols, both qualitatively and quantitatively. There are specific column requirements needed to ensure separation of methanol from ethanol. Temperature programming is utilized in gas chromatography for fast, selective separation (Skoog et al. 1998). Optimization of column temperature and carrier gas flow rates is therefore necessary to ensure the efficiency of a gas chromatographic technique.

One major advantage of the gas chromatographic technique is the elimination of reaction steps. Since methanol can be detected directly on the column, there is no need for any further reactions with the methanol as is required with the other techniques described above. Methanol generated from the demethoxylation of pectin can therefore be detected easily on a polyethylene glycol column without any further modification. In this work, this approach is used to quantify the activity of pectinesterase. The versatility of this method also makes it efficient for rapid prescreening of potential inhibitors of pectinesterase.

2.6 Inhibition of Pectinesterase activity

The control of pectinesterase activity has been the subject of study because of its implications in the modification of the texture of fruit and vegetables (Javeri and Wicker,

1991). It has also been found to act as a destabilizing agent of pectin materials in fruit and juice concentrates (Seymour *et al.*, 1991).

Inhibitors decrease the rate of an enzyme-catalyzed reaction. Control of the enzymatic activity of pectinesterase is of importance in regulating the amount of methanol generated from the deesterification of pectin. The amount of methanol in any food product needs to be regulated for obvious health reasons associated with the toxicity of methanol. Several authors have attempted inhibiting the activity of pectinesterase.

Veersteg *et al* (1978) were able to show that pectinesterase is inhibited by polygalacturonic acid. Fayyaz *et al* (1995) also showed that papaya pectinesterase was competitively inhibited by polygalacturonic acid. They calculated the inhibition constant as 0.019 mg/mL. Lineweaver and Ballou (1945) suggested a cation-carboxyl complexation of the acid was responsible for the inhibitory behavior of polygalacturonic acid. They also suggested that at higher pH, the cations were less effective because PE was less protonated and therefore had less of a tendency to form an inactive complex with an anion inhibitor.

Pectinesterase has also been inactivated by low pH. Owusu-Yaw *et al* (1988) used hydrochloric acid to lower the pH of orange juice from 3.75 to 2.0. Pectinesterase in the juice was completely inactivated at the low pH. This method of inactivation is, however, not applicable in the fruit brandy industry. In order to inactivate the enzyme while fermenting the fruit mash, very low pH needs to be avoided. Very low pH results in the inactivation of the yeast as well, and therefore pectinesterase activity is inhibited, but this process also stalls fermentation.

Hall (1966) studied the effect of tannic acid on tomato pectinesterase. He was able to obtain complete inhibition with 5 x 10^{-5} molar concentration of tannic acid using 0.05 per cent pectin as the substrate. Isolation of an inhibitor of pectinesterase from Kiwi fruit has also been reported by Balestrieri et al. (1990). The inhibitor completely inhibited pectinesterase in the pH range between 3.5 and 7.0. The inhibitor was tested on a variety of pectinesterases from various sources including orange, tomato, potato, banana and apple pectinesterases.

The most frequently used technique to inhibit the activity of pectinesterase is by thermal inactivation of the enzyme. Nakagawa *et al.*, (1970) completely inactivated purified pectinesterase by heating between 65 and 90°C. Labib *et al.*, (1995) observed stepwise inactivation of mango pectinesterase activity, which was attributed to a possible presence of more than one type of pectinesterase. Heat inactivated pectinesterase did not regenerate upon frozen storage.

Ion exchange membranes have also been used to evaluate their effect on pectinesterase-pectin complexes. Chen *et al.*, (1998) used both anionic and cationic ion exchangers to bind pectinesterase and pectin. They observed that at pH 7.2, anionic exchange disks had little binding of pectinesterase, but its binding to a cationic membrane was significantly higher at the same pH.

Interactions between pectinesterase and ion exchangers depend on several factors, including the net surface charge on pectinesterase. Other factors include pH, ionic strength, and the nature of the ion (Karlsson *et al*, 1989). One of the most important factors that determine protein binding to ion exchange membranes is pH, since it affects the effective charge on both the protein and the ion exchanger.

Theoretically, pectinesterase is positively charged at pH less than 8.0 while pectin is negatively charged at pH values above 4.0 (Charnay *et al.*, 1992; Lineweaver and Ballou, 1945). Under such conditions, positively charged pectinesterase can combine with pectin at the ionized carboxyl groups.

In understanding the effect of cations on the activity of PE, the pH of the assay needs to be considered. At lower concentrations, cations displace pectinesterase from the inactive pectin- pectinesterase complex and stimulate activity. At higher concentrations, cations competitively bind carboxylic acid groups and act as competitive inhibitors of pectinesterase activity. This competitive displacement theory is probably not the only factor involved in binding and release of pectinesterase from pectin complexes, but is a considerably reasonable explanation of the effect of ions on pectinesterase activity.

In this study a variety of inhibitors are tested to determine their effect on the activity of pectinesterase. Most of the inhibitors used are known chelating agents that should significantly bind to a positively charged protein such as pectinesterase.

3. MATERIALS AND METHODS

3.1 Methanol Analysis

3.1.1 Fermentation

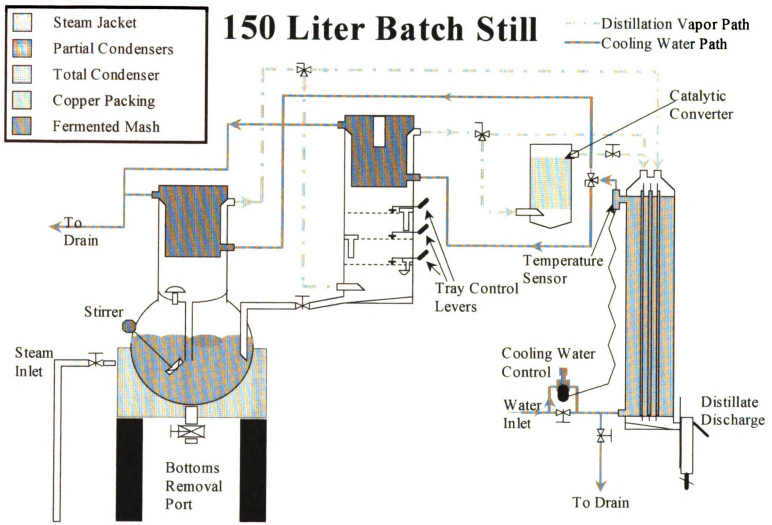
A variety of fruits were used to monitor the fermentation process, namely cherries, pears, peaches and plums. The fruits were stored frozen and had to be thawed before crushing. They were then crushed using a stem crusher and pumped into a 200-gallon fermenter. 300g of yeast (*Prise de Mouse*) were added to 600 mL of 45°C distilled water and left for 15 minutes to activate the yeast. The activated yeast was then added to the fruit in the fermenter and continuously stirred. The temperature of the fermenter was maintained at 15°C using a water chiller. Fermentation progress was monitored for a period of 10 days. Dissolved sugars in the broth give an indication of the fermentation progress and this was monitored on a daily basis using a refractometer. The pH of the broth was also monitored on a daily basis.

For monitoring the generation of methanol during fermentation, the samples were centrifuged for 2 minutes at 1400 rpm and filtered through 0.5μm filter paper to remove all the solid particles. 0.5 μL of each sample were then injected into the gas chromatograph.

3.1.2 Distillation

Distillation was performed using a Christian Carl still shown in figure 3.1. The still is equipped with three trays, a partial condenser and a catalytic converter. Once the brix of the fermented broth reached a steady value, fermentation was assumed to be complete.

Figure 3.1 Schematic of a 150 L Christian Carl still


Steam Jacket
Partial Condensers
Total Condenser
Copper Packing
Fermented Mash
150 Liter Batch Still
Distillation Vapor Path
Cooling Water Path
Catalytic Converter
To Drain
Temperature Sensor
Tray Control Levers
Stirrer
Steam Inlet
Cooling Water Control
Water Inlet
Distillate Discharge
Bottoms Removal Port
To Drain

The mash was then pumped into the still and continuously stirred for the duration of the distillation. Cooling water was circulated through the entire system before distillation began. Steam was used to heat the fruit mash and a pressure of 0.3 to 0.4 bar was maintained throughout the distillation. The number of trays to be open or closed, as well as the engaging of the "catalytic converter" varied from one distillation run to the other. The "catalytic converter," is made up of a high surface area copper packing material thought to strip cyanide out of the distillate and therefore reduce the amount of urethane in the spirits.

The distillate started condensing at approximately 90% v/v alcohol and distillation was continued until the alcohol level dropped to 45% v/v alcohol. The distillate collected after this level contains a significant amount of higher alcohols and generally does not have good taste qualities. The distillates were collected as different cuts in order to quantify the methanol at each stage in the distillation. Four-500 mL samples were collected to represent the heads, followed by five 1-liter samples to represent the hearts and finally 1 liter was collected as tails. The steam was then turned off, the still emptied and cleaned for the next run.

3.1.3 Analysis of Distillates.

Each of the different cuts was diluted with distilled water in order to obtain an alcohol content of 40 % v/v. This is the minimum alcohol content that meets the definition of a fruit brandy. One mL of each cut was sampled into vials for analysis.

Analysis was performed by gas chromatography using a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector (FID) and auto sampler. A Stabilwax® column from Restek Corporation was used for the separation of the alcohols. The stationary phase of this column is polyethylene glycol and is particularly good for the separation of alcohols. The run conditions on the GC were as follows:

Column:	30m, 0.32 ID, 0.5 μm Stabilwax®
	0.5 μL split injection
Oven temp:	40°C (hold for 1 minute); to 190°C at 2.5°C /min.
	190°C (hold for 5 minutes)
Injector temp:	240°C
Detector temp:	255°C
Carrier gas:	Helium at 30 cm/sec
Split ratio:	65:1
Total GC run time:	65 minutes

The high final column temperature was used to ensure that most of the other higher boiling alcohols were removed from the column at the end of the run. Very small amounts of methanol relative to ethanol are present in the sample. A typical gas chromatogram is shown in figure 3.2. The major component of fruit brandy is ethanol and this is reflected on the chromatogram. The other major components detected are

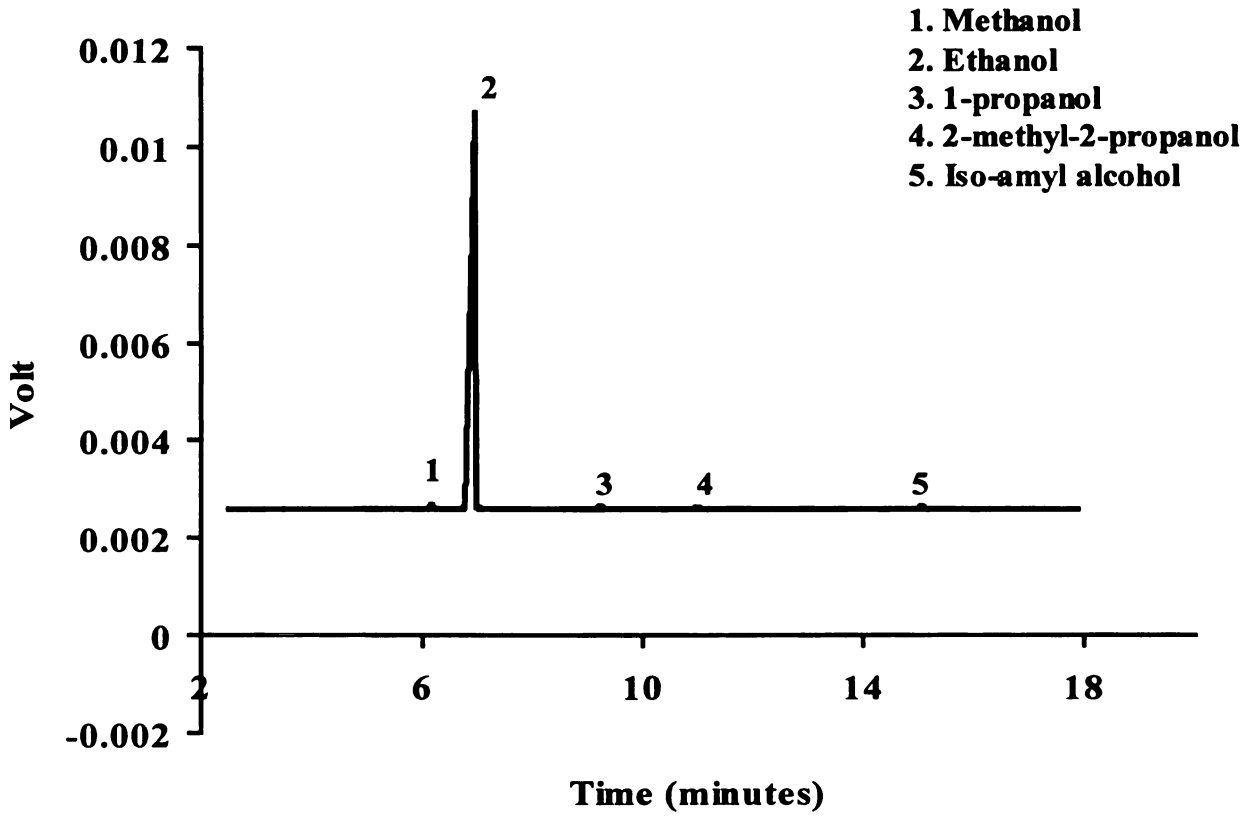


Figure 3.2 Typical gas chromatogram of fruit distillates. The GC run conditions were as follows: Oven temp: 40°C (hold for 1 minute); to 190°C at 2.5°C /min (hold for 5 minutes), Injector temp: 240°C, Detector temp: 255°C, Carrier gas: Helium at 30 cm/sec, Injected volume: 0.5 μL.

methanol, acetaldehyde, isopropanol and isoamylalcohol. The compound of major interest in this work was methanol. As is evident in the chromatogram detection of methanol was achieved with this temperature programming on the column.

3.1.4 Quantification of Methanol

In order to quantify the amount of methanol in the distillates, a calibration curve was generated. Calibration samples were made using methanol, ethanol and water mixtures in order to duplicate the matrix system in the distillate samples. The overall ethanol content of all samples was 40% and the methanol concentration was varied from 0 to 1% v/v. Each calibration sample was injected into the GC and the methanol quantified.

A calibration curve was set up using the chromatographic peak areas. Analysis of the chromatographic data was done using Class-VP software from Shimadzu. Quantification of methanol in all other samples was determined relative to this external standard calibration curve.

3.2 Pectinesterase Activity

3.2.1 Measurement of PE activity

Purified commercial pectinesterase (EC 3.1.1.11) enzyme and apple pectin purchased from Sigma Chemical Company were used in this investigation. The manufacturer specified pectinesterase activity as 130 units per mg. One unit of pectinesterase is defined as the amount of enzyme that will liberate one μmole of methanol per minute. Concentration of 4 mg/mL of pectin was selected due to solubility limitations. This was the maximum amount of pectin that could easily dissolve in water. Pectinesterase was used at a concentration ranging from 0.1 mg/mL to 0.4 mg/mL. A stock solution (21.6 mg/mL) of the enzyme was diluted with phosphate buffer (pH 7.0), the enzyme's optimum pH, to give the final concentrations used. Pectin substrate (0.5 mL) was incubated with 0.5 mL of pectinesterase for a period of time and the methanol released quantitated. All incubations were done at room temperature. The run conditions on the GC were similar to those used for analysis of distillates, except for the oven temperature and GC run time which were:

Oven temp:	40°C to 100°C at 3°C /min. (hold for 0.5 minute)
	to 190°C at 20°C/min.
Total GC run time:	25 minutes

3.2.2 Temperature Dependence

To test the stability of the enzyme at various temperatures, a fixed incubation time of 5 minutes was chosen. The temperature ranged from 20°C to 90°C. The enzyme was heated in a water bath and maintained at the respective temperature for five minutes before being added to the substrate. An enzyme to substrate ratio of 1:1 was incubated for five minutes, and then 0.5 μL of each sample was injected into the GC. The amount of methanol in each sample was quantitated.

3.2.3 Thermal Inactivation of Pectinesterase

Thermostability of pectinesterase was tested by heating in the temperature range between 60 and 75°C. For cherry juice samples, 10 mL samples were heated to 30°C, 55°C, 70°C and 90°C. The samples were kept in water baths at the respective temperatures for 5 minutes before sampling. They were then centrifuged and filtered before being introduced to the gas chromatograph.

3.3 Inhibition of Pectinesterase

Once the activity of pectinesterase was established, the next step was to study potential inhibitors for this enzyme. Several potential inhibitors were identified based on previous literature reports or similarity in functional groups that the enzyme targets in pectin. The specific inhibitors tested included tannic acid, polyacrylic acid, polymaleimide, ethylenediamminetetraacetic acid (EDTA) and citric acid.

3.3.1 Tannic Acid

Tannic acid was purchased from Sigma Chemical Company. This was used on cherry fruit juice samples instead of commercial pectin. The appropriate amount of tannic acid powder was added to 500 mL of cherry juice samples to make a final concentration ranging from 0 to 1 mM. Yeast was also added to each sample to monitor the effect of tannic acid on fermentation. The sample without any tannic acid served as a control. Every 24 hrs, 2 mL samples were centrifuged and filtered with a 0.45 μm filter paper to remove any particles that might clog the GC column. Brix measurements were then taken and methanol content for each sample determined by GC. These measurements were taken until the fermentation ceased and samples were kept in the cold room (15°C) during the fermentation period.

3.3.2 Polyacrylic acid

Polyacrylic acid with a molecular weight of approximately 2100 was purchased from Sigma Chemical Company. For this study, commercial apple pectin and purified pectinesterase were used as the substrate and enzyme, respectively. The sodium salt of polyacrylic acid was tested as an inhibitor at a concentration of 1 % w/v. The same inhibitor concentration was used over a range of pectin solutions (1- 4 mg/mL) in order to understand the dependence of the inhibition process on substrate concentration. A 1:1:1 mixture of pectinesterase, pectin and polyacrylic acid was incubated for 5 minutes at room temperature. One half μL of each sample was injected into the GC to quantitate methanol. Control samples for each pectin concentration, without polyacrylic acid were

also incubated for 5 minutes before injection. A comparison for each pair gave an indication of the extent of inhibition.

3.3.3 Polymaleimide

Polymaleimide was selected as a potential inhibitor because it possesses carboxylate functional groups that are a likely target for pectinesterase. For preliminary studies, 1% w/v sodium polymaleimide solution was prepared. The polymaleimide used was synthesized in our laboratory. A 1:1:1 mixture of pectin, pectinesterase and polymaleimide was incubated for 5 minutes. Various pectin concentrations ranging from 1 to 4 mg/mL were used and similar control samples without polymaleimide were also incubated for 5 minutes at room temperature. The extent of inhibition was determined as the difference in methanol concentrations between the samples with and without polymaleimide.

Since the 1% w/v solution showed promising results, lower concentrations of polymaleimide were tested. Solutions of 0.25% and 0.5% w/v were tested in a similar manner as the 1% w/v polymaleimide inhibitor.

3.3.4 Citric Acid

Citric acid has both low pH and carboxyl groups. These are desirable qualities for an inhibitor of pectinesterase and therefore its inhibitory effect was tested. Commercial pectin and pectinesterase were used for this study. One percent w/v citric acid was used with pectin concentration of 4 mg/mL. Increasing volumes (0.1 to 0.5 mL) of 1% w/v citric acid were added to the pectin-pectinesterase mixture. The mixtures of pectin,

pectinesterase and citric acid were each incubated for 5 minutes at room temperature. One half μL of each sample was injected into the GC to quantitate methanol.

3.3.5 Ethylenediamminetetraacetic Acid (EDTA)

EDTA is a strong chelating agent and its ability to inhibit the activity of pectinesterase was tested. Pectin substrate concentrations used ranged from 1 mg/mL to 4 mg/mL and pectinesterase concentration was 0.432 mg/mL. 1% w/v solutions of di-sodium EDTA were incubated for 5 minutes at room temperature with PE and pectin. The total incubated volume was 1.5 mL consisting of 0.5 mL of each constituent. Control samples were prepared in a similar manner except that the inhibitor was not added to the reaction mixture. One half μL of each sample was injected into the GC to quantitate the methanol produced.

4. RESULTS AND DISCUSSION

4.1 Composition of Fruit Brandy

In order to monitor the progress of fermentation, the brix measurements were taken every 24 hrs for a period of days. The pH of the fermented mash was also taken in order to keep track of any changes that might affect the functioning of the yeast. As is evident in Table 4.1 the fermentation was virtually complete in 9 days. The remaining dissolved sugar is sorbitol, which is not digested by yeast.

Table 4.1 Brix values of fermented mash of different fruits. Fruit mash stirred in a 200 gallon fermenter maintained at 15°C by a water chiller. 300g *Prise de mouse* yeast used for each fermentation.

Day	Plum	Peach	Cherry
1	13.0	10.3	13.7
2	12.6	9.7	13.4
3	11.1	9.2	13.3
4	8.8	9.0	12.6
5	6.8	7.1	10.5
6	6.0	4.7	6.6
7	6.1	4.4	6.4
8	6.2	4.5	6.3
9	6.2	4.4	6.0
10	6.2	4.4	6.0

4.2 Methanol in Fruit Brandy

4.2.1 Quantitative Analysis.

The amount of methanol in fruit distillates is regulated by the FDA at 0.35% v/v in the drinkable product (40% v/v ethanol), so the initial step in this study was to quantify the methanol content of the fruit brandy. In order to do the quantitative studies, a methanol calibration curve was generated. The calibration curve that was used to quantitate methanol throughout this study is shown in figure. 4.1. The linear plot has a correlation coefficient of 0.9987 and the lower detection limit of 0.003% v/v corresponds to 0.023 μg/mL.

Methanol generated during fermentation was monitored (figure. 4.2). The plot shows a very small increase in methanol during fermentation. This implies that most of the methanol present in the final distillate is already in the fruit mash even before the onset of fermentation. Addition of yeast does not favorably affect the activity of pectinesterase.

A typical batch brandy distillation has three main components based on the product recovered: the heads, hearts and tails. Heads, the first 2000 mL recovered from the 150 L Christian Carl still, constitute the low boiling components such as acetaldehyde and methanol. The typical alcohol content for this cut is above 80% v/v. The hearts, which is the main drinkable product and comes out next, consists mainly of ethanol and ranges between 55% v/v to 80% v/v alcohol content. This is typically diluted to at least 40 % v/v to meet the legal definition of a fruit brandy. The tails are rich in higher boiling components such as fusel oils, which are alcohols with more than two carbons and

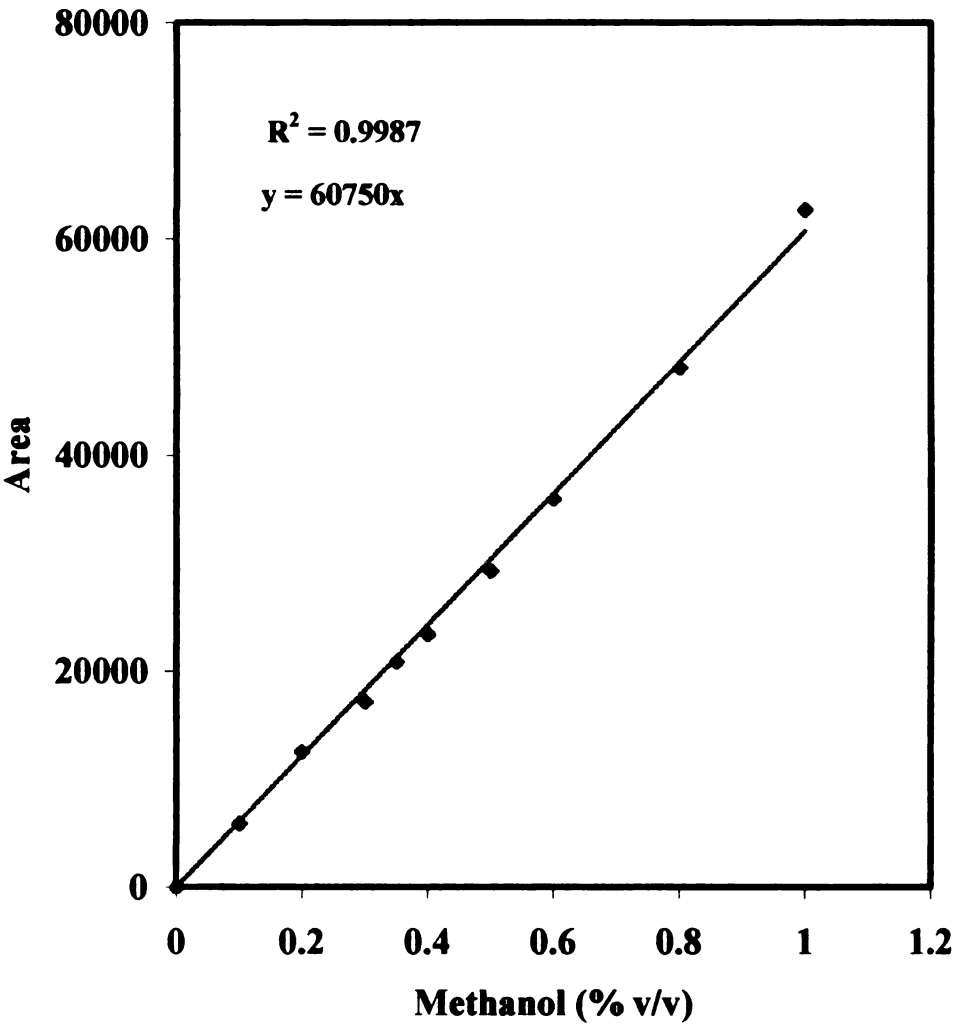


Figure 4.1. **Methanol calibration curve. Area corresponds to chromatographic peak area. The GC run conditions were as follows: Oven temp: 40°C (hold for 1 minute); to 190°C at 2.5°C /min (hold for 5 minutes), Injector temp: 240°C, Detector temp: 255°C, Carrier gas: Helium at 30 cm/sec, Injected volume: 0.5 μL. All other methanol concentrations were determined relative to this external standard curve.**

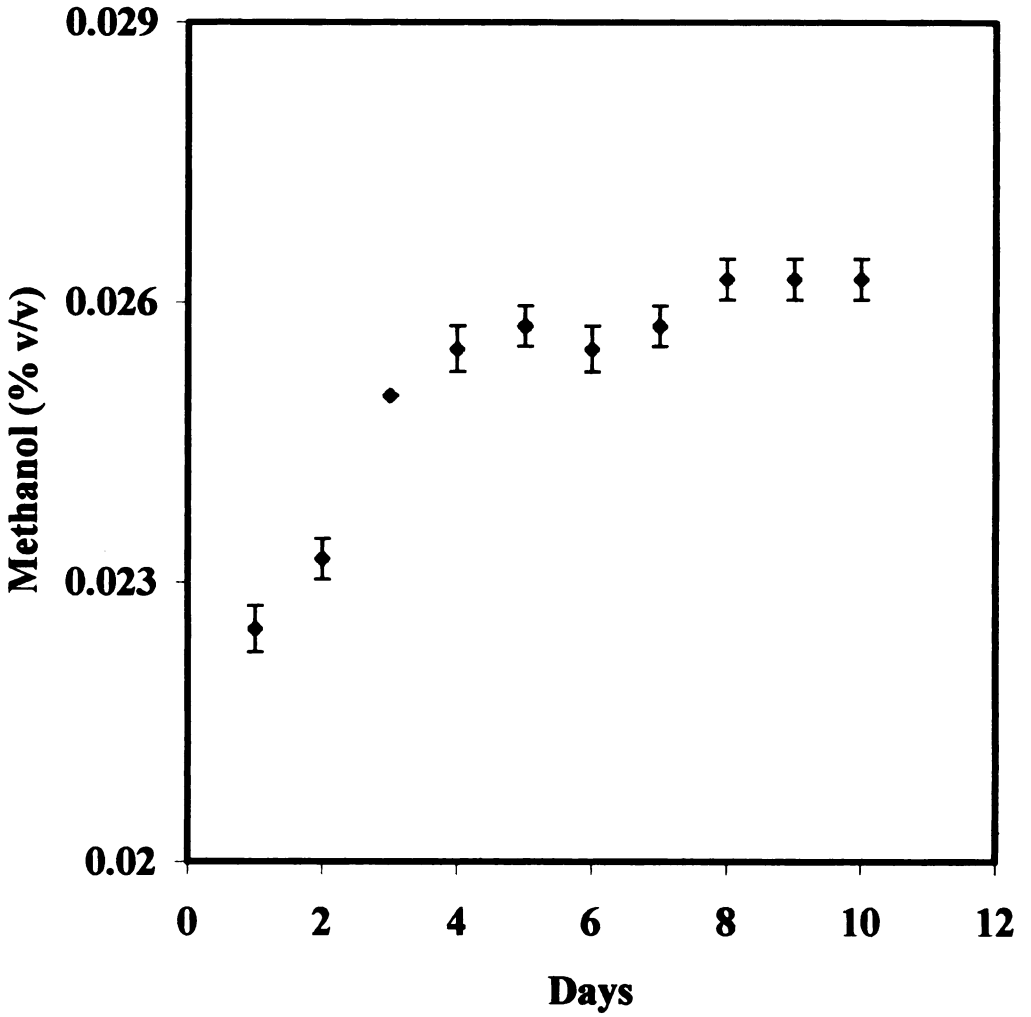


Figure 4.2. **Generation of methanol during fermentation. Montmorency cherries fermented at 15°C with *Prise de mouse yeast*. Analysis of methanol was done every 24 hours. Samples were filtered and centrifuged before injecting into the GC.**

typically contain less than 55% alcohol. This cut is usually either discarded or re-distilled to improve its quality attributes.

Methanol is a low boiling alcohol (65°C) relative to ethanol and would naturally distill mostly in the heads and hearts cuts. A typical methanol profile is shown in figure. 4.3. It is evident that the highest level of methanol is present in the heads cut. Surprising is the fact that the methanol in the tails cut is slightly higher than that in the hearts. Methanol content of distillates from the same fermentation batch also varied.

The major component of fruit brandy is ethanol. The yeast used in fermentation converts glucose into ethanol and carbon dioxide. The conversion is a 1:2 ratio such that for every 1 mole of glucose, 2 moles of ethanol are generated. Acetaldehyde is present in fruit brandy and is generated from the oxidation of ethanol with catalysts such as copper. The distillation still is made out of copper, and this copper surface catalyzes the production of acetaldehyde during distillation. Cracked pits are thought to increase the amount of urethane and benzaldehyde in fruit brandy. Benzaldehyde is a positive flavor compound, but urethane is a carcinogen. Benzaldehyde is produced through the hydrolysis of amygdalin, which is present in most stone fruits.

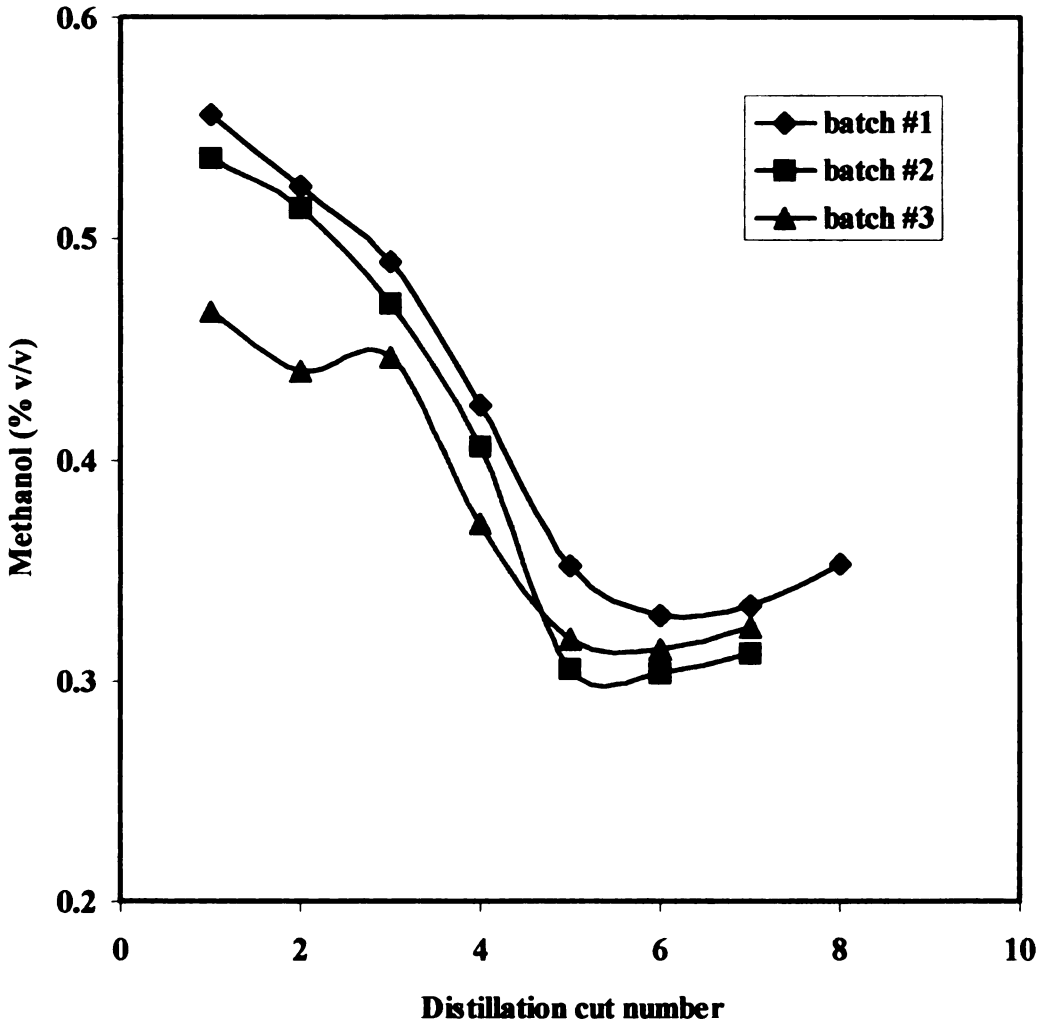


Figure 4.3. **Methanol profile during distillation. Different distillation batches from the same fermentation batch. Distillation performed with all 3 trays engaged. Distillation cut number corresponds to different volumes of distillate samples collected in the course of the distillation. Results shown are for cherry fruit brandy.**

4.2.2 Fruit Varieties

Methanol in fruit brandy is generated from the fruit pectin. It has been reported that different fruits have different amounts of pectic substances. Table 2.1 shows an example of the total pectic substances in different plants.

Table 4.2 Amount of Pectic substances in various plants.[2]

Fruit	Pectic substances (%)
Apple	14.9
Cherries	11.4
Beets	13.2
Orange	16.8

After distillation, each cut was sampled for methanol. The results of methanol analysis for various fruit distillates are shown in figure 4.4. The results are plotted for an average of three distillation batches for each fruit. The amount of methanol in the distillates varied from fruit to fruit, but generally the highest amount of methanol was found in plums on an average basis. The amount of methanol varied from batch to batch even within the same fruit type. The methanol content also varied with distillation conditions such as the number of trays closed and use of the catalytic converter.

[2] **Data obtained from *The Pectic Substances* by Z.I. Kertesz.**

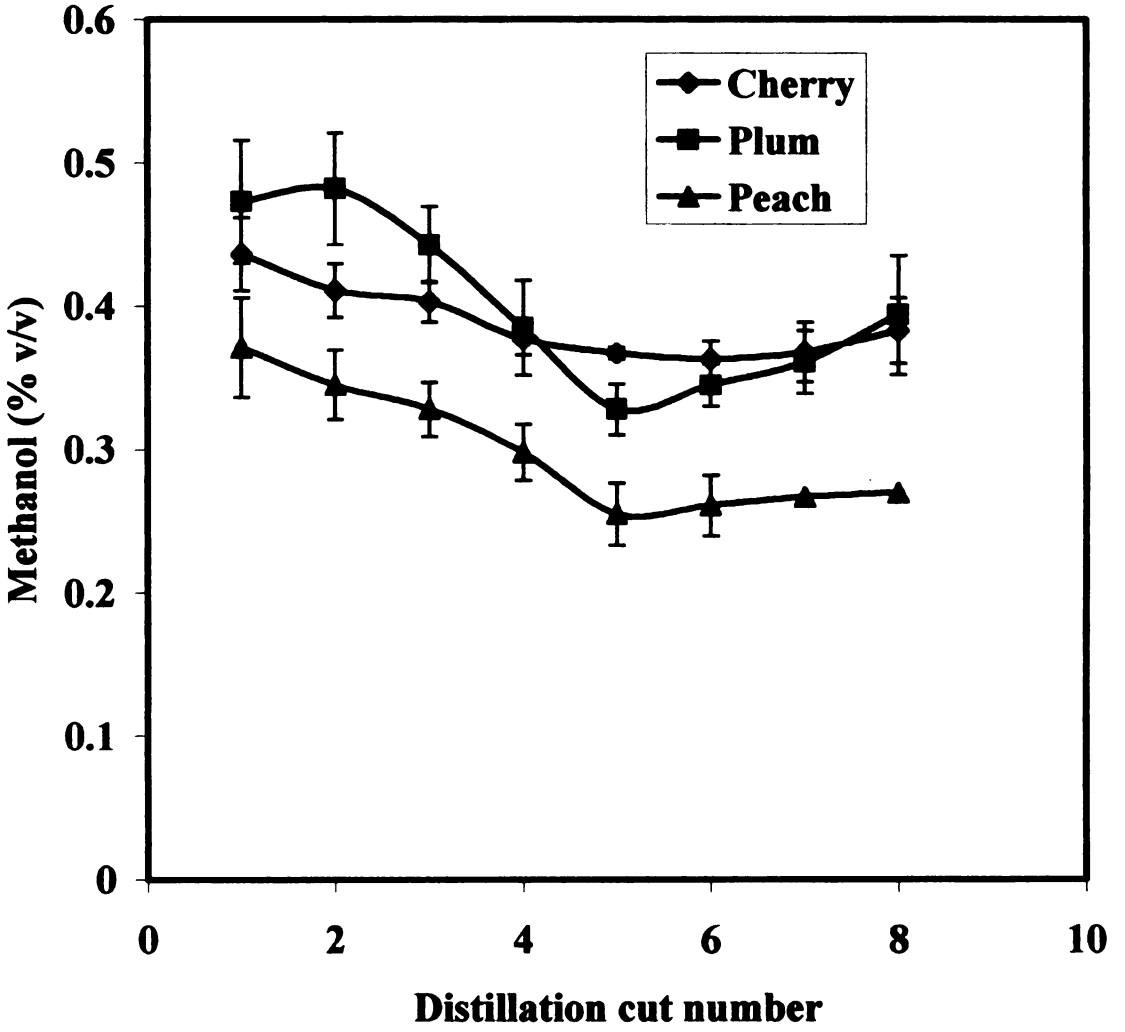


Figure 4.4. **Methanol profile among different fruit varieties. Fermented at 15°C with *Prise de mouse yeast.* Methanol content average of three distillation batches for each fruit. Distillation performed with all 3 trays engaged.**

4.3 Activity of Pectinesterase

4.3.1 Enzyme Kinetics.

The magnitude of enzymatic activity is usually proportional to the concentration of the enzyme. This proportionality is well exhibited during the earliest stages of the reaction. The linear relationship between enzyme concentration and rate of reaction is common in many enzyme-catalyzed reactions.

During the early stages of an enzymatic reaction, and for very short time intervals, the substrate concentration can be assumed constant. This is particularly true in cases where the substrate is present in excess, and the amount of product formed is proportional to reaction time. In order to understand the kinetics of pectinesterase, the substrate-enzyme reaction was monitored over a period of one hour. Figure 4.5 shows the activity of pectinesterase with time. From this figure, it is evident that the reaction is linear for the first 20 minutes and then it significantly slows down after 40 minutes. The reaction seems to get to completion after releasing 0.049% v/v methanol.

The concentration of substrate used in this study was 4 mg/mL pectin, and the maximum methanol released was 0.387 mg/mL. On this basis, the concentration of pectin-bound methanol is 0.096 mg/mg pectin, corresponding to 9.6% methoxy content in the pectin. The citrus pectin used had a methoxy content of 10% w/w as specified by manufacturer. The incomplete demethoxylation of pectin by pectinesterase is in agreement with results from other laboratories (Mangos, 1997), which have shown that

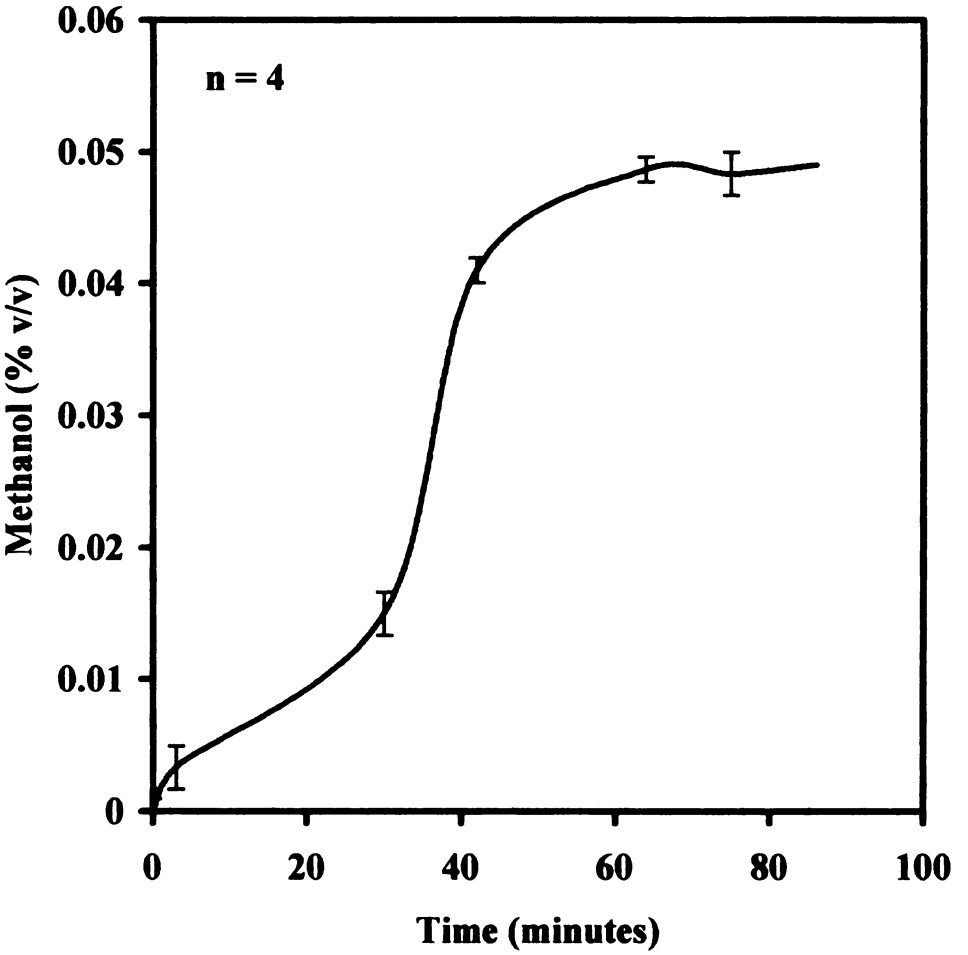


Figure 4.5. **Activity of pectinesterase as a function of time. Pectin concentration (4 mg/mL) and 0.432 mg/mL pectinesterase concentration. Pectinesterase prepared in phosphate buffer pH 7.0. Incubation at room temperature. 0.5 μL of each sample injected into the GC after incubation for the period of time shown.**

pectinesterase from higher plants demethoxylates blocks of methoxylated polygalacturonic acids and ester groups next to free carboxyl groups. Fungal pectinesterase on the other hand is capable of demethoxylating methyl esters that are not present in blocks. The results obtained here are similar to those obtained using a spectrophotometric assay that gave a methoxy content of 9.56% for citrus pectin and orange pectinesterase.

4.3.2 Effect of Temperature

Pectinesterase activity was measured at different temperatures to establish the thermostability of the enzyme. Four mg/mL citrus pectin was used as the substrate and the assay was carried out at pH 7.0, reported in the literature as the optimum pH for pectinesterase. The temperature range was from 20°C to 90°C, the broad range where pectinesterase is activated and subsequently denatured. Each sample was incubated for a period of 5 minutes at the respective temperature.

Figure 4.6 shows the results for the effect of temperature on the formation of methanol. The region below 50°C is where the enzyme is activated in accordance with normal enzyme behavior. Beyond 55°C, the enzyme is denatured and the activity declines dramatically. Heat-inactivation of the chemical reaction occurs according to the Arrhenius law.

To determine the order of the inactivation reaction, the measured methanol values for the inactivation region were plotted versus time. Under the experimental conditions

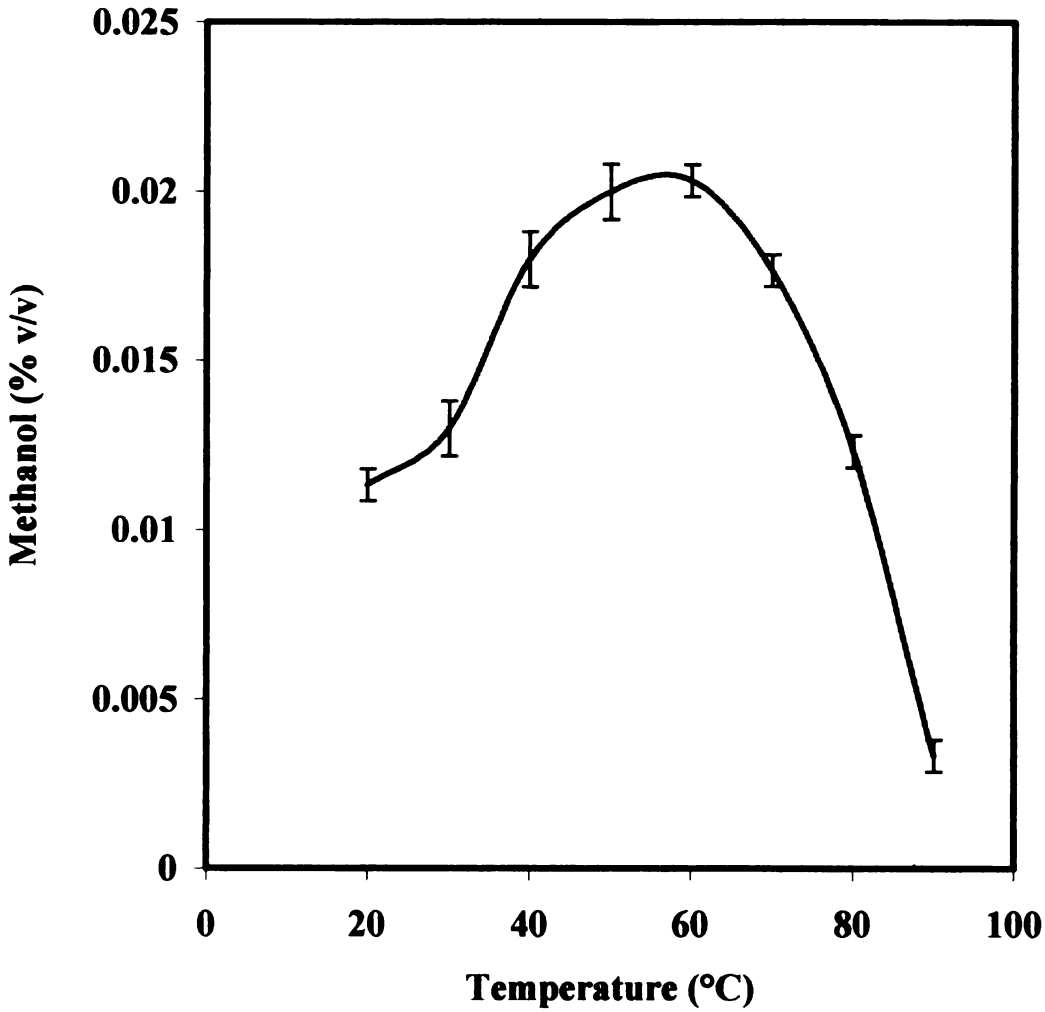


Figure 4.6 **Effect of Temperature on the formation of methanol. Pectin concentration: 4 mg/mL, Pectinesterase: 0.432 mg/mL. Pectinesterase prepared in phosphate buffer pH 7.0. The samples were incubated at the respective temperature for 5 minutes before injection into the GC.**

the data fit a straight line on semi-log coordinates and were described by a first order function. Figure 4.7 illustrates the normalized inactivation rate curves for pectinesterase.

Table 4.3 Inactivation rate constants for pectinesterase at various temperatures. Rate constants evaluated from straight line plots of equation (3).

Temperature (°C)	Rate constant $k \times 10^3$
60	2.2
65	2.5
70	2.8
75	3.2

The inactivation rate constants were determined from equation (3). Table 4.3 shows the rate constants as they were evaluated from the straight line plots at different temperatures. Computed values of the rate constant are similar to that reported in the literature. It is evident that the higher the temperature the higher the rate constant which results in steeper curves.

Another way to express the influence of temperature on the rate constant is the well known Arrhenius equation (4) rewritten as:

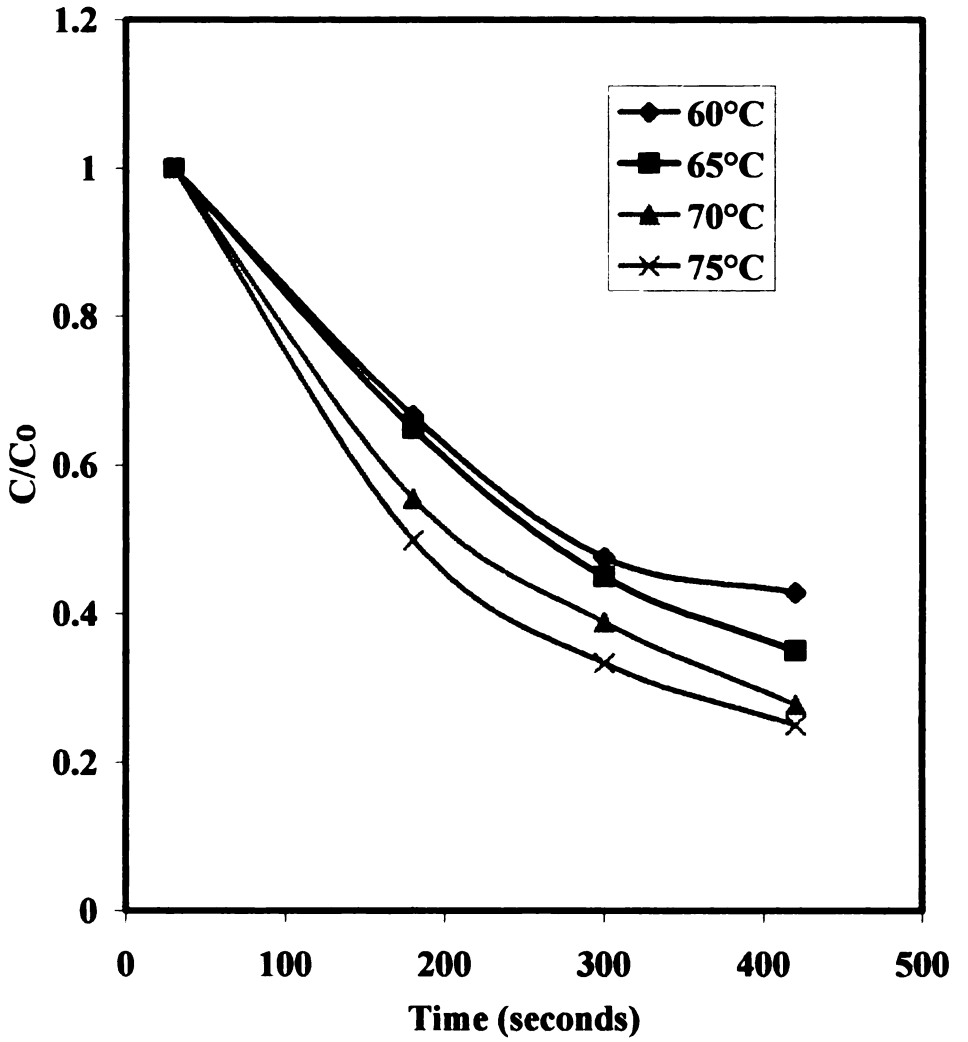


Figure 4.7 **Normalized inactivation rate curves for pectinesterase. Pectin: 4 mg/mL, pectinesterase: 0.432 mg/mL. Pectinesterase prepared in phosphate buffer pH 7.0. One half μL of each sample injected into the GC after incubation for the period of time shown. Initial methanol concentration (Co) determined after 30 seconds of incubation.**

$$\ln k = \ln A - E_a/RT \qquad (5)$$

where,

k = reaction rate constant,

A = Arrhenius constant

E_a = Activation energy

T = Absolute temperature

The Arrhenius plot for pectinesterase inactivation at pH 7.0 is illustrated in figure 4.8. The activation energy was calculated to be:

$E_a = 5697$ cal mol^{-1}

To explain the magnitude of the activation energy, the structure of the enzyme must be taken into consideration. Enzymes are complicated protein molecules and their catalytic properties are associated to their highly ordered tertiary structure. The tertiary structure is maintained primarily by weak noncovalent bonds that must be broken during the rate determining step. The activation energy found here falls in the range generally accepted for pectinesterase. This range is from 5600 to 7644 cal mol^{-1}(Fayyaz *et al*, 1995). The relatively small activation energy indicates that the enzyme molecule has a very delicate structure.

4.3.3 Thermal Inactivation of Pectinesterase in Cherries

The amount of methanol present in the cherry juice sample was measured at different temperatures under natural pH conditions. The samples were maintained at the specified temperature for 5 minutes. The results (figure 4.9) show a sudden drop in the amount of

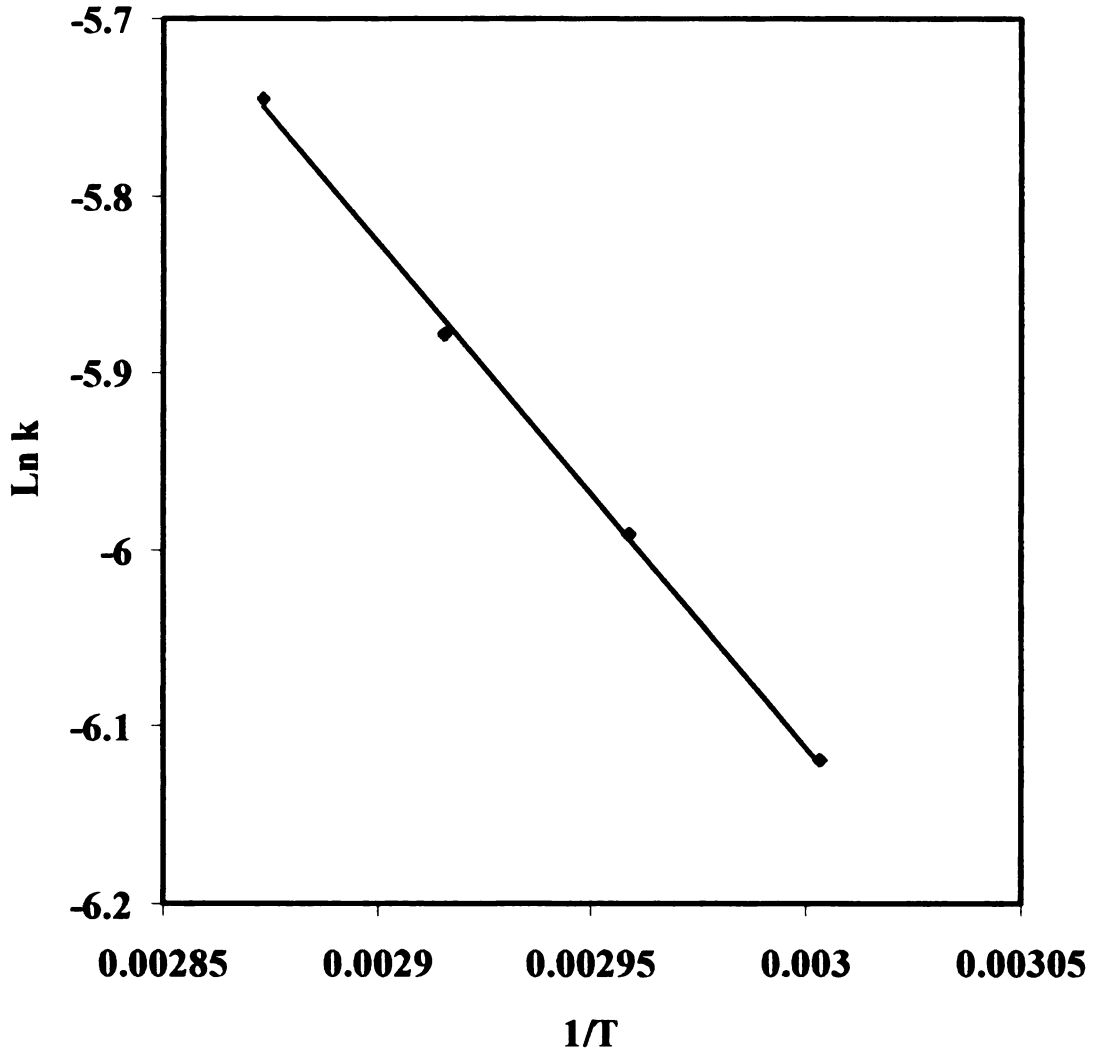


Figure 4.8 **Arrhenius plot for pectinesterase inactivation. Pectin: 4 mg/mL, pectinesterase: 0.432 mg/mL. Pectinesterase prepared in phosphate buffer pH 7.0. Activation energy was calculated from the slope to be 5697 cal mol^{-1}.**

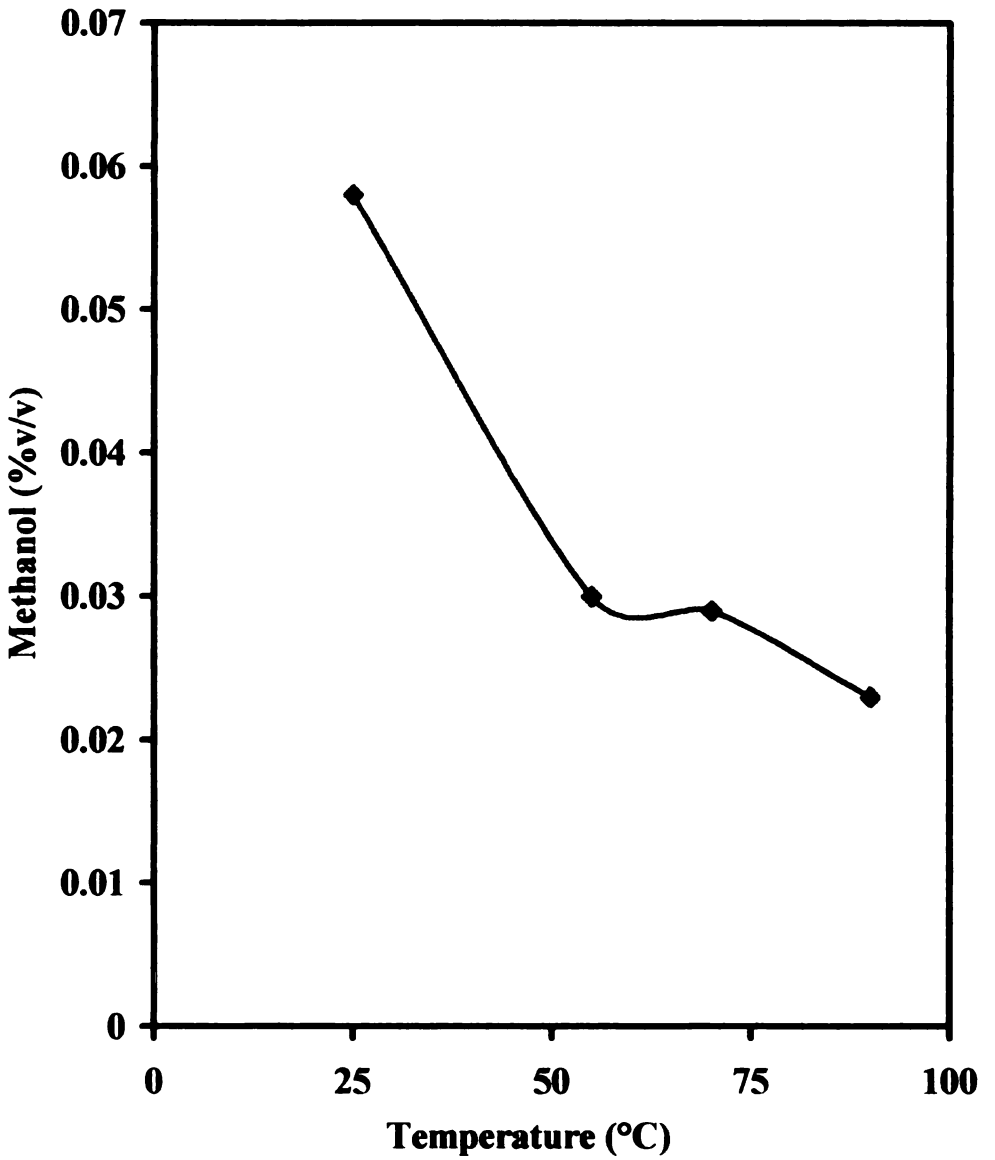


Figure 4.9 **Thermal Inactivation of Pectinesterase in cherry juice samples. Five hundred mL cherry juice heated for 5 minutes at the respective temperature. Samples centrifuged and filtered before injection into the GC.**

methanol at 55°C. At temperatures above this, low levels of methanol are observed, indicating that PE has been substantially inactivated. Heated samples were cooled to room temperature and further measurements were taken, but the inactivation of PE was irreversible therefore no change was observed in the results. These observations are in accordance with normal enzyme inactivation behavior.

4.4 Inhibition of Pectinesterase

Studies were conducted to test the ability of different compounds to inhibit pectinesterase. Initial studies were done using citrus pectin as the substrate that was incubated with pectinesterase from orange peel, both purchased from Sigma Chemical Company. The fact that theoretically pectinesterase is positively charged at pH 7.0 was used to study its complexation with inhibitors. Most of the inhibitors tested are well known chelating agents that should be able to bind to a positively charged protein such as pectinesterase.

4.4.2 Effect of Tannic Acid

Tannic acid was tested for its effect on pectinesterase using cherry juice samples as a source of pectinesterase. The results shown in figure 4.10 indicate a loss in pectinesterase activity for tannic acid concentration of 0.25 mM. However, an increase in tannic acid concentration did not have an inhibitory effect on pectinesterase.
The effect of tannic acid on pectinesterase was studied by Hall (1966) and found to inhibit pectinesterase with extremely high losses in pectinesterase activity. However, this

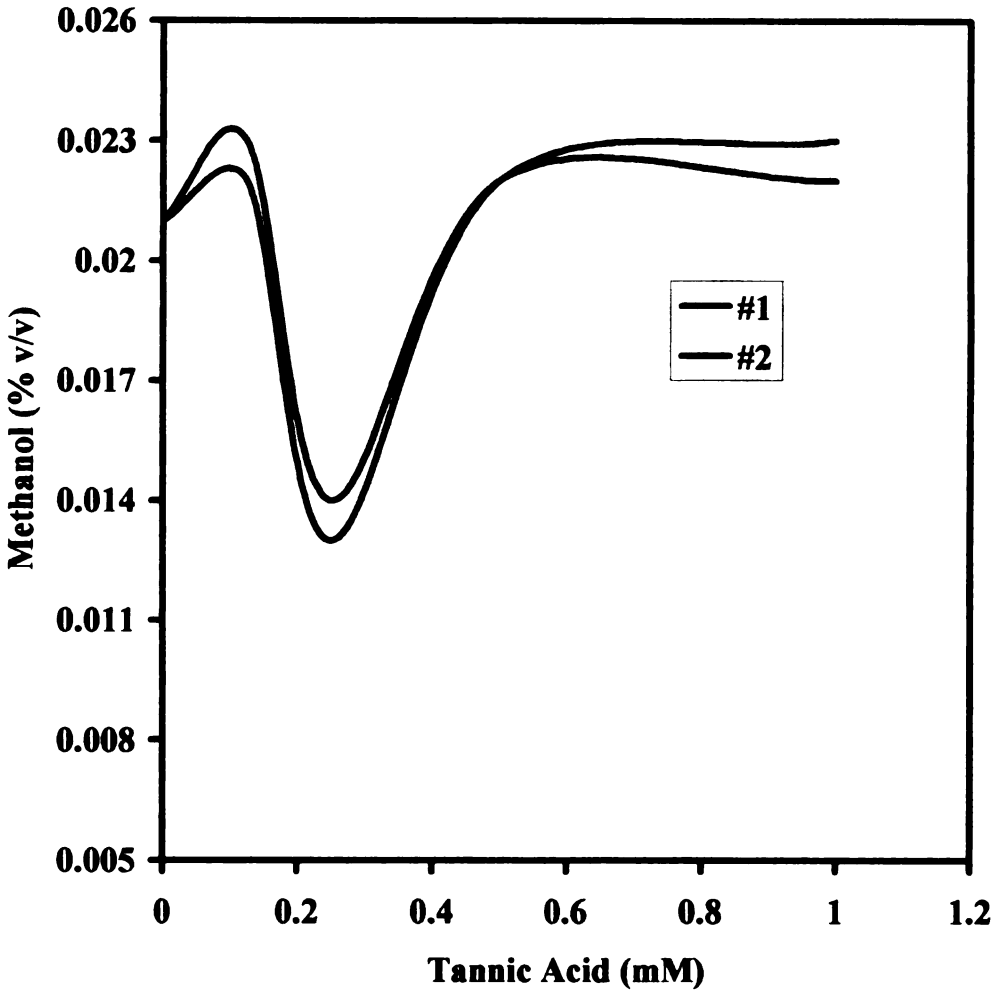


Figure 4.10 **Effect of tannic acid on the activity of pectinesterase. #1 and #2 represent two separate batches of cherry juice incubated with tannic acid. Cheery juice samples fermented with *Prise de mouse* yeast in the cold room (15°C). The final pH of the samples ranged from 3.82-4.70.**

study was carried out on a model system of purified commercial pectinesterase and pectin. Results obtained in this study indicate that fruit juice samples may contain compounds that interfere with the inhibitory effect that tannic acid has on pectinesterase, but further studies need to be conducted to ascertain this.

4.4.2 Effect of Polyacrylic Acid

The role of carboxylates in the inhibition process was investigated using polyacrylic acid. One percent w/v solutions of the sodium salt of polyacrylic acid were mixed with pectinesterase and pectin, and incubated for 5 minutes. Comparison was made between methanol concentrations of samples with and without the inhibitor. Figure 4.11 shows the effect of polyacrylic acid on the reaction. It is worth noting that in all cases some decrease in methanol generated after incubation was observed. The best inhibition, 45%, was obtained using 4 mg/mL pectin as the substrate. This is a significant change in pectinesterase activity and is very useful in applications where the objective is to minimize and not eliminate methanol. In the fruit brandy industry, methanol contributes to good flavor qualities but the amount needs to be regulated for health reasons. Therefore, the kind of inhibition obtained with polyacrylic acid is ideal for this kind of application.

Polyacrylic acid was selected as a potential inhibitor due to its carboxylate nature. As discussed previously, pectinesterase has an overall positive charge at pH 7.0, the pH at which all the inhibition studies were conducted. Use of Polycarboxylates therefore allows pectinesterase to complex with the inhibitor and ultimately reduces the activity of the enzyme.

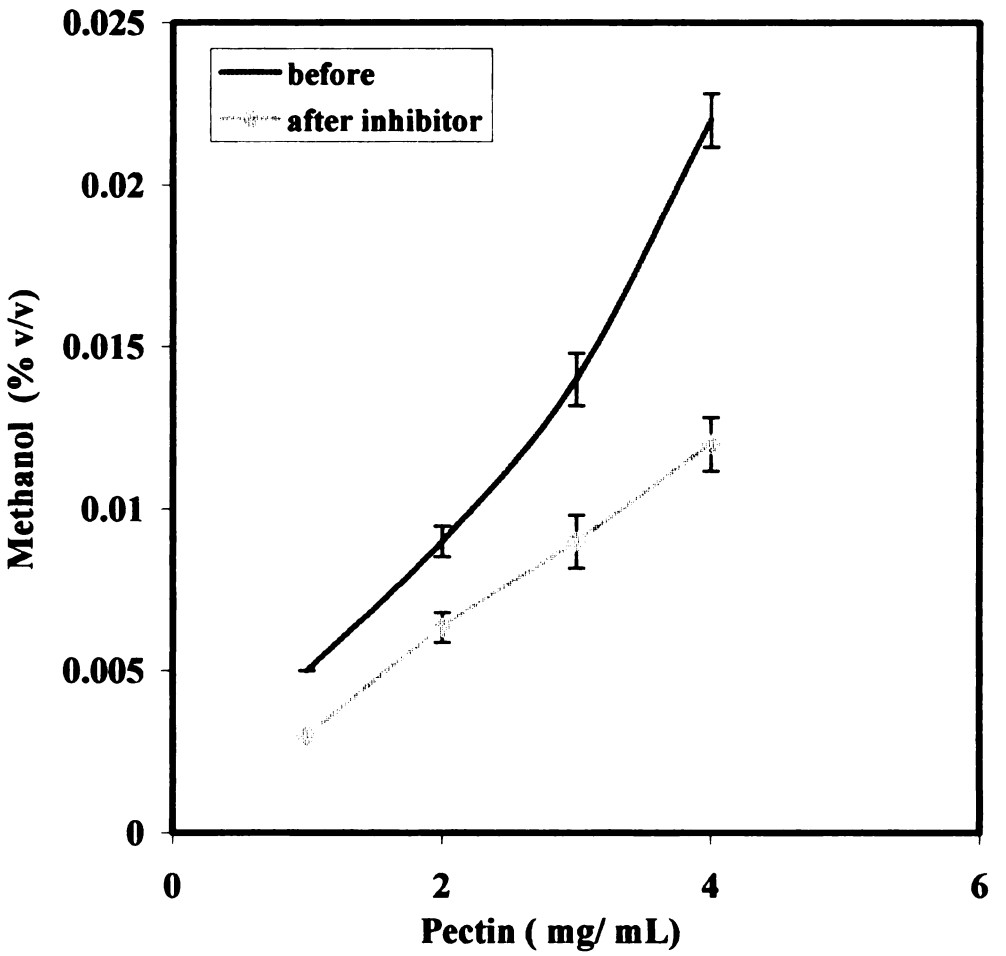


Figure 4.11 **Effect of polyacrylic acid on the activity of pectinesterase. Pectinesterase concentration: 0.432 mg/mL, prepared in phosphate buffer pH 7.0. One half μL of each sample injected into the GC after incubation at room temperature for 5 minutes. Inhibition range: 30-45%.**

4.4.2 Effect of Polymaleimide

Based on the results obtained with polyacrylic acid, it was proposed to conduct further studies using polymaleimide, which has similar properties but also possesses the advantage of being biodegradable.

The effect of polymaleimide on the activity of pectinesterase was studied in order to ascertain its inhibiting capability. Preliminary experiments were carried out with 1% w/v solutions of the sodium salt of polymaleimide. Solutions of citrus pectin, pectinesterase and polymaleimide were incubated for a period of 5 minutes. Results for this experiment are shown in Figure 4.12. The average inhibition was 35%, close to that obtained with polyacrylic acid.

It was then decided to reduce the amount of polymaleimide used in the inhibition in order to establish the minimum amount that would inhibit pectinesterase. The same incubation reaction was carried out under lower concentrations of polymaleimide. The results (figure 4.13) indicate that a concentration as low as 0.25% w/v of polymaleimide is capable of inhibiting PE. This is very promising, since lower concentrations of the inhibitor would greatly enhance its applicability.

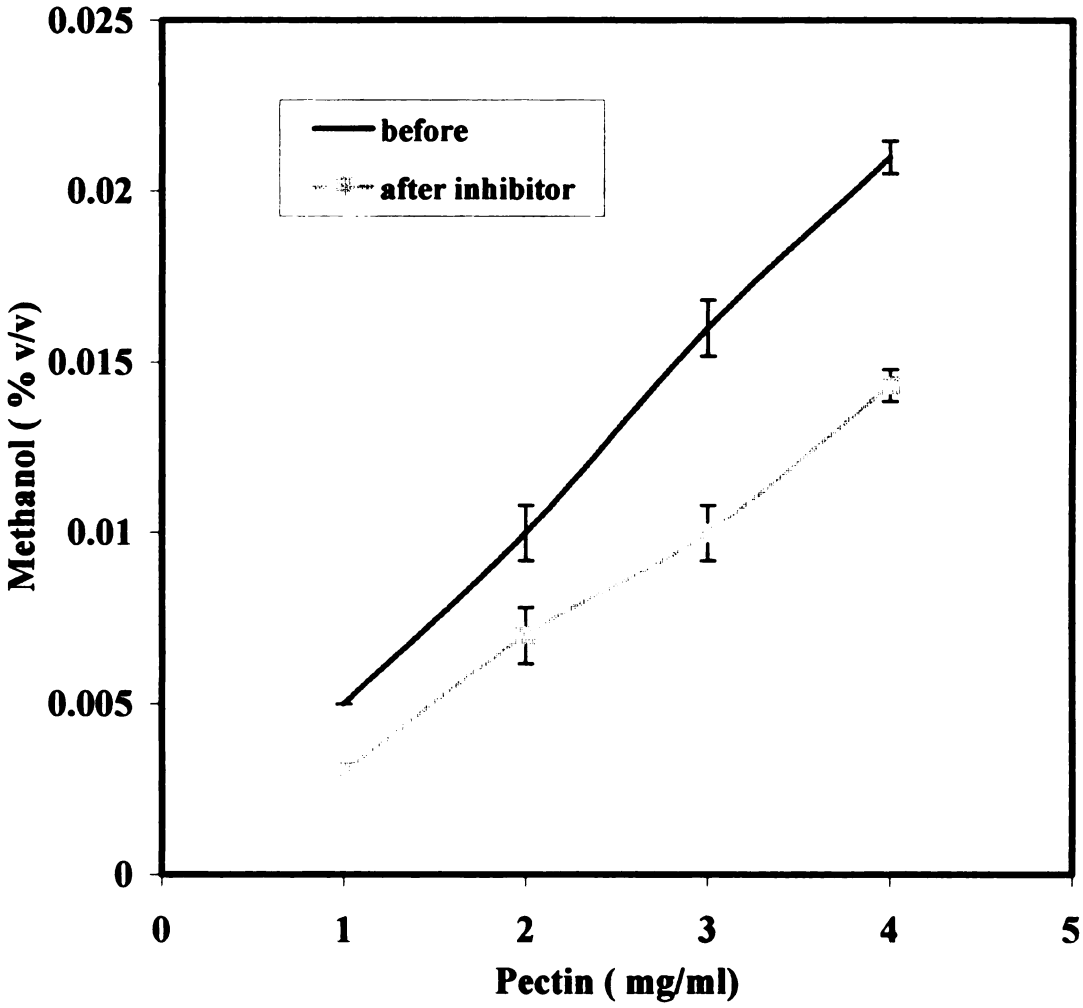


Figure 4.12 **Effect of polymaleimide on the activity of pectinesterase using 1% w/v sodium salt of polymaleimide. Pectinesterase concentration: 0.432 mg/mL, prepared in phosphate buffer pH 7.0. One half μL of each sample injected into the GC after incubation at room temperature for 5 minutes. Inhibition range: 30-40%.**

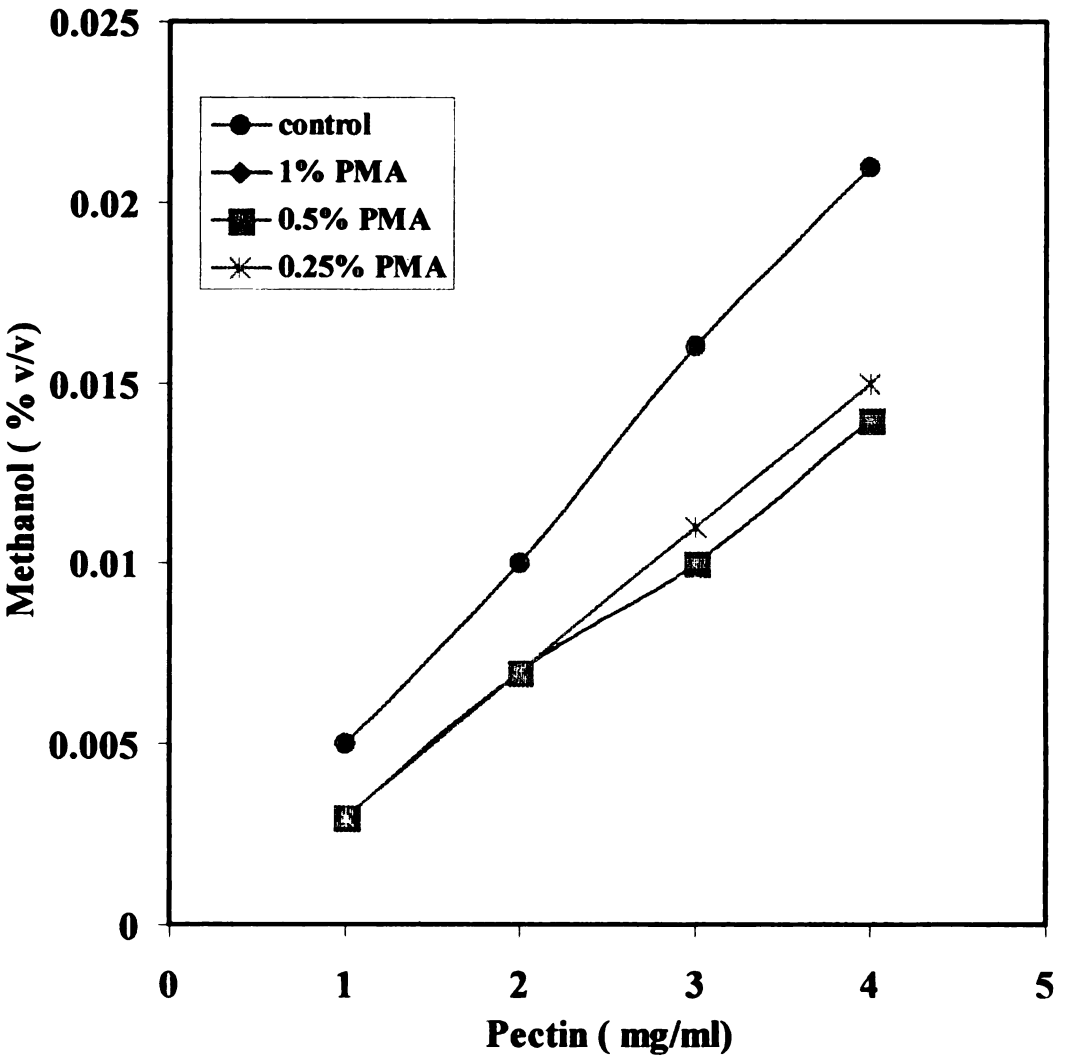


Figure 4.13 **Effect of Polymaleimide on the activity of pectinesterase. Concentrations of polymaleimide: 0.25, 0.5, 1% w/v. Pectinesterase concentration: 0.432 mg/mL, prepared in phosphate buffer pH 7.0. One half μL of each sample injected into the GC after incubation at room temperature for 5 minutes.**

4.4.3 Effect of EDTA

Considering the performance of polyacrylate and polymaleimide, it was decided to test the inhibiting properties of a known chelating agent. Ethylenediamminetetraacetic acid (EDTA) was selected for this study. EDTA has six electron donating sites that make it a very good ligand. One percent w/v solutions of the sodium salt of EDTA were incubated with pectinesterase and pectin for a period of 5 minutes. Control samples for each incubated one were also done in order to determine the extent of inhibition.

The results are shown in Figure 4.14. The highest inhibition was observed for 4 mg/mL pectin substrate. The overall effect of EDTA on the inhibition process is very similar to that observed with both polyacrylic acid and polymaleimide. This suggests that chelating effects of the inhibitors used can explain a reasonable amount of the inhibition process.

4.4.4 Effect of Citric Acid

Citric acid has carboxylate groups that have been shown to inhibit pectinesterase in the tests performed above. It also has a low pH that can be used to reduce the overall pH environment of the enzyme. Different volumes of citric acid were added to the incubation mixture and its effect monitored. The results (Figure 4.15) show that 0.5 mL of the 1% w/v citric acid solution reduced the activity of pectinesterase to an extremely low level.

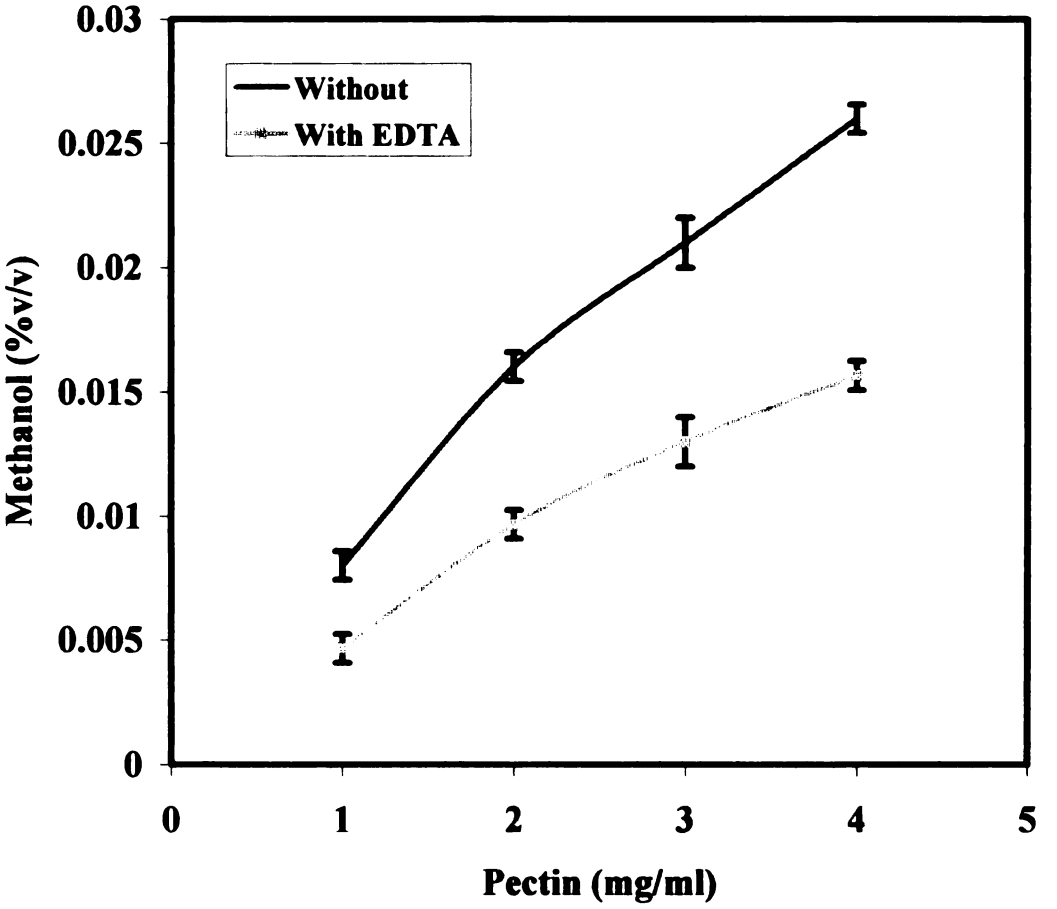


Figure 4.14 **Effect of EDTA on the activity of pectinesterase. EDTA concentration: 1% w/v. Pectinesterase concentration: 0.432 mg/mL, prepared in phosphate buffer pH 7.0. One half μl of each sample injected into the GC after incubation at room temperature for 5 minutes. Inhibition range: 38-41%.**

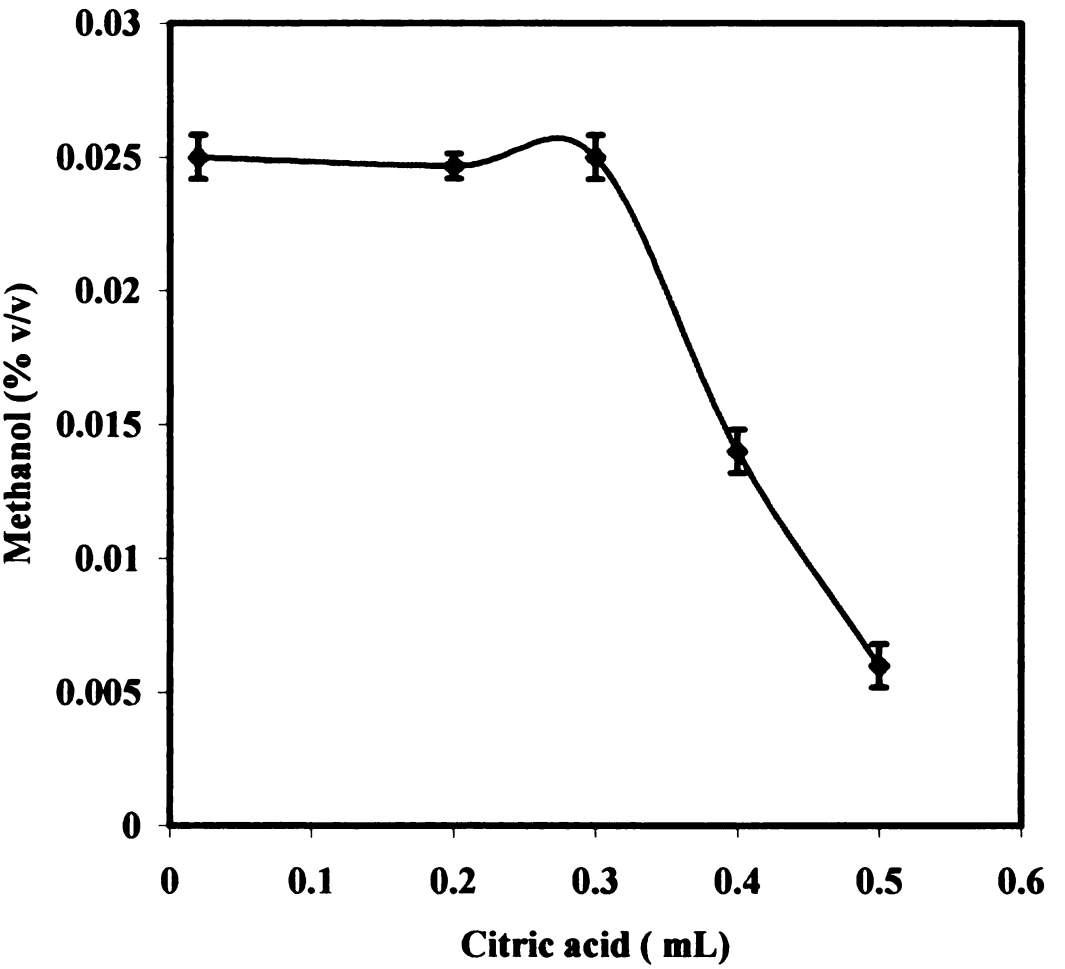


Figure 4.15 **Effect of Citric Acid on the activity of pectinesterase. Citric acid: 1% w/v, pectin concentration: 4 mg/mL. Pectinesterase concentration: 0.432 mg/mL, prepared in phosphate buffer pH 7.0. Resulting pH of samples: 3.12-3.38**

5. SUMMARY AND CONCLUSIONS

The amount of methanol in fruit brandies routinely exceeds the 0.35 %v/v legal limit set by the FDA. This is particularly true for cherry brandy, which was found to have the highest amount of methanol on average, compared to the other fruit brandies. The highest concentration of methanol was recovered in the heads cut during the distillation for all the different varieties of fruit brandy.

Results obtained from monitoring the amount of methanol during fermentation showed no major increases in the methanol content of the fermented mash. This indicates that most of the methanol is produced well before the initialization of fermentation of the fruit.

Kinetics of the activity of pectinesterase were studied. Commercial purified pectinesterase was used at a concentration of 0.432 mg/mL. It was tested at different temperatures with various concentrations of pectin substrate. The inactivation rate constant at 70°C was found to be 2.8×10^{-3} s^{-1}. The activation energy for the inactivation reaction at pH 7.0 was calculated from the Arrhenius plot to be 5697 cal mol^{-1}.

Inactivation of pectinesterase enzyme in cherry juice was also studied. The enzyme lost more than 50% of its activity at 55°C. The regeneration capability of the enzyme at room temperature was studied as well. Further tests upon cooling of the enzyme showed that the thermal inactivation was irreversible. Inactivation by lowering of pH to 2.0 was successful in eliminating the activity of pectinesterase, but the yeast required for fermentation was also destroyed, therefore this approach is not very useful.

Inhibition of pectinesterase by various compounds was studied as well. Very promising results were obtained with the potential inhibitors tested. The inhibition was tested using commercial purified pectinesterase and apple pectin. All the potential inhibitors tested showed a capability to slow down the activity of pectinesterase.

As indicated by inhibition studies conducted with polymaleimide, very low concentrations of the inhibitor are needed to reduce the activity of pectinesterase. This is a positive attribute for fruit brandy application, since using small amounts of inhibitor implies that the aroma and taste of fruit brandy will not be significantly affected.

All the inhibitors tested were anionic in nature. The positive results obtained for the inhibition tests suggests that there is some enzyme-inhibitor complexation taking place. The pH at which all the inhibitions were conducted was favorable for this kind of complexation.

The best inhibition ability was observed with citrate, which is a well-known chelating agent. This might explain the affinity of the inhibitor to pectinesterase but further tests need to be conducted to ascertain this.

6. RECOMMENDATIONS FOR FUTURE WORK

It has been established in this work that methanol in fruit brandy can exceed the legal limits. Various fruits were used to study fermentation and distillation procedures. To understand the general trend in methanol content among fruit brandies, differences in fermentation as well as distillation need to be tested for any influence on the overall methanol content in the distillates. A difference in the set up of the distillation trays on the still will cause a change in the separation process, and this will either pre-concentrate or dilute the amount of methanol in a particular stage of distillation.

The kinetic studies of pectinesterase were conducted using commercial pectin and pectinesterase. This is a model system, since in practice pectin co-exists with pectinesterase. The kinetic studies need to be conducted under this condition so as to get a clear understanding of the mode of action of fruit pectinesterase. However, since pectinesterase activity increases dramatically during the post harvest period when the fruit tissue is damaged, the kinetic studies need to be done immediately before, and during this period. This will also allow the kinetic studies to be conducted at the native pH of the fruit pectin. The effect of all the other dissolved sugars in the fruit such as sucrose and glucose, which have been speculated to aid in the inhibition of pectinesterase activity, should also be ascertained.

Testing of potential inhibitors produced very promising results. Most of the inhibitors were tested at a concentration of 1% w/v. Further reduction in concentration for polymaleimide inhibitor showed that concentrations as low as 0.25% w/v were able to inhibit pectinesterase by the same magnitude as the 1% w/v concentration. Lowering of

the concentration of the other inhibitors needs to be done in order to determine the optimum concentration for each. The inhibition studies also need to be transferred to fruit samples as opposed to the commercial pectin and pectinesterase already used in this work. Kinetic studies of the inhibition process also need to be conducted to obtain the inhibition rate constants and understand the rate of inhibition for each inhibitor.

The formation of a pectinesterase-inhibitor complex was suggested as the mechanism by which the inhibition was taking place. However, a deep understanding into the mechanism of inhibition was not obtained. Use of various spectroscopic techniques will aid in getting deeper insight into the mechanism of inhibition. Infrared Spectroscopy can be used to monitor the break up of the methyl ester bond in pectin that generates methanol. Since there is a strong band for ester carbonyl at 1760 – 1745 cm^{-1} and COO^- band at 1640 – 1620 cm^{-1}, these can be used to monitor the deesterification process. As methanol is generated there should be a decrease in the carbonyl ester as the COO^- band increases. These data can give supporting evidence of the inhibiting effect of the inhibitors, and may be, show a difference in the extent of inhibition for each. The IR studies may be conducted for the fruit samples too, to determine the effect of the fruit juice matrix on the deesterification process. Proton Nuclear Magnetic Spectroscopy can also be used to monitor the inhibition process. Structural information obtained from this technique can be used to gain insight into the chemical changes taking place during the inhibition process. Comparison of the proton shifts of the pure inhibitor and those of the inhibitor-pectinesterase mixture should be able to give an indication of the chemical sites involved in the inhibition process.

As discussed previously, the ultimate aim of this work is to reduce the methanol content of fruit bandy. All the inhibitors tested in this study were used with commercial pectinesterase and pectin. It is therefore necessary to test these inhibitors directly in fruit juice samples. The fruit samples will contain other compounds that may or may not aid in the inhibition of pectinesterase.

Ultimately, the inhibited samples need to undergo the fermentation process as well as distillation. The methanol content of these samples will then be compared to the uninhibited samples to ascertain the applicability of the inhibitors in the fruit brandy industry.

7. APPENDIX

GAS CHROMATOGRAMS FOR VARIOUS SAMPLES

The gas chromatogram of various samples with different magnification to show the chromatographic peak of interest. The chromatograms are for distillates, fermented mash and the pectinesterase-pectin model system. Comparison of the inhibited and uninhibited sample chromatogram is also shown.

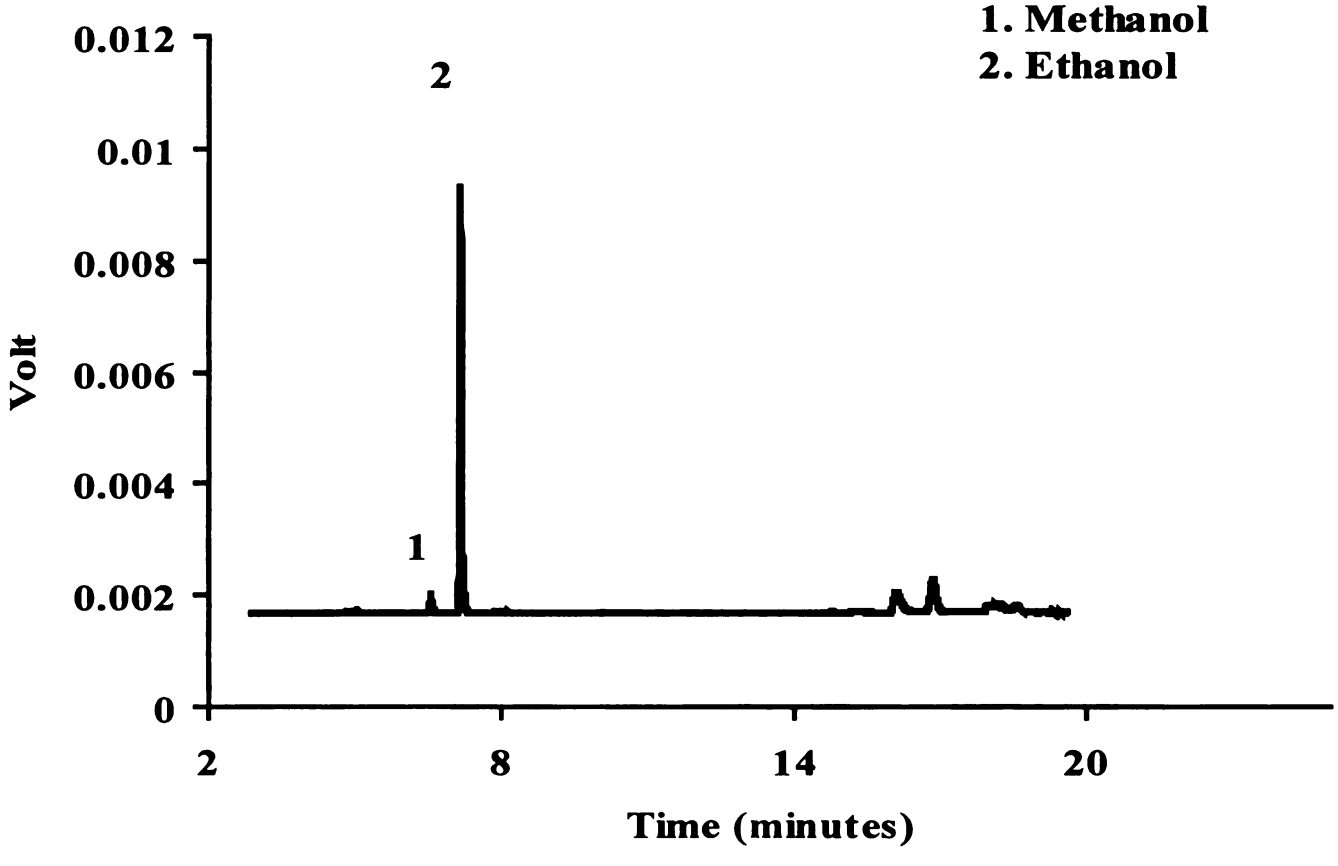


Figure A-1. Gas chromatogram of fermented fruit mash samples. The GC run conditions were as follows: Oven temp: 40°C (hold for 1 minute); to 190°C at 2.5°C /min (hold for 5 minutes), Injector temp: 240°C, Detector temp: 255°C, Carrier gas: Helium at 30 cm/sec, Injected volume: 0.5 μL.

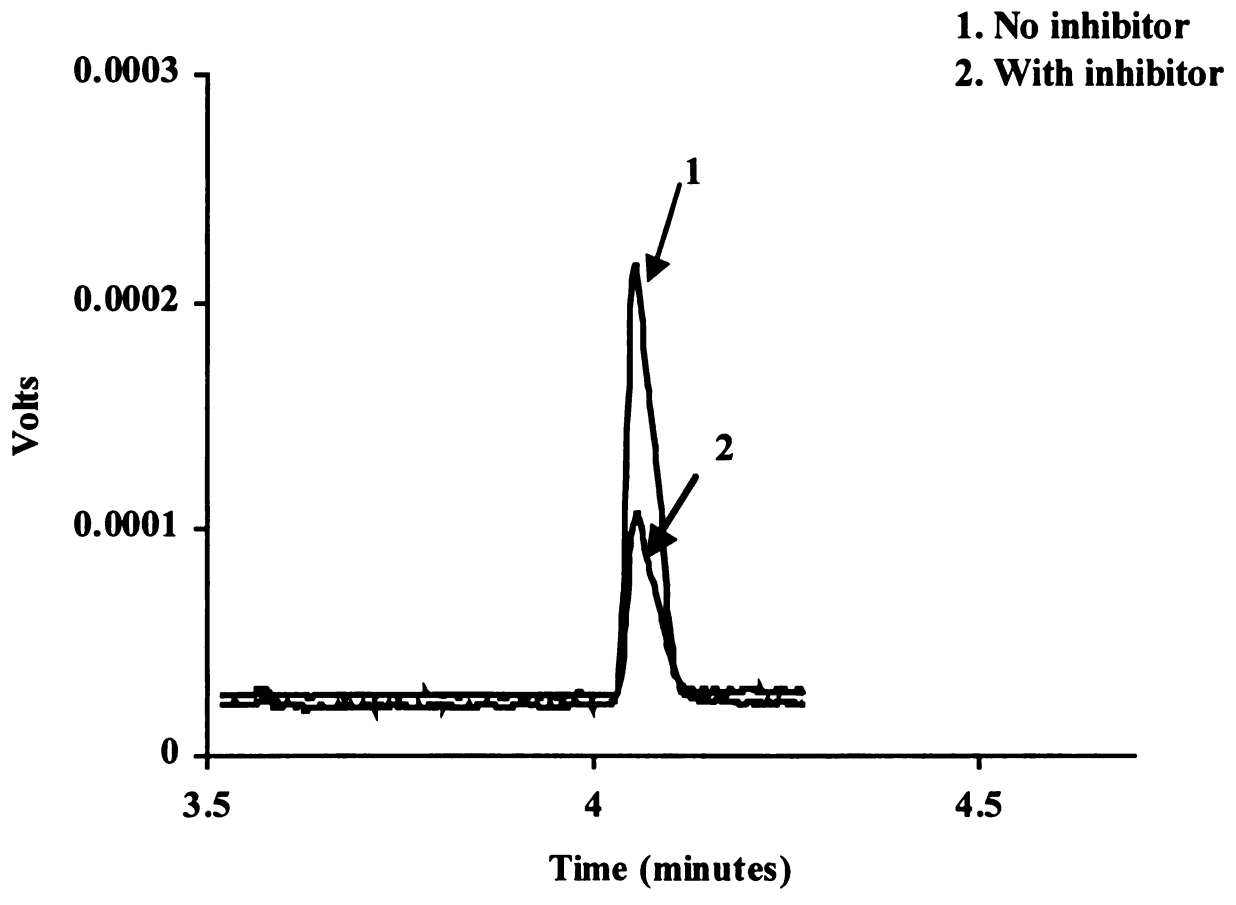


Figure A-2. **Gas Chromatogram for pectin-pectinesterase sample with 1% w/v EDTA inhibitor and without any inhibitor. Samples were incubated for 5 minutes before analysis. The GC temperature programming was as follows: Oven temp: 40°C to 100°C at 3°C/min (hold for 0.5 min) to 190°C at 20°C /min.**

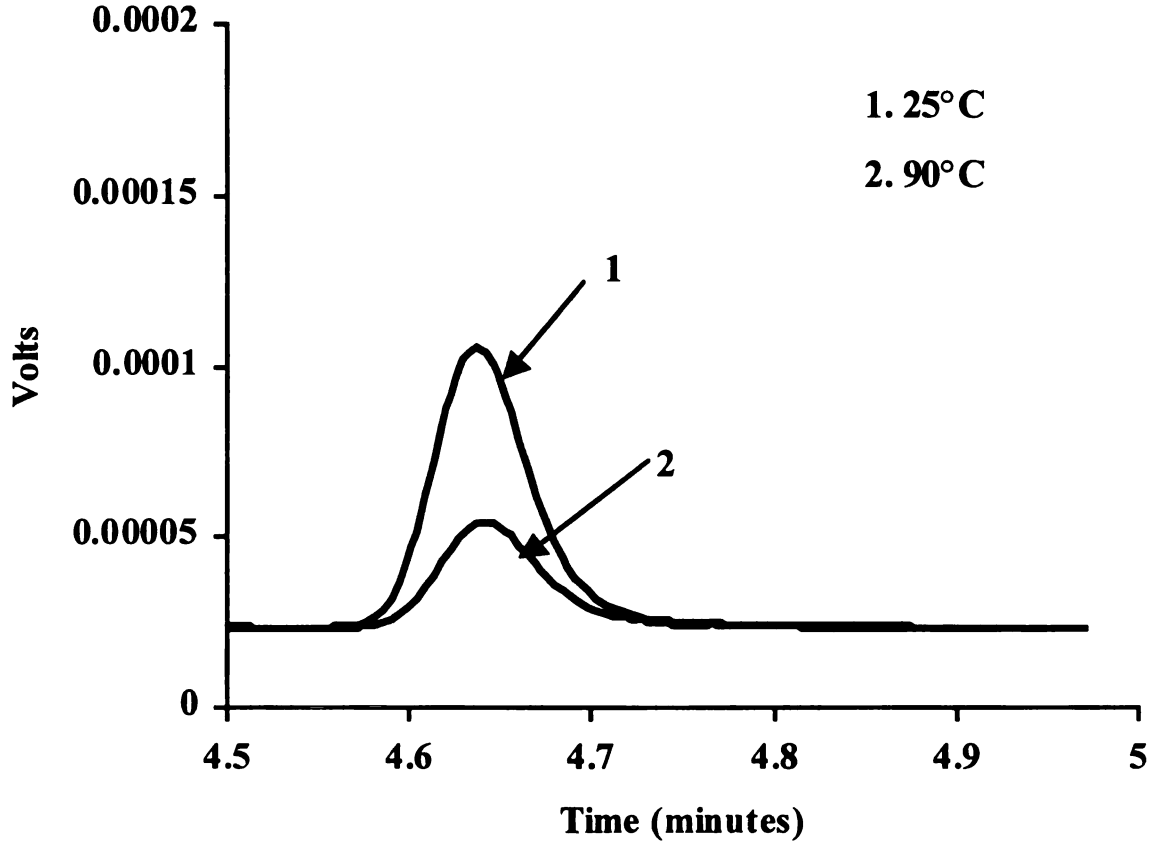


Figure A-3. **Gas chromatogram of thermally inactivated sample (90°C) and sample at room temperature (25°C). Samples were incubated for 5 minutes before analysis. The GC temperature programming was as follows: Oven temp: 40°C to 100°C at 3°C/min (hold for 0.5 min) to 190°C at 20°C /min.**

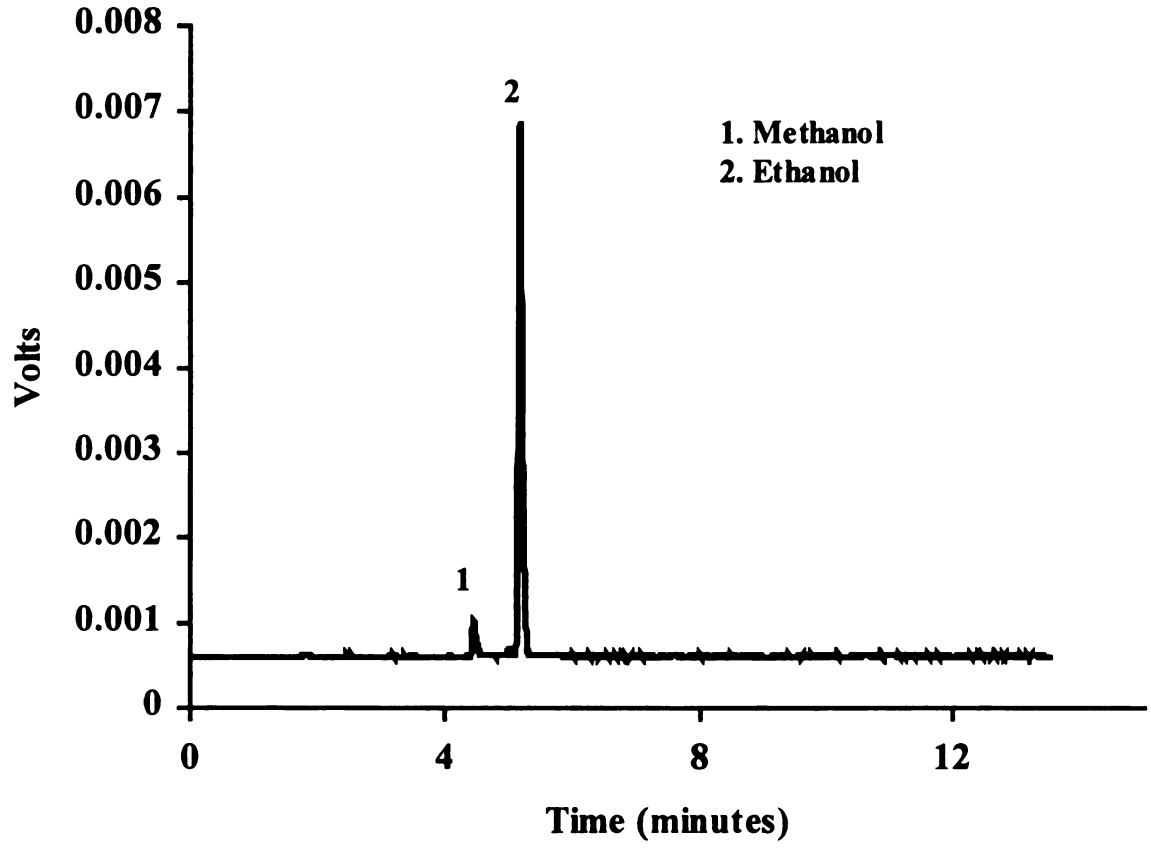


Figure A-4. **GC Chromatogram of Pectin-pectinesterase sample. The GC temperature programming was as follows: Oven temp: 40°C to 100°C at 3°C/min (hold for 0.5 min) to 190°C at 20°C /min. Injector temp: 240°C, Detector temp: 255°C, Carrier gas: Helium at 30 cm/sec, Injected volume: 0.5 μL.**

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