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THE EFFECT OF TEMPERATURE AND THE USE OF LIQUEFACTION ENZYMES ON THE CONGENER CONCENTRATIONIN IN DISTILLED FRUIT BRANDIES

By

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ABSTRACT

THE EFFECT OF TEMPERATURE AND THE USE OF LIQUEFACTION ENZYMES ON THE CONGENER CONCENTRATION IN DISTILLED FRUIT BRANDIES

By

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The first goal of this research was to examine the effects brought about by changing the temperature at which yeast fermentations for the production of fruit brandy are conducted. The distillates from fruit fermented at 10, 15, and 20 °C were analyzed by gas chromatography and the congeners of interest were analyzed. These congeners included fusel alcohols, methanol, carbonyls, and esters. Temperatures of 15 and 20 °C are found to be optimal for brandy yeast fermentations. Concentrations of congeners studied were consistent with those found in the literature. The second goal was to use liquefaction enzymes to maximize the ethanol yield. Fermentations that were performed showed that ethanol production was not affected by the use of these enzymes, but the methanol concentration increased dramatically above the legal limit. This is crucial because the limit of the methanol in alcoholic beverages is strictly regulated for health reasons. Therefore, the use of liquefaction enzymes in the treatment of mashes must be approached with caution.

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Introduction

1.1 Michigan Fruit Brandy Industry

Brandy is the product obtained by the distillation of wine or fermented juice of any fruit.[1] There exists many production methods that differ from country to country. For instance, continuous stills are frequently used in the production of California brandy, but the brandies of French Cognac and Armanac are distilled exclusively by batch pot stills without rectification. In Michigan, the production of fruit brandy has been slow though the need for such an outlet for fruit existed. The consumption of fruit in the state has remained the same despite the increase in production in certain fruit such as apples, cherries and plums. This excess fruit production has prompted the farmers and wine distilleries' owners to request the state to enact a new law regarding brandy production. A major change in Michigan's legislation in 1996 enabled Michigan wineries to sell distilled products out of the tasting rooms directly to customers and an annual license of $100. This change has had a dramatic impact on the industry and distillation became economically feasible. The number of distilleries involved in the distilling of fruit spirits increased from zero in 1996 to seven in 2000.

As a new emerging product, the brandy produced in Michigan has to be of good quality in order for the wineries to be able to compete with European brandy products and be profitable. Hence, extensive research work has to be done on many aspects of the fermentation and distillation of fruits. In this work, we have two objectives: the first is running fermentations at different temperatures (10, 15 and 20 °C) with different fruits to look for differences in congener concentrations and ethanol yield; the second involves the use of liquefaction enzymes as fermentation aides and study their effects on the end

products mainly ethanol and methanol. Congeners are compounds such as fusel alcohols, aldehydes, ketones, and carrbonyls that contribute to the flavor of an alcoholic beverage.

1.2 The Distillation Process

1.2.1 Mashing of the Fruit

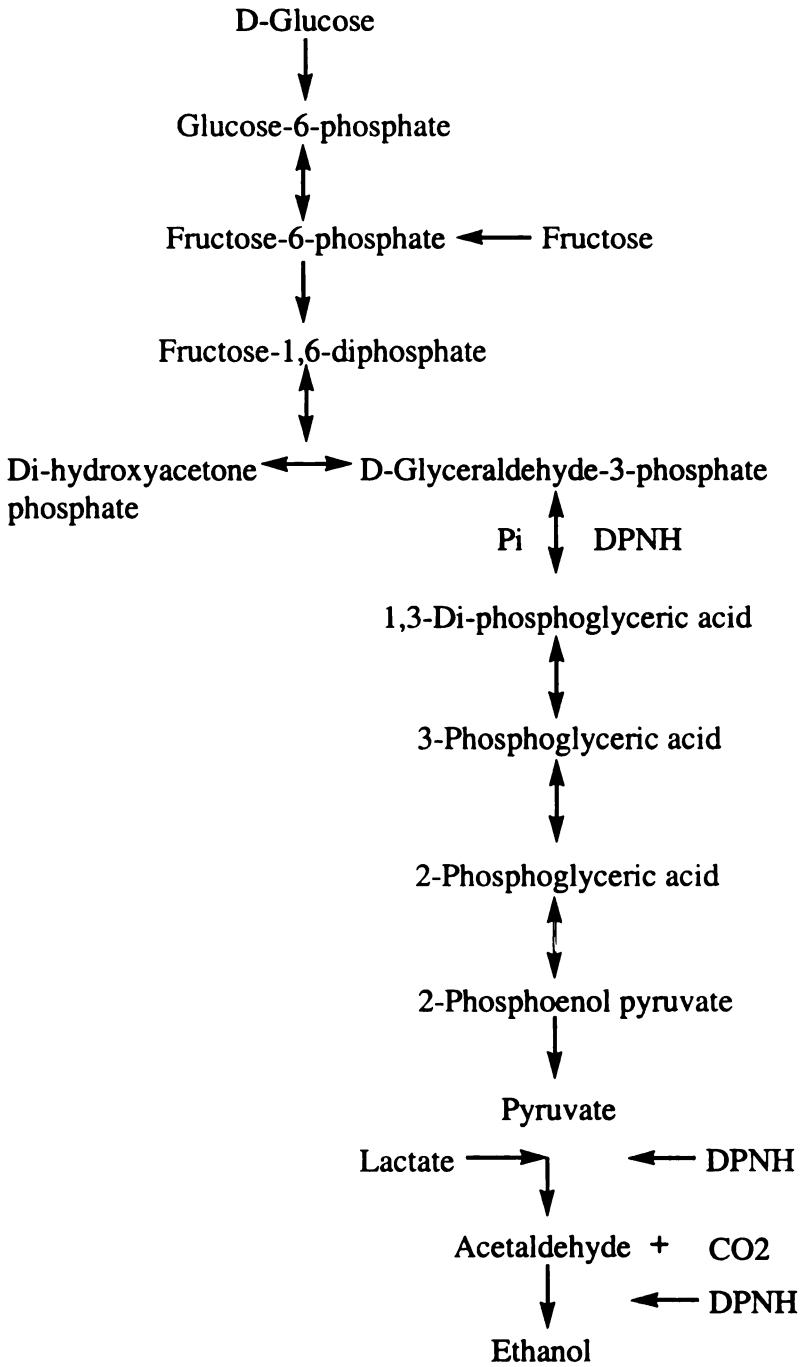
The first step in production of brandy requires mashing fresh fruit. The fruit has to be washed and damaged fruit must be discarded. A general principle is that the fermentation proceeds better and is more complete depending on the maceration of the raw fruit material.[2] This is done in several ways depending on the fruit that is being mashed. Squeezing by hand, using a wooden pestle or rolling mills are mainly used when crushing stone or berry fruits. Ratz mills are often used for the crushing of seed fruit.[2]

1.2.2 Fermentation of the Mash

After mashing the fruits, the fermentation is initiated by adding a known amount of yeast, which is in most, cases a strain of *saccharomyces cerevisiae*. Fruit fermentations usually start aerobically as there is air in the fermentation vessel, and once that air is exhausted yeast switches to anaerobic metabolism, which produces the desired product, ethanol. The pathway by which yeast metabolizes common sugars, such as glucose or fructose, under anaerobic conditions is referred to as the Embden-Myerhof-Parnas scheme. By means of this set of reactions involving phosphorylations and dephosphorylations, oxidations and reductions, as well as isomerizations, yeast produces as its principal end products ethanol and carbon dioxide.[3] This can be seen in Figure 1.1. The main fermentation equation is:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

Figure1.1. The Embden-Meyerhof-Parnas metabolic scheme showing the intermediates in the production of ethanol from glucose.[3]



The beginning of the fermentation can easily be identified by the bubbling in the fermentation top caused by the production of CO_2 and a significant increase in temperature can be noticed. The optimal fermentation temperature for fruit brandy is between 15 and 20 °C.[2] This temperature has to be kept under control throughout the course of the fermentation. Also, the pH of the mash should be kept between the range of 2.8 and 5.2.[2] Different acids or bases can be added to the mash to keep the pH in this range, and some of these chemicals can be useful as nutrients for the yeast especially nitrogen and phosphorous containing compounds. Outside this pH range, yeast will not be able to ferment the sugars properly.

Refractometry is an optical measurement method used to measure the amount of sugars still present in the fermentation medium based on the refractive index and this is usually about 2-3g sugar/L. High performance liquid chromatography (HPLC) can be used to monitor the amount of sugars and ethanol produced during the fermentation using either an UV-VIS or a refractive index detector (RI). Fermentations usually last for a period of two weeks but no significant changes occur if the fermented mash is stored airtight for longer periods of time.

1.2.3 Distillation of the Fermentation Mash

Distillation is the technique used to separate and select by heat volatile components from a liquid mixture.[4] The distillation of fruit brandy is more complex than a simple binary distillation between ethanol and water. For example, wine contains approximately 300 volatile compounds. Each volatile component will distill according to these criteria: boiling point, equilibrium relationship to water or alcohol, and the variation of alcohol content in the vapor during the distillation.

Different opinions exist regarding the ideal form of the distillation stills and the apparatus itself, but copper is the material used for the manufacture of the still. Copper is a very good heat conductor, shows optimal resistance to fruit acids, and affects the quality of brandy positively by catalytic chemistry on the surface.

The heating source can be a water or oil bath, steam, or electrical power. The time that it is required to distill the mash depends on the amount of the mash, the rate of heating, and the intensity of heating. Distillation proceeds as follows, the still is filled with mash up to 65-75 % v/v and antifoaming agents are added.[2] As the still is heated, the alcohol in the mash vaporize and proceeds into the trays. Depending on the volatility of the components vaporized, some will condense back to the still and the remainder will proceed to the spirit tube. This tube is connected to the condenser, which completely condenses the volatile components. The distillate is collected in three fractions: the heads, hearts, and the tails. The hearts are kept and then diluted to produce 40 % alcohol. The heads and tails are discarded or sometimes they are re-distilled. The hearts are then diluted with pure water to produce 40 % alcohol product that is ready for consumption. Figure 1.2 shows the distillation apparatus that is manufactured by Christian Carl in Germany and is in use our lab for large distillations (150L). Usually for a 150L batch, the first two liters of the distillate are the heads, the following eight or nine liters are the hearts, and the last couple liters are the bottoms.

1.3 Temperature Effects on the Fermentation Products

1.3.1 Types of Yeasts

Temperature is undoubtedly one of the most important environmental parameters

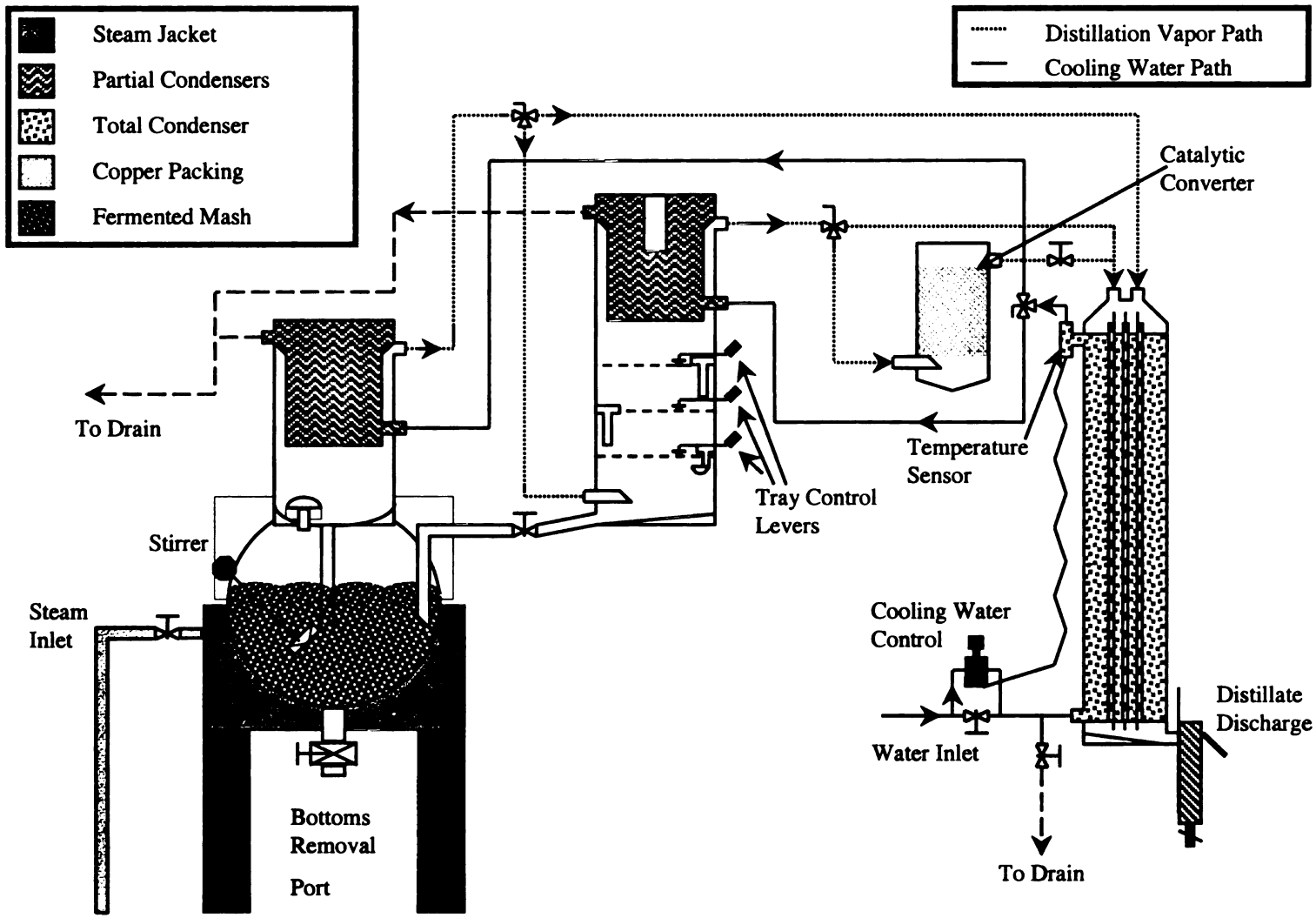


Figure 1.2. The distillation set-up used in our lab for large distillations (150L).[5]

influencing all activities of microorganisms. It is standard practice to study yeast metabolism at temperatures around 25 – 30 °C, even though these temperatures may not be strictly relevant when considering the natural habitats of many species of yeasts.[6] Yeast may be classified on the basis of their temperature limit for growth. The terms "psycrophile", "mesophile", and "thermophile" have been used to classify microorganisms into different thermal domains. A yeast that can grow in the temperature range between 5 and 10 °C would be classified as psychrophilic yeast. Thermophilic yeasts are ones that are capable of growth at temperatures at or above 50 °C. Certain yeasts that can grow between 20 and 46 °C are also termed thermophilic. Mesophilic yeasts are the most abundant and have temperature range for growth at or below 0 °C and up to 48 °C. *Pris de Mousse,* which is mostly used in brandy fermentations, is a *Sacch. Cerevisiae* yeast and it is considered mesophilic.

Very few reports exist on temperature induced changes in psychrophilic and thermophilic yeasts. It is suggested that changes in plasma-membrane composition, especially the increase in polyunsaturated fatty-acyl residues at low temperature, is responsible for the effects on solute transport across the membrane.[7] Also, the rate of glucose uptake at different temperatures is related to the composition of the unsaturated fatty –acyls in all types of yeasts.[8] An important trend found by many authors is that yeasts adjust the membrane fatty-acyls composition with temperature and the lower the growth temperature the more unsaturated the membrane fatty –acyls composition.[6] This is crucial because it will enable cell membranes at lower temperatures to remain sufficiently fluid to allow proper functioning of metabolic processes. This temperature

adaptation by the yeast metabolic pathways will ultimately affect the composition and amounts of the end products of these pathways.

1.3.2 Temperature Change and Congener Formation

The fermentation is an exothermal process wherein an increase in temperature of the mash is observed in the beginning of the fermentation. The optimal fermentation temperature for fruit brandy is between 15 and 20 °C and for mashes that are difficult to ferment it is a little higher.[2] It is generally known that higher fermentation temperatures aid in making the fermentations proceed faster, but it can have an adverse effect in that this higher temperature can promote the growth of undesired yeasts or bacteria. Some of these microorganisms include film-forming yeast, wild yeasts, and molds. The growth of these microorganisms contributes negatively to the aroma of the final product because of the production of chemicals such as butanoic acid. The production of ethanol by yeast is associated with the production of a wide variety of fermentation products, which contribute to the final flavor of the beverage, either as organoleptic compounds, or precursors of organoleptic compounds which are produced in subsequent maturation or distillation processes. These organoleptic compounds are called congeners and in this work we will be using both terms when talking about them.

Research on the aroma of the alcoholic beverages has shown that the aroma composition of alcoholic beverages consists of several hundred distinct chemical compounds.[9] Table 1.1 shows the number and type of compounds present in alcoholic beverages. These compounds are present in most types of alcoholic beverages but in different concentrations. Organoleptic compounds produced by yeasts can be classified

into five categories: alcohols, esters, aldehydes and ketones, sulphur containing compounds, and organic acids.

Table 1.1. The number of aroma compounds present in alcoholic beverages.[9]

Class	# Observed	Class	# Observed
Alcohols	38	Hydrocarbons	41
Acids	80	Nitrogen compounds	11
Esters	118	Sulphur compounds	18
Carbonyl comounds	41	Lactones	11
Acetals	17	Sugars	4
Phenols	41	Unclassified compounds	11

Concentration of organoleptic compounds present are not, however, the only parameter to be considered in evaluating the flavor of a beverage, since different compounds have different sensory odor thresholds. Table1.2 shows the flavor thresholds of some organoleptic compounds. It is clear that although the higher alcohols are the most abundant group of organoleptic compounds, they have odor thresholds ten times higher than the esters and a thousand times higher than the carbonyl compound diacetyl. Hence, their contribution to the overall flavor of alcoholic beverages is not the most important on a per mass basis.

1.3.3 Formation of Fusel Alcohols

Fusel alcohols are alcohols with more than two carbons. The most important are 1-propanol, 2-methy-1-propanol, 1-butanol, 2-methyl-1-butanol, and 3-methyl-1-butanol. Most of these can be derived from the carbon skeletons of common amino acids. Formation of these fusel alcohols is thought to be independent of the raw materials used in the mash because the formation of these longer chain alcohols can occur in whiskeys, tequila, and gin. These fusel alcohols can be produced either from amino acids or from sugars. Figure 1.3 shows the steps involved in producing some of these alcohols. The

	Flavor Threshold	Range of Concentration
Alcohols	**(ppm)**	**in beer (ppm)**
ethanol	1400	———
1-propanol	800	7.5-13.8
2-propanol	1500	0.2-2.4
1-butanol	450	———
2-methylpropanol	200	8.6-56.6
2-butanol	16	———
2-methylbutanol (optically active amyl alcohol)	65	7.0-23
3-methylbutanol (iso-amyl alcohol)	70	27-122
2-phenethanol	125	5.0-27
Acids		
acetic	175	150-280
propionic	150	5
butyric	2.2	0.6-3.3
iso-butyric	30	0.7-3.3
caproic	8	2.2-5.8
caprylic	13	3.3-8.2
capric	10	0.1-2.0
phenylacetic	2.5	0.93
lactic (D+L)	400	28-400
Esters		
ethyl acetate	33	8.2-47.6
n-butyl acetate	7.5	0.23
iso-butyl acetate	1.6	0.03-0.25
phenylethyl acetate	3.8	0.1-1.17
ethyl butyrate	0.4	0.09
ethyl caproate	0.23	———
ethyl caprylate	0.9	0.08-0.01
ethyl caprate	1.5	———
ethyl lactate	250	0.1
Aldehydes and Ketones		
acetaldehyde	25	2.5-24.4
iso-butyraldehyde	1	0-0.024
acetone	200	1
pyruvate	300	10-220
diacetyl	0.15	0.5-2.0
Sulphur compounds		
dimethyl sulphide	50	0-0.144

Table 1.2. Flavor thresholds of selected organoleptic compounds. The range of concentration found in beer is included for references purposes.[10]

effect of the temperature is not always consistent, but several reports of research indicate that the maximum production of higher alcohols occurs at intermediate temperatures of about 24 °C. A study done on the fermentation of grape juice shows that different alcohols are affected differently when temperature is changed between 10 and 32.2 °C.[11]

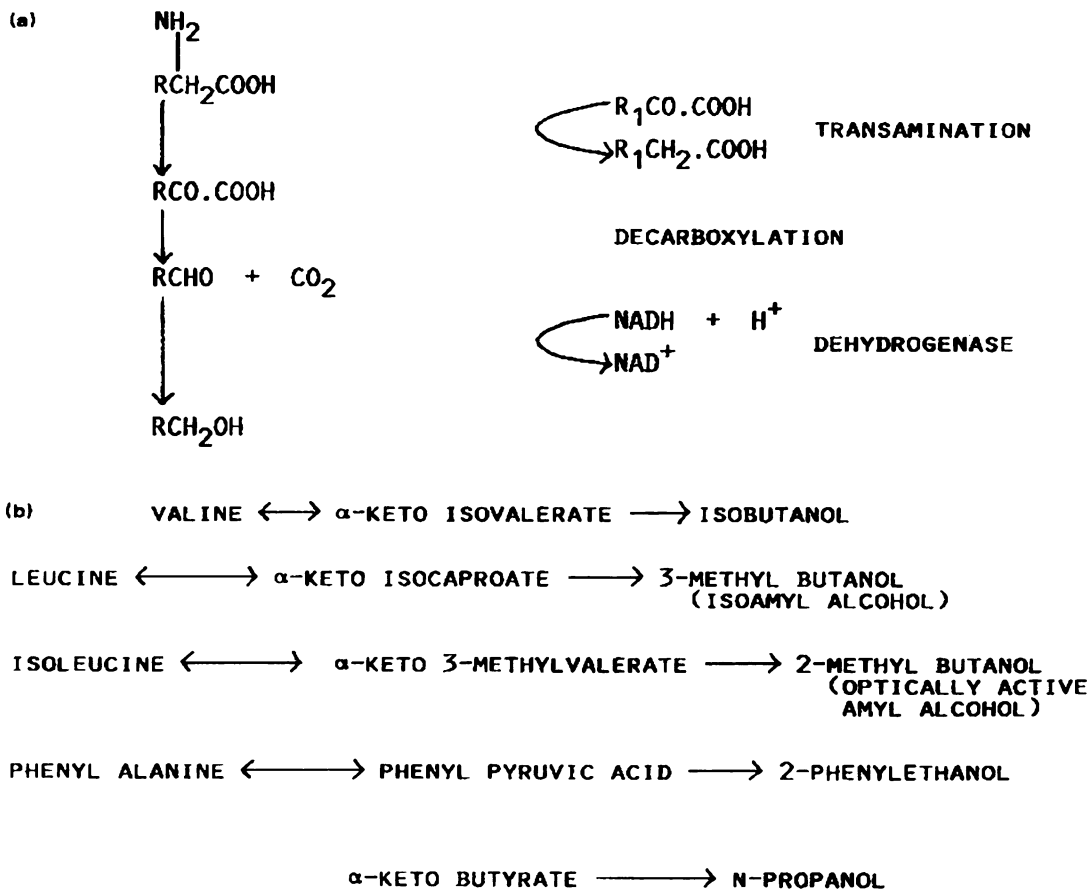


Figure 1.3. Formation of higher alcohols from amino acids and the corresponding keto acids. (a) Biochemical sequence: (b) relationship between selected amino acids and the corresponding higher alcohols.[10]

1.3.4 Formation of Esters

Esters are numerically the largest group of organoleptic compounds found in

alcoholic beverages.[10] Most common esters are produced by the yeast during the fermentation stage in addition to the esters formed during distillation and storage. Figure 1.4 shows the formation of esters and medium-chain length fatty acids by yeasts. Since ethanol is the most abundant alcohol, the ethyl esters are the most abundant followed by isoamyl and propyl esters. Acetate is the most abundant acid formed by yeast during fermentation, so acetate esters of ethanol and higher alcohols are the most abundant.

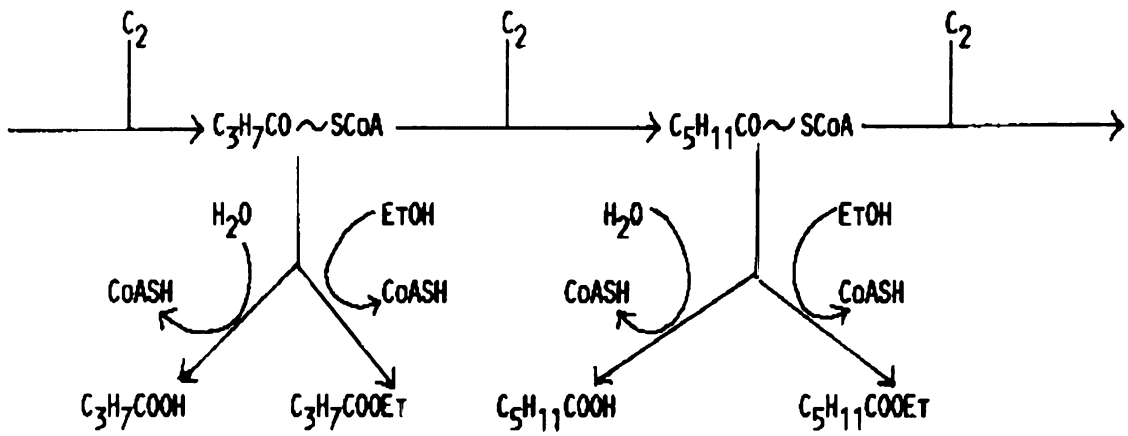


Figure 1.4. Formation of esters and medium-chain length fatty acids by yeasts.[10]

Temperature doesn't have an effect on the ester formation, but as mentioned earlier, it will affect the level of unsaturated fatty acids in the membrane and the fermentation medium and this affects the amount of esters formed. Esters also formed during aging where higher alcohols and ethanol are converted to the corresponding esters.

1.3.5 Formation of Carbonyl Compounds

The carbonyl compounds consist of aliphatic and aromatic aldehydes and ketones. Aldehydes are synthesized by yeasts as intermediates in the formation of alcohols through the decarboxylation of keto acids. Acetaldehyde is quantitatively the most

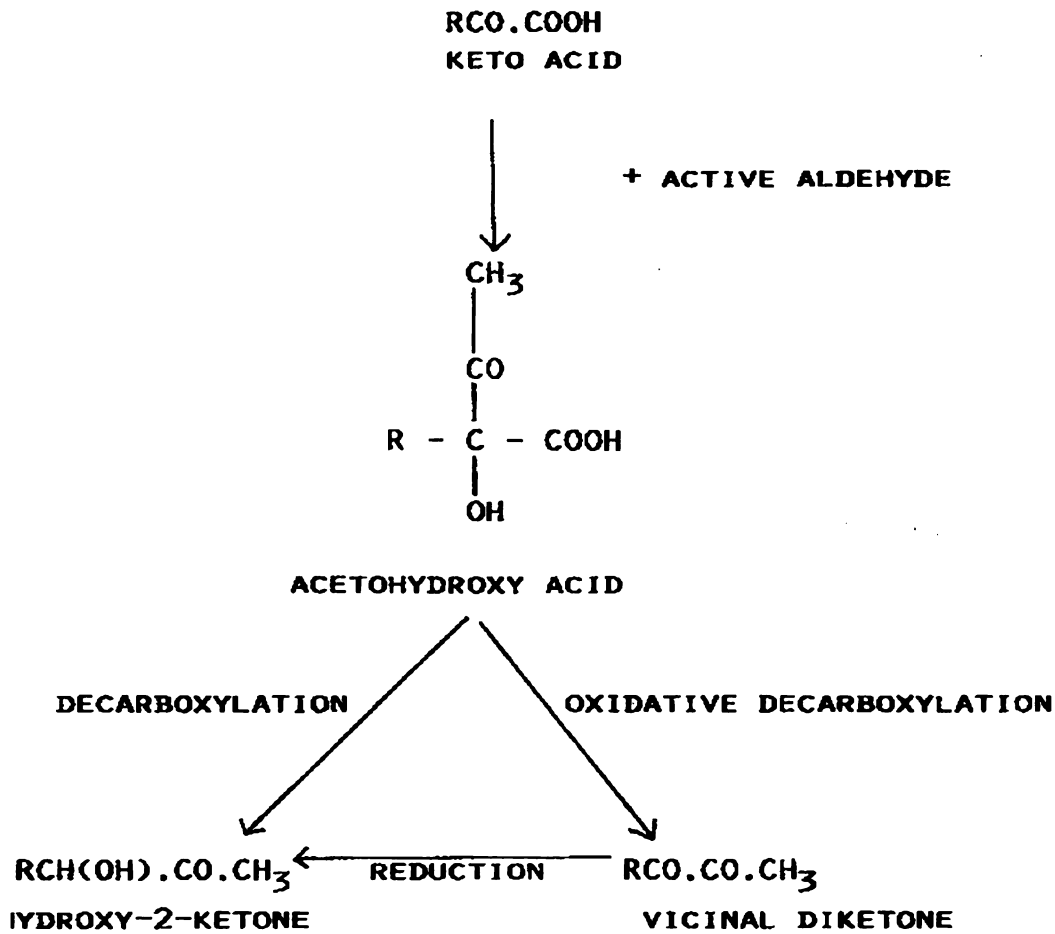


Figure 1.5. The formation of vicinal diketones and 3-hydroxy-2-ketones.[10]

compound in this group. Aldehydes are intermediates in the biosynthesis of alcohols; most of the parameters that influence the production and amount of the latter also influence the aldehyde formatio. Ketones are also intermediates in the biopathway for higher alcohol formation. They are also involved in the formation of vicinal and 3-hydro-2-ketones as seen in Figure 1.5.

Studies to determine the parameters that affect ketone formation were focused on determining the factors that influence acetohydroxy acid production. Increase temperature could cause a decrease in acetohydroxy formation and hence a decrease in the concentration of the vicinal diketones and 2,3-pentadione.[10]

1.4 Methanol Regulation and Liquefaction Enzymes (Pectinesterase and Polygalacturonase)

1.4.1 Methanol Regulation

Many types of 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. The average methanol content of fresh orange juice is 34mg/L, while fresh grapefruit juice contains 27 mg/L.[12] Table1.3 shows the amount of methanol present in some juices.

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

Table 1.3. Methanol content in fruit juices and beverages[12]

As mentioned earlier, methanol is present in fruit and is a problem for the fruit brandy industry. The methanol levels that are low in fruit juices become much more significant when the fruit mash is distilled. During distillation, separation of alcohols from the fruit mash always results in the concentration of methanol in the product. Excessive methanol present in the human body can have severe effects on many organs and in some cases could lead to the person's death. The level of methanol in many products is tightly regulated for that reason. The United States Enviromental Protection Agency recommends a minimum acute toxicity concentration of methanol in drinking water at 3.9 parts per million.[12] The EPA has also set the permissible exposure limit in air to 200 parts per million for an 8-hour time weighted average.

This regulation extends to the alcoholic beverages and especially for brandies because they are made from fruit. In Europe, the production of fruit brandy has had many guidelines and regulations for many years.[2] For example, in Germany the cherry brandy methanol content is set at 400 mg/100mL absolute alcohol while for the brandy from Bartlett pears at 790 mg/100mL absolute alcohol.[2] In Austria, the maximum allowed content of methanol in brandy is 1000 mg/100mL absolute alcohol.[2] In the United States of America, the bureau of Alcohol, Tobacco, and Firearms has set the permissible amount of methanol in brandy to 0.35 % v/v. This corresponds to 700 mg/100mL absolute alcohol.

The source of methanol is the pectin present in the fruit. Pectin is esterified by methyl groups and the naturally occurring presence of the enzyme pectinesterase and other liquefaction enzymes in fruit is responsible for deesterifying the pectin to release

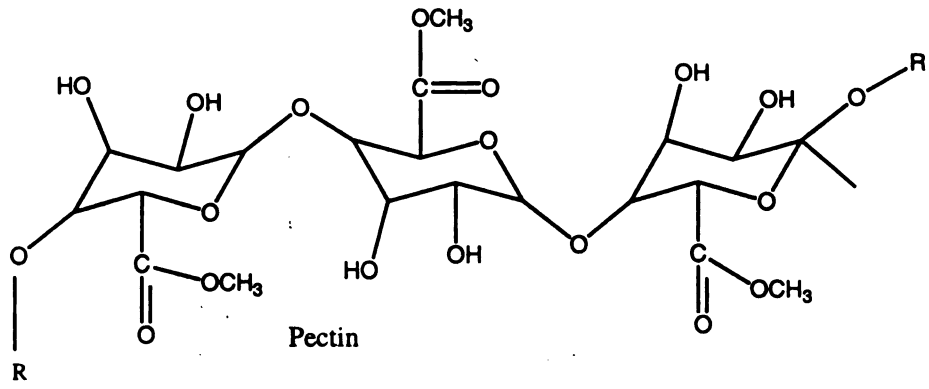
methanol. The activity of these enzymes increases as the fruit ripens and the tissue is damaged.[2]

1.4.2 Sources and Nature of Pectin

Pectic substances are carbohydrate derivatives that are widely distributed in plant tissues where they occupy intracelluar space. The pectic substances consist of a number of compounds, depending on the degree and type of enzymatic action. The parent compound in the intact immature tissue is protopectin, an insoluble substance located primarily in the middle lamella that serves as the glue to hold cells together and in the cell walls.[13] Pectin (polymethylgalacturonate) is the soluble polymeric material in which at least 75% of the carboxyl groups of the galacturonate units are esterified with methanol. Pectic acid (polygalacturonic acid) is the soluble polymeric material in which all the methoxyl groups are removed from the galacturonate units. Pectinic acids contain >0 and <75% methylated galacturonate units. Cell walls contain approximately 60% water and 40% biopolymers. Pectins make up 20-35% of the polymers.[14] Figure 1.6 shows the structure of pectin.

Pectin is polysaccharide whose main component is D-galacturonic acid, joined by means of α - (1$\rightarrow$4) glycosidic linkages. The galcturonic 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 is a wide range for the degree of esterification and this depends on the species, tissue and maturity.[15]

Stucture of Pectin



Mode of Action of Pectinesterase

E——N Represents the enzyme

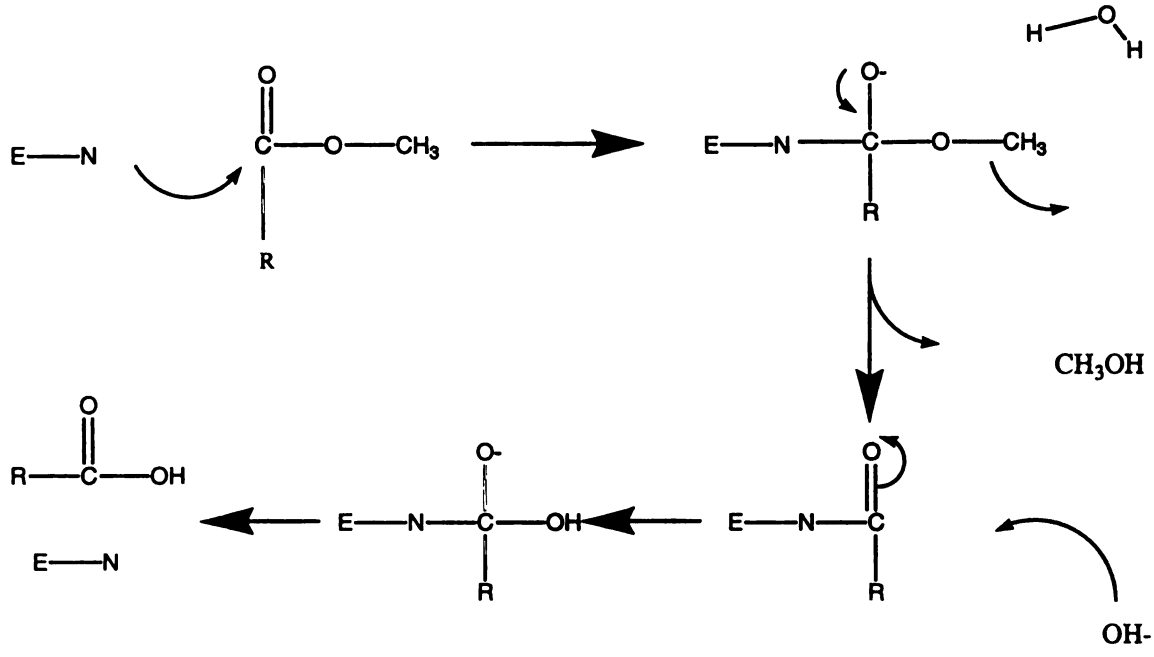


Figure 1.6. The structure of pectin is shown in the top of the figure and the bottom part shows the action of pectinesterase.

1.4.3 The Pectolytic Enzymes

The pectolytic enzymes include one esterase (pectin methylesterase); six polygalagturonases and four lyases.[16] Pectolytic enzymes are widely distributed in higher plants and microorganisms. They are not found in higher animals, but are found in some protozoa, nematodes, and insects. Enzymes that catalyze the formation of pectin from its water insoluble precursor 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.[17]

Microbial pectolytic enzymes are known to play key roles in plant pathogenicity and in much of fruit and vegetable spoilage involving rotting. Pectolytic enzymes are commercially important in a number of industrial processes including retting of flax and other vegetable fibers, extraction, clarification, and depectinization of fruit juices, extraction of vegetable oils and maceration of fruits and vegetables to give unicellular foods.[18] The use of these enzymes has become indispensable for the fruit and vegetable technology and for the production of wines in many places, yet it is not clear how suitable they are for the treatment of mashes in distilleries.[2] The use of enzymes in the in the treatment of mashes improves the capability to pump mashes in the fermentation phase because the insoluble pectin is also decomposed. The advantages of a fast liquefaction of the mash are apparent. When the mash is treated with the liquefying enzymes, the viscosity of the mash drops sharply and it becomes much easier to pump it

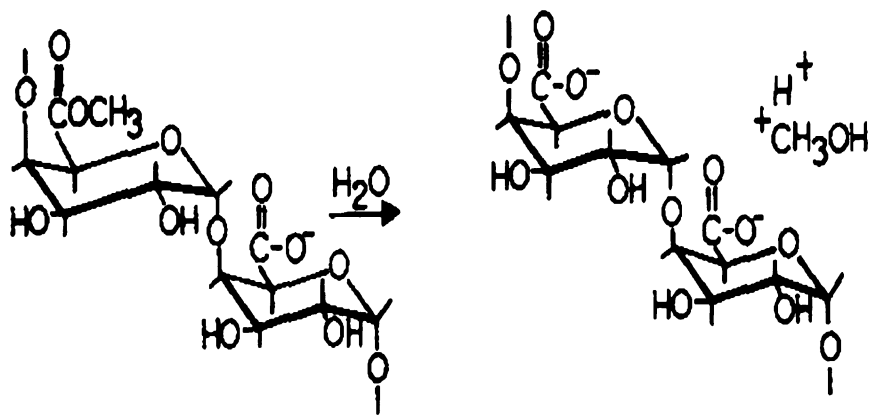
and in a much shorter period of time. Also, the speed of the fermentation is much higher and this is very important because we can obtain the same yield of alcohol in a shorter period of time.

Despite their many advantages in the fruit industry, the use of these enzymes could pose a problem for the brandy industry. Some researchers say that the methanol content of the mashes treated with enzymes does not change compared to the ones which are not treated because the formation of methanol takes place through pectimethylesterase which is already present in the fruit.[2] This result could vary considerably from fruit to fruit depending on the amount of enzyme present and the type of treatment applied. No major changes in the alcohol amount produced or in the sensory properties of the distillates were noticed.[2]

Yet, in the production of fruit brandy a slight increase in the production of methanol could have a negative impact on the brandy because the level of methanol is tightly regulated in alcoholic beverages. So, the production of brandy low in methanol content is highly desirable. This study will focus mainly on two types of enzymes that were provided by the German company Erbsloh that specializes in making products for the brandy industry and also for the production of enzymes for different industries. The first one is Fructozym M that is a pectinase and is commercially available, and the other three are polygalacturonases that are not commercially available. Ethanol, methanol and other compounds will be looked at as they are produced and compared. The next two sections will explain in detail the enzymatic action of these two types.

1.4.4 Pectin Methylesterase

Pectin methylesterase is synonymous with pectinesterase, pectase, pectin methoxylase, pectin demethoxylase, and pectolipase. Pectin methylesterases have been detected in a variety of plants. Most of the fruits have been reported to have more than one isozyme of pectin methylesterase. Orange pectin methylesterase is a well-studied enzyme.



$$\text{Polymethylgalacturonate (pectin)} + H_2O \xrightarrow[\text{methylesterase}]{\text{pectin}} \text{polygalacturonate} + \text{methanol} + H^+ \quad (1)$$

Figure 1.7. The strcuture and reaction catalyzed by the enzyme pectin methylesterase.[15]

Two isozymes have been reported, each having a molecular weight of 36,200 Da. The two isozymes have different optimal pH values unlike the isozymes found in banana pulp that both exhibited optimal activity at the same pH of 7.5. Structural studies have shown that the enzyme is a glycoprotein formed by a single low molecular weight polypeptide.[19]

Generally, the isoelectric point for this enzyme is between 7 and 11.[20]

Pectin methylesterase acts upon pectin to remove the methoxyl groups from the 6-carboxyl group of the galacturonate unit by hydrolysis as shown in equation 1. The enzyme is a carboxylic acid esterase, belonging to the hydrolase group of enzymes. The products of the reaction are: (1) a further deesterified pectin which eventually becomes a pectinic acid and finally a pectic acid; (2) methanol; (3) a H^+ from the ionization of the newly formed carboxyl group.

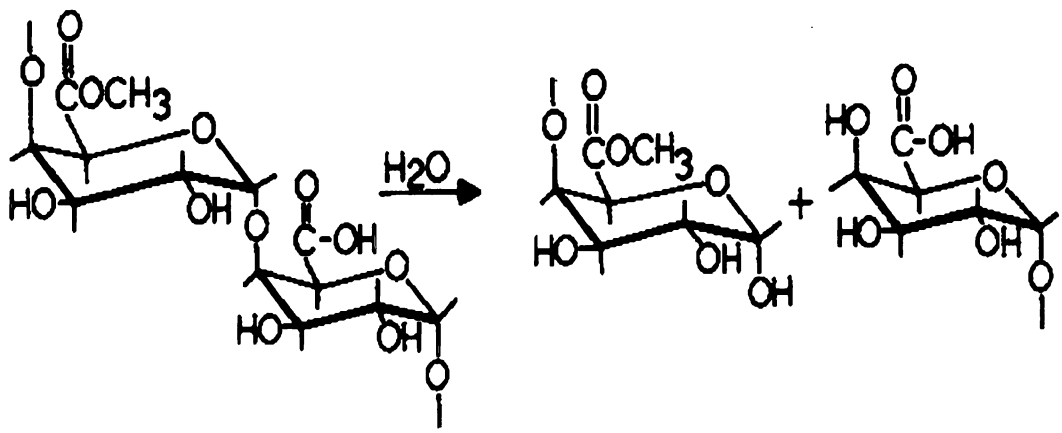
The effect of pH on pectin methylesterase has also been studied. Some researchers used the fluorescent probe 8-anilinonaphtalene-1-sulfonate (ANS) to determine the surface hydrophobicity of the enzyme.[21] ANS is non-fluorescent in water and other polar enviroments, but highly fluorescent in non-polar enviroments or when bound to hydrophobic sites on proteins. Changes in protein structure that result in an increase in hydrophobicty can be estimated by the change in fluorescence of ANS. If pH causes change in the hydrophobic sites of the protein, the structural change is detected by ANS fluorescence. Based on this technique, pectin methylesterase molecules were found to be more hydrophilic at neutral and alkaline pH than at acidic pH values. Current studies of the enzyme activity indicate that the degree of activity can be enhanced by the presence of cations. The stimulatory effect of NaCl on pectin methylesterase varies considerably. 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. Another study reported similar effect on mango pectin methylesterase by NaCl.[22] The maximum pectin methylesterase 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 the enzyme. It is worth noting that the cations increased the activity of the enzyme, but were not a requirement for its activity.

The distribution of methoxyl groups along the pectin chain is also important for pectin methylesterase. While the enzyme attacks completely methylated pectin slowly, the rate of deesterification increases to a maximumat 50% methylation.[16] Pectin, partially deesterified previously with pectin methylesterase, results in a slower rate than the equivalent degree of deesterification by alkali treatment. Some reports show that the optimum reaction temperature for this enzyme to be 65 °C and it declines after 70 °C.

1.4.5 The Polygalagturonase Enzyme

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→) polygalagturonate.[23] The pattern of degradation is either random (endopolygalacturonase) or terminal (exopolygalacturonase). Endopolygalagturonase usually prefers substrates with a low degree of esterification, typically less than 20%.[21] Exopolygalacturonase generally acts on deesterified pectin.[22] It was found that within short reaction times, random cleavage of polygalacturonate resulted in large decrease in viscosity. Very little change in viscosity was observed in the case of terminal cleavage. Figure 1.7 shows the action of the polygalacturonase enzyme.



$$\text{Polygalacturonate} + n\text{H}_2\text{O} \xrightarrow{\text{endopolygalacturonase}} \text{oligogalacturonates} + \text{galacturonate}$$

Figure 1.7. The action of endopolgalacturonase that hydrolyzes internal glycosidic bonds of polygalacturonate.

Materials and Methods

2.1 Materials

Several types of fruit were obtained from for the fermentations. The fruit selected was fresh and had no signs of damage. The types of fruit chosen were: cherries, California peaches, plums, apples (gala, jonathon, red delicious, and granny smith), Bartlett pears. Fermentations were conducted in 800 mL Fleakers® obtained from Dow Corning. The rubber tops on the Fleakers® were fitted with a U-shaped tubes that functioned as Air Locks. Ordinary lab glassware was used for all tasks need. The mash was placed in a 1000 mL round bottom flask before distillation. Enzymes used in the fermentation were four types.

1. Spirizm FM is a liquid, concentrated pectolytic enzyme preparation
2. VP 0956/2 is a purified liquid polygalacturonase from Aspergillus niger, activity 3-fold higher than Spirizm FM. Good activity up to pH 5.
3. VP 0996/2 is a liquid polygalacturonase from Aspergillus niger, activity 2-fold higher than Spirizm FM and good activity even up to pH of 6.
4. VP 0996/9 is a powdered polygalacturonase from Rhizopus oryzae, activity 8-fold higher than Spirizm FM and good activity up to pH 5.5.

A simple distillation apparatus was used and duplicates were generally performed. Figure 2.1 shows the simple distillation apparatus. The round bottom flasks were halfway immersed in silicone oil. Heating was applied using a hotplate/stirrer manufactured by Dow Corning (model PC-420).

The fermentations were performed in temperature controlled chambers that were bought from Sears (model: Galaxy # 253.19501992, $9ft^3$). To keep precise temperature

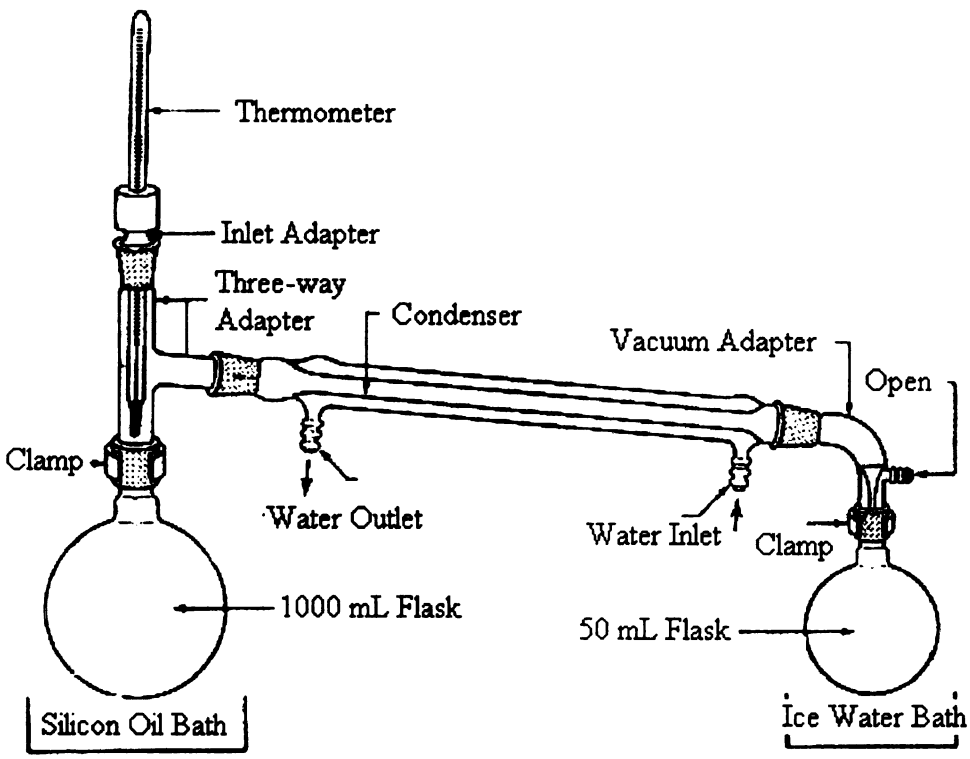


Figure 2.1. The distillation apparatus used to distill the mashes.

control, the electrical cord from the freezer was connected to a CN77000 controller from Microomega™. The parameters of the controller were set to achieve temperature control at the desired temperature and deviation no more than 1 °C.

The distillates were stored in 100-mL plastic vials. One mL from each sample was put in Gas Chromatography sample vials (model: C4010-88) from VWR Scientific. Samples were run on a Shimadzu GC 17A Gas Chromatograph with a Stabilwax® column from Restek Corporation. Helium, from BOC Gases, was the carrier gas and hydrogen and oxygen tanks were used for the flame. Chemicals used for the preparation of standards and also ethanol and methanol were bought from Sigma Aldrich Co. HPLC water was used for all dilutions.

2.2 Experimental Procedure

2.2.1 Preparation of the Fruit and the Yeast

The fruit was washed with water and all stems, leaves, and/or vines were removed from each piece of fruit. Fruit, except cherries, were cut with a pairing knife and then put into a five-gallon plastic bucket. The fruit pieces were then crushed with a wooden pestle. After the Fleakers® were cleaned thoroughly with water and soap, they were placed on a mass balance and it was tared. Seven hundred grams of fruit that was mashed was scooped into the Fleakers®. This was done in triplicate for each fruit. The Fleakers® were then quickly capped with their tops until it was time to add the yeast. The same procedure was followed at each fermentation temperature 10, 15, and 20 °C.

Three tenth of a gram of yeast was weighed. This amount is a little higher than the 0.28 grams that is recommended for thick and viscous mashes.[2] This larger value was chosen because the fermentations were not stirred during the course of the fermentation

so excess yeast helped insure good initiation of the fermentation. The yeast was placed in 50 mL beakers filled with 20 mL of distilled water at 40 °C. The beaker was placed in a water bath at 40 °C for 15 minutes to ensure complete swelling of the yeast. The suspension was added to the Fleaker® as evenly as possible and the contents were stirred for 5 minutes to ensure mixing of the yeast and the fruit pieces. The Fleaker® was capped with its top and placed in the chest freezer. The procedure was repeated for each fermentation at each temperature.

2.2.2 Enzyme Preparation

Liquefaction enzymes were added in some experiments and the solution was prepared separately. The recommended dosage for berries and stone fruit is 20 – 50mL/ Kg of mash at 15 °C.[23] The amount used in our fermentations was 0.35 grams/700 grams of mash for each enzyme (we used a relatively high value because our fermentations were not stirred). The enzymes and 3.15 mL of distilled water were combined to form a 10% solution. The procedure was repeated with each of the enzyme types except for the 0996/9. In this case, we weighed 0.35 grams because the enzyme was in powder form. The liquefaction enzyme solution was evenly added to its respective Fleaker®. The contents were kept at room temperature for 30 minutes. The yeast preparation was added and the Fleaker® placed in the chest freezer at a temperature of 15 °C. Controls were run which had no enzyme added to them and were done in triplicates. One Fleaker® of each enzyme was prepared except for the gala apples experiment where two sets were prepared.

2.2.3 Fermentation

Fermentations were run anareobically, which requires minimum exposure to oxygen. After the mash, yeast, and enzyme were added, the rubber cap with its air lock was firmly put on the Fleaker®. This air lock allowed the release of CO_2 that was generated from the fermentation. The pH of the fermentation was measured daily in each Fleaker® using pH paper and was in the range of 3.0 to 4.5. Refractive index measurements were taken. The temperature of the Fleaker® in the chest freezer was maintained at the specified temperature using a MicroOmega controller. The On/Off controller received power from a wall- mounted outlet. The controller was placed adjacent to the chest freezer. The thermocouple was connected to the On/Off controller and the temperature probe was placed in a beaker filled with water inside the freezer. This was done to prevent fluctuations in the thermocouple temperature and to model the situation in the Fleaker® as much as possible. The chest freezer received power from the controller, and hence the controller controlled the power input to the chest freezer. If the temperature inside the freezer was below the set point, the On/Off controller would not allow power to enter the chest freezer. Hence, the compressor will not be able to run and the temperature inside rises. If the temperature inside rose above the set point, the On/Off controller would allow power to enter the chest freezer and the compressors will work to bring the temperature down. This kept an excellent temperature control on the fermentation in the Fleakers® and a deviation of only a tenth of a degree Celsius was achieved. Fermentations were run for two weeks.

2.2.4 Distillation

The contents of the Fleaker® were placed in a 1000 mL round bottom flask and a small amount of an anti-foaming reagent was added. The flask was half immersed in silicone oil bath placed on a hot plate. Figure 2.1 shows the distillation apparatus. A silicone oil bath was used because it is possible to heat it over 100 °C without the oil boiling. The silicone oil bath used in these experiments typically reached temperatures of around 115 °C. Distillates started vaporizing at about 70 °C and the distillate was collected in a 50 mL round bottom flask and later transferred to a plastic or glass vial depending on the volume. At 99 °C, the distillation was stopped to prevent excess water from boiling over. After the distillation was complete, the apparatus was disassembled and each part washed with water and soap and rinsed and placed in an oven for drying.

2.2.5 Distillate Analysis

One mL from each sample was placed in a GC vial and was run on the gas chromatograph within 48 hours after distilling to prevent evaporation of highly volatile samples and any additional aging from occurring in the samples. The GC that was used for analysis was a Shimadzu GC-17A equipped with a flame ionization detector (FID) and auto sampler. A Stabilwax® column from Restek Corporation was used for all the experiments. This column had a stationary phase made of polyethylene glycol. The run conditions of 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)

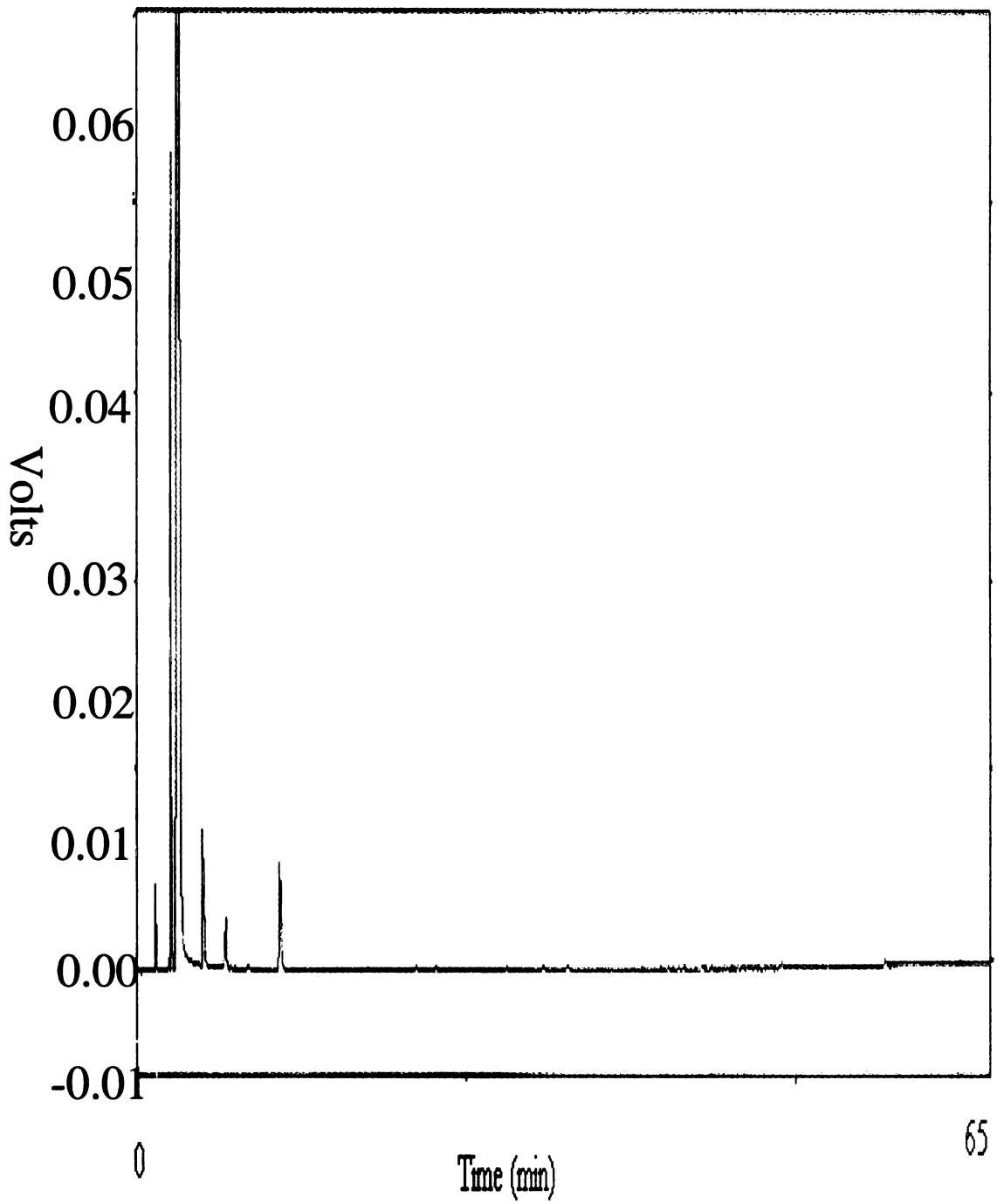


Figure 2.2. A chromatogram obtained from the gas chromatograph for distillate from gala apples.

Compound	Boiling Point (°C)	Retention Time (minutes)
Acetaldehyde	21	1.61
Acetone	56.5	1.95
Ethyl Formate	54.3	2.02
Ethyl Acetate	77	2.54
2-Methyl-2-Propanol	82	2.68
Methanol	64.7	2.83
Ethanol	78.3	3.23
2-Methyl-2-Butanol	101.9	4.47
2-Butanol	94	4.77
1-Propanol	97	5.14
2-Methyl-1-Propanol	107.7	7.41
1-Butanol	118	8.55
3-Methyl-1-Butanol	132	10.98
2-Methyl-1-Butanol	128	11.65
1-Pentanol	138	12.83
1-Hexanol	156.5	17.68
1-Heptanol	176	22.64
Furaldehyde	161.7	23.1
Benzaldehyde	179	25.63
1-Octanol	194.5	27.55
Benzyl Alcohol	205	41.68

Table 2.1. Organoleptic compounds (congeners) that were looked at and their retention times in minutes and their boiling points.

Injector temp: 240 °C

Detector temp: 255 °C

Carrier gas: helium at 30 cm/sec

Spilt ratio: 65: 1

Toatl 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. A typical gas chromatogram is shown in Figure 2.2.

The initial step to determine the components present in our chromatogram was to run standards of chemicals and figuring their retention times. Retention times in triplicate were obtained for the following compounds: acetaldehyde, acetone, ethyl formate, ethyl acetate, 2-methyl-2-propanol, methanol, ethanol, 2-methyl-2-butanol, 2-butanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, furaldehyde, bennzaldehyde, 1-octanol, and benzyl alcohol. Retention times were run in triplicate and averaged to increase the accuracy of the analysis. This qualitatively identifies peaks present in the chromatogram. Usually, compounds that low boiling points will elute first from the column followed by compounds with higher boiling points, but there are exceptions to this general rule.

2.2.6 Ethanol Calibration

An ethanol Calibration curve was generated. The following concentrations of ethanol were prepared using 100% ethanol and HPLC water: 25, 27, 30, 33, 35, 38, 40, 43, 45, 47, 50, 53, 55, 57, and 60%. Each of these concentrations (%v/v) was run five times using the same conditions as mentioned earlier. From the chromatograms obtained,

the peak areas were integrated and averaged. Plotting the areas versus the % ethanol we obtain a calibration curve as seen in Figure 2.3. The equation of this curve is then used to calculate all the other concentrations of other compounds.

2.2.7 Quantification of Methanol

The amount of methanol produced in these experiments needed to be calculated as accurately as possible. A calibration curve was constructed by plotting concentration of methanol in mg/100 mL in 40 % ethanol solution versus the areas under the peak obtained from the gas chromatograph. The concentrations chosen were: 79, 118, 158, 197, 237, 276, 316, 355, 395, 474, and 513 mg/100 mL in 40 % ethanol solution. Each was run five times and the average area was used in the plot. Figure 2.4 shows the methanol calibration curve along with error at each point.

2.2.8 Quantification of the Congeners

The quantity of these congeners were calculated using the ethanol calibration curve, Figure 2.3. First the fraction of each chemical including ethanol was calculated by dividing the compound integrated area by the total area. The ethanol concentration in our sample was calculated by using the area of the ethanol peak and putting it in the calibration curve equation. This gave us a value in v/v units. Using ethanol density, we converted this value to one that has units of g/mL. Then using this concentration of ethanol and the fraction of it in the samples, we calculated the concentration of the other compounds by equal ratios. In this procedure, we estimated the density of the compounds to be very close to that of ethanol and that ethanol acted as an internal standard. The values were then converted into units of mg/100 mL in 40 % ethanol solution. The methanol concentration was calculated using the methanol calibration

curve Figure 2.4. Using the methanol density, this v/v concentration was converted into mg/100 mL in 40 % ethanol solution. This procedure for calculating the methanol concentration was repeated for all the samples. The conversion into the unit mass/volume in 40 % ethanol solution was to make it easier to compare between samples and also because the brandy that is sold is usually at 40 % ethanol. The volume of the distillate from each fermentation was recorded. Using this volume and the % ethanol in that sample, we calculated the volume of 40% ethanol produced in mL. This value was needed to calculate the yield of pure alcohol produced from each fermentation.

Figure 2.3. Ethanol Calibration Curve to Determine the Concentration of Ethanol in the Distillates

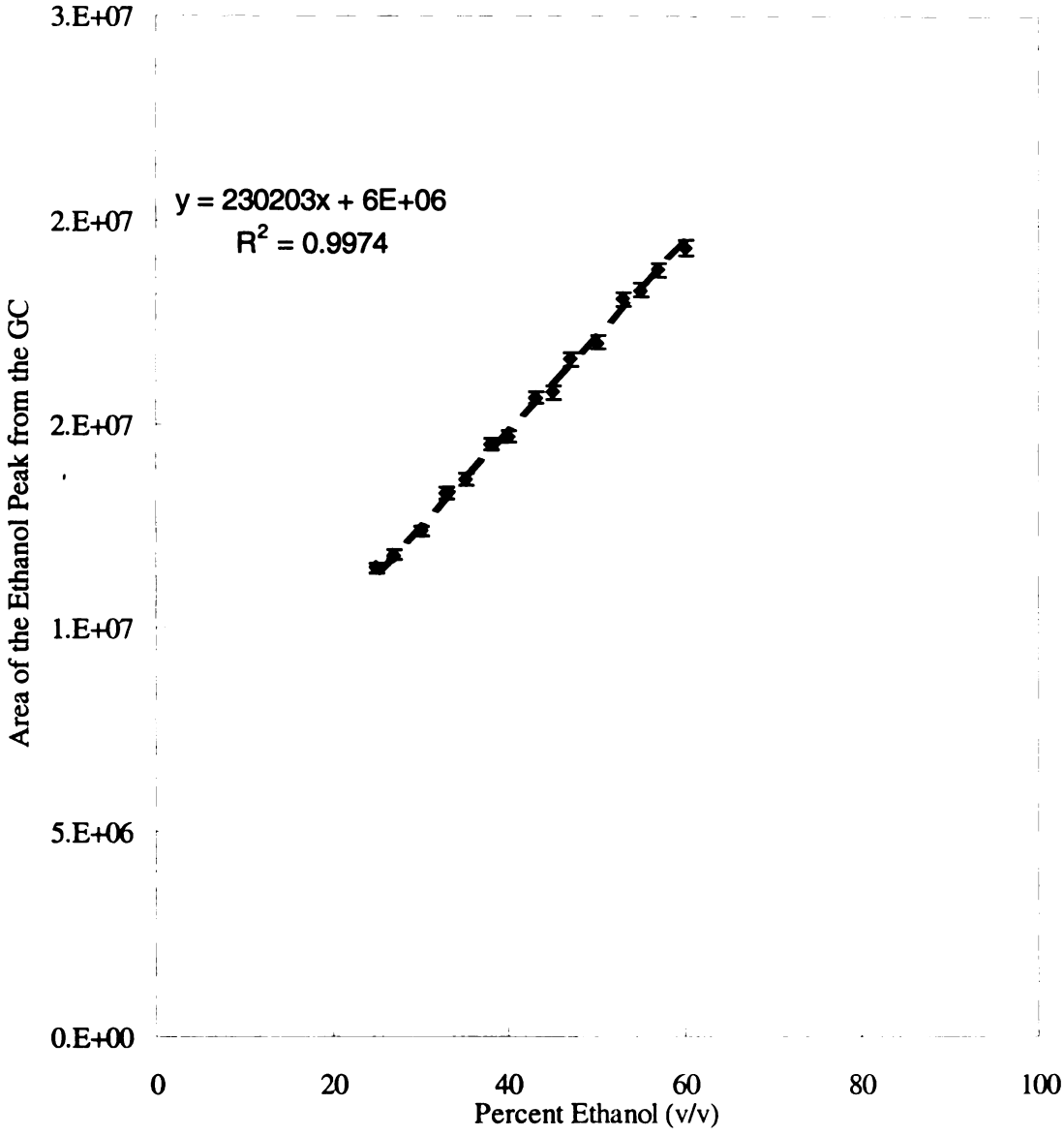
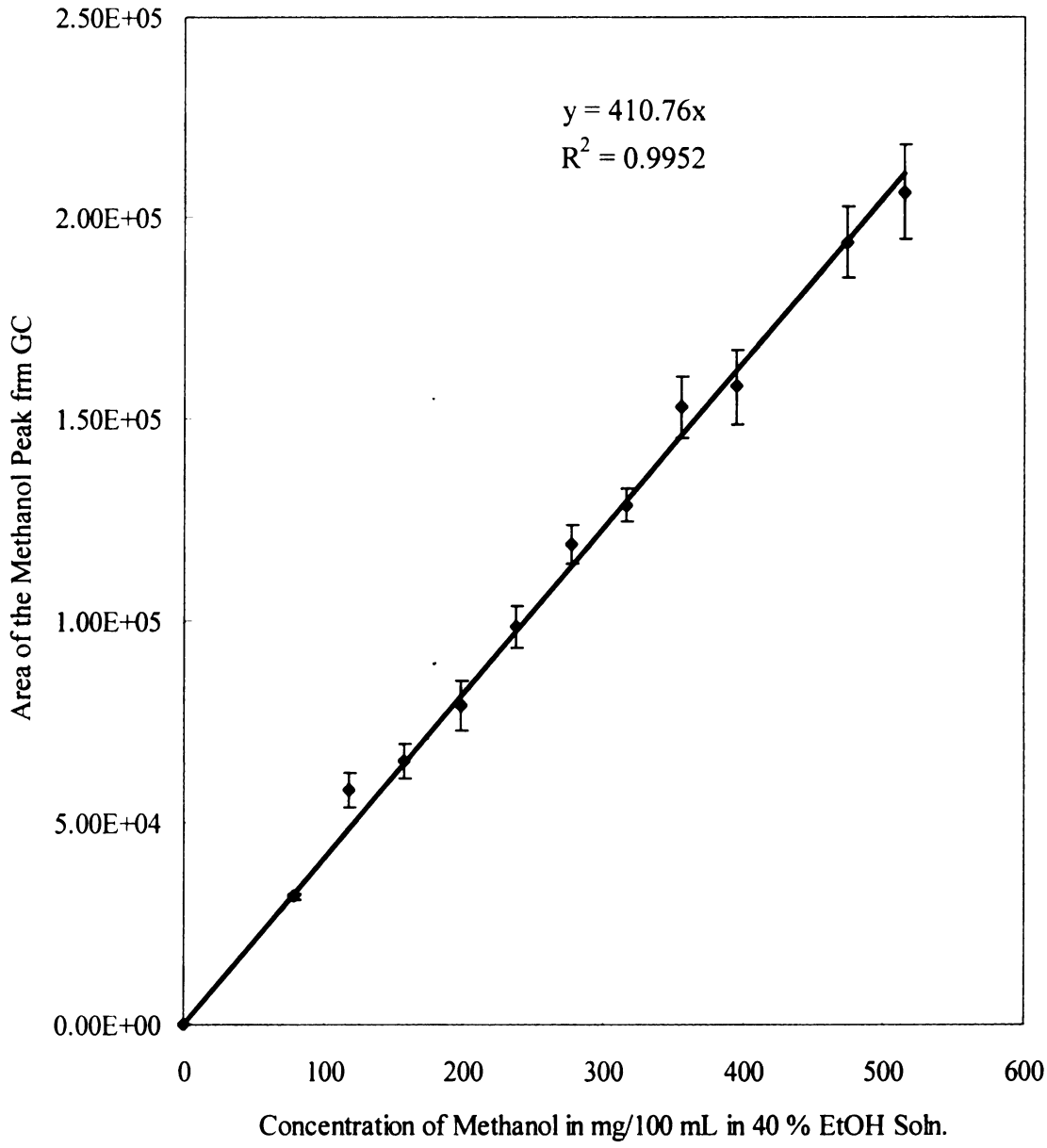


Figure 2.4. Methanol Calibration Curve to Determine the Concentration of Methanol in the Distillates



Results and Discussion

3.1 Analysis of the Distillates Using the Calibration Curves

One of the two main objectives of this study was to analyze the products of the distillation in order to quantify organoleptic compounds present and compare their concentrations at 10, 15 and 20°C. The flavor of an alcoholic beverage depends on a large part on the types of these organoleptic compounds present and their concentration. Organoleptic compounds, also called congeners, include a very large number of compounds. Table 1.1 shows the distribution of the compounds among different chemical categories[3].

This huge number of compounds makes it impractical to look at each single compound quantitatively in one study due to many reasons. In the current work, 21 compounds were analyzed in the distillates. These compounds were chosen because they include the more important compounds reported in previous studies, and their concentration is high enough for gas chromatography detection.[2, 3, 9, 10] Some of these chemicals were not detected gas chromatography due to their low concentration or absence. Also, some additional peaks were present in the chromatogram were not identified. The compounds chosen and their respective retention times and boiling points can be seen in Table 2.1. The retention times were used so we can qualitatively identify them in our chromatograms, and the standards for these chemicals were ran under the same conditions as our samples from the distillates. Usually, lower boiling compounds elute first in gas chromatography, but that is not always the case when separating alcohols as seen in Table 2.1. This is due to the interaction between the ethanol, water, and the compound. The interaction of the compound in ethanol and water also plays a

role. The compound that interacts more with ethanol will have a shorter retention time because it is vaporized with ethanol.

3.2 Congeners Present in Distillates from Fruit Fermented at 10 °C

The congeners were put in four categories: fusel alcohols, methanol, carbonyls, and esters. The volume of 40 % ethanol produced from each fruit was also determined. The main fusel alcohols present were: 2-methyl-2-butanol, 1-propanol, 2-methyl-1-propanol, 1-butanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and 1-hexanol. Large concentration of fusel alcohols are in general a negative flavor component as they have a damp cloth smell and impart a bad taste to the alcoholic beverages. They also can impart bitter harsh aftertaste. Sometimes, compounds that exhibit bad taste or aroma when present alone, could have a more pleasant aroma when in a mixture. Though, as can be seen from Table 1.2, fusel alcohols have a high threshold value and need to be in large concentrations to cause a change in the flavor of the product. The most abundant fusel alcohols were 1-propanol and 3-methyl-1-butanol. As seen from Tables 3.1 and 3.2, 1-propanol and 3-methyl-1-butanol are the most abundant fusel alcohols. So, it is a very good approximation when comparing our values to those in Tables 3.1 and 3.2. Tanner and Brunner[2] have done extensive work on fruit spirits, and it is desirable to compare the experimental values with theirs. For the fusel alcohols, the range of concentrations measured ranges from 832.5 mg/100 mL for Bartlett pears to 2245 mg/100 mL in granny smith apples, Table 3.3. The values in our tables are in mg/100 mL in 40 % ethanol solution, but were converted to mg/100 mL in absolute alcohol (A.A.) to compare them. Tanner and Brunner[2] reported 304 mg/100 mL for the pears and 468 mg/100 mL for apples, but did not specify what kind of apples were studied. Our

Compound	Kirsch (Cherries)	Zwetschgen (Plums)	Bartlett pear	Marc	Brandy	Apple
Methanol	417	931	1546	1560	58	503
Ethyl acetate	350	280	200	393	61	99
1-Propanol	888	138	69	41	52	59
2-Butanol	28	0	28	3	1	0
2-Methyl-1-Propanol	42	50	43	98	108	106
1-Butanol	0	5	19	0	1	0
2-Methyl-1-Butanol	12	28	34	57	50	
3-Methyl-1-Butanol	80	131	111	114	214	303
Acetaldehyde	5	15	10	72	7	13
Total acid (acetic acid)	100	33	90	45	40	55

Table 3.1. Analysis sample from Tanner and Brunner[2] that shows different fruit with concentrations of several congeners (all values are in mg/100 mL A.A.)

Alcohol	% Vol	42
Methanol	mg/100 mL A.A.	460
Ethyl acetate	mg/100 mL A.A.	658
Total ester	mg/100 mL A.A.	750
Aldehyde	mg/100 mL A.A.	90
1-Propanol	mg/100 mL A.A.	1252
2-Butanol	mg/100 mL A.A.	71
2-Methyl-1-Propanol	mg/100 mL A.A.	63
1-Butanol	mg/100 mL A.A.	1
2-Methyl-1-Butanol	mg/100 mL A.A.	12
3-Methyl-1-Butanol	mg/100 mL A.A.	88
Total acid (calculated as acetic acid)	mg/100 mL A.A.	220
Volatile acid (calculated as acetic acid)	mg/100 mL A.A.	180
Extract (dry residue)	mg/L	165
Hydrogen cyanide	mg/L	45
Copper	mg/L	6
Furfural	mg/100 mL a.A.	2

Table 3.2. Analysis of a cherry sample from Tanner and Brunner.[2]

	Concentration of congeners and volume of ethanol produced from the different fruits mg/100mL in 40 % ETOH				mL of 40 % ETOH
Fruit	**Fusel alcs.**	**Methanol**	**Carbonyls**	**Esters**	**Ethanol**
Plums	432	107	13.7	47.0	73.0
Peaches	372	365	21.6	9.50	24.0
Bartlett Pears	333	409	17.4	28.0	29.0
Cherries	348	222	11.3	29.5	27.0
Gala apples	324	180	75.4	0	22.0
Granny Smith apples	898	104	9.70	133	98.0

Table 3.3. Experimentally determined concentrations of congeners and ethanol present in distillates after fermentation at 10 °C as determined by gas chromatography.

are higher than theirs by almost four folds. As for the methanol, it is often regarded as a positive flavor component, but in the United States the legal permissible limit for methanol in alcoholic beverages is 0.35 %v/v or 700 mg/100 mL (A.A.). So, if the concentrations are higher than this value, it is considered that methanol would contribute negatively since it would be over the legal limit. Tanner and Brunner[2] in reported methanol concentrations that ranged from 58 to 1546 mg/100 mL, Table 3.1. In the current work, Table 3.3, values ranged from 260mg/100 mL in granny smith apples to 1022 mg/100 mL in Bartlett pears. All fruits except peaches and pears had values lower than the legal limit. Tanner and Brunner[2] showed a concentration of 1546 mg/100 mL for their Bartlett pears, higher than the value reported here.

The most common carbonyls present were acetaldehyde and acetone. They are more important than the fusel alcohols in their contribution to the flavor because of their much lower threshold limit, Table 1.2, and are considered to be a negative flavor component. Concentrations ranged from 24.3 mg/100 mL in granny smith apples to 188 mg/100 mL in gala apples, Table 3.3. Tanner and Brunner[2] (Table 3.1) report concentrations that range from 5 to 72 mg/100 mL. The current is higher than theirs for an example, a value of 43.5 mg/100 mL was obtained for pears while their value was 10 mg/100 mL. The most abundant ester identified in the current work was ethyl acetate and in a very few samples ethyl formate was present. These compounds impart a fruity taste to the flavor and aroma of an alcoholic beverage. They also have lower threshold limits than most other compounds. The range goes from 0 mg/100 mL in gala apples to 333 mg/100 mL in granny smith apples. These values are higher than those in Table 3.1. The amount of pure alcohol produced was determined to measure the efficiency of the

			Yield (liters of pure alcohol	
	Sugar Content		per 100 Kg raw material)	
Raw Material	**Variance**	**Mean**	**Variance**	**Mean**
Apples	6-15	10	3-6	5
Apricots	4-14	7	3-7	4
Pears	6-14	9	3-6	5
Blackberries	4-7	5.5		3
Gentian roots	5-13		3-5	
Windfalls (seed fruit)	2-5			2.5
Yeast deposits			2-5	
Blueberries	4.5-6	5.5		3
Rasberries	4-6	5.5		3
Elderberries	4-6	5		3
Currants	4-9	red 4.5 black 6.5		3.5
Pomace	2-4		2-3	
Cherries, sweet	6-18	11	4-9	6
Peaches	7-12	8		4.7
Plums	6-15	8	4-8	
Quinces	4-8		2.5-4	
Marc	2-4			3
Juniper berries(dried)		20	10-11	
Topinambour	13-18		6-8	
Grapes	9-19	14	4-10	8
Zwetschgen(plums)	8-15	10	4-8	6

Table 3.4. Sugar content and alcohol output of various raw material from Tanner and Brunner.[2]

fermentations. Values obtained were compared to values in Table 3.4 from Tanner and Brunner[2]. Volume of ethanol produced was 4.2 L for plums, 1.4 L for peaches, 1.7 L for Bartlett pears, 1.5 L for cherries, 3 L for gala apples, and 5.6 L for granny smith apples. These volumes would be obtained if we used 100 kilogram of raw material. These values are considerably lower than the values in Table 3.4. Some of them are close or lie within the variance of values presented there.

3.3 Congeners Present in Distillates from Fruit Fermented at 15 °C

The same method of analysis was followed for interpreting the results at 15 °C. Fusel alcohols were present in higher concentrations than at 10 °C and higher than values in Table 3.1. The range goes from 1062 mg/100 mL for Bartlett pears to 2502 mg/100 mL in plums and the values are presented in Table 3.5. The concentration found in plums doubled at 15 °C and also increased for the other fruits except for the granny smith apples where it decreased slightly. All methanol concentrations were under the legal limit except for the peaches and Bartlett pears, which is consistent with the results at 10 °C. Carbonyls were present in the same concentrations as at 10 °C with range from 22 mg/100 mL for plums to 111 mg/100 mL for peaches and Bartlett pears. Only Bartlett pears and peaches show a dramatic increase in their concentrations. The range here is a little higher than Table 3.1. The esters concentrations ranged in value from 1.6 mg/100 mL in Bartlett pears to 315 mg/100 mL in plums. Most fruits had an increase in their concentration except for the granny smith apples where it dropped slightly. The values in this work are very comparable to those in Table 3.1 except for peaches and red delicious apples. The pure alcohol yield was as follows: 5.1 L for plums, 1.4 L for peaches, 3.4 L for Bartlett pears, 3.9 L for gala apples, 4.1 L for granny smith apples, and 2.6 L for red

	Concentration of congeners and volume of ethanol produced from the different fruits				
	mg/100mL in 40 % ETOH				mL of 40% ETOH
Fruit	**Fusel alcs**	**Methanol**	**Carbonyls**	**Esters**	**Ethanol**
Plums	1001	105	8.79	126	90.0
Peaches	287	710	44.4	59.8	25.0
Bartlett pears	425	420	44.2	0.640	59.0
Gala apples	747	81.0	15.3	50.6	69.0
Granny Smith apples	651	128	10.2	51.7	72.0
Red Delicious apples	600	127	18.0	21.5	46.0

Table 3.5. Experimentally determined concentrations of congeners and ethanol present in distillates after fermentation at 15 °C as determined by gas chromatography.

delicious apples. Some of these values are higher than those obtained at 10 °C and closer to the values in Table 3.4.

3.4 Congeners Present in Distillates from Fruit Fermented at 20 °C

Fusel alcohols range from 1588 mg/100 mL for plums to 1977 mg/100 mL in Bartlett pears, Table 3.6. These values are higher than those at 10 °C and close to the values at 15 °C. The range is higher than that of Table 3.1 and for plums it is almost 5 times more. The methanol concentrations range from 178 mg/100 mL for red delicious apples and 720 mg/100 mL for Bartlett pears. All values are under the legal limit except for the pears where it is slightly higher. The carbonyls range from 26.8 mg/100 mL for Bartlett pears to 380 mg/100 mL for red delicious apples. The concentrations range in this work were higher than the range of values in Table 3.1. Esters range from 0 mg/100 mL for granny smith apples to 205 mg/100 mL for plums. This value for plums is close to the one in Table 3.1, which is 280 mg/100 mL. There is no clear trend in values for each fruit as a function of temperature. Production of pure alcohol was as follows: 2.9 L for plums, 4.1 L for Bartlett pears, 7.7 L for gala apples, 5.8 L for granny smith apples, and 6.2 L for red delicious apples. These values are mostly within the range of Table 3.4, and apples in the present study have productions closest to the mean presented in Table 3.4 while plums and Bartlett pears have values that are closer to the lower values of the range in Table 3.4.

3.5 Comparison of Plums, Pears, and Gala Apples at the Three Temperatures

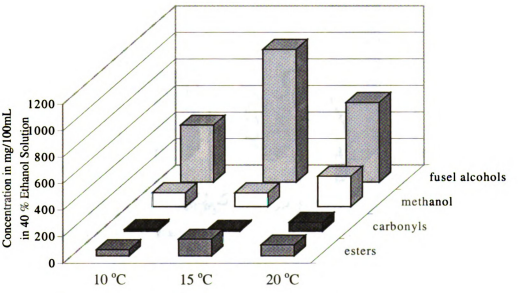
Figures 3.1, 3.2, and 3.3 show the production of the congeners at the different temperatures for three types of fruit: plums, Bartlett pears and gala apples. The data were analyzed to determine which is the optimum fermentation temperature for each fruit. The

	Concentration of congeners and volume of ethanol produced from the different fruits				
	mg/100mL in 40 % ETOH soln.				mL of 40 % ETOH
Fruit	**Fusel alcs**	**Methanol**	**Carbonyls**	**Esters**	**Ethanol**
Plums	602	230	67.0	82.0	51.0
Bartlett Pears	784	288	10.7	0.560	72.0
Gala apples	727	179	22.3	22.1	134
Granny Smith apples	664	182	20.8	0.000	102
Red Delicious apples	783	71.4	152	79.6	109

Table 3.6. Experimentally determined concentrations of congeners and ethanol present in distillates after fermentation at 20 °C as determined by gas chromatography.

concentrations of the congeners were compared as well as the statistical results obtained from performing the Duncan's Multiple Range Test on the data. Standard deviation was also calculated for each concentration as seen in Table 3.7. The Duncan test is essentially done to reduce the comparison-wise error rate to reduce the escalation in experiment-wise error. The values are arranged horizontally and vertically from smallest to largest and the difference is calculated. The difference between two values is compared to the least significant range for two adjacent treatments which is calculated based on the variance, significance level α, and q which is based on the degrees of freedom. α was chosen to be 0.05 and the degrees of freedom were 4. From Table 3.7, the best fermentation temperature for plums is 15 °C. This is because there is a higher concentrtaion of fusel alcohols and esters, very low concentration of methanol and cabonyls, and the largest volume of ethanol. Though, it can be seen that certain values at the three temperatures had no statistical difference and more than one temperature could have been chosen. As for the Bartlett pears, 20 °C would be chosen by looking at the same criteria as before. For gala apples, 15 or 20 °C could be the best temperature to run the fermentation at. Also, these results show that there doesn't exist a stringent temperature at which brandy yeast fermentation should be run. As can be seen from the Duncan's test, that many values are statistically similar at two temperatures and also if they were different it is not exactly clear the extent they have on the final flavor of the alcoholic beverage. These data could be further analyzed by looking at different compounds in each category of congener and calculating their flavor thresholds from Table 1.2.

Figure 3.1. Analysis of Congeners from Plums Fermented at Different Temperatures



Temperature at which the fermentation was done

Figure 3.2. Analysis of Congeners from Bartlett Pears Fermented at Different Temperatures

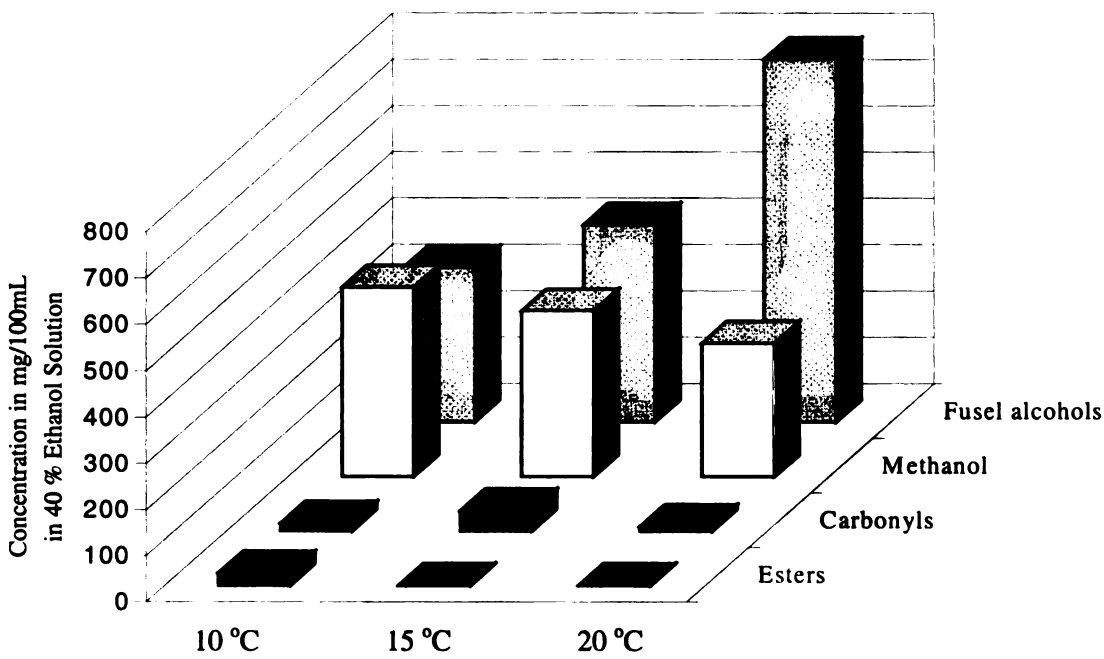
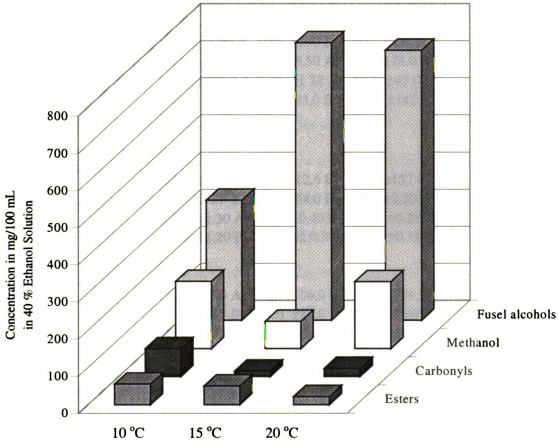


Figure 3.3. Analysis of Congeners from Gala Apples Fermented at Different Temperatures



Fruit	Concentration of congeners and the error in each measurement		
Plums	**Temperature °C**		
Congener± error	10	15	20
mg/100 mL in 40% ETOH			
Fusel alcs	432 ±14.80 A	1001 ±150 B	602 ±270 A
Methanol	107 ±18.6 A	105 ±5.50 A	230 ±78.0 B
Carbonyls	13.7 ±0.850 A	8.79 ±1.38 A	67.0 ±47.0 B
Esters	47.0 ±1.17 A	126 ±33.0 B	82.0 ±142 A
Bartlett pears			
Fusel alcs	333 ±3.90 A	424 ±12.6 B	784 ±157 C
Methanol	409 ±31.5 B	420 ±54.0 B	288 ±2.30 A
Carbonyls	17.4 ±1.30 A	44.2 ±0.40 B	10.7 ±0.250 A
Esters	27.9 ±1.20 B	0.640 ± 0.20 A	0.56 ±0.12 A
Gala apples			
Fusel alcs	324 ±32.0 A	747 ± 84.0 B	727 ±184 B
Methanol	180 ±11.7 B	81.0 ±17.6 A	179 ±4.80 B
Carbonyls	75.4 ±26.0 B	15.3 ±2.00 A	22.3 ±13.4 A
Esters	56.1 ±15.4 A	50.6 ±6.90 C	22.1 ±31.0 B

Table 3.7. The amount of congeners present in distillates from plums, pears and gala apples that were fermented at the three temperatures. The error is the standard deviation calculated from the three runs for each fruit at each temperature. The data in this table were used to plot fig. 3.1, 3.2, and 3.3. The letters A, B and C in the table represents the value obtained from performing the Duncan Range Test Method with an α of 0.05 and 4 degrees of freedom. If two values have the same letter, it signifies that they are statistically similar.

3.6 Fermentation with the Addition of Different Liquefaction Enzymes

The enzyme experiments were done with four different fruits: gala apples, Bartlett pears, red delicious apples, and granny smith apples. The Same amount of each different enzyme was added to each respective fermentation. All fermentations were run under the same conditions and the mashes were treated with the enzymes in the same manner.

The enzymes are used because they cause rapid degradation of the pectin and thus resulting in a quick liquefaction of mash and improved pumpability. Also, they are recommended because they cause a faster and easier onset of fermentation and complete fermentation of mash for an optimal alcohol yield. All fermentations were run for 14 days and noticeable change occurred in the viscosity of the mash between the enzyme treated and the non-enzyme treated mash. There were barely any fruit pieces left in the mash treated with the enzymes. All types of enzymes used produced the almost the same amount of liquefaction as observed qualitatively. The temperature used for all fermentations was 15 °C. This temperature was chosen because it is in the range recommended by many researchers and experts in this field.[2] Controls were run in each experiment in which no enzymes were added. Congener concentrations were compared for the different enzyme treatments, although, the main focus was the amount of ethanol and methanol produced by each enzyme treatment.

3.7 Congeners and Ethanol Present in Distillates from Gala Apples Fermented at 15 °C with Different Liquefaction Enzymes Added

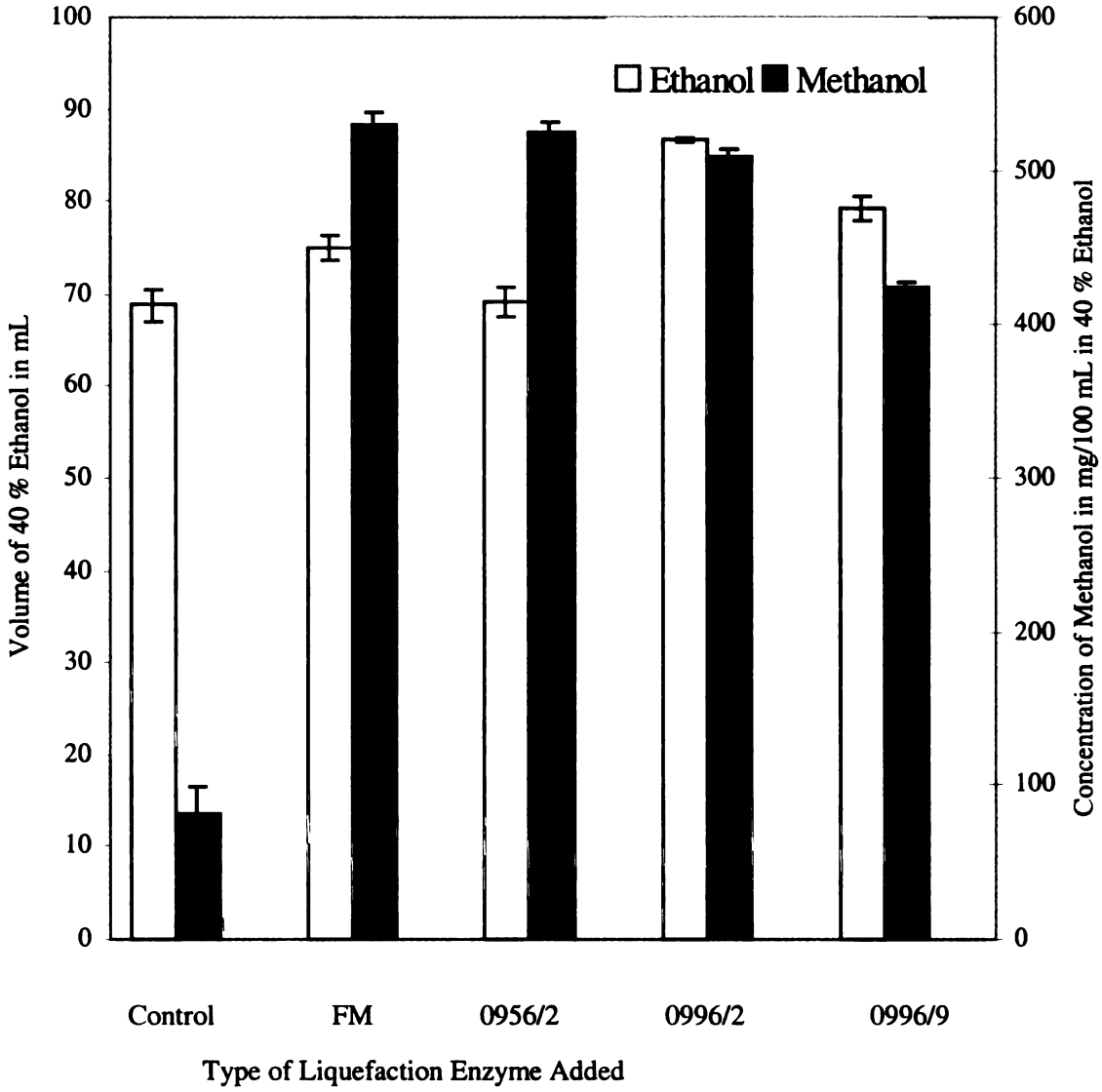
Each particular fermentation was designated by the name of the enzyme that was used to treat the mash. The amount of fusel alcohols present range from 1257 mg /100 mL in the fermentation with FM enzyme added to it, to 1867 mg/100 mL in the

fermentation with no enzyme added. The methanol concentration shows a dramatic increase from the control to the enzyme treatment ranging from 182 to 1450 mg/100 mL (A.A.) in the FM treatment. The enzyme treatment that produced the lowest concentration of methanol was 0996/9. Carbonyl concentration was 38 mg/100 mL in the FM, to 43 mg/100 mL in 0956/2 and 0996/2. Esters were only present in the control and at a concentration of 127 mg/100 mL. Ethanol yield of pure alcohol was as follows: 3.9 L for the control, 4.3 L for FM, 3.9 L for 0956/2, 4.9 L for 0996/2, and 4.5 L for 0996/9. All of these values were in the range presented in Table 3.4. The fermentations where enzymes were added did not produce more ethanol as hypothesized. Figure 3.4 shows the production of 40 % ethanol and the concentration of methanol in each sample and Table 3.8 presents the concentrations of the congeners. The trend is that ethanol production goes down slightly as enzymes were added while the methanol concentration went up drastically.

3.8 Congeners and Ethanol Present in Distillates from Bartlett Pears Fermented at 15 °C with Different Liquefaction Enzymes Added

The same analysis was followed in the Bartlett pears experiment. Fusel alcohols concentration was lower in the pears for all samples than their production from gala apples. It was consistent with the concentrations present at 10 and 15 °C but lower than the value at 20 °C. All values are presented in Table 3.9. Values ranged from 755 mg/100 mL in 0956/2 to 1060 mg/100 mL in the control. Table 3.1 shows a concentration of 304 mg/100 mL for bartlett pears and it is lower than the value we obtained. Methanol concentrations in all samples was above the legal limit and it ranged from 896 in the control to 1400 mg/100 mL (A.A.) in the 0996/2. The final value of

Figure 3.4. Volume of 40 % Ethanol Produced and Concentration of Methanol in Gala Apples Fermented at 15 °C with Liquefaction Enzymes Added



	Concentration of congeners and volume of ethanol produced from fruits with liquefaction enzymes added				
Fruit					
Gala apples	mg/100mL in 40 % ETOH				mL of 40 % ETOH
Enzyme	**Fusel alcs**	**Methanol**	**Carbonyls**	**Esters**	**Ethanol**
No enzyme	747	81.0	15.3	50.6	69.0
FM	503	530	15.3	0	75.0
0956/2	582	525	17.3	0	69.0
0996/2	549	508	17.5	0	87.0
0996/9	653	424	16.2	0	79.0
Bartlett Pears					
No enzyme	424	421	44.2	0.65	59.0
FM	336	588	34.6	84.5	68.0
0956/2	302	583	32.7	82.9	71.0
0996/2	354	623	36.1	89.9	61.0

Table 3.8. Experimentally determined concentration of congeners and ethanol present in distillates after fermentation at 15 °C with liquefaction enzymes added as determined by gas chromatography.

1400 mg/100 mL is close to the value presented for Bartlett pears in Table 3.1 which is 1546 mg/100 mL. Tanner and Brunner[2] did not specify if any enzyme treatment was used for the mashes. Carbonyls concentrations ranged from 82 mg/100 mL in 0956/2 to 110 mg /100 mL in the control. All samples' concentrations are very close to each other and far from the values in Table 3.1. Ester concentrations ranged from 1.6 mg/100 mL in the control to 225 mg/100 mL in the FM and the 0996/2 samples. This increase is positive because it adds to the positive component of the aroma of an alcoholic beverage. This value is also close to the one presented in Table 3.1, which is 200 mg/100 mL. Ethanol yield of pure alcohol goes as follows: 3.4 L for the control, 3.9 L for FM, 4.1 L for 0956/2, and 3.5 L for 0996/2. All values are very similar and are inside the range of values presented in Table 3.4. Figure 3.5 shows the ethanol yield at 40 % and the methanol concentration of each sample, and it is clear that ethanol production stayed the same while the methanol concentration increased.

3.9 Congeners and Ethanol Present in Red Delicious Apples Fermented at 15 °C with Different Liquefaction Enzymes Added

Fusel alcohol concentrations ranged from 1145 mg/100 mL in 0996/9 to 1600 mg/100 mL in the control. The concentration was higher for the control than for the other samples and was higher than the value reported in Table 3.1. Methanol concentrations jumped from 38 in the control to 1238 mg/100 mL (A.A.) in 0996/9 and was higher than 700 mg/100 mL (A.A.) in the other samples, Table 3.9. Methanol concentration reported in Table 3.1 for apples is 503 mg/ 100 mL and between both values determined experimentally. Carbonyls concentrations ranged from 27.3 mg/100 mL in 0956/2 to 64 mg/100 mL. Table 3.1 presented a concentration of 13 mg/100 mL, lower than the

Figure 3.5. Volume of 40 % Ethanol Produced and Concentration of Methanol in Bartlett Pears Fermented at 15 °C with Liquefaction Enzymes Added

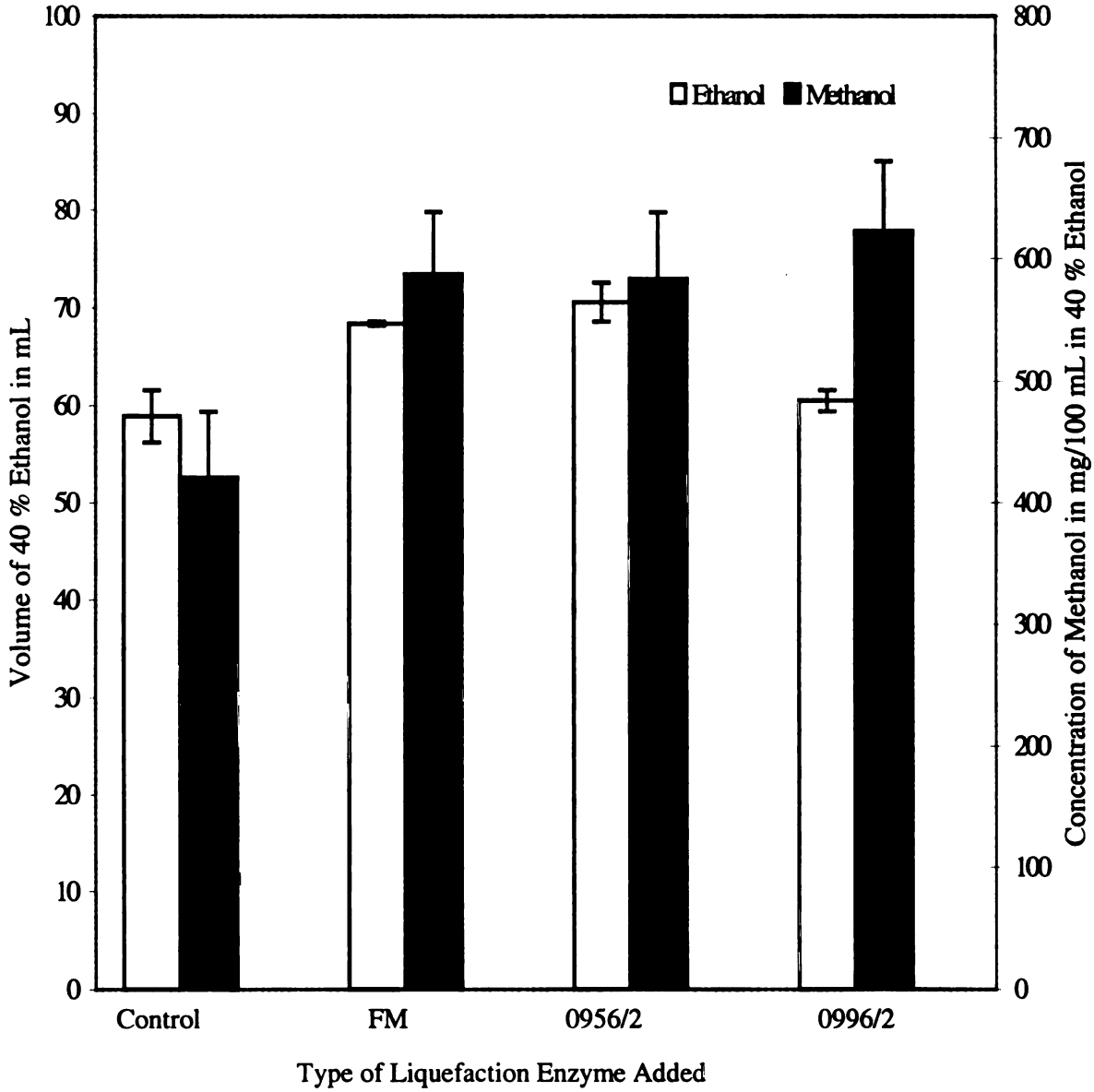
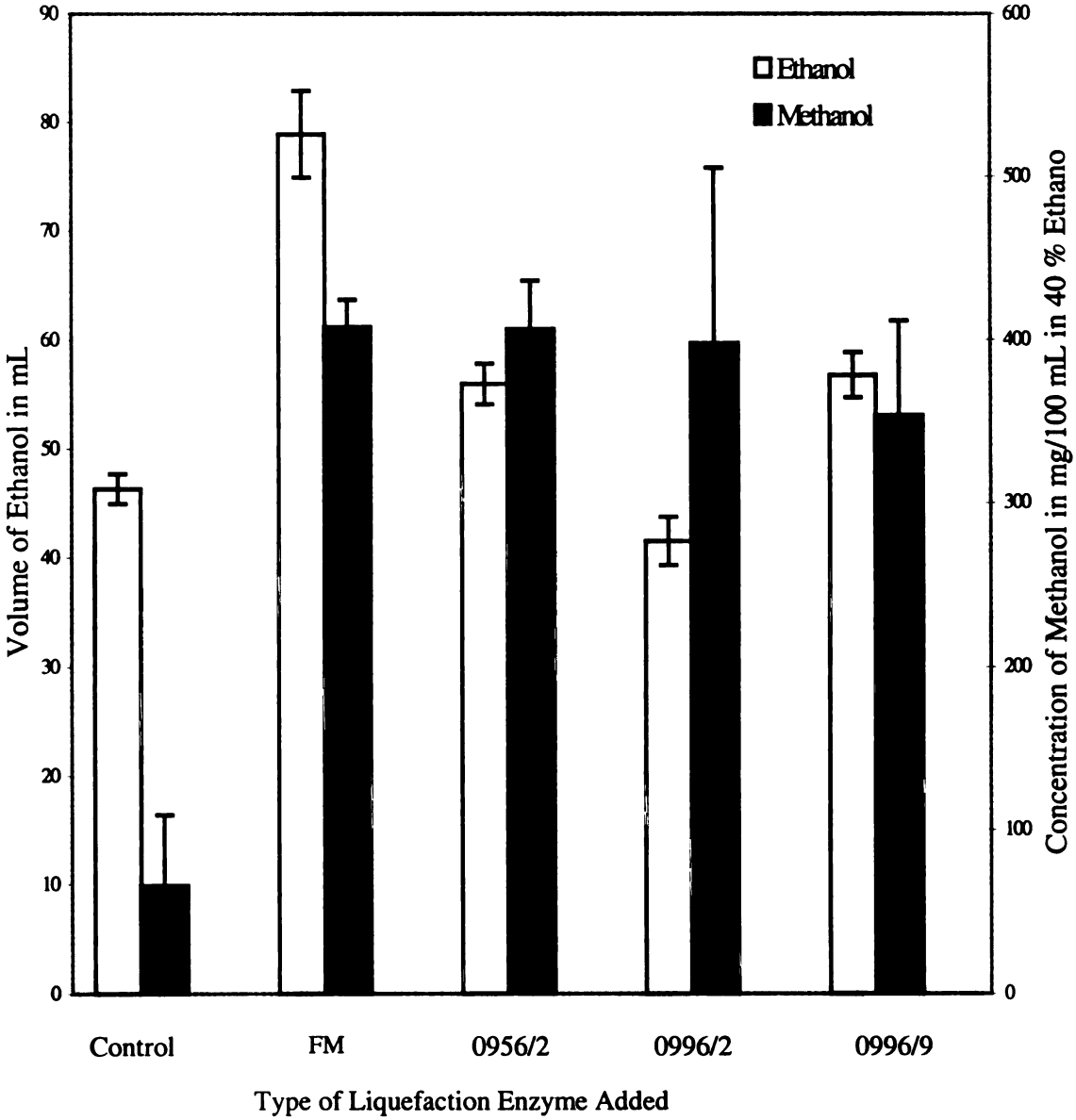


Figure 3.6. Volume of 40 % Ethanol Produced and Concentration of Methanol in Red Delicious Apples Fermented at 15 °C with Liquefaction Enzymes Added



	Concentration of congeners and volume of ethanol				
Fruit	**produced from fruits with liquefaction enzymes added**				
Red Delicious apples	mg/100mL in 40 % ETOH soln.				mL of 40 % ETOH
Enzyme	**Fusel alcs**	**Methanol**	**Carbonyls**	**Esters**	**Ethanol**
No enzyme	642	66.0	22.9	79.5	47.0
FM	463	408	11.6	38.6	84.0
0956/2	569	407	15.4	64.1	58.0
0996/2	552	398	26.6	63.5	44.0
0996/9	458	354	12.7	47.0	59.0
Granny Smith apples					
No enzyme	651	145	10.2	51.7	73.0
FM	583	501	38.2	66.2	89.0
0956/2	732	583	50.9	118	64.0
0996/2	555	506	12.7	42.9	93.0
0996/9	765	488	12.3	62.0	86.0

Table 3.9. Experimentally determined concentration of congeners and ethanol present in distillates after fermentation of red delicious apples and granny smith apples at 15 °C with liquefaction enzymes added as determined by gas chromatography.

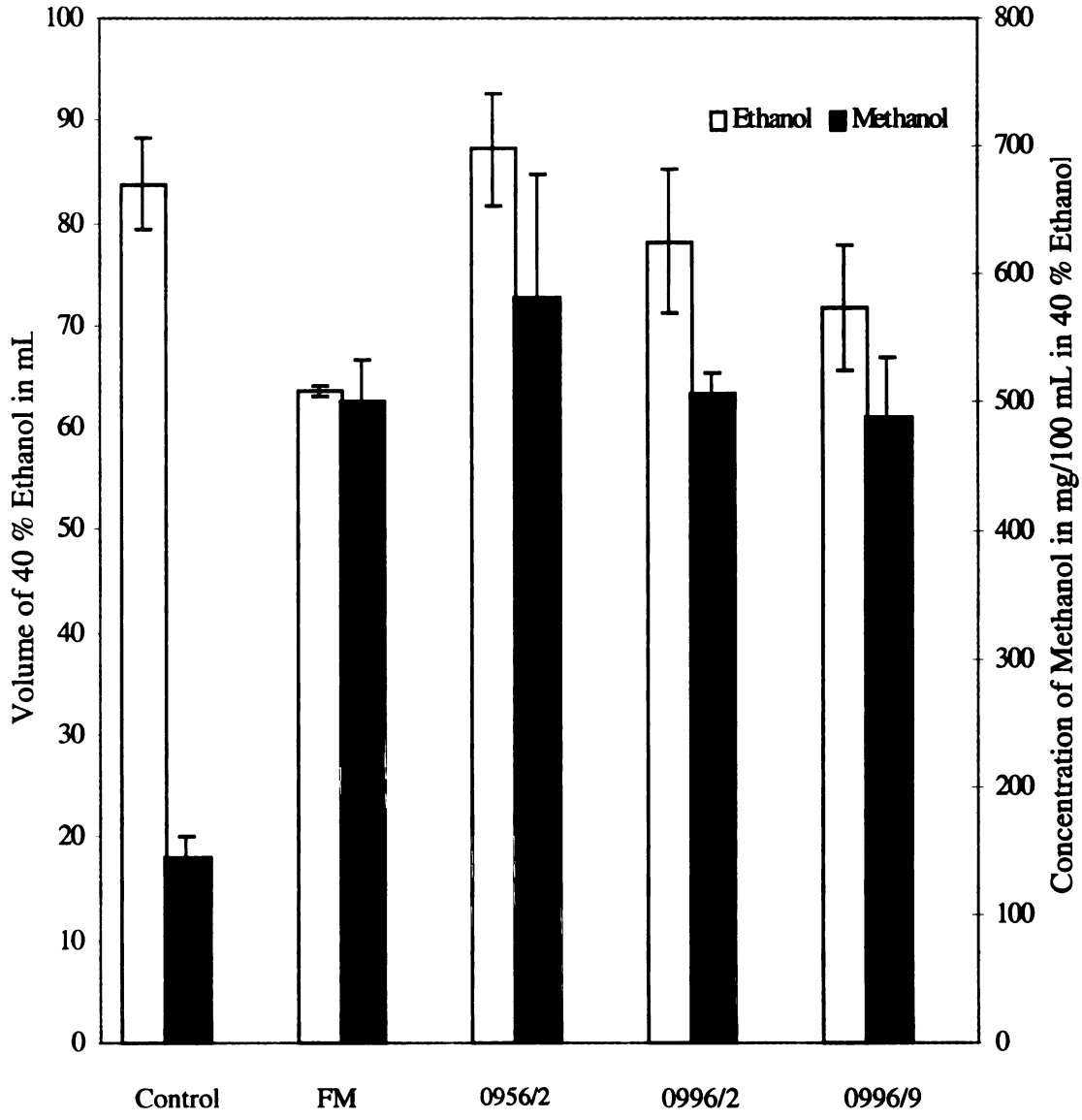
current values. Esters ranged from 96.5 mg/100 mL in FM to 198 mg/100 mL in the control. The concentration of esters went down in the samples treated with the enzymes and this will lower their contribution to the positive components in the aroma. Ethanol yield of pure alcohol was: 2.7 L for the control, 4.8 L for FM, 3.3 L for 0956/2, 2.5 L for 0996/2, and 3.4 L for 0996/9. These values are within the range of Table 3.4. The production increased in the most of the fermentations treated with enzymes, but not greatly. Figure 3.6 shows the production of ethanol and the concentration of methanol present in each sample.

3.10 Congeners and Ethanol Present in Granny Smith Apples Fermented at 15 °C with Different Liquefaction Enzymes Added

Fusel alcohols concentrations ranged from 1387 mg/100 mL in 0996/2 to 1915 mg/100 mL in 0996/9 as seen in Table 3.9. This is higher than the value presented in Table 3.1, which is 468 mg/100 mL. No major differences is seen between fermentations with enzymes added and the control. Methanol concentrations show a dramatic increase from 320 in the control to 1275 mg/100 mL (A.A.) in 0996/2. All methanol concentrations for fermentations that had enzymes added to them had concentrations above the legal limit. Carbonyl concentrations ranged from 25 mg/100 mL in the control to 127 mg/100 mL in the 0956/2. These values are higher than those values presented in Table 3.1. Esters ranged in concentration from 107 mg/100 mL in 0996/2 to 295 mg/100 mL in 0956/2. The control had a concentration of 130 mg/100 mL. So, it is within the range and no clear trend could be seen for the ester concentration. Production of pure ethanol was: 4.2 L for the contol, 5.1 L for FM, 3.7 L for 0956/2, 5.3 L for 0996/2, and 4.9 L for 0996/9. All values are within the range presented in Table 3.4. Figure 3.7

shows the concentration of methanol and the production of 40 % ethanol for all samples. No advantages are gained by treating the mash with enzymes.

Figure 3.7. Volume of 40 % Ethanol Produced and Concentration of Methanol in Granny Smith Apples Fermented 15 °C with Liquefaction Enzymes Added



Computer Simulation

4.1 ChemCAD Batch

ChemCAD Batch® is a commercial batch distillation program marketed by Chemstations®. Common applications of this software include: modeling existing batch column equipment, exploring alternative processes for existing products, design of new equipment, and validation of thermodynamics using batch column equipment. In this study, ChemCAD Batch® was used to model the distillation of a fruit mash using the experimentally determined congener concentration. The main congener of interest is methanol and it is useful to track the concentration of the methanol as it is distilled. These results could aid in knowing when to make the cuts and eliminate as much of the methanol as possible. It also can allow a preliminary assessment as to whether methanol can be removed by distillation. The ethanol profile in the distillate will be looked at as well.

To start the simulation, it is assumed that ethanol is present at 8% v/v in the mash before the start of the distillation. The concentrations of the congeners at 40 % ethanol were used to find the concentrations in 8 % v/v of ethanol. The remainder of the mash composition was assumed to be water. All these values were entered into the software as the pot charge. The mash was assumed to be at 25 °C and 1 atmosphere. The column selected had four trays, a partial condenser, and a total condenser. Also, the flow rate was set at 0.333 Liters/minute, and a reflux ratio of 1.5 was chosen as determined experimentally.

The profile for ethanol and methanol in the distillate volumes was determined. The distillate from gala apples was chosen including distillates that had liquefaction

enzymes added to the mash. Figure 4.1 shows the ethanol profile. It starts at 80 % v/v and goes down to almost 29 % v/v. Usually, the first three cuts, which are called the heads, are discarded. The hearts, which are the middle cuts, are saved and then later diluted to produce the brandy used for consumption. After the concentration of the ethanol drops below 40 % v/v, the collected distillate called the tails is discarded. All distillates showed the same trend for the ethanol concentration and there was no difference whether it was an enzyme treated mash or not. The values that were used to obtain Figure 4.1 are very close to each other and that is why we are not able to differentiate the five series clearly. The Methanol profile shown in Figure 4.2 shows the methanol concentration in % v/v versus the distillate volume from gala apples. The control which had no enzymes added to the mash had methanol concentrations much lower than the enzyme treated mashes. All enzyme treated mashes had methanol concentration above the legal limit of 0.35 % v/v.

The trend in the profile is the same for all distillates. The concentration starts low and peaks in the middle and then it goes back down. So, the middle, which is the hearts, has the highest concentration of methanol.

Figure 4.1. Ethanol Profile in the Distillate from Gala Apples Fermented at 15 °C with Different Liquefaction Enzymes Added as Determined Using ChemCAD Batch Simulation

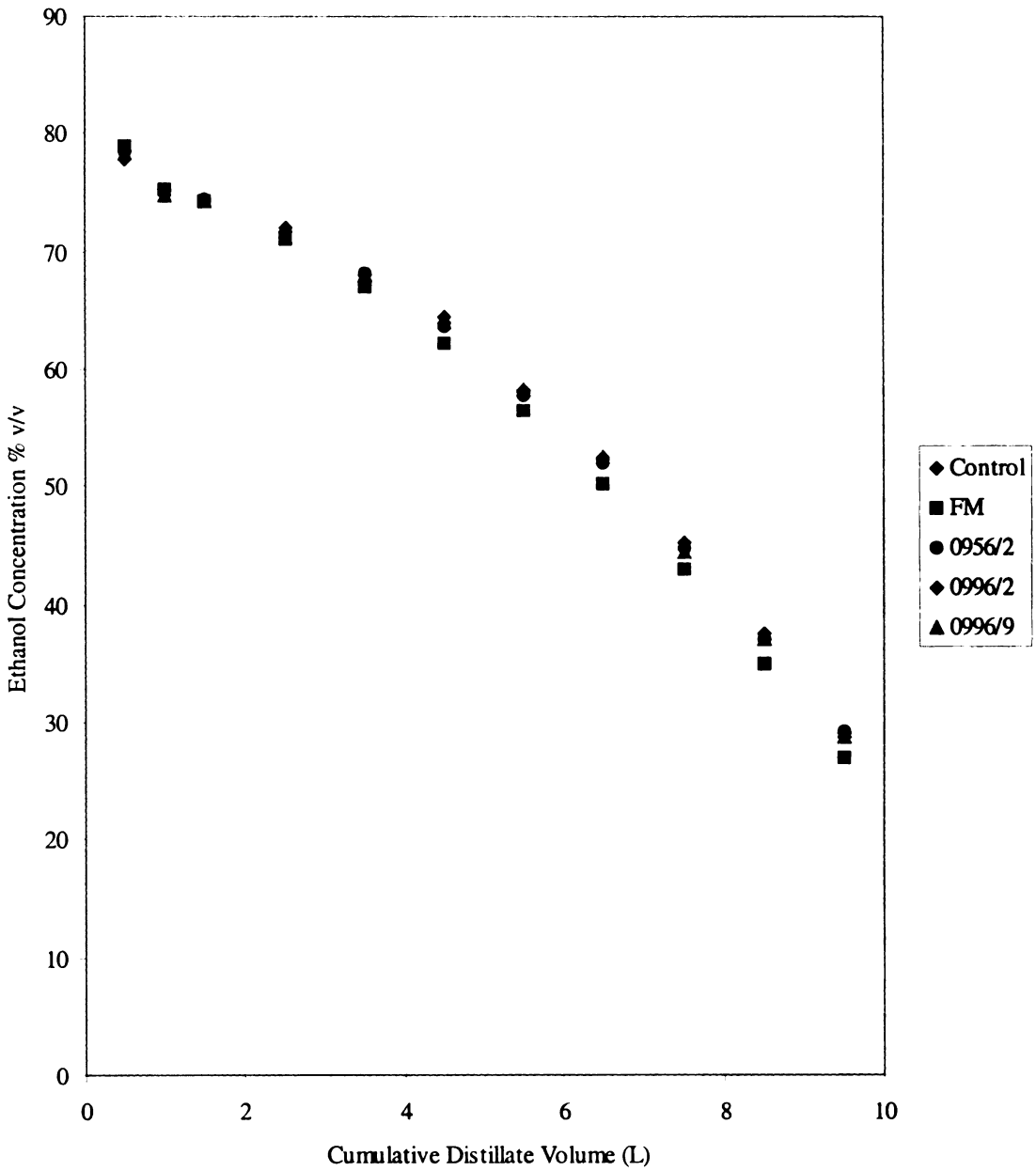
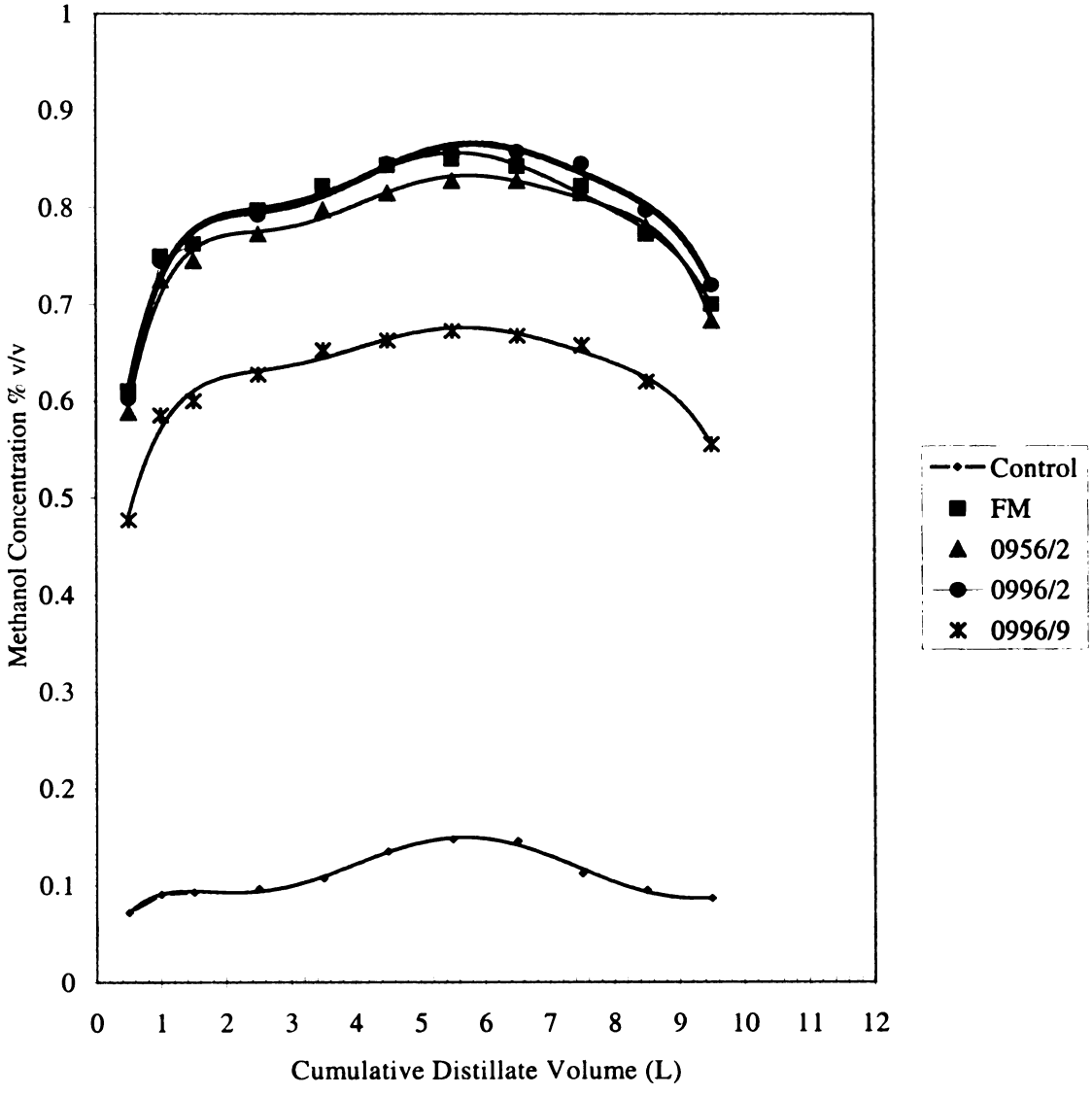


Figure 4.2. Methanol Profile in the Distillate from Gala Apples Fermented at 15 °C with Different Liquefaction Enzymes Added as Determined Using ChemCAD Batch Simulation



Conclusion and Summary

This research work was undertaken to improve understanding of two key areas in the production of brandy from fruits. The temperature at which the fermentations are done has not been studied quantitatively in relation to the amount and type of congeners and ethanol produced. In this research work, we compared our results with those of Tanner and Brunner[2] who have done extensive research on fruit distillation and production of brandy. The results obtained from the distillates of the fermentations that were ran at 10, 15, and 20 °C showed relatively consistent results regarding the production of these congeners and ethanol. The fusel alcohols at all three temperatures were within the range that Tanner and Brunner mentioned and that was 200 – 3000 mg/100 mL. These values could vary a great deal depending on the quality of the fruit used. Esters and carbonyls were studied and they were present in higher concentrations than what was reported by Tanner and Bruner. Yet, this increase in these values or those of the fusel alcohols are not the only parameter to be considered when evaluating the flavor of an alcoholic beverage. As seen in Table 1.2, different compounds have different sensory odor thresholds. These odor thresholds and evaluation of the contribution of a compound to the overall odor of a product are difficult areas of study. The production of ethanol was a little higher from fermentations at 15 and 20 °C, but that was not true for all the fruit. Methanol was below the legal limit for mostly all fruit except Bartlett pears and peaches at all fermentation temperatures.

Liquefaction enzymes can be used in the manufacture of brandy for optimal yield of alcohol and intensification of fruit aroma. The enzymes used in this study, are the commercially available Spirizym FM, which is pectin methyl esterase and three

polygalacturonases. Our results showed that for the four fruits studied, methanol concentrations increased dramatically when the mash was treated with any of the four enzymes. The concentration of methanol went well above the legal limit. As for ethanol, the production did not change and in some cases the fermentations with the enzymes in them had lower ethanol production. The increase of methanol was expected since the action of enzymes results in the release of methanol from the pectin and from extensive degradation and liquefaction of the mash. There was no trend regarding the change of the congeners' concentrations. In some cases certain compounds were produced more but most values were in a range that is very comparable in certain fruit to values presented by Tanner and Brunner.[2] The large increase in methanol concentration makes the use of liquefaction enzymes a very questionable practice for fruit brandy production.

Recommendation for Future Work

This work presented new information that would help in producing brandy of good quality. The organoleptic compounds that are present in alcoholic beverages are huge as seen by Table 1.1. The compounds that were analyzed here are only a fraction of the total number. Other detection methods such as gas chromatography/mass spectrometry could be used to detect compounds that are present in very low concentrations, but are of importance because they contribute significantly to the aroma of the final product. The use of enzymes could further be studied by using other enzymes that are commercially available in the market and study their effects. After treating the mashes with the liquefaction enzymes, enzymes could be deactivated by heat or a chemical method to see if methanol production could be lowered and still retain the advantages of liquefaction.

LIST OF REFERENCES

1. Valaer, P. 1939 Brandy. *Industrial and Engineering Chemistry* **31**, 339–53.

2. Tanner, H. Brunner, H.R. Fruit Distillation Today 3rd edition. Heller Chemical and Administration Society, Germany (1982).

3. Pomper, S. Biochemistry of Yeast Fermentation. The Bakers Digest. 32-38 (1969).

4. Guymon, J.F. James F. Guymon Lecture : Distillation in Alambic. *American Journal of Enology and Viticulture*. **41**, 90–103 (1989).

5. Claus, M. An Investigation of Relationship Between Tray Usage of the Still and Congener Compound Concentration in Distilled Fruit Spirits. M.S. Thesis. Department of Chemistry, Michigan State University, East Lansing, MI (2001).

6. Rose, A.H. and Harrison J.S. The Yeasts. Yeasts and the Enviroment. Vol **2**. 42–55 (1987)

7. Farrall, L. and Rose, A.H. (1967b). *Annual Review of Microbiology* **21**, 101.

8. Morton, H.C., Watson, K., and Streamer, M. (1978). *FEMS Microbiology Letters* **4**, 291.

9. Suomalainen, H. and Lehtonen, M. The Production of Aroma Compounds by Yeast. *Journal of Institutional Brewing* **85**, 14–156 (1978).

10. Berry, D.R., Stewart, G.G., and Russell, I. Yeast Biotechnology. 346–367

11. Ough, C.S., Guymon, J.F., and Crowell, E.A. Formation of Higher Alcohols During Grape Juice Fermentation at Various Temperatures. *J. Food Science* **31**, 620–625 (1966).

12. Cleland, J.G. and Kingsbury, G.L. Multimedia Enviromental Goals for Enviromental Assessment. U.S. Enviromental Protection Agency : EPA-600/7-77136b, E-28, November 1977.

13. Jarvis, M.C. The Proportion of Calcium –Bound Pectin in Plant Cell Walls. *Planta* **154**, 344-346 (1982).

14. Van Buren, J.P. The Chemistry and Technology of Pectin. pp 2-5, Academic Press, New York, (1991).

15. Fogarty, W.M., and Kelly, C.T. Microbial Enzymes and Biotechnology. pp 137-143

16. Pilnik, W., and Vorgan, A.G.J. (1993). Pectic Enzymes in Fruit and Vegetable Juice Manufacture. In : Enzymes in Food Processing. 3rd ed., T. Nagodawithana and G. Reed, eds., Academic Press, New York and San Diego.

17. Alonso, J., Rodriguez, M.T., and Canet, W. Purification and Characterization of Four Pectinesterases from Sweet Cherry (Prunus avium). *J. Agric. Food Chem.* **44**, 572-577 (1996).

18. Sun, D., and Wicker, L. pH Affects Marsh Grapefruit Pectinesterase Stability and Conformation. *J. Agric. Food Chem.* **44**, 3741-3745 (1996).

19. Labib, A.A.S., El-Ashwah, F.A., Omran, H.T., and Askar, A. Heat Inactivation of Mango Pectinesterase and Polygalacturonase. *Food Chemistry* **53**, 137-142 (1995).

20. Burns, J.K. The Polygalacturonases and Lyases (1991) in *The Chemistry and Technology of Pectin.* (walter,R.H.,ED). Pp 165-184, Academic Press, New York.

21. Archer, S.A. Pectolytic Enzymes and Degradation of Pectin Associated with Breakdown of sulphated Strawberries. *J. Sci Food Agr.* **30**, 711-723 (1979).

22. ERBSLOH GEISENHEIM. Getranke-Technologie Gmbh & Co. KG