The effect of distillation conditions and molasses concentration on the volatile compounds of unaged rum

Sumedha Mulye

Supervisor: Dr Rothman Kam

A research project submitted to
Auckland University of Technology
in partial fulfilment for the degree of
Master of Science
2019

School of Science

Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'The effect of distillation and molasses concentration on the flavour of unaged rum' contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Name: Miss. Sumedha Sanjay Mulye

Date: 31/01/2019

Signed:

Acknowledgement

Firstly, I would like to express my deepest admiration to my supervisor, Dr Rothman Kam, for his supervision, guidance and immense support during the course of my thesis. He always had a helping hand whenever I ran into any trouble or had questions about my writing or research. He allowed this paper to be my own work and thought process but manoeuvre me in the right direction whenever I was lacking confidence in whatever I was doing. Without Dr. Kam's immense patience, reluctance to give up and enthusiastic personality, I would not have been able to complete this thesis.

Secondly, I would also like to thank Dr. Chris Pook, without his years of experience in operating the analytical instruments at Auckland University of Technology (AUT), the final part of this thesis could not have been completed. I would also like to thank Adrian Owens for teaching me how to analyse my results.

Finally, I must express my very profound gratitude towards my mother for providing me with immense love, support and continuous encouragement throughout the difficult course of this thesis. Thank you.

Sumedha Mulye
Auckland University of Technology
August 2018

Abstract

Caribbean rums are made from blackstrap molasses and during the production process, four critical stages can affect the flavour of rum. These four stages include fermentation, distillation, aging and blending. During the distillation process, a copper alembic pot is used. The rum distillate are collected in three fractions or ’cuts‘; heads, hearts and tails. Major compounds in the head fraction contain ethanol, ethyl acetate and acetaldehyde, the latter being undesirable at high concentration. The heart fraction is generally of commercial value because it contains ethanol, esters and fusel alcohols that gives the rum the unique aroma. The tail fraction usually contains high concentration of fatty acids that gives off rancid and unpleasant odour. In industry, an experienced distiller will use sensorial approach (taste and smell) to determine these cuts. To minimise such subjective approach, this study proposed to use gas chromatography fitted with a flame ionisation detector to monitor specific compounds (congeners) in the rum distillate. By understanding the congener profiles, it is possible to determine when to make appropriate cuts objectively. The head fraction was determined to be the first 400ml of the total rum distillate of 3000ml. At this point the concentration of acetaldehyde starts to drop below the flavour threshold of 200 mg/L. Propionic acid, owing to its high boiling point, are collected at the later stage of distillation as the ‘tails’ fraction. According to the findings from this study, the tail cut is made at 2600 ml of distillate collected as the concentration of propionic acid starts rising rapidly above the odor threshold of 60 mg/L. High concentration of propionic acid in the rum distillate would give a sweaty-like and rancid odor. In addition, it was found that rum that has been distilled twice had higher purity in ethanol and lower concentration of undesirable congeners like acetaldehyde and propionic acid. However, the desirable ethyl ester congeners were also removed during after the double distillation process.

During the fermentation stage, a commercial rum yeast from a strain of *Saccharomyces cerevisiae* was used to ferment a mixture of varying molasses and dextrose concentration in the rum must (solution before fermentation). It was found that the raw material also affected the congeners of the distilled rum. Using the solid phase microextraction (SPME) with GC-MS technique, it was found that 1-butanol 3 methyl-acetate, octanoic acid ethyl-ester, acetic acid 2-phenylethyl ester, decanoic acid ethyl ester, octanoic acid ethyl ester, 3-methylbutyl ester, dodecanoic acid ethyl ester were the most abundant esters in rum distillate. However, there

were no obvious trend to correlate molasses concentration in the must to the ester profiles of rum. The results from this study also showed that the amino acids in the blackstrap molasses did not have any impact on the concentration of esters in the final rum distillate.

Table of Contents

List of Figures

List of Tables

List of abbreviations

w/w	weight by weight
% ABV	alcohol by volume
TSAI	total sugar as invert
IUPAC	International Union of Pure Applied Chemistry
ATP	adenosine triphosphate
FID	flame ionisation detector
GC	gas chromatography
SPME	solid phase microextraction
v/v	volume by volume
acetyl CoA	Acetyl coenzyme A
Tukey HSD test	Tukey's honest significant difference test

1 Introduction

Alcohol made from the fermentation of molasses is called “rum” (Pino 2007). Molasses is a viscous residue obtained at the last stage of the manufacturing process of raw sugar (Argyrios and Spyros 2015). Apart from rum production, molasses can also be used in other foods like cookies or pies, barbecue sauces, ginger bread and etc, because of its unique characteristic sugary flavour and sweetish aroma (Xu, Liang et al. 2015, Franitza, Granvogl et al. 2016). Molasses consist of 50% sugar by dry weight mainly sucrose, glucose and fructose (Xu, Liang et al. 2015). It is commonly used in baked products, animal feed additives and fermentation feed stock to produce rum. In general, there are three types of rum derived from sugar cane extract and each rum type has its own unique production process (Broom 2003). Type 1 – Rhum agricole is commonly produced in the French West Indies which involves distillation of fermented fresh sugar cane juice to obtain rum that retains the vegetal characteristic of cane. In type 2 - Cachaça, sugar cane juice is cooked and concentrated into syrup followed by fermentation and distillation. The fermented syrup can be stored for long periods so that distillers can distil Cachaça throughout the year and not be restricted by the harvest time. Type 3 – Rum, involves the use of molasses, a by-product from the manufacturing of crystallized sugar. Molasses are typically sold to distillers to ferment and distil into high quality rum. The overall flavour of rum is determined by the source of molasses, different distillation techniques, ageing and blending process.

The fermentation process differs from distiller to distiller as each use specific yeast strains of their choice. The strain of yeast, *Saccharomyces cerevisiae*, plays a vital role during the fermentation process which typically lasts for 3 to 4 days. Before fermentation, water, nitrogenous nutrients and additional sugars such as glucose, fructose and sucrose must be added to ensure healthy growth of yeast (Fahrasmane and Ganou 1998). Some producers add small amount of sulfuric acid to accelerate hydrolysis of sucrose to achieve efficient fermentation (Varzakas and Tzia 2015). Ammonium sulphate is also added by some producers to provide nitrogen which facilitates the growth of yeast. During distillation, alcohol in the fermented broth (wash) is evaporated, re-condensed and collected giving raw spirit. The distillation process is either carried out in pot stills or in column stills. Pot stills are predominantly employed in industry because of its simple design and ease of set up whereas column stills have an added complexity by having multi layered stages which allows different chemical compounds to be separated.

Finally, the prime step of aging and blending is carried out. Aging of distillate is usually done in oak casks, which are previously used for Bourbon, whiskey or brandy, accompanied by dilution with purified water by adjusting the desired alcohol percentage to 37-40% by volume (Franitza, Granvogl et al. 2016). If the ageing process of rum in oak cask are skipped, the resulting liquor is named as white rum and then if necessary it is coloured with caramel (Varzakas and Tzia 2015). When rum is aged in oak cask for more than three years it is known as 'old rum' whereas rum aged for less than 3 years is known an 'traditional rum'. Ageing influences the final colour of rum (Broom 2003). It is known that the ambient temperature can affect the ageing process of rum which are aged for one year minimum in the hot, humid Caribbean weather whereas at least 5 years in the colder climate of Scotland and England. During the entire aging process, the rum penetrates into the oak barrel's pores to facilitate the extraction of characteristic aroma compounds from the oak. The infusion of aromatic compounds from oak during the ageing process can have great influence on the final flavour of rum. The extracted characteristic compounds provides the rum with complex flavour and aroma notes such as vanilla, coconut, orange peel, coffee, spices, and fresh oak (Broom 2003), refer to **Table 1** for a list of common aromatic compounds found in aged rum. Finally, the blending process is carried out by expert distillers, who uses sensory approach (smell and taste) to mix and blend different types of rum to produce the final consistent product. Blending creates further complexity in the flavour of rum by mixing rum of different age, style and grade.

Table 1. Common aromatic compounds found in aged rum (Franitza, Granvogl et al. 2016).

Names of chemical compounds in rum	**Corresponding flavour**
Vanillin	Vanilla
γ-nonalactone	Coconut
Linalool	Orange peel
Ethyl butanoate	Fruity
4-hydroxy-2,3-dimethyl-2H-furan-5-one	Spice
3 methyl butyl acetate	banana
Ethyl octanoate	Fruity, green
Acetic acid	Vinegar
3-(methylthio) propanal	Cooked potato
Butanoic acid	Sweaty
Phenylacetaldehyde	Honey

2-methoxy phenol	Smoky
4-ethyl phenol	Phenolic
Decanoic acid	Soapy, Musty
Methyl propanol	Malty
Phenyl acetic acid	Beeswax, honey

Currently, the biggest challenge in the rum industry is to know when to make appropriate fraction or 'cuts' during the distillation process as it directly influence the flavour and taste of rum. At the moment this process is done by an experienced distiller who uses smelling and tasting to determine the cuts. The drawback of this method is that it is highly subjective and relies solely on the experience of the distiller which can introduce variability. Therefore, a more objective and consistent methodology to determine the correct time to make the head, heart and tail cuts would remove any subjective variability.

Another aspect in the rum making process that is not well documented is how the molasses concentration in the must (sugary solution before fermentation) affects the flavour of rum. It is known that raw materials play an important role in influencing the flavour of rum (de Souza, Vásquez et al. 2006). There are a number of studies done on characterizing the volatiles (Suomalainen and Lehtonen 1979, Pino 2007, Franitza, Granvogl et al. 2016) in the final aged rums but not much have been reported on how the concentration of molasses in the must can influence the volatiles in the distilled rum before ageing.

2 Literature review

2.1 History of rum

Cultivation of sugarcane crop has its origin traced back to 2000 years ago in Asia (Medeiros, de Matos et al. 2017). Among different alcoholic beverages, rum was the first spirit obtained from sugarcane crop giving it an historical significance. The origin of sugarcane cultivation belonged to warm temperature regions of south and southeast Asia, Polynesia and Melanesia (Lea and Piggott 2012). Gradually the cultivation of cane crop spread around the world including Madeira, Spain, Cyprus and Sicily towards third century A.D. In 1493 (15th century), Christopher Columbus was the one to introduce the sugar cane crop to the Caribbean. Barbados and Santo Domingo in the Caribbean were the innovators of the modern-day rum production. Around the 17th century, due to colonization of other islands by British, French and Dutch, this spirit gave great symbolic identity to the Caribbean region.

It is documented that the Portuguese in Pernambuco and Bahia in Brazil, were the ones who first manufactured molasses derived from sugarcane and fermented it into an unnamed spirit (Medeiros, de Matos et al. 2017). Molasses is the by-product of the manufacturing process of raw sugar from sugarcane sap. Upon diluting the molasses with water and fermenting this mixture, the distillation process that follow produces a spirit that is known as rum in today's language. However, the exact origin of the word 'rum' is unknown, but it may be the abbreviation of the word 'saccharum' meaning sugar in Latin and 'rumbullion' a word reported from the islands of Barbados meaning "kill-devil spirit" because of the harshness of the beverage.

During the 18th century, rum was a cheap distilled spirit that was associated with people of low social economic status (Lea and Piggott 2012). Back in those days rum had poor quality and was distilled and consumed by slaves who worked in the sugarcane plantation. In the late 18th century, pirates would frequently stock rum in their ship because it was a cheap alcoholic beverage and hence drove the popularity of rum to the point it became a trade currency for commodities like cotton, tobacco, gunpowder, gun and slaves.

2.2 Production of molasses

The production of molasses starts at the sugar mill (Broom 2003). The sugarcane crop is chopped, crushed and milled to extract the juice. The juice is boiled into syrup consisting of 30% (w/w) sugar followed by a clarification process, where some crystalized sugar is added as nucleation sites and the syrup is boiled again under vacuum. The sugar crystals will start to develop and are separated once cooled, by centrifugation. This entire process is repeated twice to obtain maximum yield from the cane juice. At the end of this process, a blackish brown, sticky, thick liquid is known as molasses.

Commercial rum distillers usually import molasses from Brazil, Venezuela or Guiana (Broom 2003). In the past, one of challenges that rum producers faced was not getting the ideal 52% percent sugar in molasses. As the sugar industry continuously improve their technology, they are able to increase the yield of sugar extraction and hence leaving less sugar in the molasses. Unfortunately, this hampers the amount of alcohol obtained from each ton of fermented molasses. On average, distillers can approximately obtain 4 to 5 tonnes of spirits with 57% alcohol by volume (ABV) per one hundred tonnes of milled sugarcane. Before fermentation, pH level of the molasses is inspected at the distillery, if needed, the pH is adjusted to between pH 4.4 to 4.6, as it is the preferable range for yeast to grow. The level of dilution of molasses by water depends on which style of rum is prepared. Usually best grade rum are those which are aged longest. For example, in Jamaica, Wray & Nephew Ltd varied their rum dilution to obtain white rum and dark rum with specific flavour of compounds.

Concentration of different components in molasses differs by the sugar cane variety, soil type, climate conditions, type of cultivation, time of harvesting, milling and sugar extracting techniques (Lea and Piggott 2012). Refer to **Table 2** for a list of constituents commonly found in molasses.

Table 2. Comparison of constituents and physical properties between good, bad and fair molasses (Lea and Piggott 2012).

Parameters	**Typical value of Molasses for Rum production**		
	Good	**Fair**	**Bad**
Brix (°Brix)	87.6	85.4	88.2
*Total sugar as Invert (TSAI)	57.97	52.91	49.93
*Sucrose	36.44	31.30	34.61
*Reducing Sugar	19.61	19.96	13.50
*Ash	7.31	9.35	11.57
*Total Nitrogen	1.10	0.60	0.45
*Total Phosphate	0.19	0.09	0.21
*Gums[a]	5.5	5.7	6.3
TSAI: Ash Ratio	7.93	5.65	4.61
Reducing sugar: Sucrose Ratio	0.54	0.64	0.39
Phosphate: Total Nitrogen Ratio	0.17	0.15	0.47
Gums[a]: TSAI Ratio	0.03	0.05	0.08
Aroma by Steam Distillation	Good	Fair	Indifferent

*Results expressed as percentage weight to weight

[a] Gums are classified under oily, waxy or greasy kind of substances which are insoluble in water and soluble in solvents such as ether, hexane and chloroform(Schoonees and Pillay 2004). They are removed in sugar factory at initial stage by clarification technique as they may create problems during refining of sugar. They form precipitate after addition of acidified alcohol.

The composition of typical molasses consists of 55% fermentable sugar, in which it contains approximately 31-36% sucrose and 20% mixture of glucose and fructose. Rise in temperature especially exceeding 60°C causes thermal degradation in molasses through Maillard reaction resulting in sugar losses. According to Chen 1985, molasses are stable even up to 45°C making it as an ideal temperature for storage (Lea and Piggott 2012). Easy storage condition and low price makes molasses a great raw material for producing spirits (Medeiros, de Matos et al. 2017).

2.3 Production of rum at an industrial level

Production of rum goes through the basic steps as with other distilled beverages. Although rum manufacturing follows the same basic steps as other distilled beverages (**Figure 1**); fermentation, distillation, ageing, blending and bottling (Medeiros, de Matos et al. 2017), the main difference stems from the 'must' preparation. A brief description on the process of manufacturing rum will be included in the following sections.

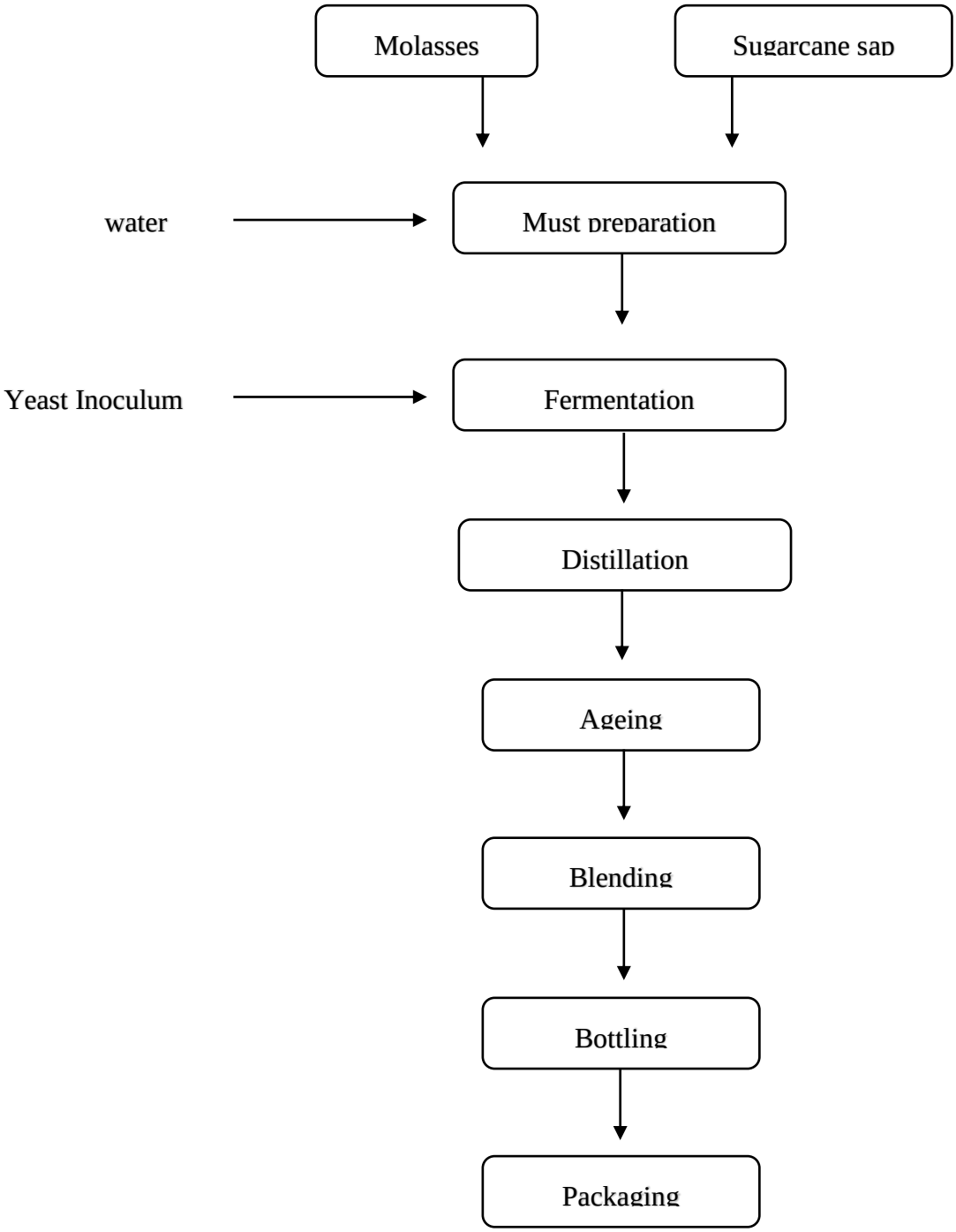


Figure 1. Flowchart for industrial rum production (Medeiros, de Matos et al. 2017)

2.3.1 Must preparation

Must preparation can be made with suspension of molasses and water (refer to **Figure 2.**) or with the fresh sap extracted from cane. Generally, rum is made up of molasses but rhum agricole is an exception, as it is purely made up of sugar cane juice, a traditional method used in French culture. During the preparation of must, some extra sucrose or glucose are added to elevate the °Brix before fermentation. Must has an average sugar concentration of 17-28 °Brix and pH ranges from 4.8 to 5.0. The must mixture can be pasteurized before it proceeds to the fermentation step to give better microbiological stability. Hindering the growth of bacteria is essential to prevent off-flavors such as hydrogen sulfide and sulphurous compounds from forming (Suomalainen and Lehtonen 1979).

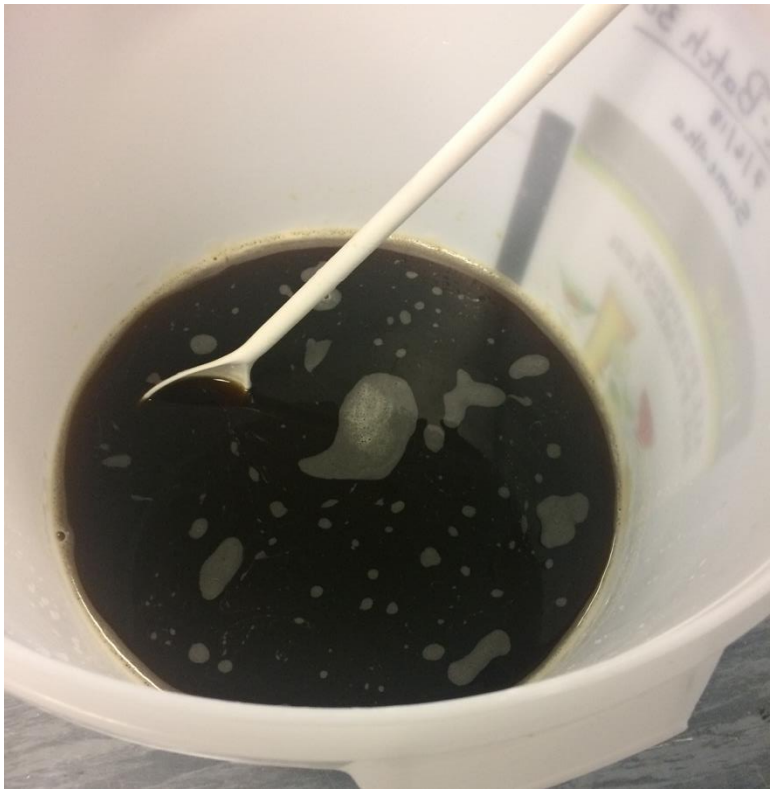


Figure 2. Must preparation with suspension of water and molasses.

2.3.2 Addition of yeast

Flavour development begins once the yeast is added to the must. Yeast is a living organism which consumes sugar and converts it to alcohol, carbon dioxide and heat. In present day, distillers do not rely on wild yeast as specific strains such as *S. cerevisiae* are readily available which can give the exact desired flavour profile (Medeiros, de Matos et al. 2017). Fermentation conditions and yeast types are two major factors that contribute to the final aroma of the rum through biochemistry. The structure and function of the yeast plasma membrane modulates the

movement of organic compounds into the yeast cell and the release of yeast metabolites from cell into the medium (Suomalainen and Lehtonen 1979). Hence the lipid composition and lipophilic nature in the yeast membrane determines the rate of penetration of organic compounds into cells. During fermentation, the yeast produces a broad range of compounds called 'congeners' but the dominant congeners are fusel alcohols (any alcohols apart from ethanol), fatty acids, esters and aldehydes.

2.3.3 Fermentation

The development of flavour congeners during fermentation not only depend on the action of yeast on sugar but also on temperature and duration. The period of fermentation affects the concentration of carboxylic acids, aldehydes and esters in rum. For example, the longer the must is left to ferment, the higher is the acidity and off-flavour compounds in the final rum. Distillers follow their own standardized time to ferment the must, which could be between 20 to 26 hours for light rum while 2 to 4 days or 1 to 3 weeks for heavier rum, depending on the ambient temperature (Medeiros, de Matos et al. 2017). **Table 3** presents the findings from literature on the major flavour compounds generated during fermentation of rum.

Table 3. Major flavour compounds developed during fermentation of rum.

Compound group	***Specific organic compound***	***Reference***
Alcohols	Butanol	(Masuda, Yamamoto et al. 1985)
	Pentan-1-ol, Propanol, 2-methylpropanol, 2-methylbutanol, 3-methyl butanol, 2-phenylethanol, Isobutyl alcohol, Amyl alcohol, Iso amyl alcohol	(Suomalainen and Lehtonen 1979)
Esters	Ethyl octanoate	(Suomalainen and Lehtonen 1979)

	Ethyl decanoate, Ethyl dodecanoate, Ethyl tetra decanoate, Ethyl hexadcanoate, Ethyl palmitoleate, Ethyl acetate, Ethyl hexanoate, Ethyl 9-hexadecenoate, Ethyl lactate, 2- & 3-methylbutyl acetate, 2-phenylethyl acetate	(Masuda, Yamamoto et al. 1985)
Acids	Acetic acid Octanoic acid, Decanoic acid, Dodecanoic acid, Hexanoic acid, Hexadecanoic acid, Butanoic acid	(Masuda, Yamamoto et al. 1985) (Suomalainen 1971)
Aldehydes	Acetaldehyde, Furfural, Nonanal	(Masuda, Yamamoto et al. 1985)

When fermentation is carried out at high temperature (35°C to 37°C) it greatly accelerates the speed of the fermentation process, however, the down side is it can easily lead to spoilage. The high temperature will allow the yeast to consume the fermentable sugars in the must quickly while exhausting it and resulting in weak must that is prone to microbiological spoilage (Broom 2003). To solve the problem the method adopted by Foursquare's Richard Seale is now followed by all rum industry. Richard Seale is known as a master distiller, blender and ambassador for all rum which are produced at their family owned Foursquare Rum Distillery in Barbados since 1926. The method consists of closed cooled fermenter which runs at 30^{o}C resulting in higher degree of control over the ferment. In order to control quick exhaustion of yeast cells, the must is drip fed into fermenter over the period of 17 hours giving yeast a controlled exposure to sugar. According to Seale, it slows down the whole fermentation process but results in good consistent wash. The whole process takes about thirty-six to forty hours giving 9 to 10% ABV in the wash.

2.3.4 Synthesis of fusel alcohol, esters, fatty acids and aldehydes during fermentation

2.3.4.1 Catabolic pathway

The metabolism of *S. cerevisiae* highly depends on nature and concentration of substrates and the state of aeration and temperature (Suomalainen and Lehtonen 1979). Studies have shown that 1 gram of yeast contains 25% dry matter which gives 3.9 m^2 of surface area, thus showing that nutrients like minerals, sugars and amino acids can penetrate into yeast cell and yeast metabolites can easily travel from the cell into the medium. It is also stated that fatty acid solubility dictates the penetration rate. The rate of penetration is also influenced by the molecular size and the degree of branching of carbon chain (Suomalainen and Lehtonen 1979).

In 1904, Felix Ehrlich found that fusel alcohols derived from amino acids (Hazelwood, Daran et al. 2008) during fermentation by *S. cerevisiae*. After separating isoleucine and characterizing it, he discovered structural similarities between this amino acid and active amyl alcohol and later on leucine and isoamyl alcohol. Ehrlich proved that production of fusel alcohol can be increased with addition of leucine or isoleucine to the yeast fermentation mixture. Furthermore, Ehrlich propounded that formation of fusel alcohol with carbon dioxide and ammonia was due to splitting of amino acids by hydrating enzyme activity. During Ehrlichment pathway, amino acids are taken up by yeast throughout the fermentation process (Hazelwood, Daran et al. 2008). α-keto acid is the end product of the transamination reaction in Ehrlich pathway. Once transamination reaction ends, the reaction is irreversible. α-keto acid under goes decarboxylation and reduction to fusel alcohols.

In the decarboxylation step, carbon dioxide is removed giving fusel aldehyde with the help of alcohol dehydrogenase (Procopio, Brunner et al. 2014). The last step reduction/oxidation of fusel aldehyde is carried out to its corresponding alcohol and acid (Romagnoli 2014). Fusel aldehyde are reduced/oxidized to fatty acids +A under aerobic conditions, whereas they are reduced to fusel alcohols under anaerobic conditions.(Hazelwood, Daran et al. 2008). A possible explanation to this step is that, the ratio of higher $NADH/NAD^+$ during anabolic growth aids reduction of fusel aldehydes (Procopio, Brunner et al. 2014). Production of fusel alcohol can substitute glycerol production in glucose. This may be reason of having positive impact on energy metabolism as it requires ATP input, as an electron sinks for excess NADH production in biosynthesis reactions.

Neubauer and Fromherz in 1911, further developed Ehrlich pathway into a more detailed metabolic scheme called the catabolic pathway (Hazelwood, Daran et al. 2008). As seen in **Figure 3,** the catabolic pathway shows that α-keto acid derived from the amino acid can be de-carboxylated into aldehyde and then subsequently reduced to fusel alcohols.

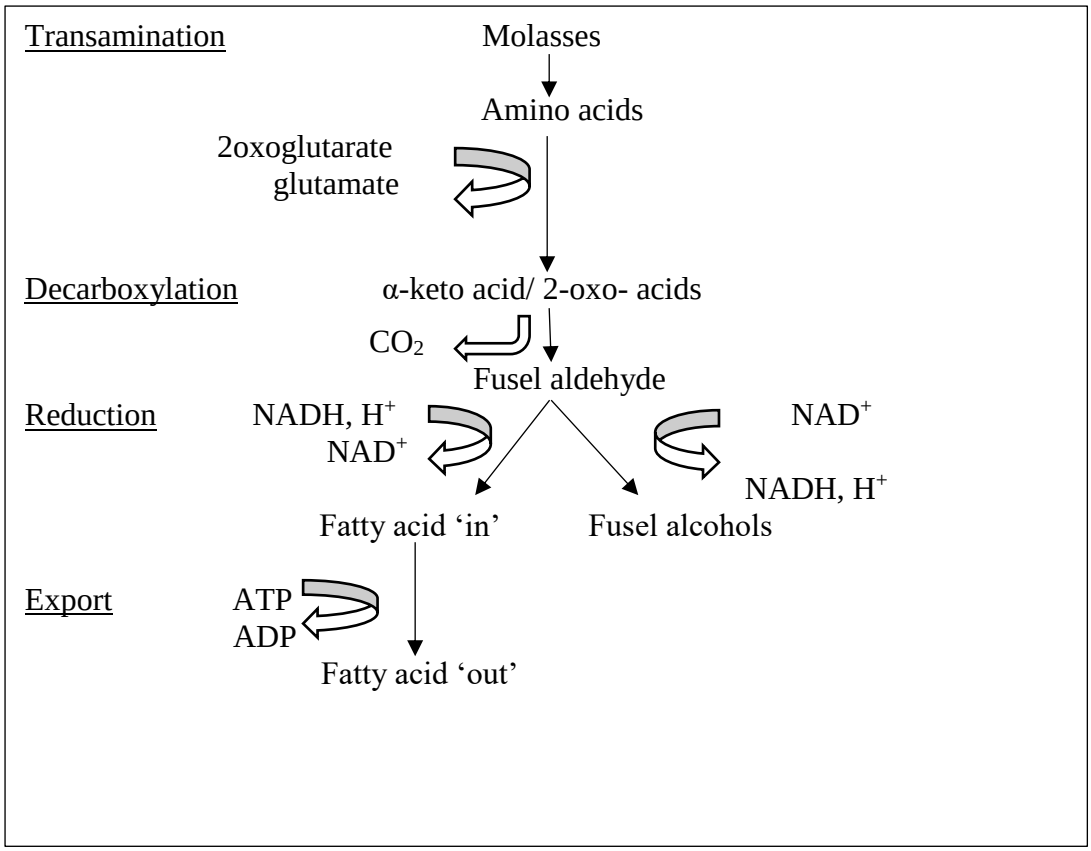


Figure 3. The Ehrlichment pathway or catabolic pathway. It consists of four intermediate steps to produce fusel alcohols from amino acids during fermentation with S. cerevisiae (Hazelwood, Daran et al. 2008).

Sentheshanmuganathan in 1960 discovered in the cell extract of *S. cerevisiae* that a number of amino acids such as aspartate, isoleucine, leucine, methionine, norleucine, phenylalanine, tryptophan and tyrosine were able to be converted to α-ketoglutarate (Sentheshanmuganathan 1960). The reaction was thought to be catalysed by aminotransferase therefore providing the initial step to the catabolic pathway (Hazelwood, Daran et al. 2008). He established the key series of reaction for the catabolic pathway which incorporates Ehrlich pathway by showing transaminase, de-carboxylase and alcohol dehydrogenase.

Fusel alcohol or higher alcohols can contribute to the flavour of rum and can be synthesized by the catabolic pathway via α-keto or 2-oxo-acids (Lodolo, Kock et al. 2008). In the cell

membrane of yeast, there are numerous amino acid transporters that are made up of polytopic membrane protein with 10-14 transmembrane segments that takes up amino acids (Lea and Piggott 2012, Popov-Čeleketić, Bianchi et al. 2016). These transporters have a range of substrate specificity where some are highly specific which means they transfer only one single type of amino acid. Throughout the fermentation process, amino acids such as the ones mentioned in **Table 4** are absorbed by the yeast cells so that the catabolic pathway can take place generating their corresponding fusel alcohols. Study done by Suomalainen 1971, tells that rum contains 600 mg/L of total fusel alcohol. During yeast fermentation isoamyl alcohol is the main fusel alcohol produced, followed by propyl alcohol, isobutyl alcohol and amyl alcohol (Suomalainen 1971).

Table 4. Amino acids and its corresponding fusel alcohols (Hazelwood, Daran et al. 2008).

Amino acid	**Fusel alcohol**	**International Union of Pure and Applied Chemistry (IUPAC) name**
Leucine	Iso amyl alcohol	3-methylbutan-1-ol
Valine	Isobutanol	2-methylpropan-1-ol
Isoleucine	Active amyl alcohol	2-methylbutanol
Methionine	Methionol	3-methylsulfanylpropan-1-ol
Tyrosine	*p*-Hydroxyphenyl ethanol	4-(1-hydroxyethyl) phenol
Tryptophan	Tryptophol	2-(1H-indol-3-yl) ethanol
Phenylalanine	2-phenylethyl alcohol	2-phenylethanol
Threonine	n-propanol	Propan-1-ol

2.3.4.2 Anabolic pathway

The anabolic pathway (refer **Figure 4**) is also another biochemical route for the yeast to produce fusel alcohols with the addition of fatty acids and aldehydes. In this pathway, the α-keto acids are produced via de novo biosynthesis of amino acids by carbohydrate metabolism (Pires, Teixeira et al. 2014). Acetaldehyde is a precursor of ethanol and is a by-product of alcoholic fermentation by yeast. During the fermentation process, small amount of branched-chain aldehydes and 2-phenylacetaldehyde are excreted from catabolism of their respective amino acids. As glucose breaks down into two molecules of ATP it gives out pyruvate which will turn itself to acetyl-Coenzyme A by oxidative decarboxylation (Denko 2008, Pires,

Teixeira et al. 2014). The presence of oxygen inside the mitochondria makes acetyl-CoA enter into the Kerb's cycle to form adenosine triphosphate (ATP). The condition when there is absence of oxygen, acetyl-CoA gets enzymatically esterified with ethanol to turn into acetate esters.

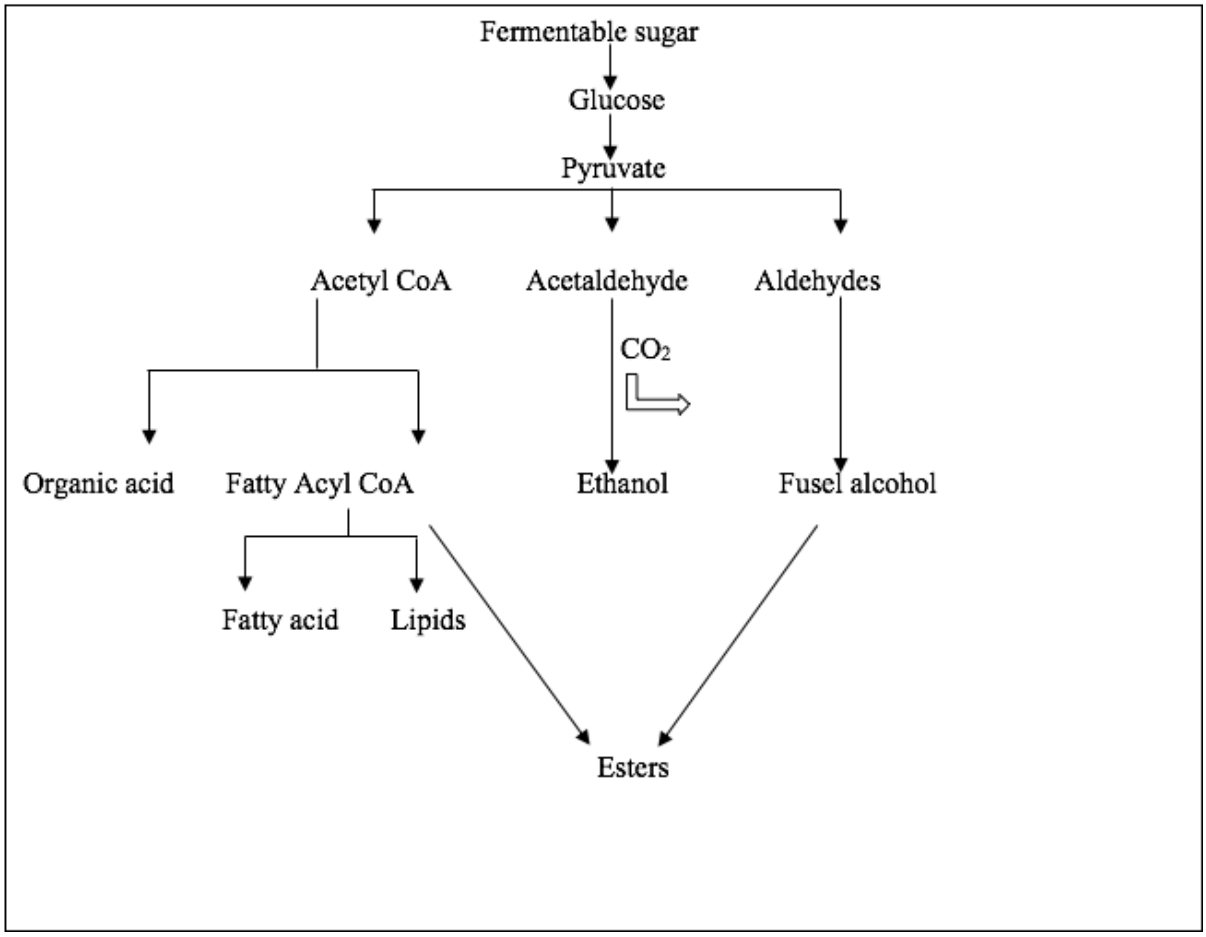


Figure 4. Anabolic Pathway. Higher alcohols are produced from the fermentable sugars as the by-product of amino acid synthesis.

The fatty acids are the largest acidic group to be produced by yeast (Suomalainen and Lehtonen 1979). Its content differs according to the type of beverage. According to Suomalainen, heavy rum like Martinique can contain 600 mg/L of its total ester concentration. The most important compounds of fatty acid synthesized by yeast are capric acid and caprylic acid followed by propionic, isobutyric, iso valeric, valeric, lauric, myristic, palmitic, palmitoleic, etc. Only rum contains 2-ethyl-3-methyl butyric acid.

Table 5. Fatty acids compounds and their (IUPAC) name.

Fatty acids compounds	**International Union of Pure and Applied Chemistry (IUPAC) name**
Capric acid	Decanoic acid
Caprylic acid	Octanoic acid
Propionic acid	Propanoic acid
Iso butyric acid	2- methylpropanoic acid
Iso valeric acid	3- methylbutanoic acid
Lauric acid	Dodecanoic acid
Myristic acid	Tetradecanoic acid
Palmitic acid	Hexadecanoic acid
Palmitoleic acid	(Z)- hexadec-9-enoic acid
2-ethyl-3-methyl butyric acid	2-methyl-3-methylbutanoic acid

In fermented alcoholic beverages esters, especially acetate esters, represent the main class of flavour metabolites. The condensation reaction between acetyl-CoA and higher alcohol by enzymic catalysis gives esters (acetate esters) (Lea and Piggott 2012). In other words (as shown in **Figure 4)**, fatty acyl CoA from 'Anabolic pathway' and fusel alcohol from 'Ehrlich pathway' together produce esters.

Fatty acid ester content differs in different beverages according to the strength of their aromas. For example, perceived strength of total ester aroma concentration in Puerto Rican rum is 50 mg/l, in contrast, Martinique heavy rum contains 600mg/l. Depending on the content of aroma compounds, rum can be classified as light or heavy rum. Most important compounds of fatty acids esters are ethyl ester, isobutyl ester and isoamyl ester. Other like ethyl hexanoate, phenethyl alcohol and ethyl esters of octanoic and decanoic acids are also present. These esters are recognized as "fruity esters" as they give sweet pleasing aroma and thus are considered positive aroma (Suomalainen and Lehtonen 1979). Suomalainen and colleagues found that the presence of yeast during fermentation highly affected the amount of ethyl esters derived from the fatty acids mentioned in **Table 5**.

2.3.5 Distillation

After fermentation, the wash contains ethanol mixed with non-fermentable sugary liquid and yeast biomass. Distillation is a separation process to extract the ethanol from the wash to produce a spirit (raw rum). The principle of distillation takes advantage of the different boiling point between ethanol and water. At a certain temperature, ethanol vapour will liberate from the water-ethanol mixture leaving water behind. The absolute boiling point of ethanol is hard to measure due to the complexity of the must which consist of other chemical compounds produced during fermentation. The ethanol vapour (the distillate) is condensed to get spirit which contains 60 to 94% ABV. The large ABV variation depends on the type of distillation unit employed. As mentioned earlier, the fermentation step produces a wide range of congeners which will also get carried over with the distillate. The congeners with boiling point lower than ethanol (e.g. methanol, acetaldehyde ethyl acetate and acetone) will come out first during the early stages of distillation. Compounds with higher boiling point with respect to ethanol will be distilled out in the later stages (e.g. propanol, acetic acids, etc) (Hazelwood, Daran et al. 2008). Ethanol is a neutral sprit, but the congeners are the main contributor to the unique aroma of rum. Since different congeners come out at different time during distillation, the distillates are collected in batches. An experienced distiller will combine different portions of the collected distillate to make the final rum as not all congeners are desirable. Distillers decide their own "cuts" i.e. the fraction of distillate to separate because these fractions contain different levels of congeners in the rum (Spaho, Dürr et al. 2013). The compounds present in "head" cut have strong, harsh solvent like aroma. Acetaldehyde, acetone, ethyl acetate and methanol are separated first (heads) from the wash because of their low boiling point. In the heads cut, methanol in particular is highly undesirable as it produces toxic metabolites after consumption (Kruse 2012, Spaho, Dürr et al. 2013). Once the compounds from head cuts are separated, a mixture of complex volatiles and esters together with ethanol will be separated which is called the "heart" cut (Spaho, Dürr et al. 2013). Heart cut is of commercial value as it contains high amount of desired flavour congeners. However, isoamyl alcohol which tends to appear in the heart cut gives unacceptable odour and bitterness in rum (Kang, Hwang et al. 2016). "Tail" cuts are not desirable in rum as they consists of volatile fatty acids such as acetic acid, butanoic acid, 2- and 3- methyl butanoic acid, propionic acid, butyric acid which impart an offensive odour, plastic like aroma and unpleasant flavour and are generally obtained towards the end of the distillation process (Bamforth and Ward 2014, Franitza, Granvogl et al. 2016).

2.3.6 Copper pot still for distillation

Originally, distillation of fermented molasses was done in alembic copper pot stills with no reflux (refer to **Figure 5**) (Lea and Piggott 2012). Previously, pot distillers were built by small scale sugarcane farmers who processed 'tafia' (cheap rum) in local parts of the Caribbean. Pot distillation is a batch system (Broom 2003) where the copper pot is specifically used to remove off flavour sulphur compounds to non-odorous salts during the distillation process (Bruno, Vaitsman et al. 2007). Common pot distillation starts with boiling the wash and collecting the vapours (Broom 2003). At this stage, distillers separate 'heart' (at roughly 72% ABV) from the collected rum distillate. The pot still produces more strong and heavier rum – rum with more ester compounds (Lea and Piggott 2012). Once the process starts, lighter compounds (having low boiling point) are first to be liberated giving out harsh, strong aroma. As distillation proceeds, heavier congeners (having higher boiling point) are released and the aroma of the alcohol changes from harsh to fruity. Gradually at the end of the process oily, leathery aroma are liberated which are musty or rancid. It is the distiller's knowledge and skill to cut the congeners to create his signature style. In **Figure 5**, the top section (vapor pipe) above coppered wooden part i.e. neck, lyne arm etc. is made from copper and brass. The lyne arm elongates from top of the still to the bottom of the retort on which rectifier is installed followed by a condenser. Retort and rectifier are optional and can be omitted depending on distiller's choice. Retort vessel consists of low or high wines (alcoholic mixture with water) whereas rectifier is a water container containing series of copper tubes. At the top of the rectifier, a vapour pipe extends and opens up in condenser.

Distillation in pot still starts with heating the wash where the hot vapour climbs the swan neck (vapour pipe) and exits in condenser via retort and rectifier. Some distillers have simple design where retort and rectifier portion are omitted. The hot vapours are passed from retort to rectifier and further to condenser by baffle plates. By controlling energy input and water supply, the quality of resulting rum can be controlled. The temperature of water in rectifier is controlled at 45-50°C. The subdued vapours finally condense congeners briefly as heads, hearts and tails. Generally, rum being collected after 1 to 2 hours with 85% abv and volume of 360-370 litres. Distillation continues till it reaches 43% abv. This technique produces a distinctive full-bodied pot still rum.

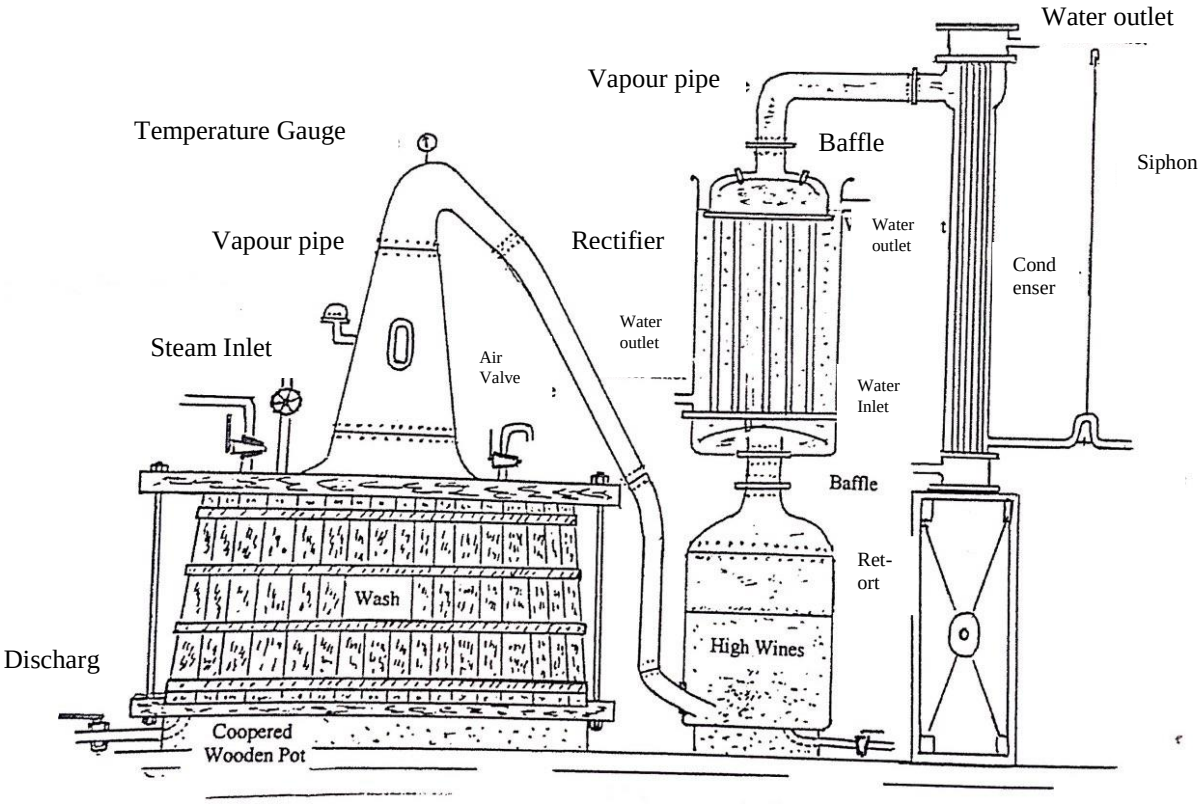


Figure 5. Single copper pot still for rum production (Lea and Piggott 2012).

2.3.7 Column still for distillation

A Scottish distiller, Robert Stein in 1827, sketched the first continuous column still design (Broom 2003). The design was further developed and patented by Aeneas Coffey, an Irish man who got recognized all over the world for his 'The Coffey (continuous) still' or 'Patent still' design. The Coffey's still design was an innovative distillation unit that is inferior to the pot still design. The primary benefit was to increase volume and decrease the cost value whereas the secondary benefit was to reduce the amount of fusel oils in the final rum. The large scale rum producers, who are engaged in several sugar plantation and have a continuous supply of molasses use continuous distillation columns (Lea and Piggott 2012). The theory behind column still distillation is that, heating the wash, converting it to the vapour form and collecting the pure spirit by mean of condensation. The Coffey column still consists of two columns; analyser and rectifier. The 1st column consists of multiple plates where each plate is a single stage distillation and 2nd column is a huge condenser (reflux). The multiple plates in analyser carry out thousands of mini distillations helping in extracting pure congeners. In **Figure 6**, The analyser comprises series of horizontal perforated copper sieve plates, compressed between wooden frame whereas the rectifier consists of series of copper coils in winded form used for

condensation of the vapour. The wash enters the bottom of the analyser and gets heated by the help of steam. Subsequently, the vapour evaporates in upward direction touching each perforated plates resulting in extraction of more and more pure alcohol. Once the vapour reaches the top of the analyser it travels down through the vapour pipe to the bottom of the rectifier. In the rectifier the coiled tubes consist of cold water which aid the hot vapour to condense it to liquid thus collecting pure spirit at the outlet.

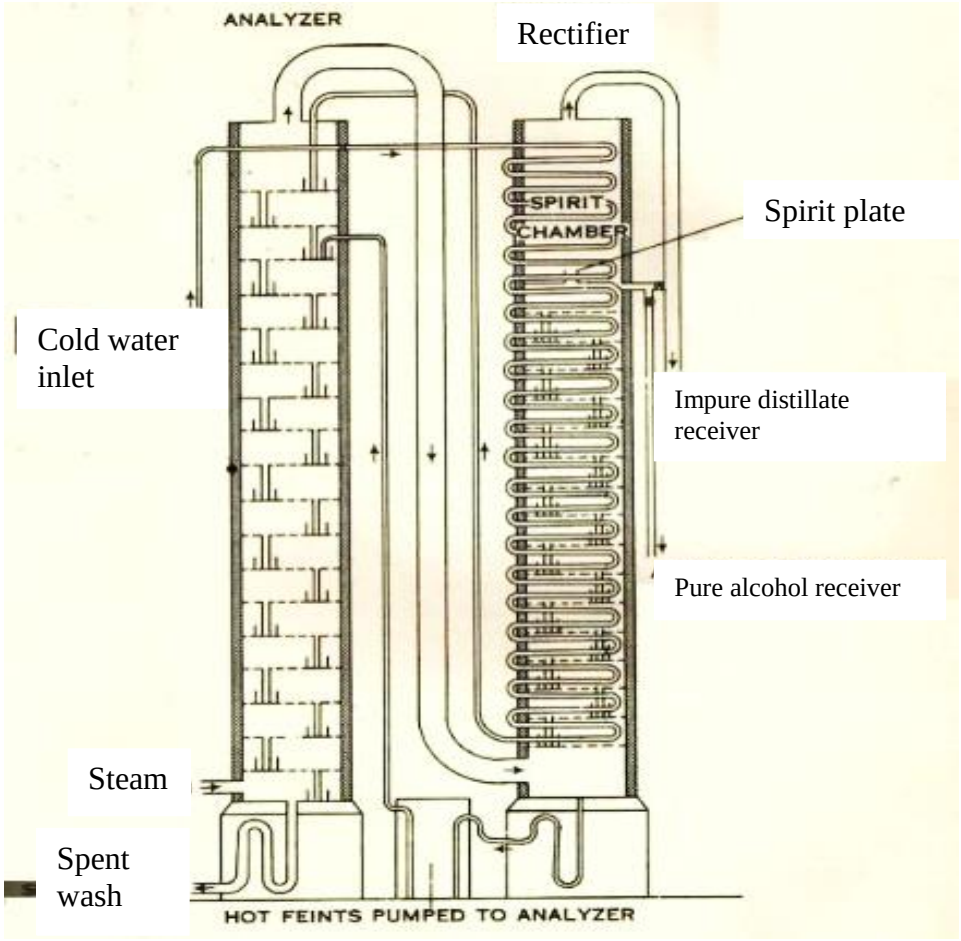


Figure 6. Column Still for production of Rum (The Whisky Exchange - AA, 2013).

2.3.8 Maturation and blending

Maturation of rum is always done in used oak barrels. Rum which are kept in oak barrels is aged in used Bourbon barrels obtained from white oak (*Quercus Alba*). Not only does it impart distinct colour to the rum but also numerous flavour compounds as detailed in **Table 1** in the Introduction section. Barrels are made by burning the inside surface through a process called "charring". Charring improves the woods physical and chemical properties, caramelizing the

sugars and make tannins and vanillin to be easily extractable. “Angel’s share” is described as the process of ageing where rum is soaked into the oak barrel pores to promote the extraction of colour and additional flavour compounds. The flavour profile of rum changes as depending on how long the rum is left in the oak barrels and the number of times the barrels have been used. There are stainless steel tanks for maturation of white rum. It consists of perforated pipe and a release valve at the top, stream of filtered air bubbles through the rum and vented out to remove unpleasant esters from rum.

Blending process is the last step to get the final perfect glass of rum. All is done by the master blender who assembles different marks and ages of rum to get consistent product. Blender uses his experience and olfactory organ to decide perfect final rum.

2.4 Types of Rum

The grades or types of rum are generally identified by the place where they are made and by flavour, colour and taste. Types such as white rum, dark rum, amber rum, over proof rums and spiced rums are found in specific parts of the world. Dark rum has its unique colour such as brown, black or red and its said to be darker than amber rum. It is made by molasses and is aged in charred oak barrels for more than two years. Gold rum also known as ‘amber’ rum are also aged in charred oak barrels but for a shorter period of time (18 months). However, the charred oak barrels must be previously used for ageing bourbon. Sometimes a more intensive golden colour is achieved by adding caramel. White rum is also called ‘silver’, ‘light’ or ‘clear’ rum has lighter flavour compared to the amber and dark rum, they are generally used in cocktails and are not drank neat. White rums are commonly kept in stainless steel vessels or casks. Charcoal filters are used to remove colour and impurities after ageing for 1 to 2 years in barrels. Over proofed rums are the most preferred rums in market of Caribbean Islands. They have more alcohol content than the standard 37-40% ABV. They contain almost 70 to 80% ABV and are generally used in punches. Spiced rum are infused with spices such as cinnamon, aniseeds, ginger, rosemary or pepper to create a variety of flavour for all tastes. They are generally dark in colour and sometimes caramel are added to enhance the sweetness.

2.5 Challenges in rum making

Currently the biggest challenge in rum industry is to know when to make appropriate “fraction” or “cuts” as it directly influence the flavour and taste of rum. Although, currently it is done by sensory approach - smelling and tasting the rum sample by experienced distillers. Therefore, the aim of the research is to objectively determine the appropriate time to collect the head, heart and tail cuts by monitoring the concentration of certain congeners during single and double distillation of rum. Apart from the production process, the raw material also plays an important role in influencing the flavour of rum (de Souza, Vásquez et al. 2006). There are number of studies done on characterizing the volatiles in the final rum products but how these final rum products were processed and treated were never reported. Therefore, as part of this research, the influence of molasses to sugar ratio in the must during fermentation on distilled rum will be investigated.

3 Project aims

The main objective of this study was to:

- To find out concentration of particular congener to be present in distillate in order to make appropriate and objective distillation ‘cuts’ for head, heart and tail fraction without the need to use sensory approach.
- To analyse the effect of molasses to sugar ratio during fermentation, on the flavour of rum

4 Experimental methodology

4.1 Preparation of non-aged rum

Rum must was prepared by mixing 3 kg of food grade blackstrap molasses (Hauraki Brewing Company Ltd, NZ) with 4 L of deionized water and 3 kg of glucose or dextrose (Davis Food Ingredients, NZ). The must was heated to 75°C to 80°C in order to fully dissolve the dextrose and molasses in the mixture (refer to **Figure 7**). The must was then transferred to a 30L plastic fermenter bucket consisting of air lock system (iMake Ltd, NZ). The must was topped up with deionized water till the 20 L mark in fermenter bucket. In the second part of the experiment the ratio of molasses and dextrose was varied. The following table displaces 5 different concentration of molasses and dextrose used for study. For each of the following type, 3 batches were prepared and their average was then analysed and plotted.

Table 6. Varying types of sugar to molasses concentration in the must before fermentation and distillation.

	Glucose content in kg	**Molasses content in kg**
Type 1.	6 Kg Glucose (Pure Glucose)	-
Type 2.	4 Kg Glucose	2 Kg Molasses
Type 3.	3 Kg Glucose	3 Kg Glucose
Type 4.	2 Kg Glucose	4 Kg Molasses
Type 5.	-	6 Kg Molasses (Pure Molasses)

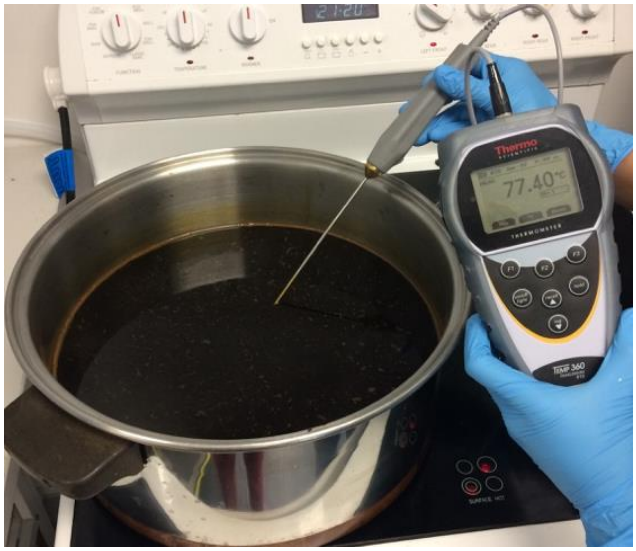


Figure 7. Suspension of water and molasses heated to 77.40°C.

Before adding the yeast, the must was cooled down to 25°C as high temperature (above 30°C) would generate undesirable flavour compounds during fermentation as mentioned in Section 3, Literature review. A commercial distiller's rum yeast of 72 g (Still Spirits, UK) was pitched into the must (refer **Figure 8**) and left to ferment for 3-4 days in a temperature-controlled room set at between 22°C to 25°C.

Figure 8. Addition of Rum Distiller's yeast into the must.

After fermentation, 130 g of clearing agents – Turbo Clear (Still Spirits, UK) which consisted of silicic acid and chitosan was added into the wash and was left to settle for 24 hours in order for the yeasts to flocculate to the bottom of the fermenter and to prevent yeast cells from carrying over to the distillation unit. The clarified fermentation solution was siphoned into a commercial Alembic pot copper still unit (iMake Ltd, NZ). The condenser was connected to a cold water source and the flow rate was adjusted to 3 L per minute (refer **Figure 9**).

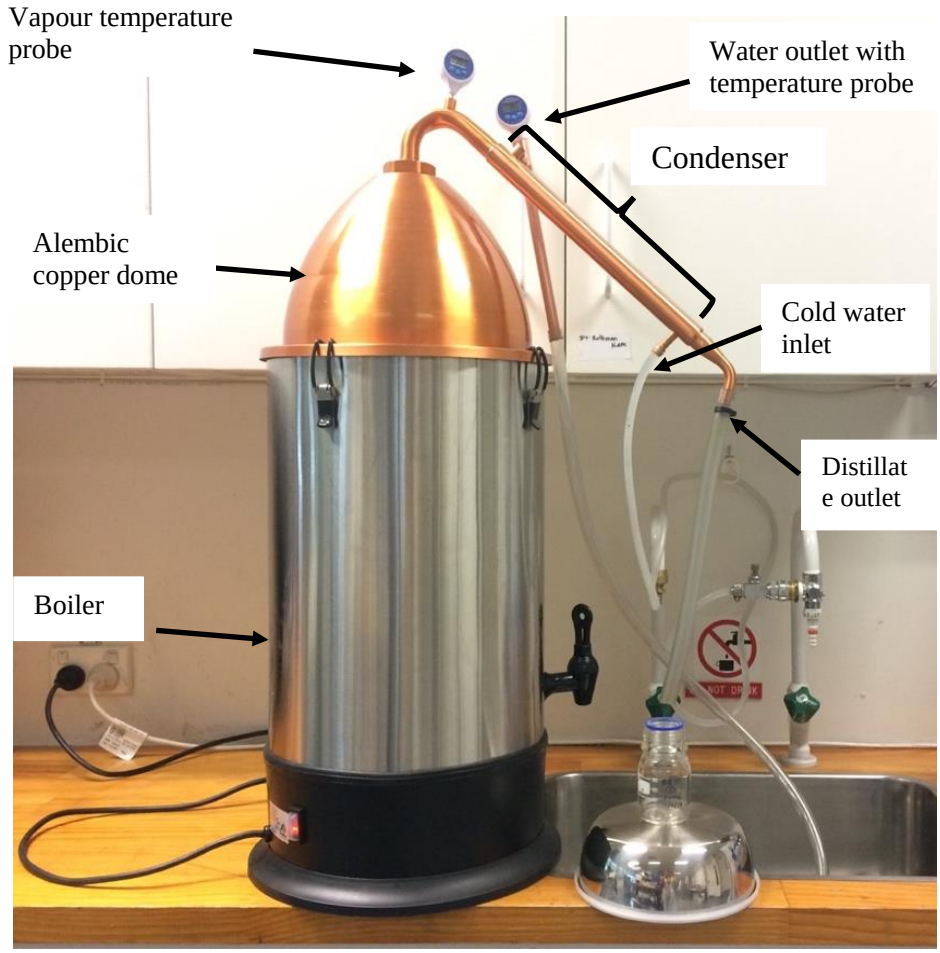


Figure 9. An Alembic copper pot still for distillation of rum.

4.2 Collection of rum distillate

During distillation, the 3 L/min water outlet from the condenser had an outlet temperature between 38°C to 39°C. The volume of distillate was fractionated into the following volumes in tandem as shown in **Table 7**;

Table 7. Volume of distillate collected in tandem (not cumulative).

Bottle number	Volume of distillate fractionated
1	100 ml
2	100 ml
3	200 ml
4	200 ml
5	500 ml
6	500 ml
7	500 ml
8	500 ml
9	500 ml
10	500 ml
11	500 ml
12	500 ml
13	500 ml

The temperature of the distillate vapour was noted down during each collection until it reached 99°C, at this point the distillation process was stopped. During each collection, the alcohol content was measured by an alcoholmeter (Alcometer n. Tralles, Still Spirits) and the pH was measured using a pH meter (Eutech, pH 700). After analyses of single distilled rum; double distillation of rum was carried out. The entire rum obtained in single distil rum was collected in distiller and distilled again giving out double distil rum. Analyses was carried out in same manner as of single distilled batches.

4.3 Analysis of distillate

4.3.1 Gas chromatography with flame ionisation detection (GC-FID)

1 ml of neat rum sample was pipetted into a 1.5 ml GC amber vial which was loaded onto a Shimadzu Autosampler (AOC-20i Autoinjector, Shimadzu). A Shimadzu GC-2010 Plus gas chromatograph with flame ionization detector (FID) was equipped with a FAMEWAX capillary column (Restek, USA) with (30m x 0.32mm ID, film thickness 0.25μm). 1.5 μL of sample was injected into the GC with split ratio of 1 to 40 with respect to the total column flow. The carrier gas used was nitrogen and column flow was 1.11 mL/min. The temperature program started at 30°C and was held for 10 min before ramping to 230°C at the rate of 10 °C/min and was held for 5min. The injector and detector temperature was 250°C.

4.3.2 Calibration curve

Calibration curves were obtained for chemicals such as acetaldehyde, acetone, ethyl acetate, methanol, propanol, iso amyl alcohol, iso butyl alcohol, phenyl ethanol, amyl acetate, acetic acid, ethyl isobutyrate, ethyl hexanoiate, octanoic acid and decanoic acid. For obtaining each chemical standard curve, 0.2560 gm of chemical was added to 60% ethanol solution. Distilled water was used to top up till the mark of 100ml conical flask.

Calibration curves were obtained by using the concentration and GC area values from the analysis. These experimental concentration and GC area were then plotted in MS Excel and the r^2 value was obtained by using the equation function available.

4.3.3 Solid phase microextraction (SPME) with gas chromatography mass spectrometry (GC-MS)

Rum samples in duplicates from single distillation and double distillation were prepared for SPME analysis. 1990 μL of rum sample was pipetted into a 10ml clear glass vial (Thermo Fisher, UK) with 0.30 g of sodium chloride (NaCl) (Fisher Scientific, UK). Addition of salt increases the extraction of chemical compounds from the sample (Pino, Martí et al. 2002). According to the study done by Pino and his colleagues (2002), the adsorption of chemical compounds increases when there is increase in salt concentration. Each vial was spiked with 10μL of 0.80 mg L^{-1} 2-chlorophenol solution as an internal standard. The vials were sealed

with a18 mm screw cap with a silicon blue PTFE white septum (Gerstel, Germany) and then vortex for 1 minute to dissolve the salt. A blank was made similarly using distilled water.
The samples were loaded into the GERSTEL Multi-Purpose Sampler and incubated for 5 minutes at 40°C and agitated for 1 minute. This was then followed by a 2 second stationary period. The volatile compounds in the rum were extracted by inserting a CAR/DVB/PDMS/ SPME fibre (Sigma Aldrich) into the headspace of the vial where the fibre was exposed for 10 minutes. The SPME fibre was then inserted into the GC injector (Agilent Technologies, 7890B GC system) which was heated to 250°C. The GC injector was operating in a splitless mode. Helium was used as carrier gas at a constant flow of 1 mL/min to separate volatiles in the GC column (Phenomenex, Nebron, ZB-1701) with (30m x 0.25mm ID, film thickness 0.15μm). The GC oven was held at 40°C for 5 minutes before being increased at a rate of 4°C/min to 200°C. The GC oven was held at 200°C for 1 minute and then increased to 260°C at a rate of 10°C/min. The GC oven was then held at 260°C for a further 5 minutes. The GC-MS results for each sample was collated by the GERSTEL MAESTRO software, and the GC-MS peaks of each sample was analysed with MassHunter WorkStation-Qualitative Analysis software (Agilent, USA). Any compounds identified in the blank sample, excluding the internal standard were ignored in the rum samples. The compound for each peak was identified through the NIST MS Spectral library and the area of the peaks was semi-quantified with respect to the normalised internal standard peak. Higher GC signal means higher concentration for that compound. The volatile compounds were separated into major and minor compounds based on their GC signal. Minor compounds were defined as having a normalised GC signal below 1.2.

4.3.4 LC-MS analysis of amino acids

In triplicates, approximately 45 ± 0.1 mg masses of molasses was placed in a 1.5 mL microcentrifuge tube. A 1.4 mL volume of 50% methanol containing 10 mg L^{-1} of 2,3,3,3-d4-alanine [d4A] was added and the tubes were vortexed until the molasses dissolved. Tubes were centrifuged at 10,000 rpm for 5 minutes at 4 °C (Z216MK, HERMLE Labortechnik GmbH, Germany). A 10 µl volume of extract was used to perform pre-column derivatisation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate following a method adapted from (Salazar, Armenta et al. 2012).

The LC-MS was an Agilent 1260 Series liquid chromatograph comprising a G1311B quaternary pump, G1329B thermostatted autosampler and a G1330B thermostatted column compartment (Agilent Technologies, Santa Clara, USA). Mobile phase A was 0.6% formic acid in ultrapure water and mobile phase B was 0.1% formic acid in acetonitrile. The column was a Phenomenex Kinetex EVO C18 column, measuring 2.1x150 mm with 1.7 µm diameter packing material, maintained at 25 °C. The chromatographic gradient was held for one minute at 1.5% B and then ramped to 13% B at eight minutes, 17% B at 15 minutes and 80% B at 16 minutes before returning to 1.5% B at 17.5 minutes. The flow rate was 300 µL min^{-1}, the total run time was 28 minutes and the injection volume was 5 µL. For detection an Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Optimum Multiple Reaction Monitoring [MRM] transitions were established using Agilent MassHunter Optimiser B06.00 software and are presented in **Appendix 6.1**. Derivatised amino acid peak areas were normalised to the recovery of d4A and quantified by reference to a dilution series of external standards prepared from a commercial amino acid mix (Sigma product A9906, Sigma-Aldrich Pty. Ltd., Sydney, Australia). Data was collected and processed using Agilent MassHunter software.

4.3.5 Statistical analysis

The result obtained were analysed using a one-way analysis of variance (ANOVA) with the help of XLSTAT version 19.7 statistical software. A quantitative analysis was performed with pairwise comparison using Tukey HSD (Honestly significant difference) test for each of the congeners in each batch.

5 Results and discussion

5.1 Effect of single stage distillation on percentage alcohol by volume and pH in rum

The percentage alcohol by volume (% ABV) and pH in the distillate was monitored throughout the distillation process of the fermented molasses wash. In addition, the temperature of the distillate vapour phase was also recorded. As shown in **Figure 10(a)**, the first drop of distillate was collected when the vapour temperature in the alembic pot reached 90.3°C. The % ABV gradually dropped to 15% when the vapour temperature reached 99.0°C when 5.1 L of distillate was condensed out. At this point, most of the ethanol have been extracted out of the wash according to the temperature-vapour-liquid phase diagram of an ethanol and water mixture (Perry, Chilton et al. 1963). Interestingly, the pH of the distillate was acidic with a starting pH of 4.2 and gradually got lower as the distillation process continued **Figure 10(b)**. The initial pH of the rum distillate in this study was similar to the starting pH found in Salvi et al. (2013)'s sugarcane spirits at 4.32 ± 0.02 (Silva, Vendruscolo et al. 2013).

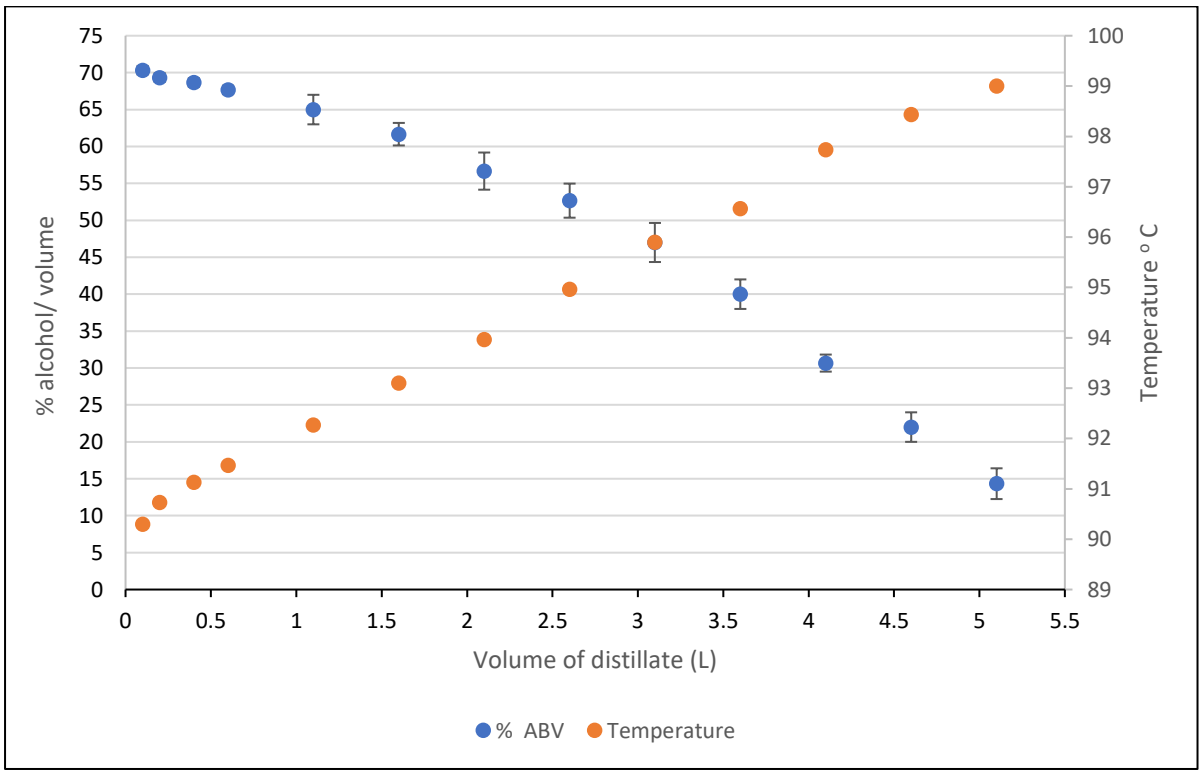


Figure 10(a). % ABV and temperature profile for single stage distillation process (n=3, batch distilled three times separately), error bars represent standard deviation.

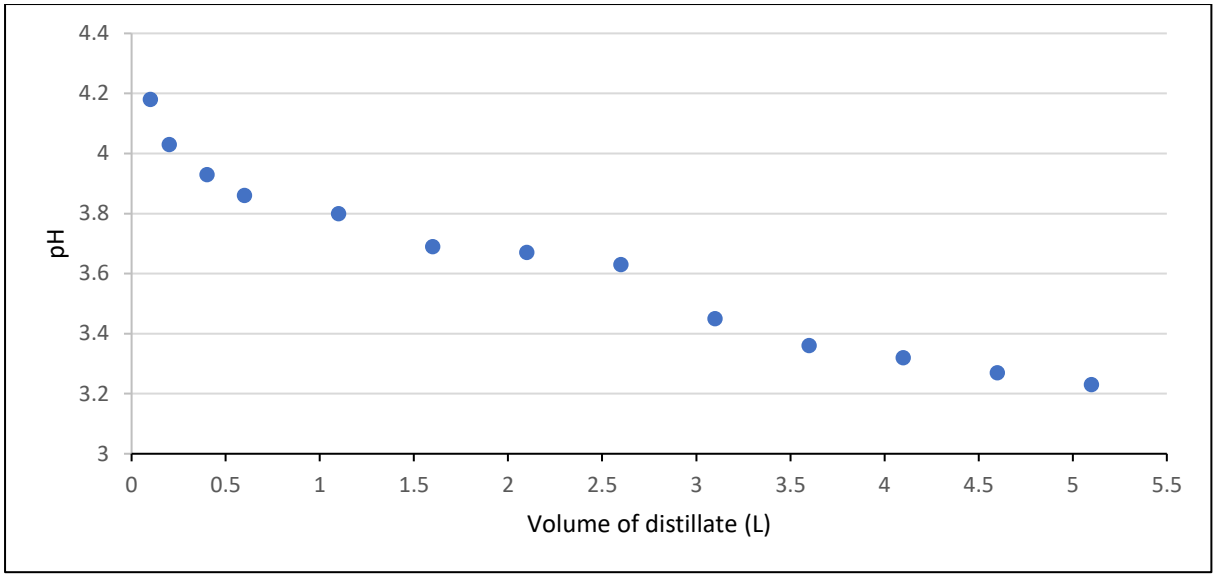


Figure 10(b). pH profile for single stage distilled rum (n=1).

5.1.1 Determining the head fraction from single stage distilled rum

A study from Claus and Berglund (2005) noted that the main congeners from the head fractions in distilled fruit brandy, apart from water and ethanol, were acetaldehyde, acetone, ethyl acetate and methanol. These compounds are known as 'low boilers' – solvents with boiling point lower than 78°C. In this study, the 3 kg molasses and 3 kg dextrose rum wash was distilled in triplicates and the fractions were subjected to GC-FID analysis. The concentration profile of the low boilers are presented in **Figure 11**.

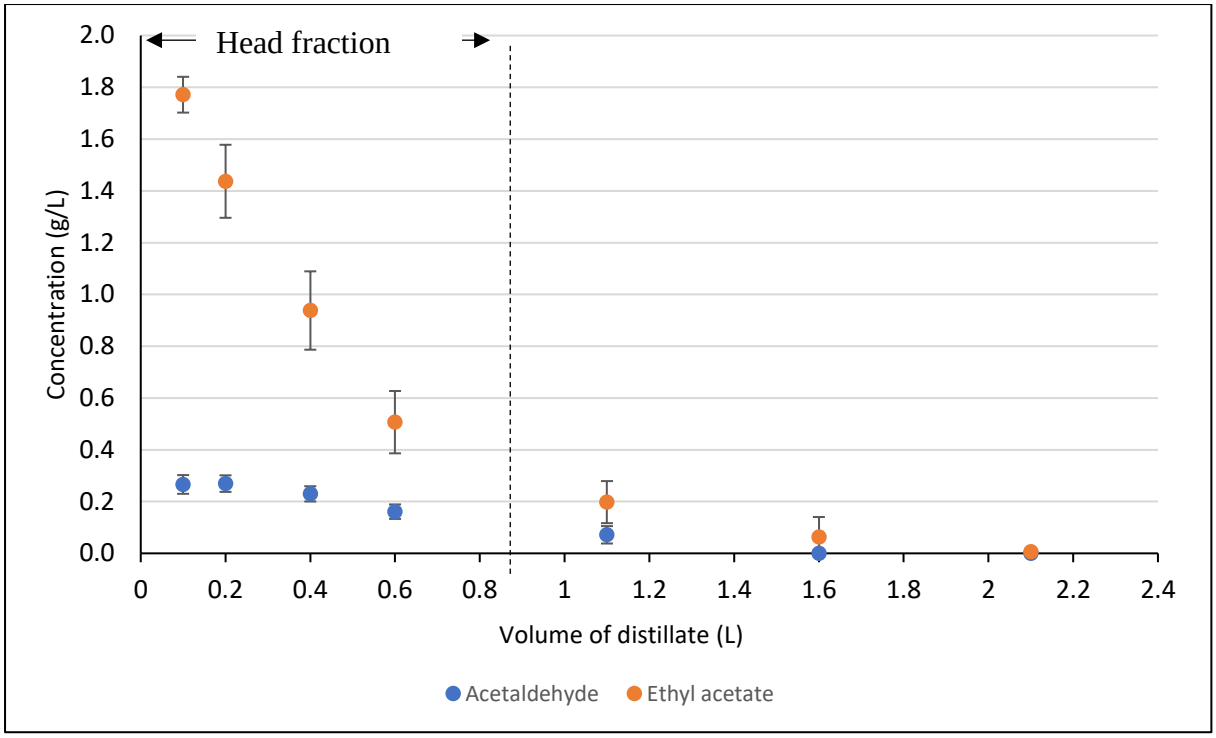


Figure 11. Concentration of low boilers during single stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose wash (n=3; significant difference between compounds, $p<0.0001$).

Acetaldehyde and ethyl acetate were detected in the first 1600 ml of the distillate collected (refer to **Figure 11**) with significant difference in concentration ($p < 0.0001$). No methanol and acetone were detected during the distillation of the rum washes. In the first 100 ml of distillate fraction, acetaldehyde had the highest concentration at 0.27 ± 0.03 g/L which decreased from 400 ml onwards. Ethyl acetate started with 1.78 ± 0.1 g/L in the first 100 ml of distillate and progressively decreased during the distillation process down to non-detectable amounts after collecting 2100 ml of distillate.

Acetaldehyde is produced as a part of the catabolic and anabolic pathways during fermentation of glucose via decarboxylation of α-keto acids and pyruvate in yeast cell respectively (Suomalainen and Lehtonen 1979). It is miscible in water and ethanol, so distillation is an effective method in separating this compound since the boiling point (20.2°C) is vastly different from water (100°C and ethanol 78.9°C). Acetaldehyde accounts for the highest amount of aldehyde in the rum distillate as reported by Nykanen and Lalli (1986). Moreover, acetaldehyde has a distinct aroma of green apple and gives out 'grassy' off-flavour when present above the flavour threshold of 200 mg/L (Lodolo, Kock et al. 2008). Hence, the ideal

time to make the head cut is after the first 400 ml distillate fraction because after this point the acetaldehyde starts to drop below 200 mg/L (**Figure 11**).

Methanol is formed by degradation of pectin which is commonly found in the cell wall of fruits (Bortoletto and Alcarde 2013). Pectin is a polysaccharide bonded by several molecules of galacturonic acid with a methanol group in its structure. In some cases, methanol can be detected in the rum wash after fermentation because of the sugar cane bagasse particles. Bagasse is a type of fiber which contains pectic substance, it can lead to the formation of methanol by *S. cerevisiae* during fermentation. However, the overall presence of methanol is low in rum as sugar cane contain low concentration of pectin as compared to grape which is shown in a study by Hodson and co-workers (2017). Hodson et al. (2007) showed that fermented wine wash can contain between 120 – 250 g/L methanol. Blackstrap molasses made from sugar cane contains no pectin and hence the no methanol was detected in the rum distillate.

Ethyl acetate is a major ester found in sugarcane beverages (Bortoletto and Alcarde 2013, Procopio, Brunner et al. 2014). It is formed by the esterification reaction between ethanol and acetic acid where its concentration is governed by ethanol and the acetyl-CoA molecule during yeast metabolism. It has an odor threshold of 17 mg/L (Salo, Nykanen et al. 1972) and a characteristic fruity aroma. Since ethyl acetate has no negative sensory impact and low toxicity, it does not contribute to the cutting of the head fraction even though it has the highest concentration during the initial stage of rum distillation.

5.1.2 Determining the tail fraction from single stage distilled rum

Apart from esters, acids are one of the important class of volatile compounds in rum (Pino, Tolle et al. 2012). According to an experiment carried out by Pino (2012), organic acids consisted around 4% of the total volatile composition in rum. Namely acetic acid, propionic acid, butanoic acid, decanoic acid, hexanoic acid 2- and 3-methylbutanoic acid are considered among the majority of volatile fatty acids in rum (Nykänen, Puputti et al. 1968, Franitza, Granvogl et al. 2016). Organic acids usually contribute to lesser amount in relation to flavour in rum as they have high odour threshold. Acetic acid in sugar cane spirits are produced via oxidation of ethanol by alcohol dehydrogenase (ADH) isoenzyme found in *S. cerevisiae* to

acetic acid during fermentation (Millan and Ortega 1988). Propionic acid is formed similarly to acetic acid except the starting compound is propanol and it isan important compound considered in tail fraction (Nykänen, Puputti et al. 1968).

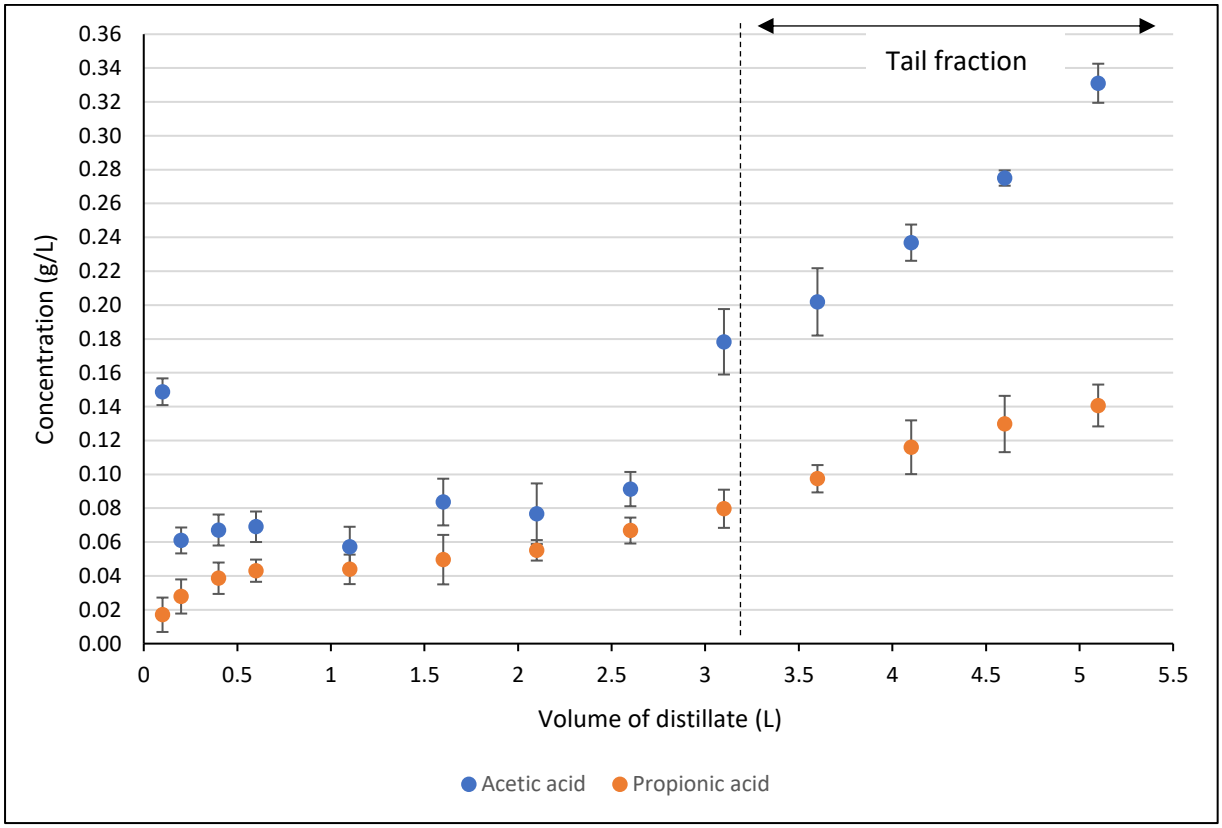


Figure 12. Determining the tail fraction during single stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose wash (n=3; significant difference between compounds, $p<0.0001$).

The fatty acids are usually distilled out at the later stages of distillation as 'tails' owing to their relatively high boiling points such as acetic acid which boils at 118.1°C and propionic acid at 141.2°C. Acetic acid have an inherit odour described as "vinegar-like" and "sweaty" whereas, propionic acid has a rancid unpleasant odour which bring out negative flavour nodes in terms of sensory quality (Franitza, Granvogl et al. 2016, Franitza, Granvogl et al. 2016). **Figure 1**2shows the rising trend for acetic acid and propionic acid during distillation. This is because acetic acid and propionic acid are more soluble in water than in ethanol in the vapor phase (Howard 1996) hence the increase in acid concentration towards the end of the distillation process. The finding is also in line with the pH profile in **Figure 10b** which helps explain the decrease in pH of the distillate over time. According to Paula et al.'s (1970) study, propionic acid has an odor threshold of 0.06 g/L which makes it easy to decide the relevant cut for the

tail fraction (Salo 1970). In **Figure 12,** the threshold for the tail cut can be made at 2600 mL, which means the rum distillate fraction from 2600 mL onwards can be considered as the tail fraction.

5.1.3 Detection of fusel alcohols and esters during single stage distillation of rum

During batch distillation, two sequential fractions; heads and tail cuts are made. The cuts are done based on acetaldehyde and propionic acid concentration in the distillate. From **Section 5.1.1**, the head fraction is determined to be the first 400 mL of distillate collected when the concentration of acetaldehyde reaches 0.200 g/L. In **Section 5.1.2** the tail fraction is all the distillate beyond 2600 mL as the propionic acid concentration in the distillate reaches 0.06 g/L (odour threshold). Therefore, the rum distillate between 400 mL and 2600 mL are the heart fraction. In general, the hearts are the only fractions that have commercial value as it contains the highest amount of positive flavour contributing to congeners and lowest level of negative congeners in the fraction. After the head fraction, a complex volatile mixture of fusel alcohols and esters are separated together with ethanol. All rums are expected to have high amount of esters and moderate amount of fusel alcohols as the esters gives rum its unique sweet tasting aroma. These have been well characterized by Franitza et al. (2016) by using sensomics approach.

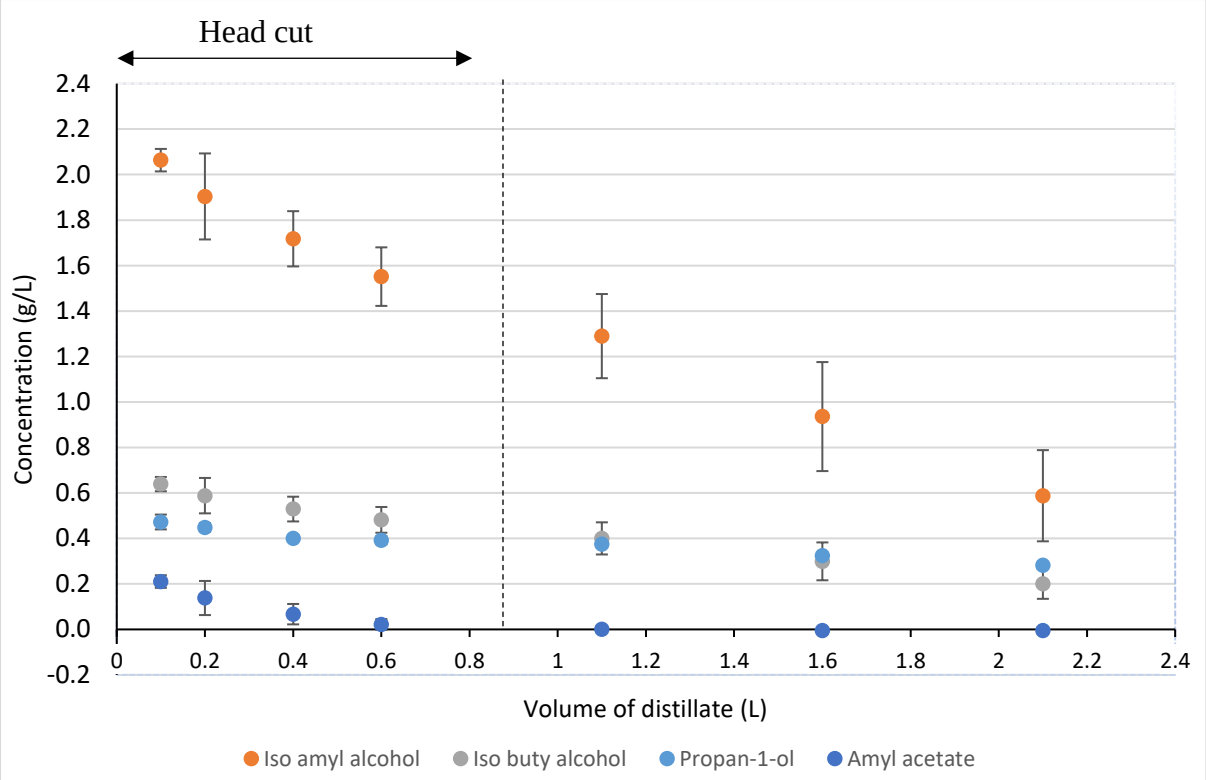


Figure 13. Determining the fusel alcohol with GC-FID during single stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose must (n=3; significant difference between compounds, $p<0.0001$).

The most important fusel alcohol to consider is iso-amyl alcohol (3-methylbutan-1-ol) because it is the main fusel alcohol synthesized during fermentation by yeast (Suomalainen and Lehtonen 1979) and in large concentration can create an unpleasant flavour due to its strong banana like odour and bitterness in the rum (Boscolo, Bezerra et al. 2000, Kang, Hwang et al. 2016). The study done by Harrison et al. (1970) gives the flavour threshold of isoamyl alcohol as 75 ppm (0.075 g/L) in beer but there is no report of the threshold in rum. Generally, fusel alcohols have damp cloth aroma which is not desirable at high concentrations during production of alcoholic beverages (Claus and Berglund 2005), however, their presence is essential as they are precursors to desirable esters during the ageing process. In **Figure 13,** the main fusel alcohols detected in the rum distillate are isoamyl alcohol followed by isobutyl alcohol and propan-1-ol. According to Sigmund (1971) beer study, the flavor threshold for isoamyl alcohol is 50 ppm whereas isobutyl alcohol and propan-1-ol is 100 ppm and 600 ppm respectively (Engan 1972). The characteristic flavor of isobutyl alcohol gives sweet whiskey taste and propan-1-ol has sharp ripe, fruity flavor (Furia and Bellanca 1971, Budavari 1989,

Christoph and Bauer-Christoph 2007). Though amyl acetate is not a fusel alcohol, it was included in **Figure 13** because the GC-FID could detect it with relatively good resolution. Amyl acetate has characteristic fruity banana-like flavor (Pires, Teixeira et al. 2014) and is generally regarded as a positive flavor compound.

As discussed in the literature review, alcoholic beverages contain different volatile compounds like fusel alcohols which can be detected by GC-FID whereas volatile compounds such as esters, congeners or carbonyl compounds are present in minute amount (except amyl acetate) which can be only detected by the more sensitive solid-phase microextraction (SPME) technique (Pino 2007). According to Pino's (2007) findings, 64 compounds were detected with SPME in aged rum. The dominant esters constituted of methyl, 3 methyl-1-butyl and phenyl esters. **Figure 14(a)** depicts the dominant ester detected in single stage distillation where the normalized response is greater than 10 and Figure 14b exhibits the esters detected in lesser concentration (normalized response is less than 10). **Figure 14(a)** shows esters detected in high concentration as compared to **Figure 14(b)**. The octanoic acid, hexanoic acid and octanoic acid exhibits gradual decreasing trend. **Figure 14(b)**. are the esters detected in lesser concentration. Though being in lesser amount they are important ethyl ester and contribute to the flavour of rum. SPME was also done on a pure blackstrap molasses sample (refer Appendix 2).

From the SPME-GC/MS results, only decanoic acid ethyl ester and naphthalene,1,2,3,4-tetrahydro-1,6,8-trimethyl- were found to be common compounds that were also identified in the single stage rum distillate samples. Therefore, it was observed that only these two organic compounds carried over from the molasses to the rum during distillation. Though being in traceable amount and not being an ethyl ester, it should be noted that SPME-GC/MS detected naphthalene,1,2,3,4-tetrahydro-1,6,8-trimethyl- which is the reason to include this compound in **Figure 14(b)**.

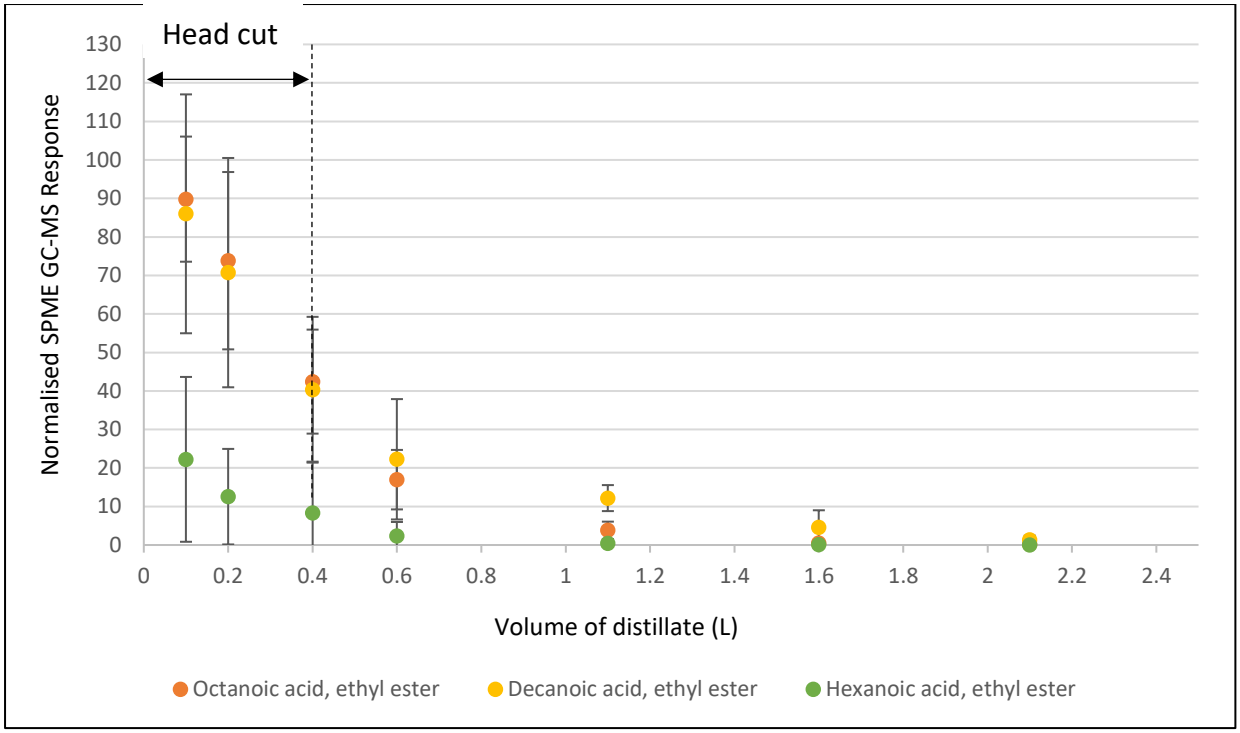


Figure 14(a). Determining the dominant esters (normalized response is greater than 10) with SPME during single stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose must (n=3), error bars represent standard deviations.

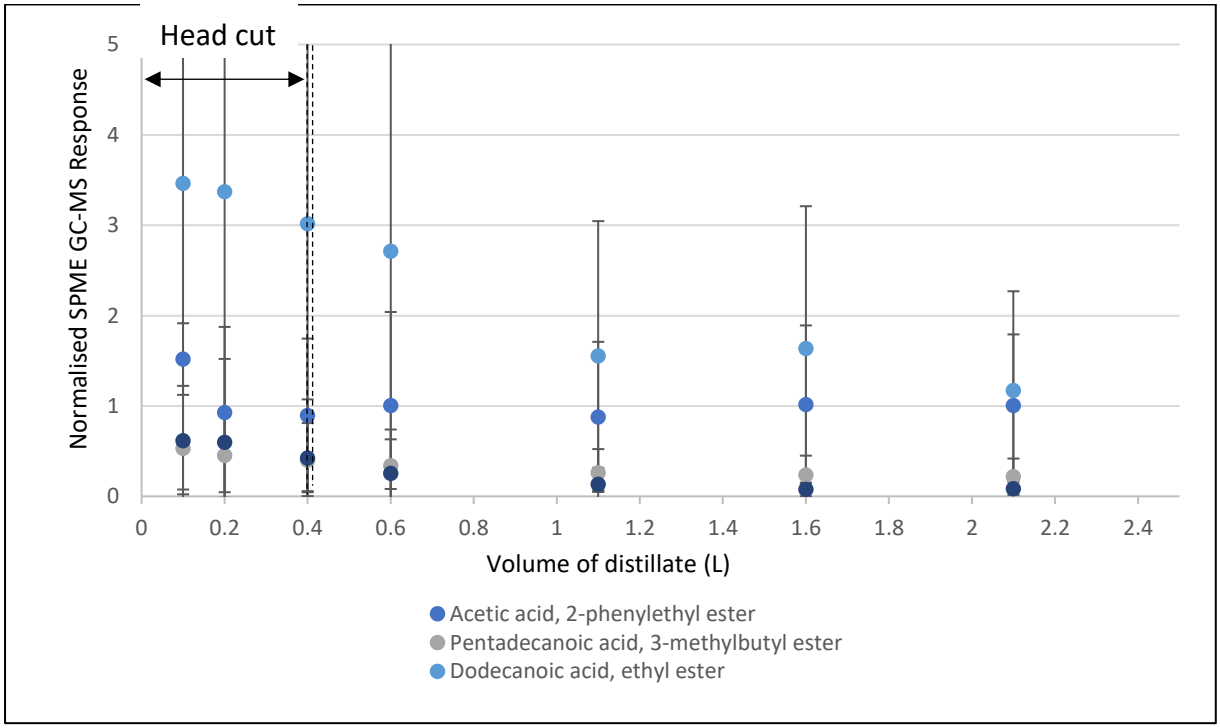


Figure 14(b). Determining the minor esters (normalized response is less than 10) with SPME during single stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose must (n=3), error bars represent standard deviations.

5.2 Effect of double stage distillation in rum

The single stage distilled rum from Section 5.1 was fractionated into various volumes as mentioned in the Methodology section. Only the hearts were collected, whereby the first 400 ml and the distillate from 2600 ml onwards were discarded. The hearts, which retained some of the desirable ester compounds and less of the undesirable congeners, were re-distilled to produce a final rum. This final rum will be termed as having to been through a double stage distillation process. The rum from the second distillation were fractionated in the same manner as the first and the alcohol content and vapour temperature were monitored (refer to **Figure 15**). Since the starting wash (heart fraction) had higher alcohol content 50 to 55% ABV, it started to boil at 83°C and hence the distillate that followed had an ABV of 87%. This is in agreement with the ethanol and water vapour-liquid equilibrium data from Coker (1963). The %ABV content in the distillate decreased slowly from 87% down to 73% in the first 2100 ml. From this point onwards, the %ABV sharply dropped to 30% in the next 1 L of collected distillate. The %ABV profile of the distillate from 2nd distillation was markedly different compared to the 1st. The %ABV gradually decreased from 70% to 60% in the first 1.6 L and down to 30% in an almost linear fashion for the next 2.5 L of distillate.

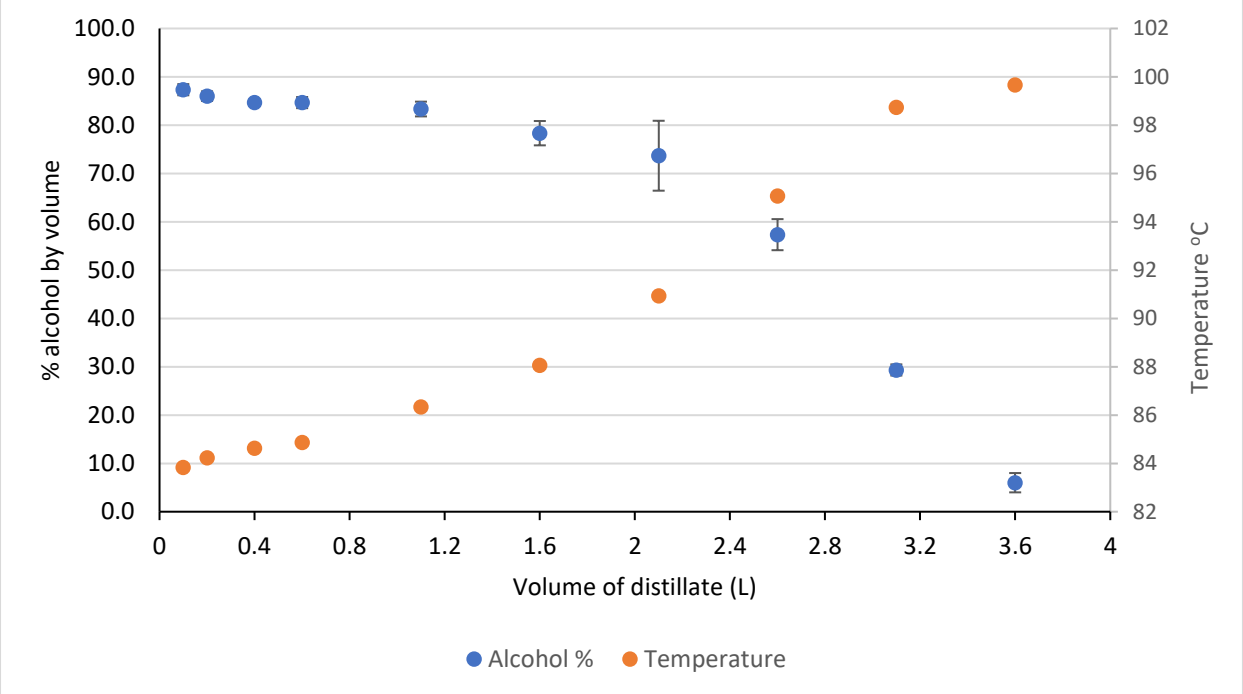


Figure 15. % ABV and temperature profile for double stage distillation of rum (heart fractions only) (n=3).

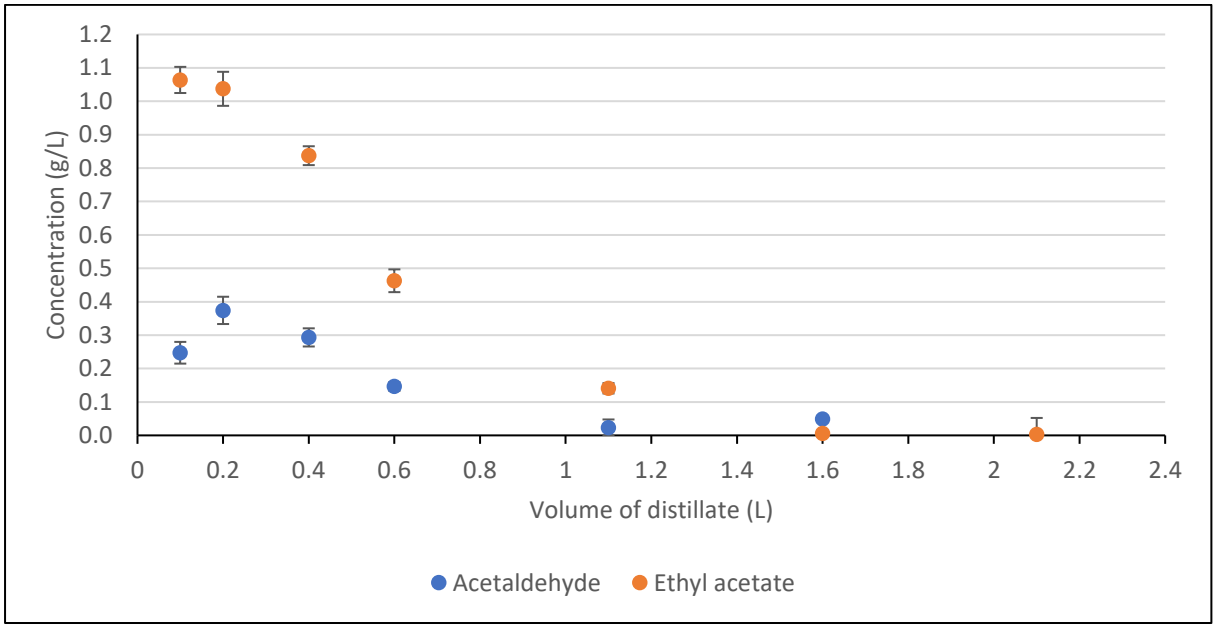


Figure 16. Acetaldehyde and ethyl acetate profile for double stage distillation of rum (n=3, $p<0.0001$).

The acetaldehyde and ethyl acetate profiles (refer to Figure 16) from the 2nd distillation process were very similar to the profiles from the 1st. The difference is that the starting concentration of ethyl acetate (1.05 g/L) is significantly lower than the first (1.8 g/L). This is expected as majority of the ethyl acetate was captured in the heads fraction from the 1st distillation process and was discarded so less ethyl acetate was carried over to the 2nd distillation.

Interestingly, propionic acid were barely detected in the 2nd stage distillate and the acetic acid profile was very different to the single distillate rum. However, one thing is certain that the concentration of acetic acid is significantly lower in double distilled rum (refer to **Figure 17**).

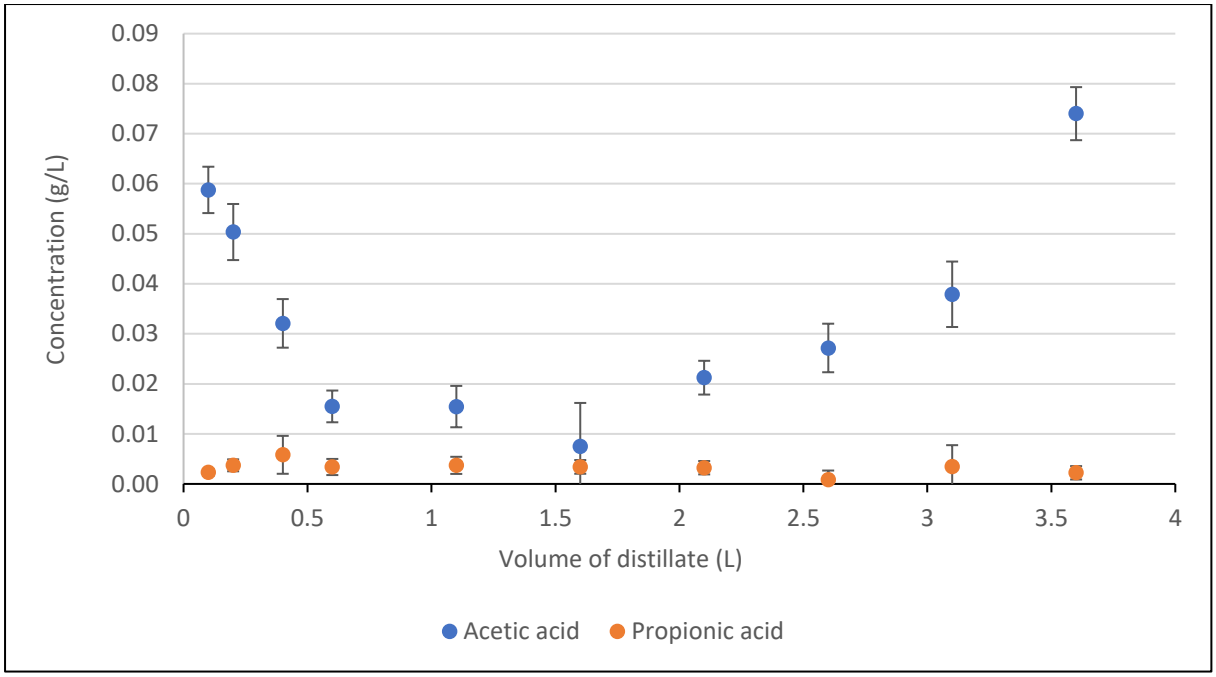


Figure 17. Acetic and propionic acid profile for double stage distillation of rum (n=3), error bars represent standard deviations.

The fusel alcohol profile in the rum from 2nd distillation were very different to the profile of the 1st. The fusel alcohol concentrations plateaued throughout the 2nd distillation process until 1100 ml (following the %ABV trend in **Figure 15**), at this point they start to decrease. This phenomenon reinforces the idea that the fusel alcohols were more soluble in ethanol than in water. The initial concentration of isoamyl alcohol in the 2nd distillate (1.10 g/L) was significantly lower than the 1st stage distillate (2.00 g/L). However, the other fusel alcohols were similar to the 1st stage distillate. This could be explained that isobutanol, 2-phenyl ethanol and propanol were more miscible in ethanol than water as during the 2nd distillation (**Figure 18**).

It is clear that the rum distillate after two stages of distillation has higher ethanol content compared to single stage distillation. The results from this study showed that the concentration of undesirable congeners such as isoamyl alcohol and propionic acid were significantly lower when the rum was distilled twice. However, the desirable ethyl ester compounds were also significantly lowered as shown in **Figure 19(a)** and **Figure 19(b)**.

5.2.1 Detecting fusel alcohols and esters during double stage distillation of rum

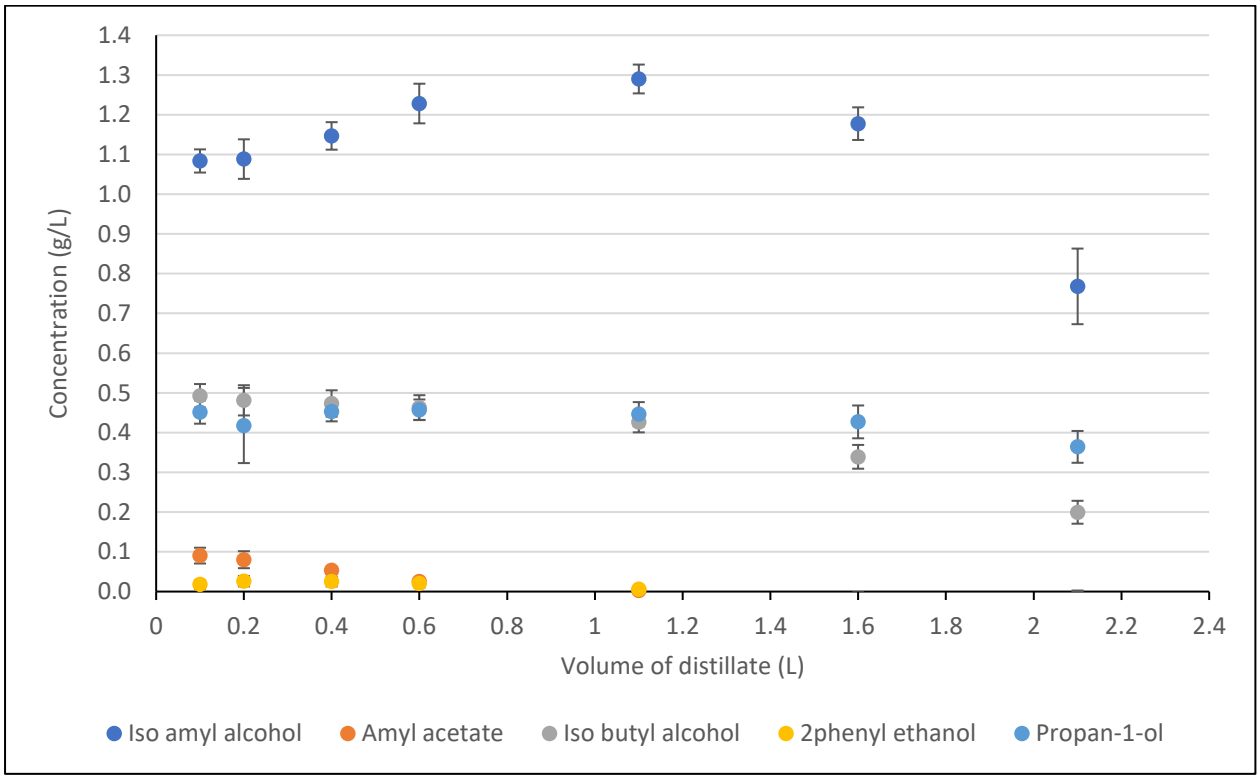


Figure 18. Determining the fusel alcohol with GC-FID during double distillation of rum (n=3; significant difference between compounds, $p<0.0001$) (n=3, $p<0.0001$).

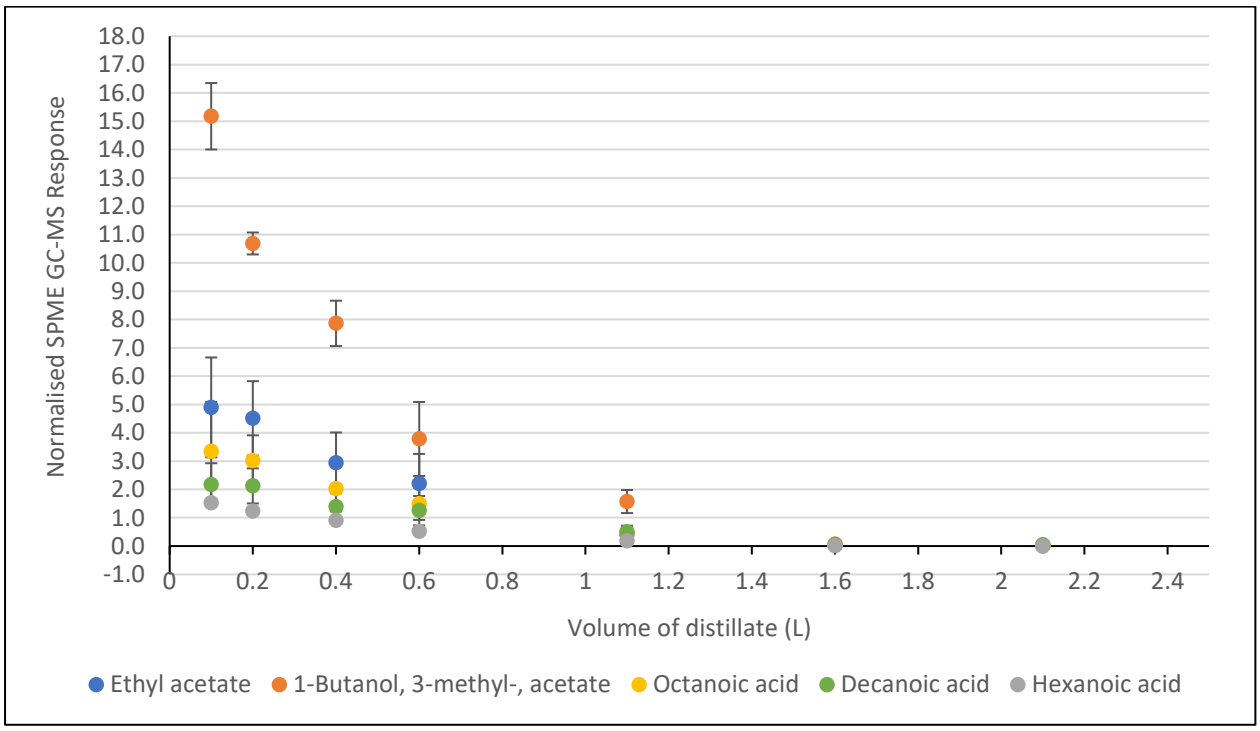


Figure 19(a). Determining the dominant esters (normalized response is greater than 10) with SPME during double stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose must (n=3), error bars represent standard deviations.

Figure 19(a) depicts the dominant ester detected in double stage distillation where the normalized response is greater than 10 and **Figure 19(b)** exhibits the esters detected in lesser concentration (normalized response is less than 10).

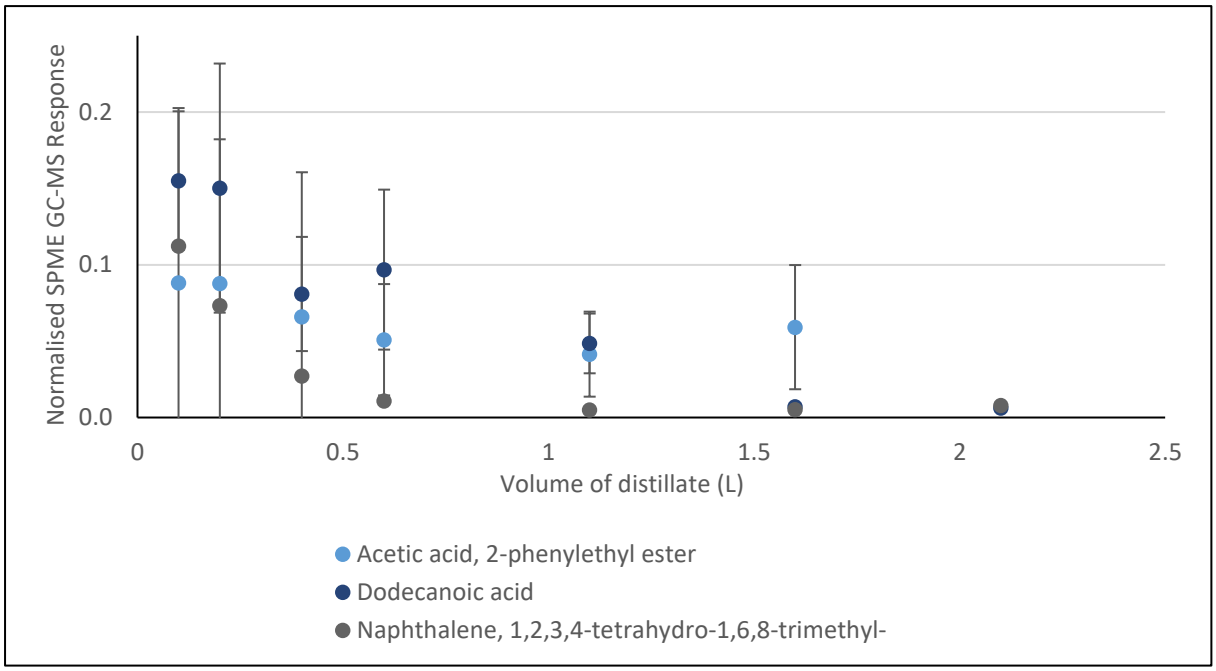


Figure 19(b). Determining the minor esters (normalized response is lesser than 10) with SPME during double stage distillation of rum by fermenting 3 kg molasses and 3 kg dextrose must (n=3, $p < 0.001$).

5.3 Effect of varying molasses to dextrose concentration in the rum must on the final distilled run

5.3.1 Detecting the amino acids in pure molasses samples.

As discussed in literature review, amino acids are important precursor to the formation of fusel alcohols in catabolic pathway. The study done by Procopio et al. (2014) described that the concentration of amino acids determines the final concentration of higher alcohols and their corresponding esters during fermentation (Procopio, Brunner et al. 2014). In the literature review, **Table 4** lists the amino acids and its corresponding esters. Amino acids and their corresponding esters co-relate with each other, if there are more amount of amino acids present in raw material more of their corresponding esters are detected in rum distillate. Amino acids were detected in the blackstrap molasses sample and are listed in Table 8.

Hashizume et.al (1996), studied the amino acid composition of Okinawa cane molasses. The amino acids detected using LC-MS in this research were similar to what Hashizume and colleagues found. Both of the studies found that aspartic acid has the highest concentration in molasses. According to the results of their study, concentration of aspartic acid was reported as 0.3740% whereas this study found the concentration of aspartic acid in blackstrap molasses to be 15660.00 ± 210.94 μM.

Table 8. List of amino acids found in molasses and respective concentration in this study (n=3).

Amino acids in molasses	**Concentration in μM**
Cystine 291	0.00
Tryptophan	18.67 ± 0.58
HO-Pro	18.00 ± 1.00
Glutamine	14.67 ± 2.08
Lysine 244	19.67 ± 2.08
Arginine	42.33 ± 4.04
Methionine	95.67 ± 6.51
Glycine	159.33 ± 24.17
Phenylalanine	342.33 ± 23.71
Tyrosine	424.67 ± 40.53
Threonine	607.33 ± 29.28
Leucine	632.67 ± 42.10
Glutamic acid	751.33 ± 99.28
Isoleucine	1271.67 ± 63.95

Serine	1532.67 ± 119.51
Proline	1882.00 ± 109.14
d4-alanine	2639.00 ± 201.72
Valine	3687.67 ± 245.93
Alanine	4986.33 ± 79.22
Asparagine	12408.00 ± 343.32
Aspartic acid	15660.00 ± 210.94

*d4-alanine is the internal standard.

5.3.2 Analysis of concentration of ester compounds in varying molasses to glucose ratio.

Rum must preparation includes the suspension of molasses and water with addition of some extra glucose to elevate the °Brix before the fermentation process. The must consists of an average sugar concentration of 17-28 °Brix. In this research, an experiment was carried out to investigate the effect of varying the glucose to molasses ratio in the rum must before fermentation on the ester profile in the rum distillate (heart fractions only). Under the Methodology section, five glucose to molasses ratio were investigated as detailed in **Table 6.** After fermentation, only the heart fractions were collected from each run. These runs were done in duplicates. The head and tail cuts were performed according to the proposed method in Section 5.1. Six dominant rum esters were monitored using SPME; 1-butanol 3-methyl-acetate, octanoic acid ethyl ester, acetic acid 2 phenyl ethyl ester, decanoic acid ethyl ester, octanoic acid ethyl ester, 3-3methylbutyl ester, dodecanoic acid ethyl ester (Suomalainen and Lehtonen 1979, Masuda, Yamamoto et al. 1985, Pino 2007, Franitza, Granvogl et al. 2016). All of this volatile compounds have a distinct aroma compounds which contribute to the overall aroma of rum.

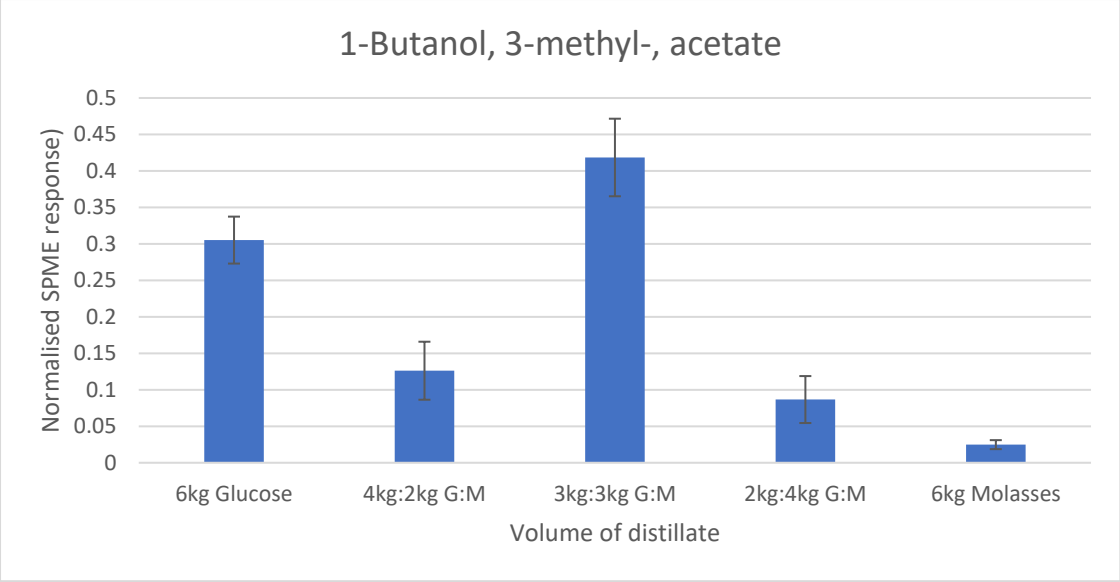


Figure 20**.** SPME response of isoamyl acetate in rum distillate from fermented rum wash of varying dextrose and molasses ratio must (n=2).

1-butanol 3 methyl-acetate or isoamyl acetate is produced during fermentation process by decarboxylation as explained in literature review. It is derived from the amino acid leucine. In **Figure 20,** displayed more presence of isoamyl alcohol with around 0.43 normalized SPME response in 3:3 glucose to molasses ratio batch, followed by the pure dextrose batch around 0.3 g/L. Least concentration could be found in pure molasses sample with around 0.03 normalized SPME response. This clearly showed that the formation of isoamyl acetate is not dependant on the amount of leucine in the rum must.

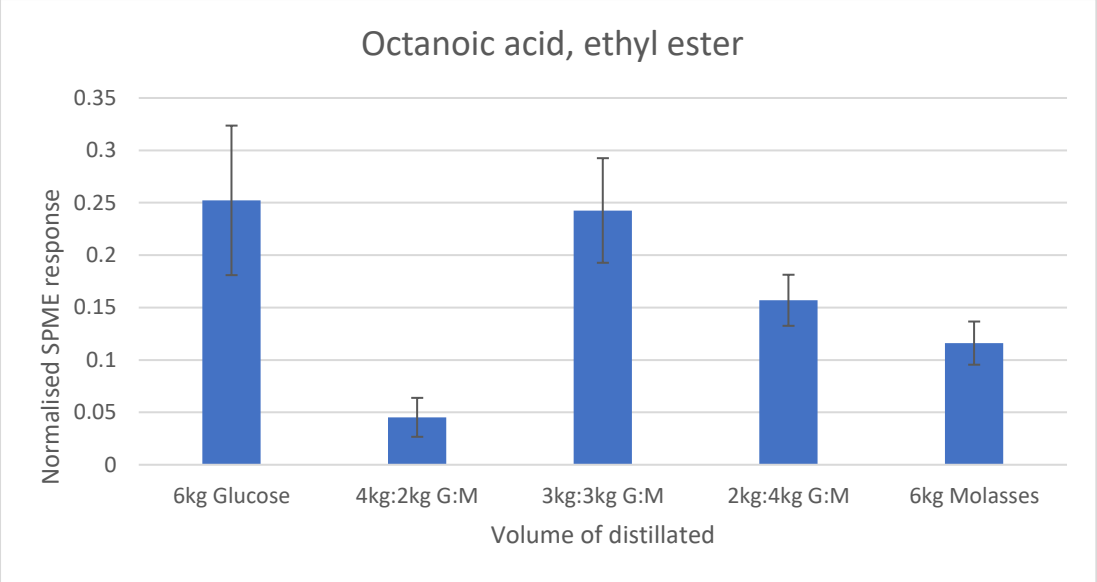


Figure 21**.** SPME response of octanoic acid ethyl ester in rum distillate from fermented rum wash of varying dextrose and molasses ratio must (n=2).

According to Suomalainen et al.'s (1979) study, large amount of ethyl esters are present in the fermentation of their white rum. It includes ethyl, isobutyl and isoamyl ester, octanoic acid ethyl ester, decanoic acid ethyl ester, tetradecanoic acid ethyl ester and hexadecanoic acid ethyl ester. Their results showed around 14.5% of the total esters were octanoic acid ethyl ester in Martinique rum. Their study also showed that octanoic acid ethyl ester concentration where higher in heavy flavour rum as compared to light flavour rum. **Figure 21** illustrates high amount of octanoic acid ethyl ester in the batch prepared using pure dextrose followed by 3:3 kg dextrose to molasses ratio. It's characteristic aroma is grape flavor with a threshold of 5µg/L(Francis 2012). The reason for low concentration of octanoic acid ethyl ester in 4:2 dextrose to molasses batch is unknown.

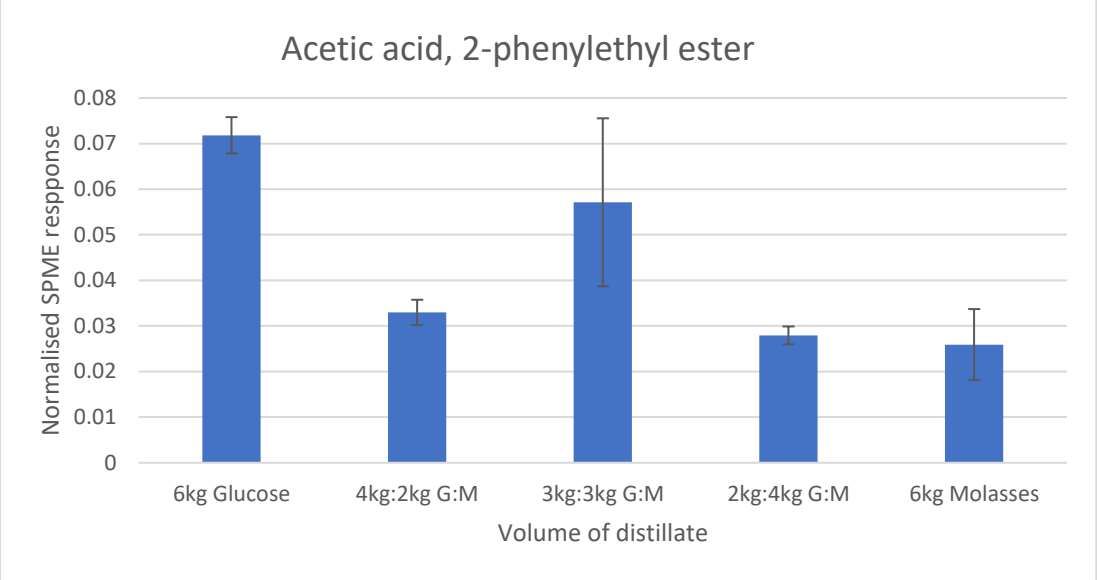


Figure 22**.** SPME response of acetic acid, 2-phenylethyl ester or 2-phenethyl acetate in rum distillate from fermented rum wash of varying dextrose and molasses ratio must (n=2).

Acetate esters chiefly contribute as flavor active compounds in alcoholic distilled beverages (Procopio, Brunner et al. 2014). Synthesis of ethyl acetate is linked to ethanol produced in anabolic pathway. Acetate esters are produced by condensation reaction between acetyl CoA and fusel alcohol which is illustrated in the literature review under anabolic pathway. Along with 2-phenyl ethanol, 2-phenethyl acetate is also detected in the study done by Pino (2012). Though in trace amount, the combined effect of volatile compounds such as ethyl acetate, isoamyl acetate, isobutyl acetate, 2-phenethyl acetate plays a prime role in affecting the flavor of distilled beverages below their threshold. **Figure 22** exhibits highest response of 2-phenethyl acetate in pure dextrose up to 0.07 normalized response in rum distillate. Whereas, least normalized response around 0.03 in pure molasses.

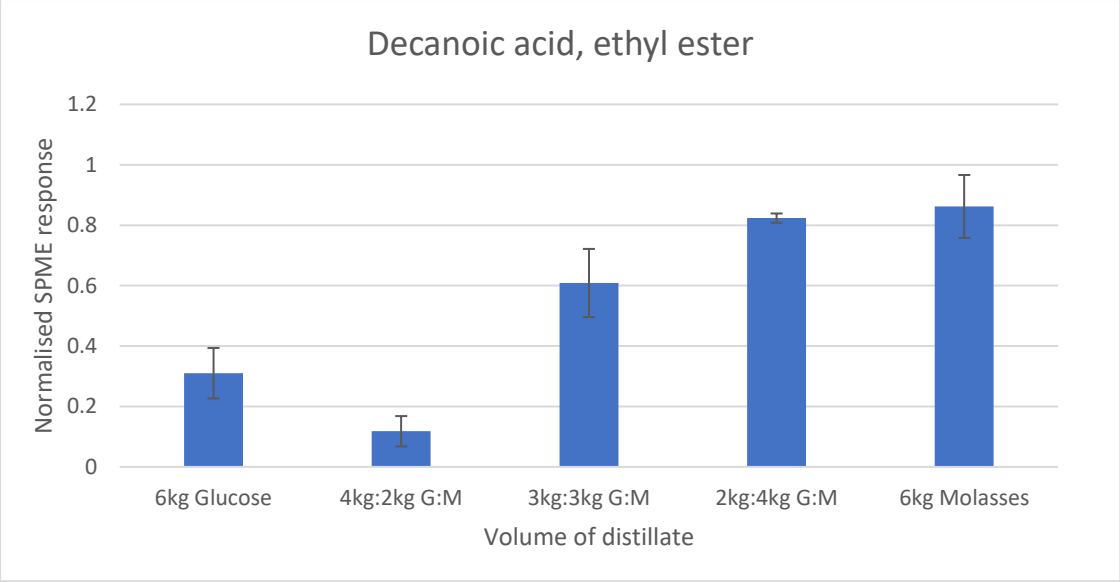


Figure 23. SPME response of decanoic acid in rum distillate from fermented rum wash of varying dextrose and molasses ratio must (n=2).

Suomalainen (1971) has detected decanoic acid as one of the higher fatty acid in rum distillate. In another study (1979), he found out the presence of 17.5 % of decanoic acid ethyl ester in one of the varieties of rum. As compared to hexanoic and octanoic acid ethyl ester, decanoic acid ethyl ester is detected in higher concentration. Odor threshold of decanoic acid is ethyl esters also detected as high as 15ppm (Salo 1970). After analyzing pure molasses sample through SPME technique, we found out high concentration of decanoic acid ethyl ester (refer appendix 2) which may explain the presence of high amount of decanoic acid ethyl ester in pure molasses 6kg batch in **Figure 23**.

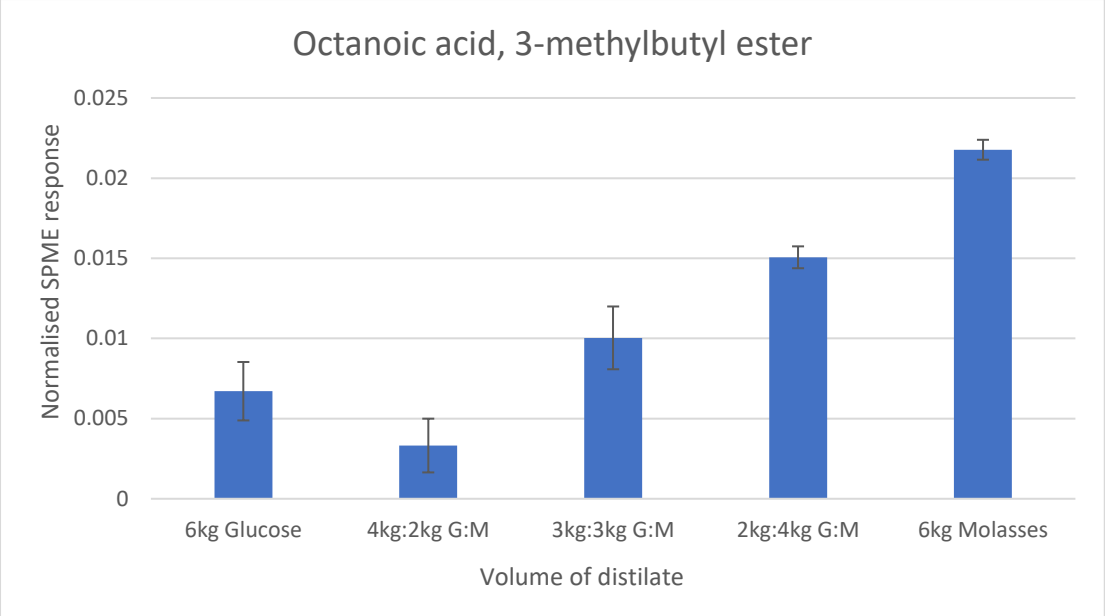


Figure 24. SPME response of octanoic acid, 3-methylbutyl ester in rum distillate from fermented rum wash of varying dextrose and molasses ratio must (n=2).

According to Procopio study (2014), ethyl octanoate along with C6 and C8 short chain fatty acid ethyl ester are accountable for much of the fruity characteristic aroma in alcoholic beverages (Procopio, Brunner et al. 2014). The odor threshold of ethyl octanoate in rum distillate is around 0.25 ppm (Salo 1970, Suomalainen 1971). Along with the production of ethyl esters of decanoic and octanoic acid, ethyl hexanoate is also observed in heart fraction of white rum produced in West Indies (Suomalainen and Lehtonen 1979). As described in literature review Jamaican rum is produced by using blackstrap molasses which is the byproduct of sugarcane sap. Liebich (1970) has research on the analysis of flavor of Jamaican rum using gas-liquid chromatography and mass spectrometry. His findings include ethyl octanoate amongst the major ethyl esters in rum (Liebich, Koenig et al. 1970). It has a woody, fatty, creamy flavor characteristic with 10 ppm odor threshold(Burdock 2016). **Figure 24** shows higher concentration of octanoic acid, 3-methylbutyl ester in batch prepared from pure molasses whereas less concentration in batch prepared with 4 kg glucose and 2 kg molasses.

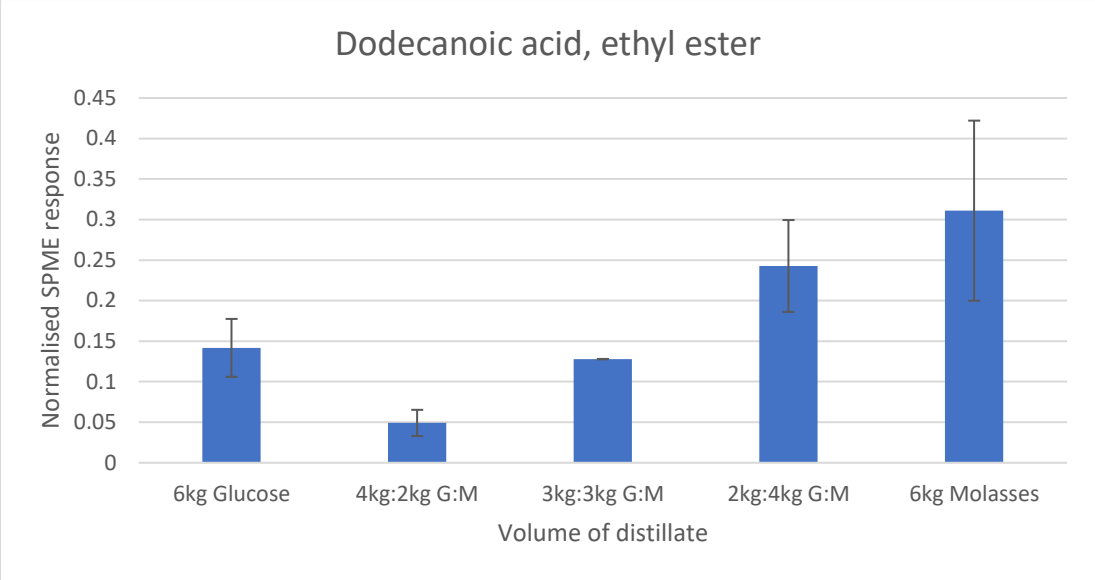


Figure 25. SPME response of dodecanoicacid ethyl ester in rum distillate from fermented rum wash of varying dextrose and molasses ratio must (n=2).

Dodecanoic acid ethyl ester comes out in heart fraction after isoamyl alcohol and before hexadecenoic acid *ethyl* ester (Suomalainen and Lehtonen 1979). Dodecanoic acid ethyl ester is considered among main volatile fatty acids as it consists of 6.5 % in Martinique rum. The organoleptic analysis carried out by Suomalainen (1970) gives <0.02 g/L of dodecanoic acid ethyl ester for odour threshold. **Figure 25** depicts <0.3 normalized response of dodecanoic acid ethyl ester in batch prepared from pure molasses whereas >0.05 normalized response in batch containing 4kg glucose and 2kg molasses.

The above study done of changing the concentration of dextrose to molasses ratio in the rum must have significant influence on the ester profile in the final distillate. However, there is no obvious trend to correlate molasses ratio to the flavour profile of rum.

6 Conclusions

This study has shown that the GC-FID technique was suitable to determine the head and tail cuts during the distillation of the rum wash. The ideal time to make the head cut is when the first 400 ml of distillate is collected i.e. 7% of the total volume. This is when the concentration of acetaldehyde starts to drop below 200 mg/L (the flavour threshold). The most suitable time to make tail cut is after 2600 ml of distillate collected as the concentration of propionic acid in the distillate starts to rise above 60mg/L, which would give the rum a vinegar-like rancid unpleasant odor.

The solid phase microextraction (SPME) with GC-MS technique, was used to determine the ester compound in the heart fraction, It was found that 1-butanol 3 methyl-acetate, octanoic acid ethyl-ester, acetic acid 2-phenylethyl ester, decanoic acid ethyl ester, octanoic acid ethyl ester, 3methylbutyl ester, dodecanoic acid ethyl ester were the most abundant esters in rum distillate.

It was found that the rum which was double distilled had higher purity in ethanol and lower concentration of undesirable congeners like acetaldehyde and propionic acid. However, the desirable ethyl ester congeners were lowered after the double distillation process.

Besides the distillation process, the molasses and dextrose ratio in the rum must also influenced the flavor of the final rum by altering the ester profiles. However, there was no clear correlation between the raw material and volatile compound profile of rum.

7 Reference

Argyrios, T. and P. Spyros (2015). Alcoholic Beverages. Handbook of Food Processing: Food Safety, Quality, and Manufacturing Processes, CRC Press**:** 319-350.

Bamforth, C. W. and R. E. Ward (2014). The oxford handbook of food fermentations, Oxford Handbooks.

Bortoletto, A. M. and A. R. Alcarde (2013). "Congeners in sugar cane spirits aged in casks of different woods." Food chemistry **139**(1-4): 695-701.

Boscolo, M., et al. (2000). "Identification and Dosage by HRGC of Minor Alcohols and Esters in Brazilian Sugar-Cane Spirit." Journal of Brazilian Chemical Society **11**(1): 86-90.

Broom, D. (2003). Rum, New York : Abbeville Press, 2003.

Bruno, S. N. F., et al. (2007). "Influence of the distillation processes from Rio de Janeiro in the ethyl carbamate formation in Brazilian sugar cane spirits." Food chemistry **104**(4): 1345-1352.

Budavari, S. (1989). The Merck Index, an encyclopedia of chemical drug, and biologicals, Merck.

Burdock, G. A. (2016). Fenaroli's handbook of flavor ingredients, CRC press.

Christoph, N. and C. Bauer-Christoph (2007). Flavour of spirit drinks: raw materials, fermentation, distillation, and ageing. Flavours and Fragrances, Springer**:** 219-239.

Claus, M. J. and K. A. Berglund (2005). "FRUIT BRANDY PRODUCTION BY BATCH COLUMN DISTILLATION WITH REFLUX." Journal of Food Process Engineering **28**(1): 53-67.

De Souza, M. D. C. A., et al. (2006). "Characterization of Cachaça and Rum Aroma." Journal of Agricultural and Food Chemistry **54**(2): 485-488.

Denko, N. C. (2008). "Hypoxia, HIF1 and glucose metabolism in the solid tumour." Nature Reviews Cancer **8**(9): 705.

Engan, S. (1972). "Organoleptic threshold values of some alcohols and esters in beer." Journal of the Institute of Brewing **78**(1): 33-36.

Fahrasmane and P. Ganou (1998). "Microbial flora of rum fermentation media." Journal of Applied Microbiology **84**(6): 921-928.

Francis, L. (2012). "Fermentation-derived aroma compounds and grape-derived monoterpenes." Appl. Microbiol. Biotechnol **96**: 601-618.

Franitza, L., et al. (2016). "Characterization of the Key Aroma Compounds in Two Commercial Rums by Means of the Sensomics Approach." Journal of Agricultural and Food Chemistry **64**(3): 637-645.

Franitza, L., et al. (2016). "Influence of the Production Process on the Key Aroma Compounds of Rum: From Molasses to the Spirit." Journal of Agricultural and Food Chemistry **64**(47): 9041-9053.

Furia, T. E. and N. Bellanca (1971). "Fenaroli's handbook of flavour ingredients." Fenaroli's handbook of flavour ingredients.

Hazelwood, L. A., et al. (2008). "The Ehrlich Pathway for Fusel Alcohol Produciton: a Century of Research on *Saccharomyces cerevisiae* Metabolism." Applied and Environmental Microbiology **74**(8): 2259-2266.

Howard, P. H. (1996). Handbook of physical properties of organic chemicals, CRC press.

Kang, H.-R., et al. (2016). "Quantitative analysis of volatile flavor components in Korean alcoholic beverage and Japanese sake using SPME-GC/MS." Food Science and Biotechnology **25**(4): 979-985.

Kruse, J. A. (2012). "Methanol and Ethylene Glycol Intoxication." Critical Care Clinics **28**(4): 661-711.

Lea, A. G. and J. Piggott (2012). Fermented beverage production, Springer Science & Business Media.

Liebich, H. M., et al. (1970). "Analysis of the Flavor of Rum by Gas-Liquid Chromatography and Mass Spectrometry." Journal of Chromatographic Science **8**(9): 527-533.

Lodolo, E. J., et al. (2008). "The yeast Saccharomyces cerevisiae–the main character in beer brewing." FEMS yeast research **8**(7): 1018-1036.

Masuda, M., et al. (1985). "Direct gas chromatographic analysis of fusel alcohols, fatty acids and esters of distilled alcoholic beverages." Journal of Food Science **50**(1): 264-265.

Medeiros, A., et al. (2017). Cachaça and Rum. Current Developments in Biotechnology and Bioengineering, Elsevier**:** 451-468.

Millan, C. and J. Ortega (1988). "Production of ethanol, acetaldehyde, and acetic acid in wine by various yeast races: role of alcohol and aldehyde dehydrogenase." American Journal of Enology and Viticulture **39**(2): 107-112.

Nykänen, L., et al. (1968). "Volatile Fatty Acids in Some Brands of Whisky, Cognac and Rum." Journal of Food Science **33**(1): 88-92.

Perry, J. H., et al. (1963). Chemical engineers handbook. McGraw-Hill handbooks, McGraw-Hill.

Pino, J., et al. (2002). "Headspace solid-phase microextraction of higher fatty acid ethyl esters in white rum aroma." Journal of Chromatography A **954**(1–2): 51-57.

Pino, J. A. (2007). "Characterization of rum using solid-phase microextraction with gas chromatography–mass spectrometry." Food chemistry **104**(1): 421-428.

Pino, J. A., et al. (2012). "Characterisation of odour-active compounds in aged rum." Food chemistry **132**(3): 1436-1441.

Pires, E. J., et al. (2014). "Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast." Applied microbiology and biotechnology **98**(5): 1937-1949.

Popov-Čeleketić, D., et al. (2016). "A plasma membrane association module in yeast amino acid transporters." Journal of Biological Chemistry **291**(31): 16024-16037.

Procopio, S., et al. (2014). "Differential transcribed yeast genes involved in flavour formation and its associated amino acid metabolism during brewery fermentation." European Food Research and Technology **239**(3): 421-439.

Romagnoli, G. (2014). The Ehrlich pathway for amino acid catabolism in yeasts, TU Delft, Delft University of Technology.

Salazar, C., et al. (2012). "An UPLC-ESI-MS/MS assay using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization for targeted amino acid analysis: application to screening of Arabidopsis thaliana mutants." Metabolites **2**(3): 398-428.

Salo, P. (1970). "Determining the odor thresholds for some compounds in alcoholic beverages." Journal of Food Science **35**(1): 95-99.

Salo, P., et al. (1972). "Odor thresholds and relative intensities of volatile aroma components in an artificial beverage imitating whisky." Journal of Food Science **37**(3): 394-398.

Schoonees, B. and V. Pillay (2004). A new filteration system for gum analysis in raw sugar and molasses. Proc S Afr Sug Technol Ass.

Sentheshanmuganathan, S. (1960). "The mechanism of the formation of higher alcohols from amino acids by Saccharomyces cerevisiae." Biochemical Journal **74**(3): 568.

Silva, F. A., et al. (2013). "Influence of the number of distillations on the composition of organic sugarcane spirit." Journal of the Institute of Brewing **119**(3): 133-138.

Spaho, N., et al. (2013). "Effects of distillation cut on the distribution of higher alcohols and esters in brandy produced from three plum varieties." Journal of the Institute of Brewing **119**(1-2): 48-56.

Spaho, N., et al. (2013). "Effects of distillation cut on the distribution of higher alcohols and esters in brandy produced from three plum varieties." Journal of the Institute of Brewing **119**(1-2): 48-56.

Suomalainen, H. (1971). "Yeast and its effect on the flavour of alcoholic beverages." Journal of the Institute of Brewing **77**(2): 164-177.

Suomalainen, H. and M. Lehtonen (1979). "THE PRODUCTION OF AROMA COMPOUNDS BY YEAST." Journal of the Institute of Brewing **85**(3): 149-156.

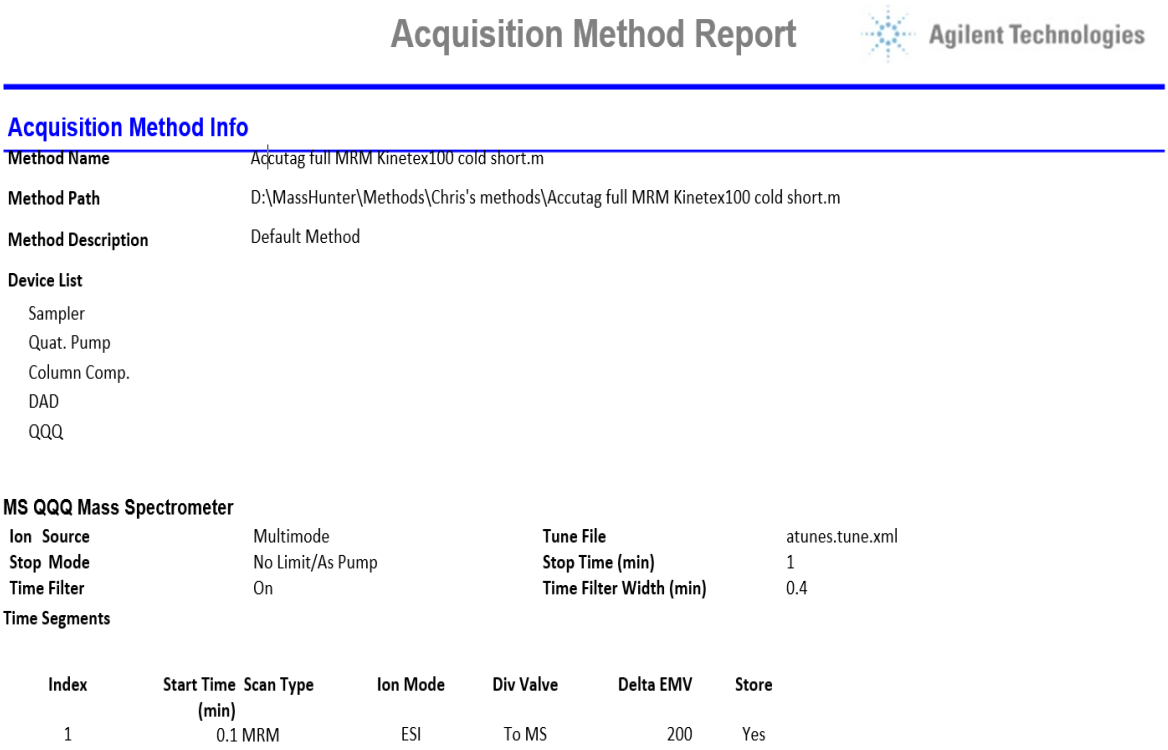
Varzakas, T. and C. Tzia (2015). Handbook of Food Processing. Boca Raton, UNITED STATES, CRC Press.

Xu, W., et al. (2015). "Determination of sugars in molasses by HPLC following solid-phase extraction." International journal of food properties **18**(3): 547-557.

8 Appendix

8.1 LC-MS analysis for amino acids

Agilent 6420 triple quadrupole mass spectrometer fitted with an Agilent Multimode Ionisation source was operated in positive electrospray mode. Optimum Multiple Reaction Monitoring [MRM] transitions were established using Agilent MassHunter Optimiser B06.00 software.



Acquisition Method Report

Agilent Technologies

Acquisition Method Info

Method Name	Accutag full MRM Kinetex100 cold short.m
Method Path	D:\MassHunter\Methods\Chris's methods\Accutag full MRM Kinetex100 cold short.m
Method Description	Default Method

Device List

Sampler
Quat. Pump
Column Comp.
DAD
QQQ

MS QQQ Mass Spectrometer

Ion Source	Multimode	**Tune File**	atunes.tune.xml
Stop Mode	No Limit/As Pump	**Stop Time (min)**	1
Time Filter	On	**Time Filter Width (min)**	0.4

Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store
1	0.1	MRM	ESI	To MS	200	Yes

Time Segment 1

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
Tryptophan	No	375.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Tyrosine	No	352.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Arginine	No	345.2	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Phenylalanine	No	336.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Histidine	No	326.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Methionine	No	320.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Glutamic acid	No	318.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
GLN	No	317.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Aspartic acid	No	304.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
ASN, ORN	No	303.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
HO-Pro, LEU, ILE	No	302.2	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
cystine 291	No	291.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Threonine	No	290.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Valine	No	288.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Proline	No	286.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Serine	No	276.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
d4-alanine	No	264.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
alanine	No	260.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Glycine	No	246.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive
Lysine 244	No	244.1	Unit/Enh (6490)	171.1	Unit/Enh (6490)	60	100	20	7	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
APCIHeater	200	200
Gas Flow (l/min)	6	6
Nebulizer (psi)	60	60
Capillary (V)	2000	2000
VCharging	0	500
APCINeedlePos	0	0
APCINeedleNeg	0	0

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	4000

Instrument Curves

Actual

Acquisition Method Report

Agilent Technologies

Name: Sampler **Model: G1329B**

Auxiliary
- **Draw Speed** 200 µL/min
- **Eject Speed** 200 µL/min
- **Draw Position Offset** 0.0 mm

Injection
- **Injection Mode** Standard injection
- **Injection Volume** 5.00 µL

High throughput

Overlapped Injection
- **Enable Overlapped Injection** No

Stop Time
- **Stop time Mode** As pump/No limit

Post Time
- **Post time Mode** Off

Name: Quat. Pump **Model: G1311B**
- **Flow** 0.300 mL/min
- **Low Pressure Limit** 5.00 bar **High**
- **Pressure Limit** 570.00 bar **Maximum**
- **Flow Gradient** 10.000 mL/min² **Primary**
- **Channel** Automatic

Stroke
- **Automatic Stroke Calculation** Yes

Compress
- **Compressibility Mode** Compressibility Value Set
- **Compressibility** 100 10e-6/bar

Stop Time
- **Stop time Mode** Time set
- **Stop time** 28.00 min

Post Time
- **Post time Mode** Off

Timetable

Timetable

	Time	Function	Parameter
1	1.00 min	Change Solvent Composition	Solvent composition A: 0.0 % B:1.5 % C: 98.5 % D: 0.0 %
2	8.00 min	Change Solvent Composition	Solvent composition A: 0.0 % B:13.0 % C: 87.0 % D: 0.0 %
3	15.00 min	Change Solvent Composition	Solvent composition A: 0.0 % B:17.0 % C: 83.0 % D: 0.0 %
4	16.00 min	Change Solvent Composition	Solvent composition A: 0.0 % B:80.0 % C: 20.0 % D: 0.0 %
5	17.50 min	Change Solvent Composition	Solvent composition A: 0.0 % B:1.5 % C: 98.5 % D: 0.0 %

Solvent Composition

	Channel	Name 1	Used	Percent
1	A	milliQ, 0.1% formic	Yes	0.0 %
2	B	MeCN, 0.1% formic	Yes	1.5 %
3	C	milliQ, 0.6% formic	Yes	98.5 %
4	D	MeOH, 0.1% formic, 10mM NH4 COOH	No	

Acquisition Method Report

Agilent Technologies

Name: Column Comp. **Model:** G1316A

Left Temperature Control
- Temperature Control Mode: Temperature Set
- Temperature: 25.00 °C
- Enable Analysis Left Temperature
  - Enable Analysis Left Temperature On: No

Right Temperature Control
- Right temperature Control Mode: Combined
- Enable Analysis Right Temperature
  - Enable Analysis Right Temperature On: No

Stop Time
- Stop time Mode: As pump/injector

Post Time
- Post time Mode: Off

Name: DAD **Model:** G4212B

Peak width	>0.10 min (2.0 s response time) (2.5 Hz)
UV Lamp Required	No
Analog Output 1	
Analog 1 Zero Offset	5 %
Analog 1 Attenuation	1000 mAU
Signals	
Prepare Mode	
Margin for negative Absorbance	100 mAU
Auto balance	
Auto balance Prerun	No
Auto balance Postrun	No
Spectrum	
Spectrum Store	None
Stop time	
Stop time Mode	As pump/injector
Post time	
Post time Mode	Off

Signals

Signal table

	Use Sig.	Signal
1	No	Signal A
2	No	Signal B
3	No	Signal C
4	No	Signal D
5	No	Signal E
6	No	Signal F
7	No	Signal G
8	No	Signal H

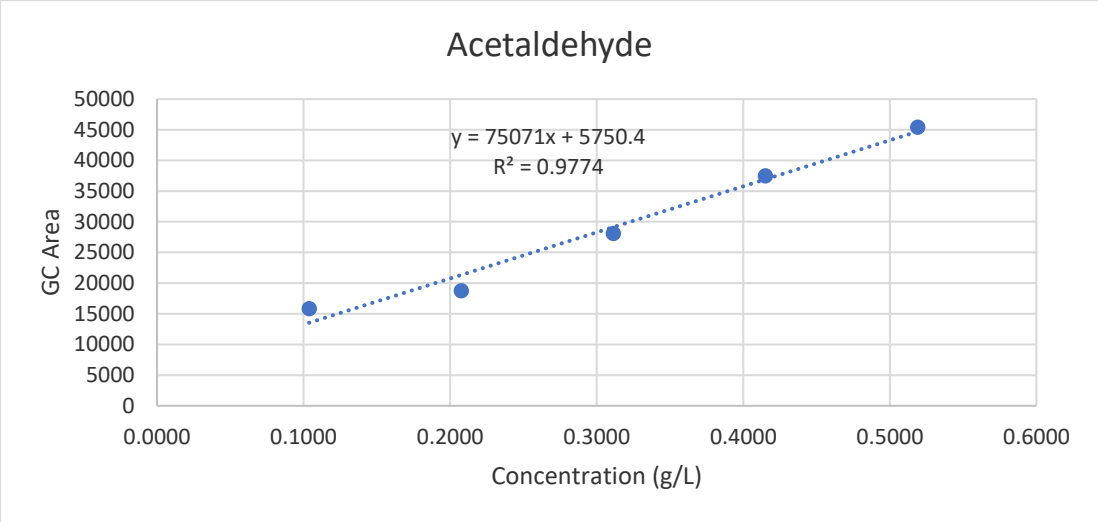
8.2 SPME analysis for pure molasses sample

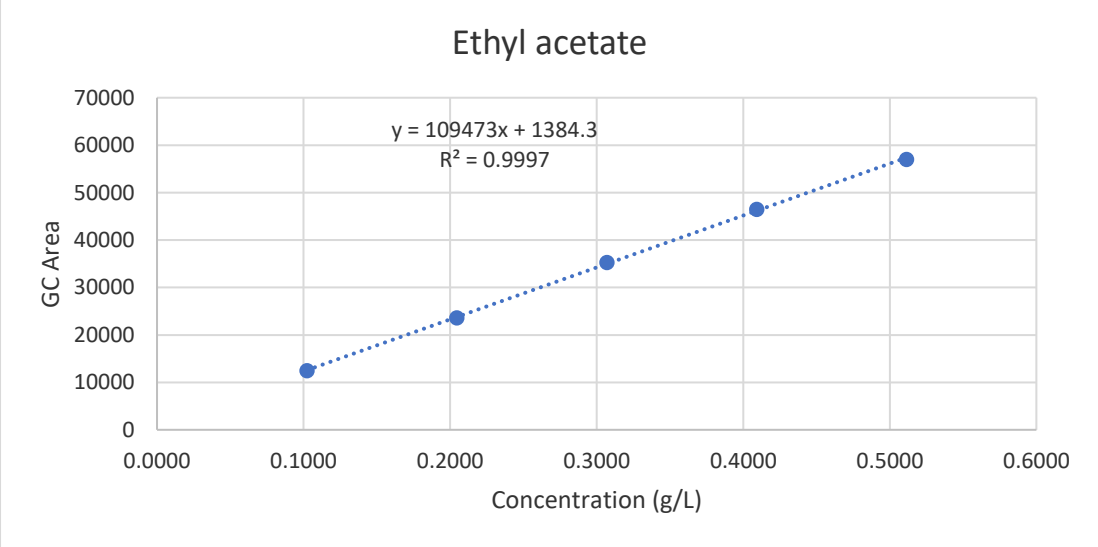
Pure molasses sample was prepared for SPME analysis. 1990 µL sample was pipetted into a 10ml clear glass vial (Thermo scientific) with 0.30 g of sodium chloride (NaCl) (Fisher scientific, UK).

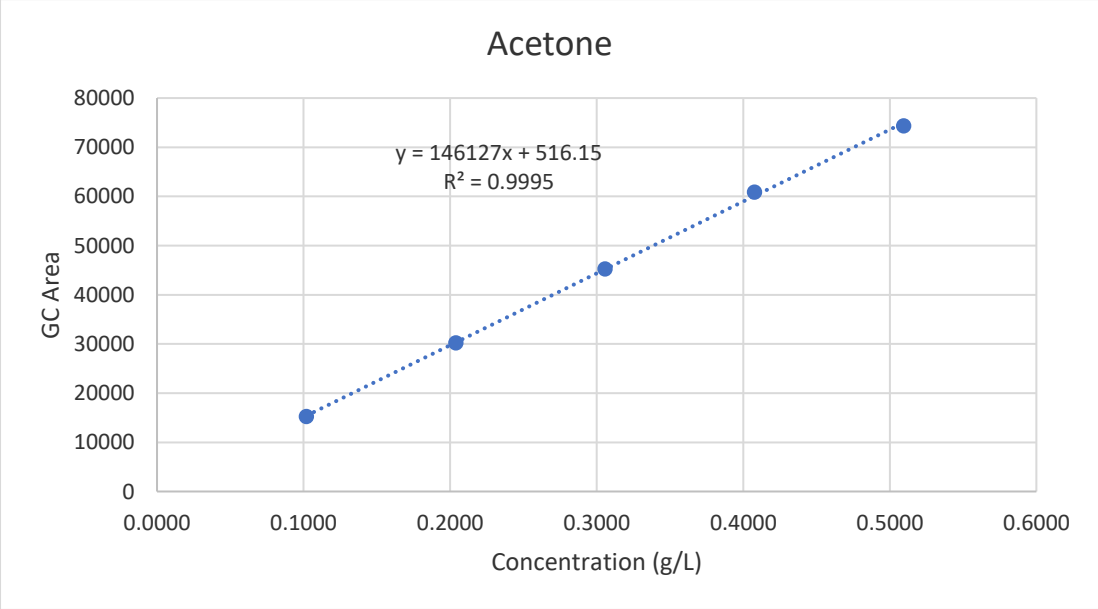
Compounds	Retention time (min)
2-Butanone	2.367
Butanal, 2-methyl-	3.104
Isoamyl nitrite	3.813
Cyclotrisiloxane, hexamethyl-	4.786
Hexanal	6.29
3(2H)-Furanone, dihydro-2-methyl-	7.43
Furfural	9.423
Oxirane, hexyl-	10.015
8-Nonynoic acid	10.334
β-Myrcene	11.391
4,6,6-Trimethyl-bicyclo[3.1.1]heptan-2-ol	11.619
Cyclohexanol, 2,3-dimethyl-	12.178
Cyclohexanol, 2,3-dimethyl-	12.269
Acetoxyacetic acid, tridec-2-ynyl ester	12.861
Isopinocarveol	13.305
Benzaldehyde	13.587
2-Pentanone, 4-cyclohexylidene-3,3-diethyl	14.883
Cyclopentanecarboxaldehyde, 2-methyl-3-methylene-	15.517

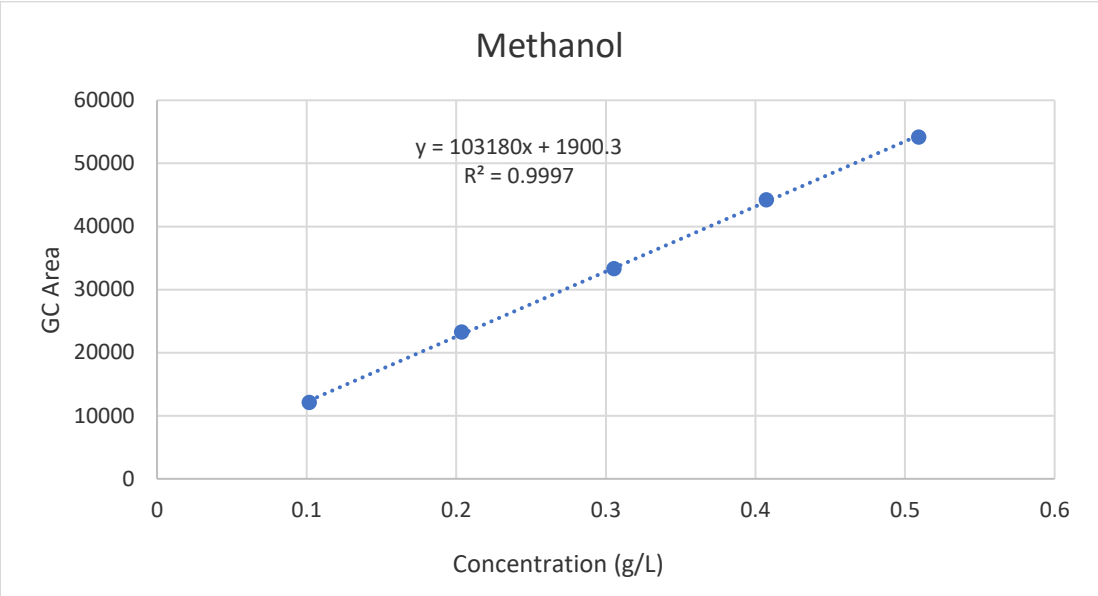
1-Hexanethiol, 2-ethyl-	15.981
Phenol, 2-chloro-	16.255
Phosphonoacetic Acid, 3TMS derivative	16.785
3,6,9,12-Tetraoxatetradecan-1-ol, 14-[4-(1,1,3,3-tetramethylbutyl)phenoxy]-	17.129
Benzeneacetaldehyde	17.361
Heptanoic acid, 2-ethyl	17.73
trans-2-undecenoic acid	17.92
trans-2-undecenoic acid	18.169
2-Naphthol, 1,2,3,4,4a,5,6,7-octahydro-4a-methyl-	18.554
Phenol, 2-methoxy-	19.246
2,5-Dihydroxybenzaldehyde, 2TMS derivative	19.35
Ethanol, 2-[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethoxy]-	19.565
Ethanone, 1-(1H-pyrrol-2-yl)-	19.934
4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one	20.427
Falcarinol	20.663
Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	21.127
Geranyl isovalerate	21.637
Benzene, 2-(2-butenyl)-1,3,5-trimethyl-	21.865
Phosphonoacetic Acid, 3TMS derivative	23.439
Phosphonoacetic Acid, 3TMS derivative	23.369
1H-Indene, 2,3-dihydro-1,1,5,6-tetramethyl-	24.148
alfa.-Copaene	24.628
Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	25.3
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-	26.447
Caryophyllene	26.601
Decanoic acid, ethyl ester	26.903
Humulene	27.798
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	27.893
γ-Muurolene	28.179
Hexadecane	30.877
Megastigmatrienone	34.879

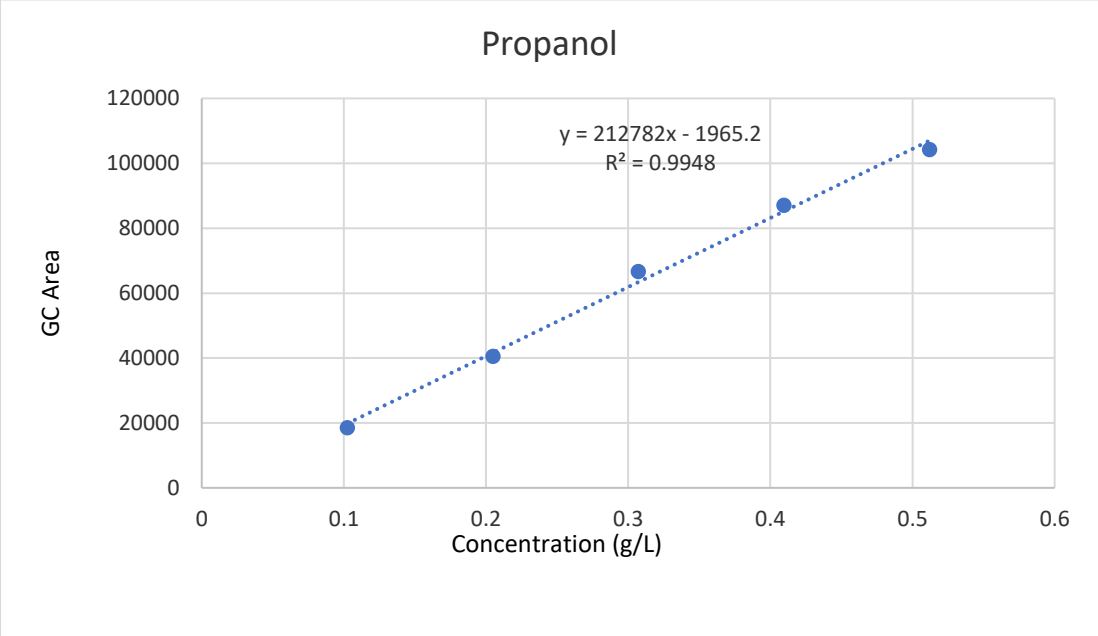
8.3 Calibration curves for considered chemicals.

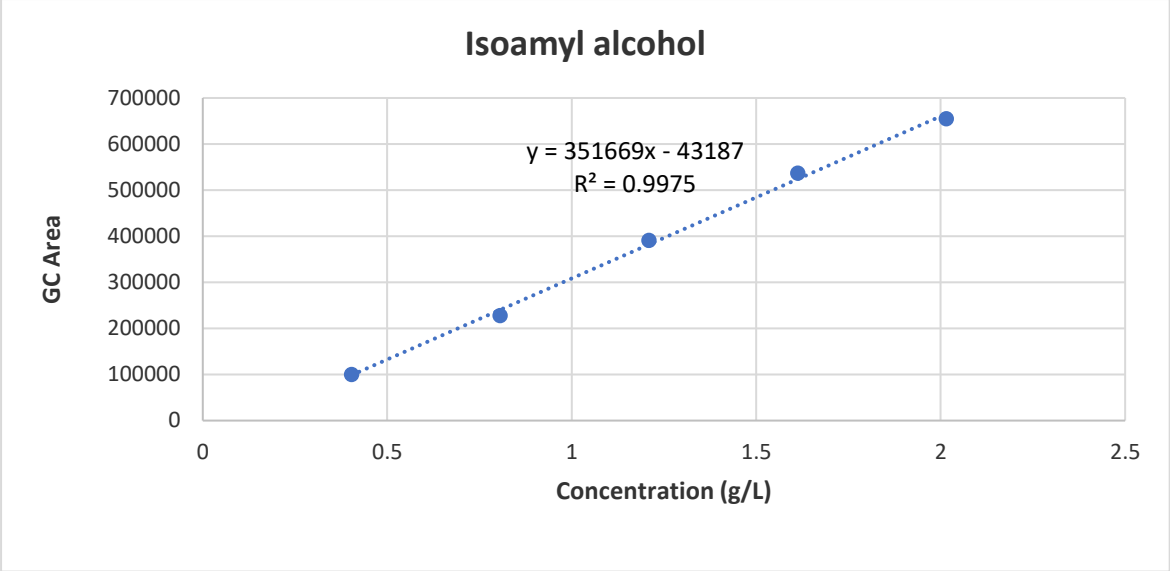


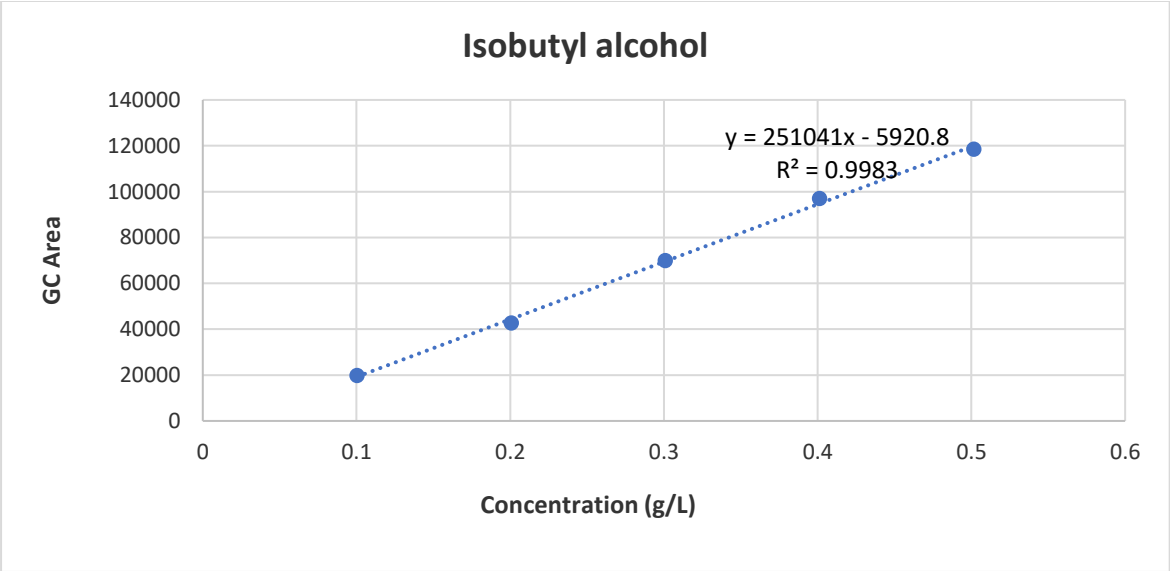


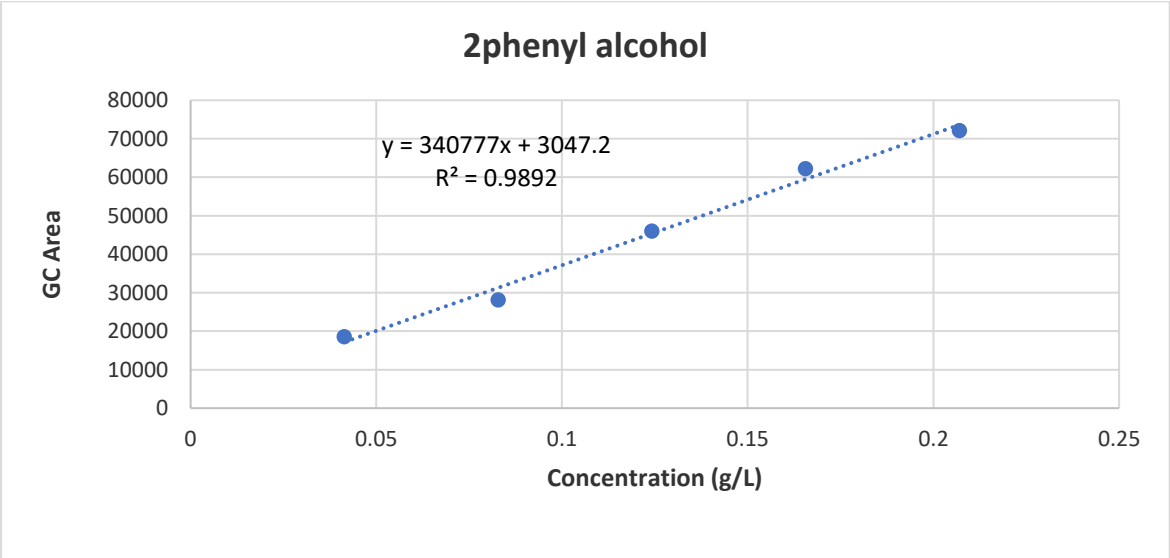


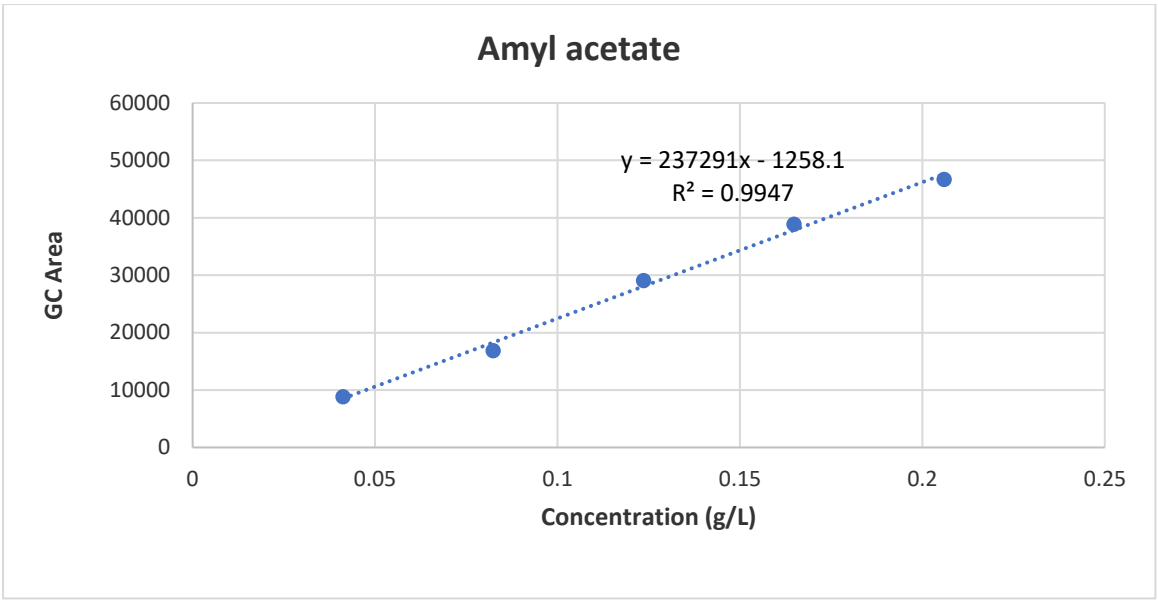

Methanol
y = 103180x + 1900.3
R² = 0.9997
GC Area
Concentration (g/L)
60000
50000
40000
30000
20000
10000
0
0
0.1
0.2
0.3
0.4
0.5
0.6

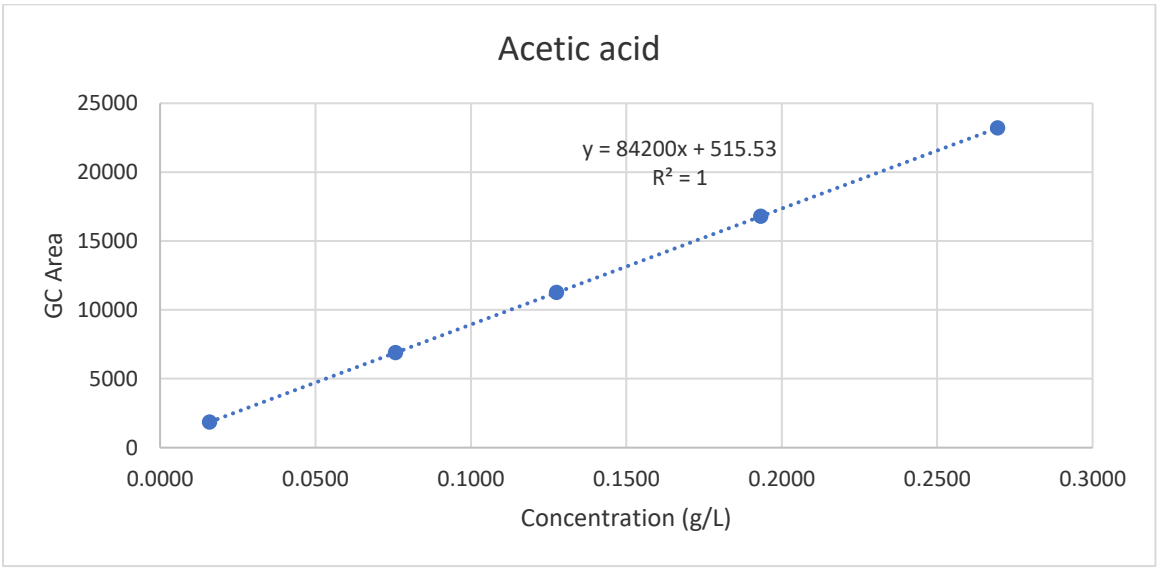

Propanol
y = 212782x - 1965.2
R² = 0.9948
GC Area
Concentration (g/L)
120000
100000
80000
60000
40000
20000
0
0
0.1
0.2
0.3
0.4
0.5
0.6

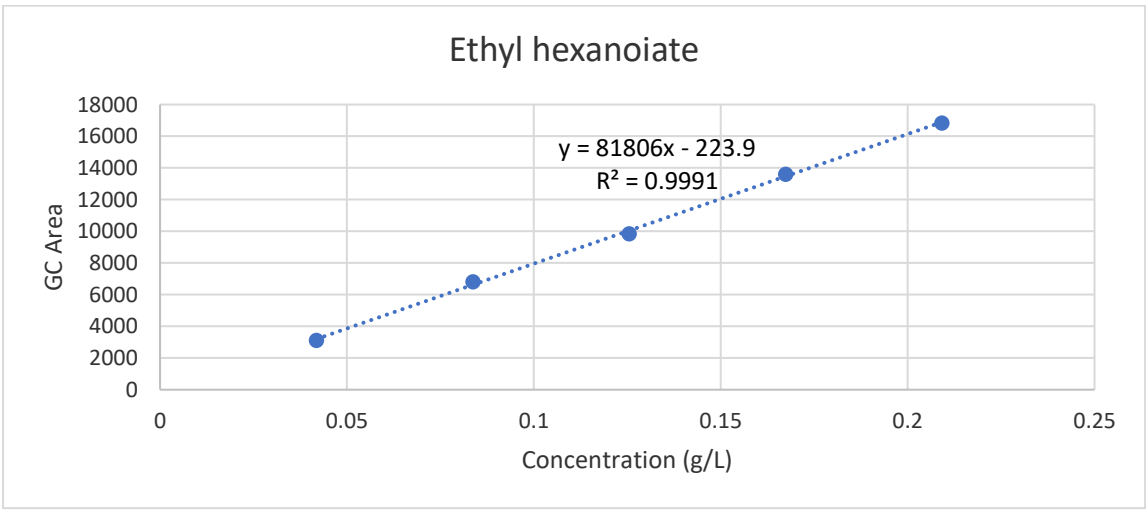

Isoamyl alcohol
y = 351669x - 43187
R² = 0.9975
GC Area
Concentration (g/L)
700000
600000
500000
400000
300000
200000
100000
0
0
0.5
1
1.5
2
2.5

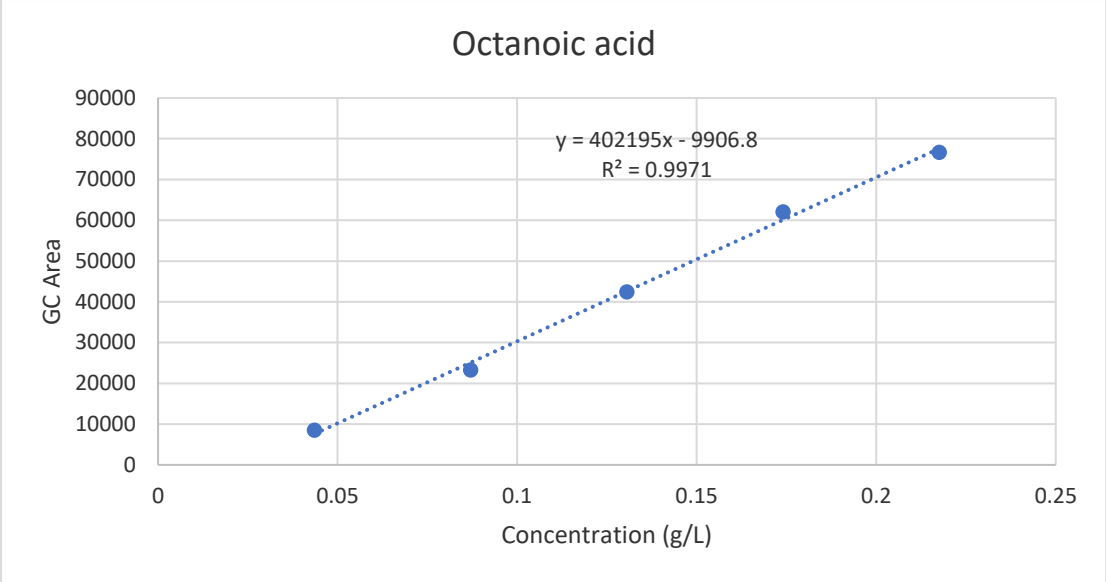

Isobutyl alcohol
y = 251041x - 5920.8
R² = 0.9983
GC Area
Concentration (g/L)
140000
120000
100000
80000
60000
40000
20000
0
0
0.1
0.2
0.3
0.4
0.5
0.6

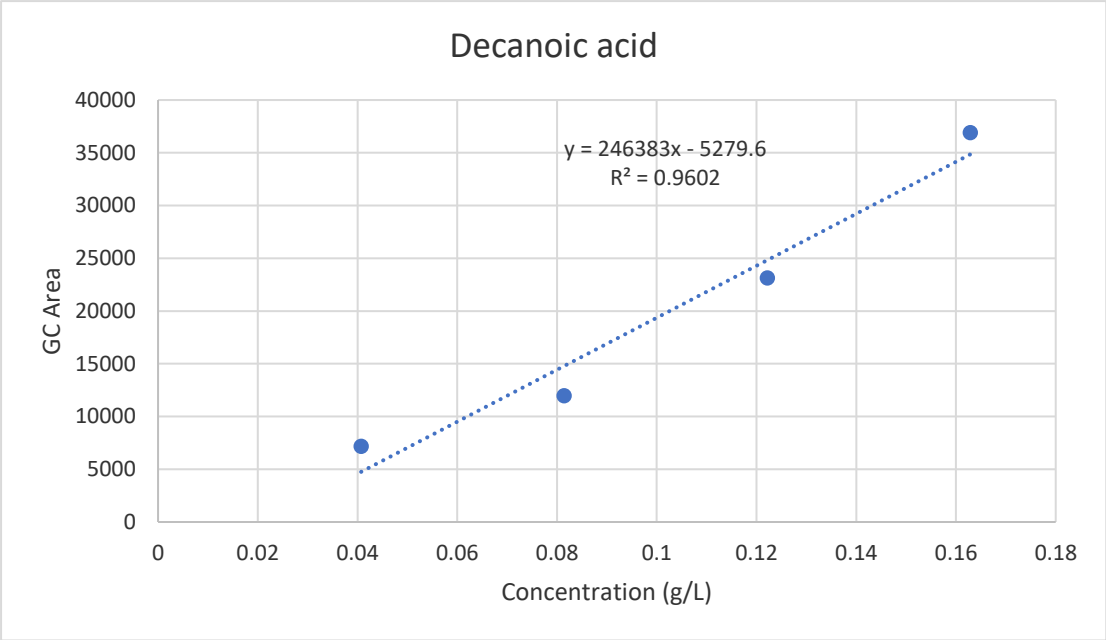

2phenyl alcohol
y = 340777x + 3047.2
R² = 0.9892
GC Area
Concentration (g/L)
80000
70000
60000
50000
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25


Amyl acetate
y = 237291x - 1258.1
R² = 0.9947
GC Area
Concentration (g/L)
60000
50000
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25


Acetic acid
y = 84200x + 515.53
R² = 1
GC Area
Concentration (g/L)
25000
20000
15000
10000
5000
0
0.0000
0.0500
0.1000
0.1500
0.2000
0.2500
0.3000


Ethyl hexanoiate
y = 81806x - 223.9
R² = 0.9991
GC Area
Concentration (g/L)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
0.05
0.1
0.15
0.2
0.25


Octanoic acid
y = 402195x - 9906.8
R² = 0.9971
GC Area
Concentration (g/L)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
0.05
0.1
0.15
0.2
0.25


Decanoic acid
y = 246383x - 5279.6
R² = 0.9602
GC Area
Concentration (g/L)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
0.18

8.4 Synonym of used chemicals in entire study.

No.	Names of chemicals (IUPAC)	Synonyms
1.	Acetaldehyde	Ethanal, Acetic aldehyde, Ethyl aldehyde
2.	Ethyl acetate	Acetic acid ethyl ester, Acetic ether, Acetoxyethane, Ethyl acetic ester
3.	Acetic acid	Ethanoic acid, Ethylic acid, Vinegar acid, Glacial acetic acid
4.	Propionic acid	Propanoic acid, Ethyl formic acid, Propionate
5.	Iso amyl alcohol	3-Methyl-1-butanol, Isoamyl alcohol, Isopentyl alcohol
6.	Iso butyl alcohol	2-Methyl-1-propanol, Isobutanol
7.	Propan-1-ol	1-propanol, propanol, propyl alcohol
8.	Amyl acetate	Pentyl acetate, n-Amyl acetate, acetic acid, pentyl ester
9.	Octanoic acid, ethyl ester	Ethyl octanoate, Ethyl caprylate, Ethyl n-octanoate, Ethyl octylate
10.	Decanoic acid, ethyl ester	Ethyl decanoate, Ethyl caprate, Ethyl caprinate, Ethyl decylate
11.	Hexanoic acid, ethyl ester	Ethyl caproate, Ethyl hexanoate, Caproic acid ethyl ester, Ethyl n-hexanoate, Ethyl butyl acetate
12.	Acetic acid,2-phenylethyl ester	Phenethyl acetate, 2-Phenylethyl acetate
13.	Pentadecanoic acid,3-methyl butyl ester	Isoamyl decanoate, Iso pentyl decanoate, Decanoic acid, 3-methylbutyl ester, Isoamyl caprate, Iso amyl decanoate (natural)
14.	Dodecanoic acid, ethyl ester	Ethyl decanoate, Ethyl caprate, Ethyl caprinate, Ethyl decylate
15.	1-Butyl 3methyl acetate	1-butyl-3 methyl-1H-imidazol-3-ium acetate
16.	Octanoic acid	Caprylic acid, Octanoate
17.	Hexanoic acid	Caproic acid, Capronic acid
18.	Decanoic acid	Capric acid
19.	Dodecanoic acid	Lauric acid, Dodycylic acid
20.	Napthalene,1-2,3,4-tetrahydro-1,6,8trimethyl-	2,5,8-Trimethyltetralin