

ADDIS ABABA UNIVERSITY  
COLLEGE OF NATURAL SCIENCES  
CENTER FOR FOOD SCIENCE AND NUTRITION



**Comparison of Volatile Flavor Compounds in Ethiopian Alcoholic  
Beers**

By

**Emnet Tegene**

Advisor:

**Dr. Ashagrie Zewdu**

A thesis submitted to Centre for food science and nutrition of Addis Ababa University in partial fulfillment of the requirement for the Degree of Master of Science in Food Science and Nutrition.

March, 2018  
Addis Ababa, Ethiopia

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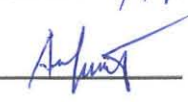
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### DECLARATION

I, the undersigned, declare that this thesis is my original work and that all sources of materials used for the thesis have been duly acknowledged.

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## List of abbreviations and acronyms

VDK	Vicinal Diketones
KAP	Knowledge, Attitude and Practice
ECD	Electron Capture Detector
GC	Gas Chromatography
HS-GC	Head Space Gas Chromatography
DMS	Dimethyle Sulphide
FID	Flam Ionization Detector
V/V	Volume By Volume
W/W	Weight By Weight
FNA	Free Amino Nitrogen
SMM	S-Methyl Methionine
BAC	Blood Alcohol Concentration
EBC	Europeans Beer Convention
ASBC	American Society Of Beer Convention
FV	Flavour Unit
TC	Technical Committees
LB	Light Beer
SB	Strong Beer
ESB	Extra Strong Beer
PPb	Parts Per Billion
PPm	Parts Per Million
MS	Mass Spectrophotometer
RSD	Relative Standard Deviation
FDA	Food and Drug Agency
Kpa	Kilo Pascal
RF	Response Factor
IS	Internal Standard
SD	Standard Deviation
QMS	Quality Management System
HACCP	Hazard Analysis and Critical Control Point

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## Abstract

*The objective of this study was to compare the level of flavor compounds of Ethiopian alcoholic beers. In addition, the knowledge, attitude and practice (KAP) study was employed to assess the level of understanding of professionals working in breweries about the flavor compounds related to beer quality and safety. A total of 220 beer samples from 11 brands were collected randomly from different retail outlets and food markets across ten sub-cities of Addis Ababa. For each brand, two beers were randomly collected from each sub city. For analysis, two composite samples were prepared by taking 20 ml from the 330 ml beer bottle and each brand were analyzed in quadruplicate. VDKs were analyzed with HS-GC-ECD method, while Acetaldehyde, Esters, Dimethyl sulfide (DMS) and higher alcohols were analyzed with HS-GC-FID method. A structured questionnaire was used to undertake KAP study. One-way ANOVA were used to evaluate the significance difference between brands at the 0.05 level of significance and Duncan HSD was used to separate the means. Volatile flavor compounds were detected in this study and they were in the range between (13.8-128.2 µg/l) for total vicinal diketones, (1.65-8.0 mg/l) for Acetaldehyde, (0.00-76.35 µg/l) for DMS, (11.75- 28.35 mg/l) for Ethyl acetate, (8.40-20.95 mg/l) for n-Propanol, (5.85-17.7 mg/l) for Iso-butanol, (0.10-3.95 mg/l) for Isoamyl acetate and (38.05-6.50 mg/l) for Amyl alcohol. This means the total VDK content of three brands of beers were above the threshold value and only two brands have acetaldehyde concentration above the threshold value. In one brand, DMS is not detected and three brands were above the threshold value. For Iso-amylacetate, ten brands were above the threshold value. Conversely, the higher alcohols n-Propanol and Iso-butanol content of all brands were in the acceptable threshold value. For amyl alcohol five brands contain above the threshold value and only one brand were above the threshold value for ethyl acetate. Majority of the alcoholic beers contain acceptable level of volatile flavour compounds except for their Iso-amyl acetate and Amyl alcohol content. In addition, level of Vicinal diketones, Acetaldehyde, and DMS in some Ethiopian alcoholic beers is significant. This shows that the maturation time during fermentation process might not be enough to reduce the level of these compounds. As a result of this, brands with significant amount of acetaldehyde might have hangover effect when consumed in large amount. By considering customer satisfaction, brewers have to take actions and assess the root causes for those flavour compounds which are above the threshold value. Moreover, knowledge of some professional employees in the beer factories are not satisfactory, especially about selected*

*flavour compounds such as VDK*

***Key words:*** *Beer, Flavour, VDK, DMS, Higher alcohols, Esters, Quality, Threshold, Ethiopia*

# Chapter One

---

## *Introduction*

# 1. Introduction

## 1.1 Background

Beer is an alcoholic beverage produced from cereals by enzymatic conversion of starch into fermentable sugars followed by a fermenting process (Damerow,2012). It is one of the most popular alcoholic beverages in the world, and it has been consumed in steadily increasing amounts in almost every country (Huimin, Hongjun, Xiuhua, & Bing, 2012). This fermented beverage was most likely created by accident thousands of years ago. Despite the massive technological growth that separates ancient brewing from today's high-tech breweries, the process in its traditional version remains entirely unchanged (Pires & Branyik, 2015).

The quality of beer is mostly judged based on its flavour and aroma profile arising from a broad spectrum of *flavour active compounds* (Bamforth & Kanauchi, 2004). *Volatile compounds* in beer are substances that contribute significantly to the organoleptic properties of beverages and thus affect product quality both in positive and negative way (Kleinová & Klejdus, 2014). The major volatile products of yeast metabolism are ethanol, glycerol and carbon dioxide, which make a relatively weak but nonetheless fundamental contribution to beer flavour. Important aroma compounds that characterize the quality and palatability of beer are, esters, *higher alcohols*, sulphur compounds, organic acids and carbonyl compounds (*aldehydes ketones*), which is originated from yeast metabolism during primary fermentation. Of all the yeast secondary metabolites responsible for the principal flavour of finished beer, *higher alcohols* and esters are deemed to be a highly significant group of flavour-active metabolites (He, et al., 2014).

Beer has been widely consumed in Ethiopia for a while and as a result the country enjoys a variety of beer brands. Historically the most popular of these brands is St. George Beer (named after the country's patron saint) which is the country's oldest brewery, established in 1922 (Sisay & Andualem, 2017). Currently Ethiopia's beer industry is comprised of seven major breweries plants. These are BGI Ethiopia, Meta Abo, Heineken, Dashen, Habesha, Raya and Zebider. Harar and Bedele are under Heineken brewery SC. These breweries brew and deliver more than fifteen commercial brand beers to the consumer (Sisay, 2015).

*VDKs* are normal products of brewery fermentations, but are generally considered to be undesirable contributors to the flavor of beers. The two major *VDKs* of concern to the brewer are *diacetyl* (*2,3-butanedione*) and *2,3-pentanedione* (Bamforth & Kanauchi, 2004). Both *VDKs* are formed from intermediates of the amino acid biosynthesis. The first intermediates in this metabolism are  $\alpha$ -acetolactate and  $\alpha$ -acetoxybutyrate. These components are discharged from the cell and undergo an oxidative decarboxylation to form *diacetyl* and *2,3-pentanedione* (Naydenova, Iliev, Kaneva, Kostov, Koprinkova-Hristova, & Popova, 2014). Their flavour is undesirable in many beer styles and it can also indicate microbial contamination, for example, by *Lactobacillus* spp., *Pediococcus* spp. or *Pantoea agglomerans* (Krogerus & Gibson, 2013).

*Dimethyl sulfide* is a volatile sulfur based organic compound derived from S-methyl methionine (SMM) which is an amino acid derivative synthesized when grain germinates during malting. No DMS is produced during germination (Barnes, 2013). *DMS* has the taste and aroma of sweet corn. This comes either from the malt, as a result of the short or weak boil of the wort, slow wort chilling or bacterial infection (Ruppel & Grecsek, 2005). *Enterobacteria* have been found to possess the ability to produce *DMS* along with several other sulfur compounds (Witrick, 2012).

During alcoholic fermentation, a sizeable quantity of esters can be produced as a result of yeast metabolism. Esters are one of the most *volatile compounds* in beer and hence impact greatly beer aroma. In moderate quantities, they can add a pleasant, full-bodied character to beer aroma. When present in excess, however, they give beer aroma an overly fruity quality, which is considered undesirable by most consumers (Zhang, Cao, Wang, & Li, 2017).

*Acetaldehyde* is an important flavor compound. It induces flavor of freshly cut green apples, grass or green leaves in beer (Engan, 1991). Higher concentrations of this metabolite are undesirable, due to the unpleasant off-taste (Ashurst & Dennis, 1998), which is characterized as "young", "green" it leaves in beer. Aldehydes are intermediate metabolites in alcohol fermentation. They could also result from alcohol oxidation. *Acetaldehyde* could also be a result of bacterial contamination of beer with microorganisms from the genes *Zymomonas* and *Acetobacter* (Petkova & Jonkova, 2010).

Several *higher or fusel alcohols*, other than ethanol are formed in beer during fermentation, among which *n-propanol*, *iso-butanol* and isoamyl alcohols (2-methyl and 3-methyl butanol)

contribute most significantly to beer by alcoholic or solvent-like aroma and a warm mouth feel. *Higher alcohols* achieve maximum concentrations during batch fermentation at a time roughly coincident with cell growth arrest and minimum free amino nitrogen (FAN) concentration. Their formation takes place by the so-called anabolic and catabolic route (Branyk, Vicente, Dostalek , & Teixeira, 2008).

## **1.2. Statement of the problem**

Success in today's highly competitive global market requires producers to be quality conscious that helps them in providing better quality products (Huimin, et al.,2012).The quality of beer is mostly judged based on its flavour and aroma profile arising from a broad spectrum of *flavour active compounds* (He, et al.,2014). Placing great emphasis on producing good quality beer products helps to ensure that the products offered to their customers are consistent, reliable and truly meet their customer's needs (Huimin , et al., 2012). Flavor is considered as the main index in evaluating the quality of beer. And it is a very important factor for both brewers and consumers in commercial market (Zhang, et al., 2017). Quality is defined as 'degree of excellence, relative nature, or kind, or character'. When the customer has chosen the quality he/she wishes to drink, he/she demands that his/her beverage shall have the 'degree of excellence' which he/she expects and this shall not change from day to day. Today the concept of quality assurance is more important (Briggs, Boulton, Brookes, & Stevens, 2004).

The enjoyment of a glass of beer may be received by many senses. Alcoholic content, nutritive value, colour, clarity, usually, the absence of haze, the formation and retention of a good head of foam, and the absence of gushing, all contribute to the enjoyment but it is the flavour, the taste and aroma, which really determine the acceptability and drinkability of the beer (Briggs, et al.,2004). Deviations from specification can result in pronounced off-flavours and consumer unacceptability.

Even if there have been large increases in the consumption frequency and quantity of beer is high in Ethiopia by referring the brewing capacity increment, from a level of just 1 million hectoliters in 2003/04, beer production has risen to nearly 10 million hectoliters by 2015/16 (Access Capital, 2010), there are no studies carried out related to *flavour compounds* of Ethiopian beers and their quantification to assure quality standards.

### 1.3. Significance of the study

- The main concern of this study is to determine and compare the level of *volatile flavor compounds* in Ethiopian alcoholic beers and recognize its level of significance which is unknown before.
- The study will create awareness for beer manufacturers and it helps to which area they should focus regarding to controlling *volatile flavour compounds* of beer to produce quality beer products.
- To disseminate the level of significance of flavour compounds to the government regulatory bodies (Ethiopian Standard Authority )
- To disseminate the level of significance and create awareness for consumers about *volatile flavor compounds* in Ethiopian alcoholic beers
- Rendering of base line data for further researches on *volatile flavor compounds* in beer.

### 1.4. Research questions

- Is there a significant amount of *volatile flavor compounds* in Ethiopian alcoholic beers?
- What is the existing KAP in brewing industries of the cause and effect of flavor compounds (*vicinal diketones*) in beer?

### 1.5. Objectives of the study

#### 1.5.1. General objective

- To compare the level of *volatile flavor compounds* among Ethiopian alcoholic beers and to assess the existing KAP about *flavor compounds* in Ethiopian beer industries.

#### 1.5.2. Specific objective

- To determine the level of *Vicinal diketons, Acetaldehyde, Easters, Higher Alcohols* and *DMS* in Ethiopian alcoholic beers.
- To compare the level of *Vicinal diketons, Acetaldehyde, Easters, Higher Alcohols* and *DMS* in different Ethiopian alcoholic beers and with international standards.
- To assess the existing KAP about flavor compounds (*vicinal diketones*) in Ethiopian beer industry.

# Chapter Two

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*Literature review*

## 2. Literature Review

### 2.1. Beer: An Overview

Manufacture of all kind of alcoholic beverages utilizes the ability of yeast to ferment sugars in to alcohol. Beer is a special kind of beverage where it is famous all over the world under the category of low alcoholic beverage. The recognized definition of beer would be an alcoholic beverage produced by the fermentation of sugars derived from malted barley and flavoured with hops (Ariyaratne, 2012).

### 2.2. Beer Styles

Although beers are brewed from similar materials, beers throughout the world have distinctive styles (Ariyaratne, 2012). In a general way, there are two classical beer styles, ales and lagers according to the type of yeast added to wort, that produce typical and distinct beer flavour profiles. These differences result from the morphologic and physiologic characteristics of the yeasts and from their performance in fermentation (Vieira, 2017).

#### a. Lager beer

Lager is by far the biggest proportion of beer sold. Its delicate flavour comes from a relatively low bitterness, the use of a bottom fermenting yeast, cold maturation and the use of a malt that is relatively under modified and lightly kilned (Ariyaratne, 2012). It ferments at lower temperature (4-12°C) for a longer period of time, the active yeast flocculates at the bottom of the fermentation vessel and, usually, *Saccharomyces uvarum*, also known as *S. carlsbergensis*, strains are used (Vieira, 2017).

#### b. Ale beer

Ales are produced mainly in the United Kingdom and the Republic of Ireland. Their flavours come from the use of well modified and biscuit flavoured malt which is sometimes highly coloured and the use of top fermenting yeast (Ariyaratne, 2012). It ferments at higher temperatures (14-15°C) for a shorter period of time, the active yeast settles on the top of fermentation vessel and, usually, *Saccharomyces cerevisiae* strains are used. These specifications lead to a highly fruity beer, due to an increased esters formation (Vieira, 2017). Ales come in various forms, bitter pale ales and mild beers (Ariyaratne, 2012).

### 2.3. Brewing Process

Beer production can be understood as a series of chemical and biochemical reactions combined with solid-liquid unit operations for the separation and clarification of the brewing wort and the final beer (de Medeiros, de Medeiros, & da Silva, 2017). The main stages in the brewing process are wort production; alcoholic fermentation and maturation; processing and stabilization of the beer. The wort transforms into beer during alcoholic fermentation and maturation. The ethanol fermentation occurs as a result of enzymatic activity of the yeast which leads to glucose conversion to pyruvate. Under anaerobic conditions the yeasts convert pyruvate to ethanol and CO<sub>2</sub>. In aerobic conditions, yeasts consume sugars, mainly for biomass accumulation and CO<sub>2</sub> production (Vassilev, et al.,2013). Wort is a complex and equilibrated solution that contains fermentable carbohydrates, amino acids and minerals and is a source of nutrients for yeast to perform alcoholic fermentation. Thus, the main goal of wort production is to prepare everything that is needed for the fermentation (Vieira, 2017).

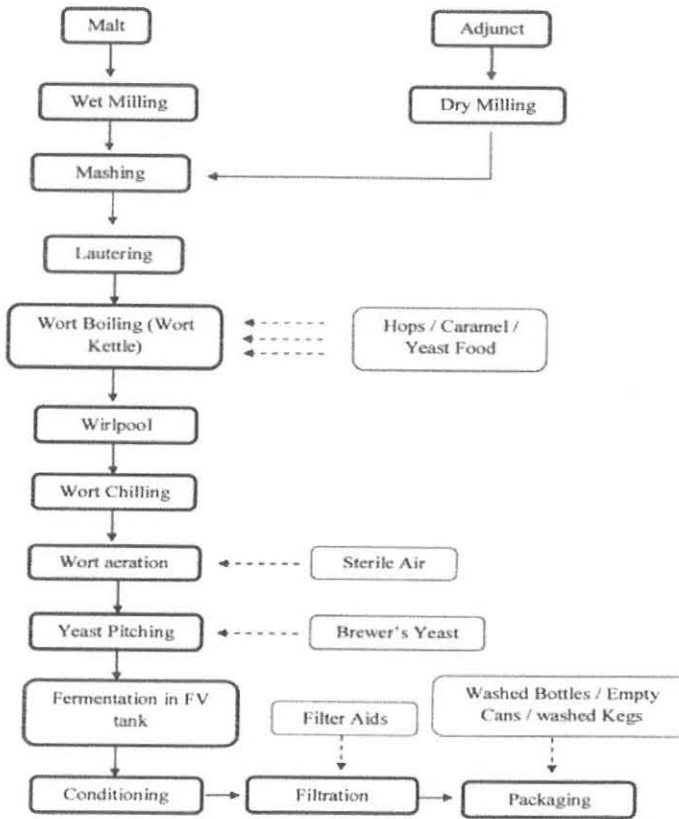


Figure 2.1 Basic flow chart of Beer production with the ingredients (Ariyaratne, 2012).

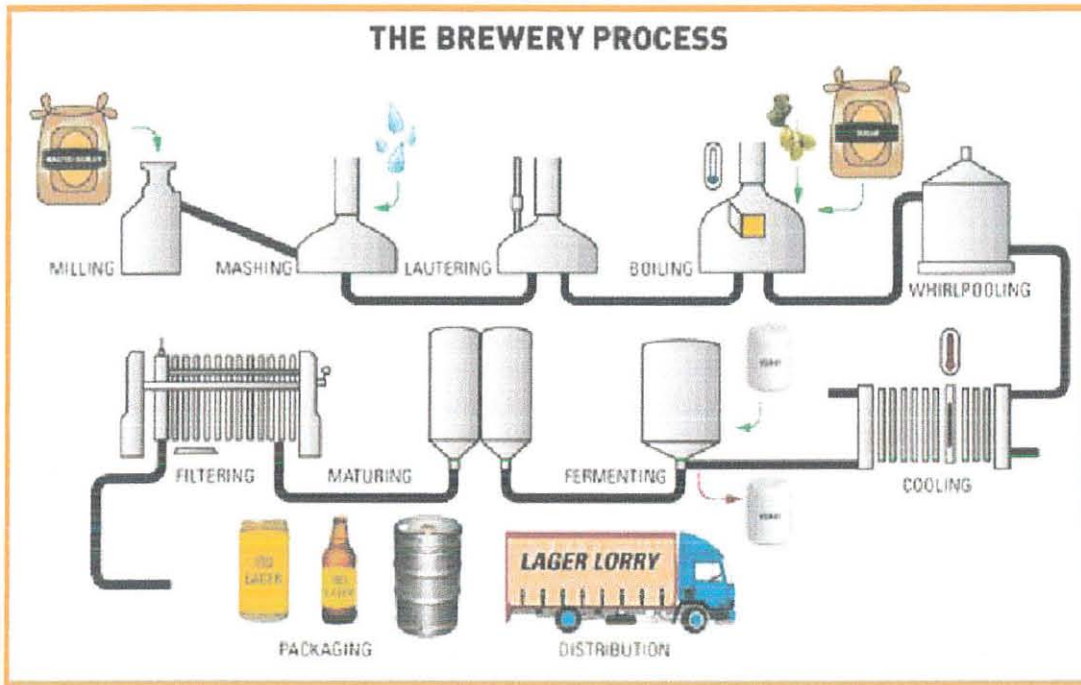


Figure 2.2 Flow diagram for beer process (Antonio & M.Luisa, 2015)

### 2.3.1. Milling

Before mashing, the malt and other grains must be milled in order to increase the contact surfaces between the brewing liquor and malt. The ground malt (with or without other unmalted grains) is called grist (Pires & Brányik, 2015).

### 2.3.2. Mashing

To initiate mashing, the grist is mixed with water (mashing-in) at a pre specified temperature to produce a slurry known as mash. Subsequently, the mash is heated to optimum temperatures of the technologically most important enzymes and allowed to rest (Pires & Brányik, 2015). This resting enzyme activity (enzymatic rest e.g.  $\beta$ glucanase-, protease-, and amylase rest) can be utilized during mashing, to liquefy carbohydrates and proteins. Mashing temperature should thus be raised slowly according to the optimal temperatures of the mashing enzymes; 40–45 °C for  $\beta$ -glucanase, 50–54 °C for protease, 62–67 °C for  $\beta$ -amylase, and 71–72 °C for  $\alpha$ -amylase (Manelius, 2017). The breakdown of starch into fermentable sugars is quantitatively the most important task occurring during mashing. At the end of the mashing, it is necessary to separate the aqueous solution of the extract (wort) from the insoluble fraction called spent grains (Pires & Brányik, 2015).

### 2.3.3. Wort Boiling

After separation from the residual solids (brewer's spent grains), the hot sugary liquid (wort) is boiled with hops. Furthermore, it is also in this stage that sugar adjuncts as sucrose, malt syrup, and sugar cane may be added as "wort extenders" to increase extract. The whole process takes from 90 to 120 min and, the crucial processes taking place during wort boiling are: inactivation of enzymes; sterilization; precipitation of proteins (hot break); evaporation of water and unwanted volatiles such as *dimethyl sulfide*, isomerization of hop  $\alpha$ -acids; and the formation of flavour compounds through Maillard reaction. After separation of hot break and cooling, the wort is aerated and it is ready for pitching (Pires & Brányik, 2015).

### 2.3.4. Fermentation and Maturation

Fermentation and maturation are the longest processes in brewing. The primary fermentation lasts between 3-6 days and the maturation up to 2 weeks depending on the fermentation type and the used equipment (Vassilev, et al., 2013). The fermentation step is critical and the most important part of the process and demands extensive control mechanisms since its success is directly affected by process conditions such as temperature, agitation, pH, oxygen input, nutrient and yeast concentrations. Fermentation can be divided into two clearly separated stages: i) the first part occurs in the presence of oxygen and is characterized by a rapid increase on cell concentration and the formation of the *vicinal diketones*, ii) second part occurs in the absence of oxygen and the yeast cells consume the *vicinal diketones* present in the wort. Beer fermentation is responsible not only for the conversion of the wort's sugary content in to beer's alcohol content, but also for the production of biochemicals by products responsible for flavour, for instance, *vicinal diketones*, *superior alcohols*, esters and carboxylic acids (de Medeiros, et al., 2017).

Maturation or lagering starts right after most of the yeasts have been removed from beer. During this process, beer rests at very low temperature for several weeks, in a highly carbonated atmosphere. Low temperatures are responsible for the precipitation of insoluble complexes, like those formed between polyphenols and proteins which, if not removed, may cause beer haze. On the other side, residual yeast cells start a secondary fermentation by reducing some beer off-flavours, like *vicinal diketones*, into less flavour active compounds. *Diacetyl* removal is one of

the main purposes of beer maturation (Vieira, 2017). Vicinal diketones (VDK) are often used as markers of the beer maturity. The beer is thus considered mature when the VDK levels have fallen under a certain level (Manelius, 2017).

**Table 2.1. Multidisciplinary factors affecting beer flavour in continuous fermentation systems (Brányik, et al., 2008).**

Factors affecting beer flavour	Comments on flavor impact
Temperature	Increasing the production of growth related by-products ( <i>VDKs</i> , <i>higher alcohols</i> , <i>acetaldehyde</i> ) and enzyme activity (esters)
Dissolved oxygen	Increasing the production of growth related by-products ( <i>VDKs</i> , <i>higher alcohols</i> , <i>acetaldehyde</i> ) and inhibiting ester formation
Residence time	Enhancing <i>diacetyl</i> removal and increasing attenuation
Wort composition	Complex (e.g. higher extract increases formation of volatiles; optimum FAN reduces <i>diacetyl</i> formation etc.)

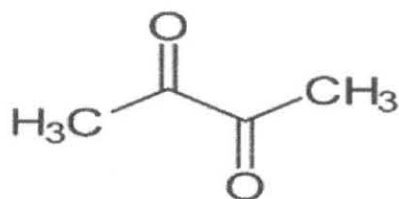
## 2.4. Flavour Compounds During Continuous Beer Fermentation

The most important process in fermentation is the conversion of sugars to ethanol and CO<sub>2</sub>. However, a great many other reactions occur during brewery fermentations with far reaching impacts on the flavour of beer. It is difficult to separate reactions occurring in fermentation from those occurring during maturation, so it is best to consider it as a continuous process. Fermentation flavour characteristics can be divided into two categories: 1) Green beer flavour substances: *diacetyl*, *aldehydes*, sulphur compounds. These are produced early in fermentations, giving beer an immature, unripe and unbalanced taste and can be biochemically removed by yeast during maturation. 2) Mature beer flavour substances: *Higher alcohol and esters*. These are responsible for the complex aroma of beer and once formed cannot be removed by yeast. Although many different types of flavour compounds are developed during fermentation, it is only those with a sufficiently low flavor threshold that will affect the character of the beer. The most important beer flavour compounds, include: *Esters* (fruity characters), *Higher alcohols* (winey characters), *Vicinal Diketones* (buttery characters), *Aldehydes* (oxidation characters), Sulphur Compounds (rotten egg and vegetal characters) (American Brewers Guild, 2016).

### 2.4.1. VDK production and reduction

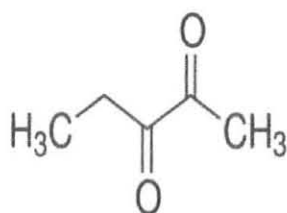
*VDKs* are normal products of brewery fermentations, but are generally considered to be undesirable contributors to the flavor of beers. The two major *VDKs* of concern to the brewer are

*diacetyl*(2,3-butanedione) and 2,3-pentanedione (Bamforth & Kanauchi, 2004). 2,3-butanedione is a simple dicarbonyl compound, has a relatively simple structure of CH<sub>3</sub>-CO-CO-CH<sub>3</sub> as showed in Figure 2.3.



**Figure 2.3. Diacetyl chemical structure**

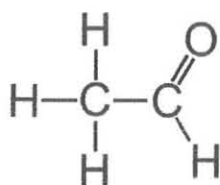
2, 3-pentanedione (Figure 2.4), is an intensively yellow compound, with the structure CH<sub>3</sub>-CH<sub>2</sub>-CO-CO-CH<sub>3</sub>. The concentrations of these two *VDKs* are of critical significance for beer flavour (Olaniran, Hiralal, Mokoena, & Pillay, 2017). For flavor profile of beer it is very important that the concentration of *VDK* to be less than flavour threshold. *Diacetyl* and *pentanedione* have similar tastes and smell, and the flavor is so characteristic that is described as "*diacetyl*". It is also described as "butter", "honey" (Elena, Muste, Tofana, & Muresan, 2014).



**Figure 2.4. 2, 3-pentanedione chemical structure**

The formation and reduction of *vicinal diketones* takes place in several stages. At first  $\alpha$ -acetoxy acids are formed, then oxidative decarboxylation of  $\alpha$ -acetoxy acids to *vicinal diketone* takes part followed by reduction of *vicinal diketones* (Horák, Culík, Jurková, Cejka, & Kellner, 2009). At the end of the main fermentation and maturation phase, diacetyl is re-assimilated and reduced by yeast to acetoin. It seems that numerous enzymatic systems are involved in the reduction of *VDKs* by brewing yeast (Olaniran, et al., 2017).





**Figure 2.6. Diagrammatic representation of Acetaldehyde**

*Acetaldehyde* from yeast metabolism as a step in the production of alcohol from glucose has a crisp green apple flavour. *Acetaldehyde* is the major *aldehyde* to consider owing to its importance as an intermediate in the formation of ethanol and acetate. *Acetaldehyde* has a flavour threshold of 10–20 mg/L and its presence in beer above the threshold value results in ‘grassy’ off-flavours. However, many tasters can detect this compound at much lower levels (Olaniran, et al., 2017).

### 2.4.3. Ester compounds

Esters comprise possibly the most important set of flavour active beer components which arise as a result of yeast metabolism. They are formed by the intracellular reaction between a fatty acyl-coenzyme A and an alcohol (Vassilev, et al., 2013). The esters of beer can be divided into two main groups. The first group comprises acetate esters such as *ethyl acetate* (fruity, solvent-like), *isoamyl acetate* (banana) and phenyl ethyl acetate (roses, honey and apple). The Second group of esters are the so called ethyl or medium chain fatty acid esters e.g. ethyl caproate and ethyl caprylate (both apple-like) (Brányik, et al., 2008). Of these esters, *ethyl acetate* is typically present in the highest concentration (Jespersen & Jakobsen, 1996), and represents approximately one-third of all esters in beer. Longer-chain fatty acid such as ethyl acetate esters (C<sub>13</sub>-C<sub>22</sub> long) are formed intracellularly by fermenting yeast cells and remain largely in the cell, although the distribution between fermenting medium and cells is dependent on the yeast species used. Being lipid soluble, ethyl esters can diffuse through the cellular membrane into the fermenting medium, thus accounting for their higher concentrations in beer (Saerens, Delvaux, Verstrepen, Thevelein, & Delvaux, 2008).

Esters are one of the more volatile compounds in beer and hence impact greatly beer aroma. In moderate quantities, they can add a pleasant, full-bodied character to beer aroma. When present in excess, however, they give beer aroma an overly fruity quality, which is considered

undesirable by most consumers. The presence of different esters can have a synergistic effect on the individual flavours, which means that esters can also affect beer flavour well below their individual threshold concentrations (Olaniran, et al., 2017).

#### 2.4.4. Higher alcohols

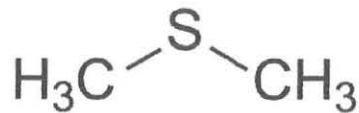
Several *higher or fusel alcohols*, other than ethanol are formed in beer during fermentation, among which *n-propanol*, *iso-butanol* and isoamyl alcohols contribute most significantly to beer by alcoholic or solvent-like aroma and a warm mouth feel (Brányik, et al., 2008). These compounds may have both positive and negative impacts on aroma and flavour. Large quantities of these higher alcohols (>300 mg/L) in beer can lead to a strong, pungent smell and taste, whereas optimal levels impart desirable characteristics (Olaniran, et al., 2017).

*n-Propanol* or *1-Propanol* ( $\text{CH}_3\text{CH}_2\text{CH}_2\text{OH}$ ) is a primary alcohol. *n-Propanol* is thought to be similar to ethanol in its effects on human body, but 2-4 times more powerful (Boulton & Quain, 2001). *Isobutanol* ( $(\text{CH}_3)_2\text{CHCH}_2\text{OH}$ ) is an organic higher alcohol present in beer. Its presence in beer is of great importance in the flavour profiles, as they generate desired fruity aromas in these products when produced in favourable amount (Chen, Nielsen, Borodina, & Kielland-Brandty, 2011). Amyl alcohol is any of the alcohols with the formula  $\text{C}_5\text{H}_{10}\text{OH}$ . The most common amyl alcohols in beer are the primary alcohols isoamyl alcohol and active amyl alcohol. It is a colourless liquid with unpleasant aroma. Its presence has been detected in trace amounts in yeast fermentation, but the biosynthetic pathway is not well understood (Cordern, 2013).

*Higher alcohols* can be synthesized via two routes: from wort carbohydrates (the anabolic route) or as by-products of amino acid assimilation (the catabolic route). At the ends of primary fermentation over 90% of the *higher alcohols* have been built, the rest arise during the maturation process (Vassilev, et al., 2013).

#### 2.4.5. Dimethyl sulfide

*Dimethyl sulfide* or its abbreviation *DMS* ( $(\text{CH}_3)_2\text{S}$ ) is an organosulphur compound. It is a water-insoluble flammable liquid. Its boiling point is  $37^\circ\text{C}$ . It has a strong smell; it smells like the smell produced from cooking certain vegetables, notably maize, cabbage, beetroot and seafood. Opinion is divided if the *DMS* is desirable in beer (Codern, 2013).



**Figure 2.7. Diagrammatic representation of Dimethyl sulfide (Codern, 2013).**

In general, however the level of *DMS* in beer is low and present under taste threshold of 50-60ppb, but other brewers get paranoid if the level rises above 20 ppb, which is below the level of nose detection. The perception of *DMS* can be masked by phenyl ethanol (Codern, 2013).

*DMS* arises in beer via two routes. First, from S-methyl methionine (SMM) which decomposes to *DMS* on heating and, second, via the reduction of dimethyl sulphide. The later reaction is catalysed by yeast during fermentation. The common precursor of all *DMS* arising in beer is SMM and this derives from green malt (grain softened by steeping in water and allowed to germinate but not yet subjected to drying) (Codern, 2013).

## **2.5. Some Physico-chemical parameters of beer**

### **2.5.1. Alcoholic Strength**

The alcohol content of a beer typically refers to the amount of ethyl alcohol (as opposed to higher or fusel alcohols). The analysis of beer for alcohol content is an important part of brewing laboratory work for quality assurance programs and for legal reporting purposes. Results, however, are subject to appreciable variation and under official methods the analyses are time consuming and expensive. Alcohol is generated relative to the amount of fermentable sugars in beer wort (Spedding, 2014).

### **2.5.2. Original Extract or Gravity**

The initial SG of wort is known as the original extract (OE) or original gravity (OG) depending upon units implied by these terms (OE is in Plato, while OG is in numerical “gravity units). OE is an expression of sugar content - grams of sugar per 100 grams of wort. [Equivalent to % weight/weight (w/w)]. In the brewing industry this is denoted as degrees Plato (°P) and to a first degree of approximation beer responds in the same way as the original solutions of sucrose and water used to derive the Plato scale. Brewers often determine or record and report this using numerical specific gravity values rather than in degrees Plato and it is this that is more correctly

the OG value. OG represents the ratio of the density of wort at 20 °C to the density of water at the same temperature.

OG often held as an indirect indicator of alcoholic strength of a beer. A higher OG (implying the higher the fermentable sugars) indicates a higher potential for conversion to alcohol. But this depends upon how fermentable the extract is - yeast can only consume the simple sugars glucose, maltose and triose (Spedding, 2014).

### **2.5.3. Apparent and Real Extract**

Brewers measure changes in density as sugars are consumed and converted into alcohol. Measurements are obscured (the true gravity is “hidden”) by alcohol (of lower density than water, sugar solutions or beer) causing “buoyancy effects” with hydrometers for example. Thus false or apparent readings of gravity are made when instruments measure beer (containing water, sugars and alcohol); hence “apparent extract”. The real extract is a true measure of remaining sugars - and proteins etc., to be exact about the nature of the extract – as determined in the absence of alcohol (removed via distillation or boiling) or compensated for the actual alcohol content via its determination and/or via algorithms in instrumentation. When yeast has finished fermenting the brewers wort, or the brewer terminates the fermentation, the final gravity reading is sometimes also called the terminal gravity (this will also be in “apparent” or “real” terms depending how measured or calculated). RE is real extract in grams per 100 grams of beer (Plato) (Spedding, 2014).

### **2.5.4. Beer color**

The visual quality of beer depends on color, clarity and foam characteristics and how these support or negate consumer expectations regarding the particular style and brand (Shellhammer & Bamforth, 2008). Beer color comes from malted barley. Different types of malt have different characteristic colors, depending on how long and at what temperature they were kilned. During the mash, color carrying molecules are dissolved into the wort. Malt extracts are just concentrated worts, so the color of extracts will depend on the malts that were mashed in order to make it. Other factors in the brewing process can also influence beer color, such as caramelization or darkening during boiling (Palmer, 2001). In some instances, beer color is modified post-fermentation using caramel color or roasted malt extracts (Shellhammer & Bamforth, 2008).

## **2.6. Contribution of flavor compounds on Hangover**

One of the most immediate and common unwanted consequences of drinking alcohol is the hangover, known to the medical world as “veisalgia.” These terms describe the group of unpleasant symptoms that may follow a night of drinking; chiefly, the headache, often accompanied by stomach discomfort, fuzzy tongue and fuzzy thinking, drowsiness and sweating (Alberta Health Services, 2014).

Based on the research to date and an exhaustive review of the biological and medical literature on the subject, it is now possible to group the causes of alcohol hangover into two main categories: indirect and direct. Indirect causes are the dehydration, low blood sugar and sleep disturbance resulting from excessive drinking. The direct cause is the production of acetaldehyde (Educ’alcool, 2012).

Brewery yeast produces acetaldehyde as an intermediate compound in the conversion of glucose to ethanol, so it is found in every beer you make, at least during primary fermentation. However, in a healthy fermentation, the yeast fully converts the vast majority of this compound to alcohol so that any residual amount falls below the flavor threshold. If the fermentation is less than optimal, the conversion of acetaldehyde to alcohol may remain incomplete, and too much will remain in the final beer. It is frequently described as having a tart flavor reminiscent of green apples and the flavor of dry cider also. In the first step when alcohol reaches to the person’s liver, his body metabolizes ethanol in to acetaldehyde. Acetaldehyde is far more toxic than alcohol, as much at 30 times more toxic. As the person drinks the buildup of acetaldehyde in his body is often linked to hangover symptoms. Eventually, the person’s body will turn acetaldehyde in to a less toxic acetic acid (Educ’alcool, 2012).

## **2.7. Flavour threshold in beers**

Many heavier beers, such as European beers, have *VDK* at higher levels than the lighter beers typically produced in the U.S. and they still maintain good flavor. *VDK* concentrations typically range from 1-50 ppb in lighter beers, but they can reach several hundred ppb in darker beers (Ruppel & Grecsek, 2005). As with many other compounds, the taste varies with concentration and is modified by the presence of other substances. The taste can be detected at a lower level in lagers than in ales, and it is reported that it is less noticeable in a beer with a strong hop or malt flavour. On the basis of threshold values and flavor units. Moreover, individuals differ in their

sensitivity to *diacetyl* and some people have to be trained to detect it (Wainwright, 1973).

**Table 2.2. Taste threshold in beer.**

Flavor compounds in beer	Taste threshold in beer	Reference
Vcinal diketones	20-40 µg/l	(Barnes, 2013)
Acetaldehyde	5-20 mg/l	(Barnes, 2013)
Dimethyl sulphide (DMS)	25- 50 µg/l	(Barnes, 2013)
Ethyle Acetate	25-30 mg/l	(Barnes, 2013)
Isoamyl acetate	1.0-1.6mg/l	(Brányik, et al, 2008)
n-Propanol	800 mg/l	(Kobayashi, Nagahisa, Shimizu, & Shioya, 2006)
Isobutanol	200 mg/l	(Kobayashi, Sato, & Fukuhara, 2007)
Amylalcohol	65 mg/l	(Kobayashi, Sato, &Fukuhara, 2007)

## 2.8. Beer in Ethiopia

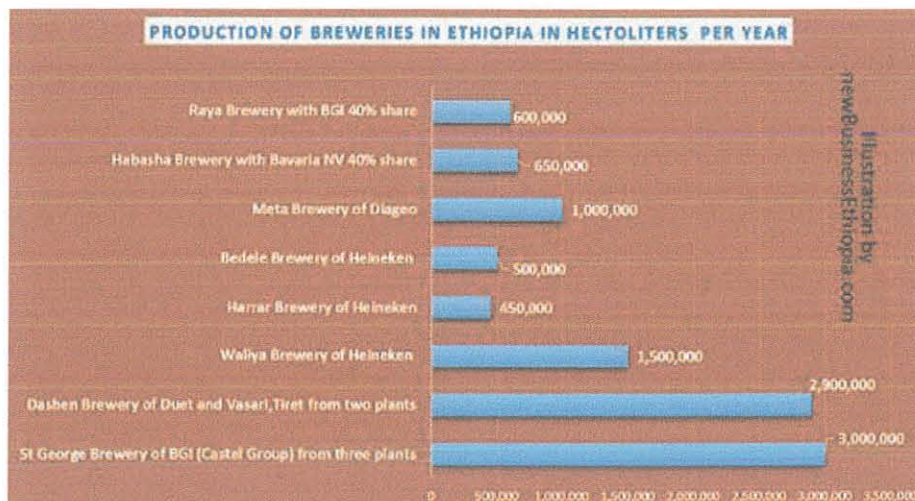
Beer in Ethiopia is the most popular alcoholic beverage. The beer industry in Ethiopia has gone through tremendous growth in the last two decades. The year 1992 is way back in time to be referred to as the old days. The first brewery was St. George Brewery, the factory started with machineries that were operated by hand and foot and work was done by direct human labor. Transporting of the raw material to the silo, fermenting of the malt, boiling the barley, filtering the beer, filling the bottles, etc. were all done manually. Basic raw materials like barley and hops were imported from Europe. During its first years, about 200 bottles (half liter size) were produced daily (Addismap, 2015).

Currently, Ethiopia has seven major breweries with more than fifteen commercial beer brands brewed and delivered to the consumer (Hahudaily, 2015). The breweries are BGI Ethiopia, Meta Abo, Heineken (Harar and Bedele), Dashen, Habesha, Raya and Zebider. They are owned by a share of foreign investors and local private producers. Except St. George Beer of BGI (French's Castel Group), all breweries in Ethiopia were owned by the government. The 42 years old brewery in the country, Meta Abo, was sold to Diago for \$250 million in June 2012 while Bedele and Harar breweries are handed over to the Dutch-based Heineken at \$178.3 million (Sisay, 2015).



**Figure 2.8. Ethiopian commercial alcoholic beer brands**

In Ethiopia the frequency of consumption and quantity of beer is high by referring to the brewing capacity increment, from a level of just 1 million hectoliters in 2003/04, beer production has risen to nearly 10 million hectoliters by 2015/16 (Access Capital, 2010).



**Figure 2.9 Production capacities of breweries in Ethiopia in hectoliter per year.**

### 2.9. Ethiopian Standards in beers

The Ethiopian Standards are developed by national technical committees (TC) which are composed of different stakeholders consisting of educational institutions, research institutes, government organizations, certification, inspection, and testing organizations, regulatory bodies, consumer association, etc. The requirements and/or recommendations contained in Ethiopian

Standards are consensus based that reflects the interest of the TC representatives and also of comments received from the public and other sources. Ethiopian Standards are approved by the National Standardization Council and are kept under continuous review after publication and updated regularly to take account of latest scientific and technological changes (Ethiopian Standard, 2013).

According to Ethiopian standard beer classified in to three groups on the basis of the ethyl alcohol content specified in Table 2.3., shall have the following grades.

**Table 2.3. Beer grade** (Ethiopian standard, 2013)

<b>Beer type</b>	<b>Grade</b>
Lighter beer	LB
Strong beer	SB
Extra strong beer	ESB

All grades of beer shall comply with the physical requirements specified in Table 2.3 and chemical requirements specified in Table 2.5.

**Table 2.4. Physical requirements of Beer** (Ethiopian standard, 2013)

<b>Characteristics</b>	<b>Requirement</b>		
	<b>Light Beer</b>	<b>Strong Beer</b>	<b>Extra Strong Beer</b>
Colour	Minimum 6	Minimum 6	Minimum 6

**Table 2.5. Chemical requirements of Beer** (Ethiopian standard, 2013)

<b>Characteristics</b>	<b>Requirement</b>		
	<b>Light Beer</b>	<b>Strong Beer</b>	<b>Extra Strong Beer</b>
Original extract (W/W%)	3.5 – 10.5	10.51 – 12.50	> 12.51
Apparent extract (W/W %), minimum	0.5	1.5	1.5
Real extract (W/W %), minimum	1.04	3.43	4.42
Ethyl alcohol content (V/V %), minimum	1.52	4.61	5.34
Vicinal di-ketone, ppm, max.	0.6	0.6	0.6

## 2.10. Determination volatile flavour compounds in beer

The analysis of *volatile compounds* in beer is important for quality control in the brewing industry (Castro & Ross, 2015). Among the most common practices to measure volatile compounds in the field of food science is headspace analysis (either static or dynamic), and subsequent GC, which may be combined with other techniques such as mass spectrometry (MS) or olfactometry. (Clark & winter, 2015).

The brewing industry standard relies on the use of headspace sampling gas chromatography with an electron capture detector (ECD) and (FID) (Bergen, 2006). The advantages of these procedures are simplicity and fast sample preparation (Horák, et al., 2009).

In this study, headspace gas chromatography (HS-GC) method was used to analyze the concentration of volatile compounds in beer. Nine compounds, i.e. *acetaldehyde*, *DMS*, *N-propanol*, *ethyl acetate*, *isobutanlo*, *Amyl alcohol*, *isoamyl acetate*, *2,3-pentanedions* and *diacetylc* were separated and quantified.

# Chapter Three

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## *Materials and methods*

### 3. Materials and Methods

#### 3.1. Location of Study Area

The study was conducted in Addis Ababa, which is the capital city of Ethiopia. The experiments were carried out from March 2017 to July 2017 in Heineken Breweries Share Company, Quality Control Laboratory.

#### 3.2 Sample and Sampling

A total of 220 beer samples from 11 brands were collected randomly from different retail outlets and food market across ten sub-cities of Addis Ababa. For each brand two beers were randomly collected from each sub city. From each brand 20 bottles were collected from 10 sub cities of Addis Ababa. For each brand two composite samples were prepared and each brand analyzed in quadruplicate. To prepare the composite sample, 10 bottles of the same brand were taken and 70ml of sample were taken from each 330ml bottle then transferred to 700 ml bottle as shown in Figure 3.1 and mixed well then labeled blindly by AAU laboratory staff Table 3.1. The composite samples were taken to Heineken brewery quality analysis laboratory and were re-coded by the laboratory staff of Heineken brewery to keep the confidentiality of the beer companies and avoid any conflict of interest. The double coding was done so that no one could trace back the code with the brand name. Finally, 250ml of sample was collected from the composite for the flavor compounds analysis and the remaining was stored in a refrigerator.



**Figure 3.1 Beer samples (a) Beer sample in 700ml sample container (b) Beer sample in 0°C water bath**

**Table 3.1 Beer coding for Volatile flavour compounds analysis**

No	Name of brand	Beer coding for VDK analysis			
		Replication		% Alcohol	Number of samples
		Coding 1	Coding 2		
1	Dashen	A001	A002	4.80%	n=20
2	George	B001	B002	4.70%	n=20
3	Waliya	C001	C002	5%	n=20
4	Zebidar	D001	D002	5%	n=20
5	Meta	E001	E002	5%	n=20
6	Habesha	F001	F002	5%	n=20
7	Heniken	G001	G002	5%	n=20
8	Harar	H001	H002	5%	n=20
9	Bedele	I001	I002	5.50%	n=20
10	Raya	K001	K002	5%	n=20
11	Castel	J001	J002	5%	n=20

### 3.3. Materials and Chemicals for VDK Analysis

#### 3.3.1 Reagents

- **Chemicals:** Ethanol 99.9% purity, 2,3-hexanedione 90% purity and distilled water were purchased from Aldrich, United states and Merck, Germany suppliers .
- **Solutions**  
**Internal standard stock solution:** 150 µl 2, 3-hexanedione in 100 ml ethanol.  
**Internal standard working solution:** 2.0 ml stock solution in 200 ml 5 % (V/V) ethanol.
- **Calibration compounds:** 2, 3-butanedione (diacetyl) 97% purity and 2, 3-pentanedione 97% purity were purchased from Aldrich suppliers.
- **Gases : Nitrogen** (grade 99.998)

#### 3.3.2. Apparatus

Thermo Scientific TRACE 1310 Gas Chromatograph, USA. Fitted with capillary columns equipped with split/splitless injection port and Electron Capture Detector (ECD).

oven temperature : 50 °C  
 limit oven temperature : 350 °C  
 injection zone temperature : 140 °C  
 detection zone temperature : 140 °C

carrier gas pressure : 20 kPa  
carrier gas flow :  $\pm 10$  ml/min  
carrier gas : N  
ECD make-up : N<sub>2</sub>  
ECD make-up pressure : 150 kPa  
ECD make-up flow :  $\pm 40$  ml/min  
ECD mode : constant current  
ECD referent current : 1.0 nA  
ECD pulse voltage : 50 mV  
ECD pulse width : 1  $\mu$ sec  
ECD temperature: 150 °C  
Flow bottom split :  $\pm 22$  ml/min  
Flow top split :  $\pm 2$  ml/min  
Automatic headspace sampler: Fisons HS-800 injection  
Autosampler incubation temp. : 40 °C  
Autosampler equilibration time : 20 min (with shaking)  
Autosampler injection volume : 0.5 - 0.75 ml  
Autosampler syringe temperature : 60 °C  
Balance, accurate to 0.1 mg  
Waterjet suction pump  
Analytical column: 50 m\*0.53 mm (i.d.) fused silica, WCOT CP Sil 8 CB,  $d_f = 1.0 \mu\text{m}$

### **3.4 Procedure for *VDK* analysis**

#### **Method Adaptation**

To evaluate the analytical performance of the instrument and validity of the method, precession and linearity was done.

#### **3.4.1 Precision**

Precision of the method was evaluated through the repeatability of the method by assaying six replicate injections of standard beer samples during the same day under the same experimental conditions to obtain an acceptable %RSD.

### 3.4.2 Linearity

Linearity was determined by injecting a series of diluted (0,250, 500,750, 1000 µg/L) standard calibration compounds of butanedione and pentanedione solutions and regression equation was found by plotting the peak area (Y) versus standard solution concentration (X) expressed in µg/L.

### 3.4.3 Accuracy

Accuracy of results was checked by using HEINEKEN Certified Reference Beer Sample that is obtained from Quality Assurance Laboratories Burgemster Smeesweg 1,2382 PH Zoeterwoude , The Netherlands. Reference number B1700550 (See appendix IV).

### 3.4.4 Standard preparation

- Internal standard stock and working solutions: Internal standard stock solution was prepared by adding 150 µl of 2, 3-hexanedione which is obtained from Aldrich in 100 ml ethanol.
- Internal standard working solution: prepared by taking 2.0 ml stock solution (internal standard stock solution) in 200 ml 5 % (V/V) ethanol. The prepared standard was stored in refrigerator for 1 week.

## 3.5. Materials and Chemicals for acetaldehyde, esters, DMS and higher alcohols analysis

### 3.5.1 Reagents

- **Chemicals:** 4-Heptanon 98% purity and n-Butanol 99% purity were purchased from Merck, Germany and Aldrich, United states suppliers respectively.

- **Solutions**

**Internal standard stock solution:** 3.0 ml 4-heptanon and 30.0 ml n-butanol in 50 ml ethanol and make up to 100 ml with distilled-water.

**Internal standard working solution:** 50.0 ml ethanol and about 400 ml distilled water in a 1000 ml volumetric flask. Pipette 20.0 ml of the stock solution and make up to 1000 ml with distilled water.

- **Calibration compounds:** Heineken Esters & Alcohols Calibration Mix were purchased from Aldrich United states suppliers.
- **Gases : Nitrogen** (grade 99.998)

### 3.5.2. Apparatus

Thermo Scientific TRACE 1310 Gas Chromatograph, USA. Fitted for capillary columns equipped with split/splitless injection port and flame ionisation detector (FID):

oven temperature : 50 °C  
 limit oven temperature : 150 °C  
 injection zone temperature : 155 °C  
 detection zone temperature : 155 °C  
 carrier gas : N<sub>2</sub>  
 carrier gas pressure : 30 - 60 kPa  
 carrier gas flow : 10 - 20 ml/min  
 flow bottom split : 10 - 30 ml/min  
 flow top split : 2 ml/min  
 air pressure (FID) : 100 kPa  
 hydrogen pressure (FID) : 55 kPa  
 Autosampler tray temperature : 5 °C  
 Autosampler incubation temp. : 40 °C  
 Autosampler equilibration time : 20 min  
 Autosampler injection volume : 0.5 - 0.75 ml  
 Autosampler syringe temperature : 60 °C  
 Analytical column: DBWaxETR column, 60 m \* 0.32 mm ID, 1µm FD fused silica.

## 3.6 Procedure for Acetaldehyde, Esters, DMS and Higher alcohols analysis

### Method Adaptation

To evaluate the analytical performance of the instrument and validity of the method, precession and linearity was done.

### 3.6.1 Precision

Precision of the method evaluated through the repeatability of the method by assaying six replicate injections of standard beer samples during the same day under the same experimental conditions to obtain an acceptable %RSD.

### 3.6.2 Linearity

Linearity was determined by injecting a series of diluted (0,250, 500,750, 1000 µg/L) standard calibration compounds of esters & alcohols calibration mix solutions and regression equation was found by plotting the peak area (Y) versus standard solution concentration (X) expressed in µg/L.

### 3.6.3 Accuracy

Accuracy of results was checked by using HEINEKEN Certified Reference Beer Sample that is obtained from Quality Assurance laboratories Burgemeester Smeetsweg 1,2382 PH Zoeterwoude ,The Netherlands. Reference number B1700550 (See appendix IV).

### 3.6.4 Standard preparation

- Internal standard stock and working solutions: Internal standard stock solution was prepared by adding 3.0 ml 4-heptanon and 30.0 ml n-butanol into 50 ml ethanol in a 100 ml volumetric flask. Make up to 100 ml with distilled-water and mixed. The prepared standard was stored in refrigerator (0-10 °C) for 1 month.
- Internal standard working solution was prepared by taking 20.0 ml of the stock solution into 50.0 ml ethanol and about 400 ml distilled water in a 1000 ml volumetric flask. Make up to 1000 ml with distilled water and mixed. The prepared standard was stored in refrigerator (0 – 10 °C) for 1month.

## 3.7 Sample preparation for volatile flavour compounds analysis

In case the ethanol percentage of the sample is less than 4 % (V/V), ethanol is added in accordance to the Table in Appendix II. Beer samples with an ethanol percentage more than 6 % (V/V) were diluted in accordance to the Table in Appendix III.

The internal standard working solution is placed in a tray with melting ice. Cold (0 – 10 °C) beer samples were slowly filled in a 250 ml graduated measuring cylinder. The excess of beer and/or foam were removed by using a water jet suction pump to obtain 250 ml. 2.0 ml of the cold (0 °C) internal standard working solution was added into an infusion bottle. Immediately 250ml of the beer sample were transferred into an infusion bottle, closed with a screw cap and rubber septum and mixed carefully.



**Figure 3.2: Sample preparation** (a) internal standard working solution in a tray with melting ice. (b) 2.0 ml of the cold internal standard working solution added into an infusion bottle, (c), beer sample were slowly filled in a 250 ml graduated measuring cylinder. (d) The excess of beer and/or foam were removed by using a water jet suction pump to obtain 250 ml. (e), each infusion bottle was kept in refrigerator (f) Auto sampler vials filled with the prepared samples.

In this way the samples were prepared one by one. After a series of samples has been prepared, each infusion bottle were kept in refrigerator closed for at least 5 minutes to prevent foaming over. Prepared samples were stored in the refrigerator for 2 days. Auto sampler vials (50% of the vial volume) were filled with the prepared samples and the analysis was run by starting the auto sampler as described in its manual. Analysis of volatile flavour compounds was carried according to European Brewery Convention (EBC, 1998).



**Figure 3.3. Beer samples in auto sampler vials on auto sampler tray**

### **3.8. GC determination and calculation for VDK analysis**

The concentrations of *Vicinal diketones* in beer were determined at parts per billion ( $\mu\text{g/l}$ ). Separation of the compounds is achieved on a fused silica WCOT CP Sil 8 CB wide bore column. For detection, an Electron Capture Detector (ECD) was used. The concentrations were calculated according to the following equation.

$$\text{Conc } y = \text{Ratio } y \times \text{R.F } y$$

Where:

Conc  $y$  = concentration of compound  $y$  ( $\mu\text{g/l}$ )

Ratio  $y$  = area of compound  $y$  / area of internal standard

R.F.  $y$  (Response Factor for compound  $y$ ) =  $1 / \text{slope calibration curve } c$

### **3.9. GC determination and calculation for Acetaldehyde, Esters, DMS and Higher alcohols analysis**

Separation of *acetaldehyde*, *dimethyl sulphide*, *esters* and *higher alcohols* is achieved on a polar capillary narrow bore column. For detection, a Flame Ionisation Detector (FID) was used. Concentration of *dimethyl sulphide* (*DMS*) expressed in  $\mu\text{g/l}$  and report to one decimal place. The results of *acetaldehyde*, *esters* and *higher alcohols* expressed in  $\text{mg/l}$  and report to one decimal place, except for the values of *isoamyl acetate* which was reported to two decimal places. The concentration is calculated as follows:

$$\text{Conc} = \text{Area component} / \text{Area ISTD} \times \text{R.F component}$$

Where:

Conc. = concentration of the concerning component (mg/l)

Area component = area of the peak of the concerning component

Area ISTD = area of the peak of the concerning internal standard

RF comp. (response Factor of the component) = 1 /slope calibration curve c

Calculation is automatically done by the chromatography software.

### 3.10 Method for Physico-chemical analysis

#### 3.10.1 Equipments

- Anton Paar DMA 38 digital density meter and alcholyzer (Austria-Europe)
- Sample vials
- Flasks



Figure 4.1. Anton Paar Beer analyzer

#### 3.10.2 Sample preparation and analysis

Determination of Ethanol, color, original extract, apparent extract and real extract were carried out by using Anton paar alcholyzer analyzing system. The samples were degassed to ensure that the samples were free of gas bubbles. The samples were transferred in to sample vials. The sample from the first vial was automatically filled into the measuring system and measured.

### 3.11. Experimental Design

Completely randomized experimental design was followed to see the level of volatile flavor compounds in industrially brewed alcoholic beer in Ethiopia.

### **3.12. Study Design for Knowledge attitude and practice**

The study design employed for the survey was a purposive sampling technique and semi-structured questionnaires used to get more information. The questionnaire evaluation was carried out for knowledge attitude and practice assessments related to quality and food safety issues of the breweries based on the purposive sampling. A total of 30 professional employees who are working in the production and quality aspects participated in the survey. The response was classified in three parts; the first part was general information about the breweries and the respondents, the second part contained quality standards and certification of the breweries and the final part was KAP related to vicinal diketones in the beer industries.

### **3.13. Statistical data analysis**

The print out results of *VDK* concentration was collected after GC quantification then the data were evaluated using one-way analysis of variance (ANOVA) and Duncan HSD post hoc test (95% of probability) was used to determine statistical significance ( $p < 0.05$ ). Statistical analysis was performed using IBM SPSS version 20

# Chapter Four

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## *Results and discussion*

## 4. Result and Discussion

### 4.1. Validation of the chromatographic method

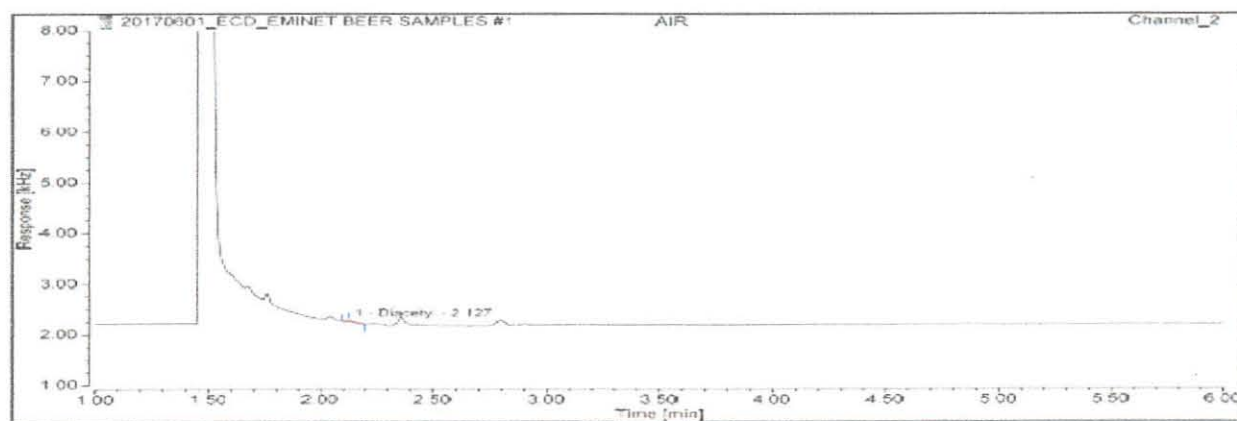
#### 4.1.1. Peak Identification by Retention time

Peak identification results are shown in Table 4.1. The retention time of *diacetyl*, *2,3-pentanedions*, *acetaldehyde*, *esters*, *DMS* and *higher alcohols* gives a good precession having range (0.01 – 0.33) %RSD, which is acceptable according to FDA standard which is less than 2% RSD. In addition to the retention time chromatographic result for blank, diacetyl, 2,3-pentanedions, acetaldehyde, esters, *DMS* and higher alcohols shown in Figure 4.1. demonstrate the qualitative aspects of identification test is more defined and acceptable.

**Table 4.1. Statistics for volatile flavor compounds retention time identification**

Volatile flavor compounds in beer	N	Mean	Std.Deviation	% RSD
Diacetyl	2	2.1300	0.00707	0.33
2,3-pentanedione	2	3.0775	0.00636	0.21
Acetaldehyde	2	3.1375	0.0063640	0.20
DMS	2	3.561	0.0056569	0.16
Ethylacetate	2	5.235	0.0028284	0.05
Isoamylacetate	2	8.4785	0.0021213	0.03
Propranol	2	7.389	0.0014142	0.02
Isobutanol	2	8.0585	0.0021213	0.03
Amylalcohol	2	9.4875	0.0007071	0.01

#### Blank Run ECD



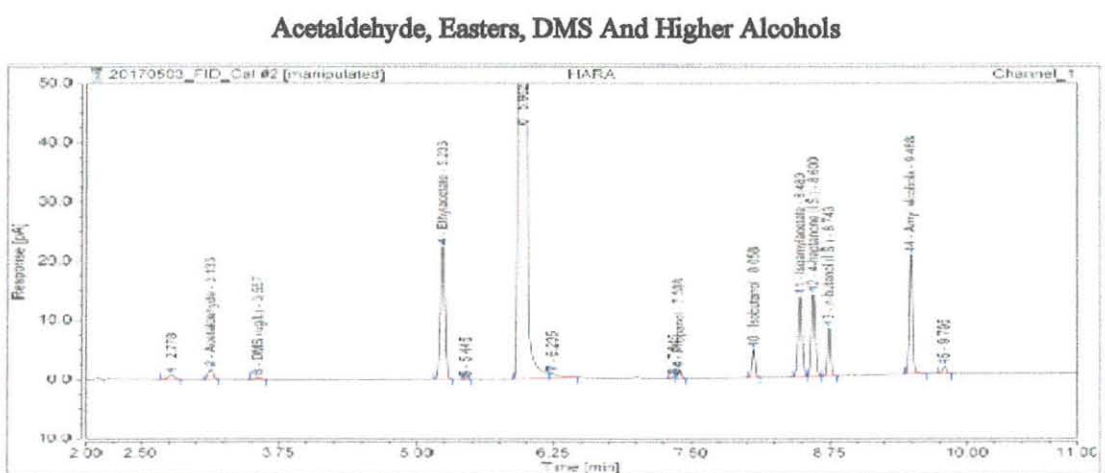
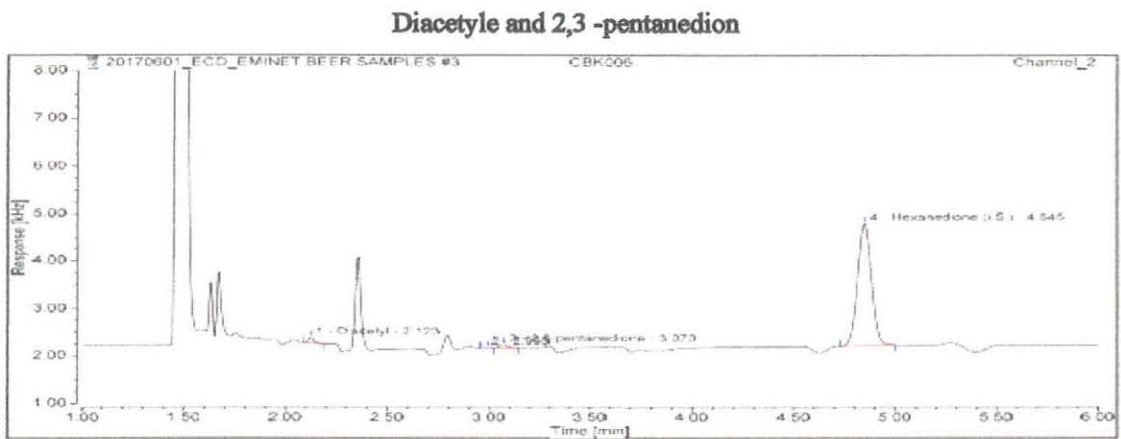
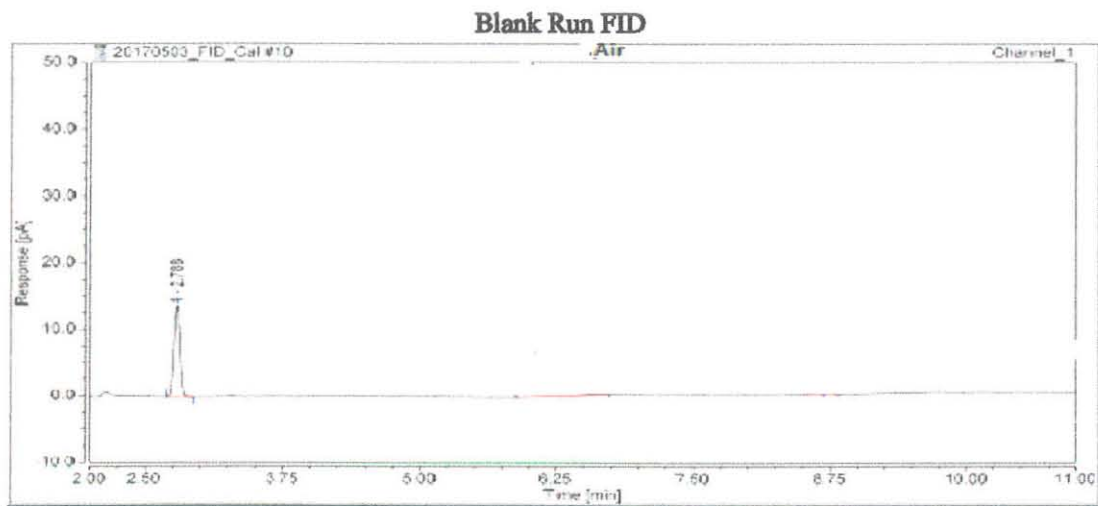


Figure 4.2. Chromatogram for volatile flavor compounds Identification

#### 4.1.2. Limit of Detection and Quantification (LOD and LOQ)

Detection performance of GC was determined by the limit of detection and limit of quantification shown in Table 4.2. LOD was determined by the amount of analyte that can be detected above baseline noise; typically, three times the noise level  $S/N > 3$  as shown in Table 4.2

**Table 4.2. Limit of Detection (LOD) and Limit of Quantification (LOQ)**

	LOD		LOQ	
	PPb	Signal to Noise Ratio (S/N > 3)	PPb	Signal to Noise Ratio (S/N > 10)
Diacetyl	0.21	3.14	0.86	11.24
2,3-pentanedione	0.03	3.12	0.54	10.74
Acetaldehyde	0.53	3.66	0.82	12.51
DMS	0.05	3.94	0.96	11.44
Ethylacetate	0.22	4.20	0.80	11.59
Isoamylacetate	0.32	4.75	0.91	12.76
Propranolol	0.26	3.75	0.85	11.43
Isobutanol	0.04	3.66	0.53	10.43
Amyl alcohol	0.24	3.12	0.58	10.82

LOQ was determined based on the amount of analyte which can be reproducibly quantitated above the baseline noise, that gives  $S/N > 10$ . The limits of quantification were in the range of (0.53 – 0.96)  $\mu\text{g/L}$  which shows satisfactory quantification of the instrument on the desired working range.

#### 4.1.3. Precision measurement

The precision was evaluated through the repeatability of the method by assaying ten replicate of standard beer samples injected during the same day, under the same experimental conditions. It shows an acceptable %RSD which had a values of  $< 0.05\%$  and  $< 3.0\%$  for retention time and peak area respectively. A precision criterion of the instrument precision (repeatability) and is normally expressed as the percent relative standard deviation for a statistical number of samples should be  $\leq 5\%$  RSD in FDA standard.

**Table 4.3. Descriptive Statistics for checking precision**

Volatile flavor compounds in beer	N	Descriptive Statistics for Peak Area			Descriptive Statistics for Retention time		
		Mean	Std.Deviation	% RSD	Mean	Std.Deviation	% RSD
Diacetyl	10	4.3083	0.05913	1.37 %	2.1308	0.00646	0.30 %
2,3-pentanedione	10	9.9050	0.05992	0.60 %	3.0792	0.00627	0.20 %
Acetaldehyde	10	0.75	0.0054772	0.73 %	3.1338	0.00402	0.13 %
DMS	10	0.10	0E-7	0 %	3.558	0.003899	0.11 %
Ethylacetate	10	0.8667	0.013663	1.58 %	5.2328	0.002317	0.04 %
Isoamylacetate	10	0.44667	0.00817	1.83 %	8.4795	0.001761	0.02 %
Propranol	10	0.05	0E-7	0%	7.3883	0.001366	0.02 %
Isobutanol	10	0.130	0E-7	0%	8.0572	0.001941	0.02 %
Amyl alcohol	10	0.57167	0.00408	0.71 %	9.4866	0.001366	0.01 %

#### 4.1.4. Linearity

Each standard calibration compounds of butanedione, pentanedione and esters & alcohols calibration mix was diluted to 0,250, 500,750, 1000µg/L standard solutions. The standard curves of butanedione, pentanedione, esters and alcohols were drafted and achieved good linearity that could be able to quantification accurately, as shown in Appendix I.

Acceptability of linearity data is often judged by examining of the correlation coefficient and y-intercept of the linear regression line for the peak area versus concentration plot. As revealed in Table 4.3. the demonstration coefficient ( $R^2$ ) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of vicinal diketones, Acetaldehyde, Dimethyl Sulphide, Esters and Higher Alcohols and their coefficient of correlation ( $R^2$ ) sited on 0.9999 and 1.000. The regression coefficient ( $r^2$ ) > 0.998 is generally considered as evidence of acceptable fit of the data to the regression line on the FDA Standard.

**Table 4.4. Linearity check for Volatile flavour compounds**

Beer flavour compounds	N(point)	Calibration Curve Equation	$R^2$
Butanedione	5	$Y = 0.0012x + 0.0071$	0.9996
2,3-pentanedione	5	$Y = 0.0078x + 0.0073$	0.999
Acetaldehyde	5	$Y = 0.046x - 0.002$	0.999
DMS	5	$Y = 0.000x - 0.001$	0.999
Ethylacetate	5	$Y = 0.117x - 0.039$	0.999
Isoamylacetate	5	$Y = 0.451x - 0.015$	0.999
Propranol	5	$Y = 0.018x + 0.000$	1.000
Isobutanol	5	$Y = 0.041x - 0.001$	1.000
Amyl alcohol	5	$Y = 0.044x - 0.003$	1.000

#### 4.1.5. Accuracy

The accuracy of this analytical method was obtained by using certified reference beer sample. As illustrated in Table 4.5 accuracy results were obtained between 90% and 110% and it is evident that the method is accurate.

**Table 4.5 Statistics for volatile flavour compounds accuracy check**

Volatile flavor compounds in beer	Reference sample concentration	N	Measured value Mean	Accuracy (%)	% RSD
Butanedione	8.1 µg/l	5	7.97 ± 0.184µg/l	98.39	2.30 %
2,3-pentanedione	4.1 µg/l	5	4.02 ± 0.043 µg/l	98.04	1.07 %
Acetaldehyde	2.6 mg/l	5	2.55 ±0.066mg/l	98.08	2.58 %
DMS	38.4 µg/l	5	38.39 ±0.048µg/l	99.97	0.13 %
Ethylacetate	25.9 mg/l	5	25.86 ±0.045mg/l	99.84	0.17 %
Isoamylacetate	3.68 mg/l	5	3.66 ± 0.01mg/l	99.46	0.27 %
Propranol	8.7 mg/l	5	8.68 ±0.04mg/l	99.77	0.46 %
Isobutanol	13.4 mg/l	5	13.39 ± 0.03mg/l	99.92	0.22 %
Amyl alcohol	62.5 mg/l	5	62.46 ±0.02mg/l	99.94	0.03 %

Values are mean ± standard deviation

#### 4.2. Physico-Chemical analysis of beer samples

The Original gravity, apparent extract, real extract, color and alcohol content (v/v) were measured using the Anton Paar Beer analyzer.

**Table 4.6. Physico-Chemical analysis of beer samples**

Beer Brand	Ethanol (V/V)	Original gravity (% W/W)	Apparent extract (% W/W)	Real extract (% W/W)	Color (EBC)
Z	5.11 ± 0.051 <sup>abcd</sup>	11.71 ± 0.056 <sup>abc</sup>	2.05 ± 0.227 <sup>b</sup>	3.91 ± 0.077 <sup>cd</sup>	8.49 ± 0.054 <sup>b</sup>
Y	4.75 ± 0.051 <sup>a</sup>	10.79 ± 0.056 <sup>a</sup>	1.79 ± 0.053 <sup>a</sup>	3.53 ± 0.058 <sup>ab</sup>	9.97 ± 0.565 <sup>cd</sup>
X	4.75 ± 0.051 <sup>a</sup>	11.86 ± 0.050 <sup>bcd</sup>	2.94 ± 1.000 <sup>c</sup>	4.70 ± 0.132 <sup>f</sup>	11.40 ± 0.080 <sup>e</sup>
W	5.19 ± 0.107 <sup>bcd</sup>	12.22 ± 0.083 <sup>cde</sup>	2.49 ± 0.771 <sup>c</sup>	4.36 ± 0.070 <sup>c</sup>	11.45 ± 0.080 <sup>c</sup>
V	4.69 ± 0.051 <sup>a</sup>	10.92 ± 0.056 <sup>ab</sup>	2.04 ± 0.227 <sup>b</sup>	3.75 ± 0.058 <sup>abcd</sup>	10.93 ± 0.082 <sup>de</sup>
U	5.03 ± 0.051 <sup>abc</sup>	11.13 ± 0.056 <sup>ab</sup>	1.60 ± 0.053 <sup>a</sup>	3.43 ± 0.058 <sup>a</sup>	11.51 ± 0.080 <sup>c</sup>
T	5.12 ± 0.051 <sup>abcd</sup>	11.42 ± 0.056 <sup>abc</sup>	1.75 ± 0.053 <sup>a</sup>	3.61 ± 0.058 <sup>abc</sup>	7.26 ± 1.000 <sup>a</sup>
S	4.95 ± 0.051 <sup>ab</sup>	11.49 ± 0.056 <sup>abc</sup>	2.16 ± 0.227 <sup>b</sup>	3.95 ± 0.213 <sup>d</sup>	9.64 ± 0.054 <sup>bc</sup>
M	5.48 ± 0.084 <sup>d</sup>	12.93 ± 0.104 <sup>e</sup>	2.69 ± 0.067 <sup>d</sup>	4.66 ± 0.070 <sup>ef</sup>	9.87 ± 0.565 <sup>cd</sup>
R	5.41 ± 0.076 <sup>cd</sup>	12.63 ± 0.079 <sup>de</sup>	2.52 ± 0.771 <sup>cd</sup>	4.46 ± 0.070 <sup>ef</sup>	13.87 ± 1.000 <sup>f</sup>
P	4.85 ± 0.051 <sup>ab</sup>	11.21 ± 0.056 <sup>ab</sup>	2.04 ± 0.227 <sup>b</sup>	3.80 ± 0.096 <sup>bcd</sup>	12.04 ± 0.080 <sup>c</sup>

Values are mean ± standard deviation and different superscripts across a column represent statistically significant difference (P<0.05).

### **Ethanol**

The necessary analyses (the minimum amount) a brewer needs are only a few. The perhaps most important analysis, already because of the current legislation, is the measurement of the ethanol content. Regarding to Ethiopian alcoholic beers, the ethanol percentage of all brands as shown in Table 4.6 is 4-6 % (V/V). Based on Ethiopian standard classification of beers by observing the ethanol content, all brands are categorized as strong beers and all are in acceptable interval.

### **Original extract/Original gravity**

As shown in Table 4.6, based on Ethiopian standard the original extract of all brands is within acceptable interval. OG is often held as an indirect indicator of alcoholic strength of a beer. Since these beers are brewed in different condition and raw materials, they have different value of extract with having different alcoholic strength. The OG of final product of beer is differ based on its style. A higher OG (implying the higher the fermentable sugars) indicates a higher potential for conversion to alcohol. But this depends upon how fermentable the extract is - yeast can only consume the simple sugars glucose, maltose and triose (Spedding, 2014)..

### **Apparent extract and real extract**

When comparing apparent and real extract value of all brands with Ethiopian standard all brands met the criterion. Since a substantial portion of the dissolved solids will be removed from the wort during fermentation by the action of yeast, so the progress of fermentation monitored by measuring the disappearance of these solids. As the fermentation time becomes longer more extracts are converted to alcohol (ethanol).

### **Color**

The color of all brands as shown in Table 4.6 is in the range 7.26 – 13.87 EBC. The higher the EBC, the darker in color the beer will be. The only thing the color of beer can really tell us is its style, as a result of the malt grain used, and a hint about the flavors we might taste (darker beer will taste more like coffee than lighter beers). Beer gets its color from the way it is brewed, which is why beers of the same style usually have similar color. When comparing color of all brands with Ethiopian standard all brands met the criterion which is  $> 6$  for all type of beer.

Among the eleven brands, brands T have the lowest value and brand R has the highest value of color. Based on this brand T is brewed with relatively light malts and is therefore very pale in

color and conversely brand R is brewed with relatively dark roasted barley. While malt is the main factor that influences color in beer, there are a few other factors that can also play a part.

#### **4.3. The level of *volatile flavor compounds* in beer**

The 11 brands of domestic alcoholic beers are listed in Table 4.7 coded as Z, Y, X....P. Each sample was run in quadruplicate according to the procedure and specification. *Volatile flavor compounds* were detected in the range between (13.8 – 128.2  $\mu\text{g/l}$ ) ; for *vicinal diketones*, (1.65-8.0 mg/L) ; for *Acetaldehyde*, (0.00-76.35  $\mu\text{g/L}$ ) ; for *DMS*, (11.75-28.35mg/L) ; for *Ethyle acetate*, (8.40-20.95 mg/L) ; for *n-Propanol*, (5.85-17.7 mg/L) ; for *Isobutanol*, (0.10-3.95 mg/L) ; for *Isoamyle acetate*, and (38.05-86.50 mg/L) ; for *Amayle alcohol*. One-way ANOVA test showed that the mean difference is significant at the  $P < 0.05$  level.

Table 4.7. Level of *volatile flavor compounds* in beer

Brand	Diacetylc ( $\mu\text{g/l}$ )	Pentanedions ( $\mu\text{g/l}$ )	Total VDK ( $\mu\text{g/l}$ )	Acetaldehyde ( $\text{mg/l}$ )	DMS ( $\mu\text{g/l}$ )	Ethylacetate ( $\text{mg/l}$ )	n-Propanol ( $\text{mg/l}$ )	Isobutanol ( $\text{mg/l}$ )	Isoamyl Acetate ( $\text{mg/l}$ )	Amyl alcohol ( $\text{mg/l}$ )
Z	27.50 $\pm$ 0.20 <sup>c</sup>	10.30 $\pm$ 0.079 <sup>dc</sup>	37.80 $\pm$ 1.00 <sup>c</sup>	1.65 $\pm$ 0.067 <sup>a</sup>	76.35 $\pm$ 1.00 <sup>g</sup>	11.75 $\pm$ 1.0 <sup>a</sup>	16.80 $\pm$ 1.00 <sup>e</sup>	5.85 $\pm$ 0.074 <sup>a</sup>	0.10 $\pm$ 1.00 <sup>a</sup>	38.05 $\pm$ 1.0 <sup>a</sup>
Y	24.65 $\pm$ 0.20 <sup>c</sup>	21.25 $\pm$ 1.00 <sup>f</sup>	45.90 $\pm$ 1.00 <sup>d</sup>	2.35 $\pm$ 0.067 <sup>abc</sup>	53.15 $\pm$ 0.074 <sup>f</sup>	24.70 $\pm$ 0.217 <sup>ef</sup>	15.15 $\pm$ 0.395 <sup>d</sup>	12.30 $\pm$ 0.186 <sup>d</sup>	2.10 $\pm$ 0.06 <sup>c</sup>	59.00 $\pm$ 0.078 <sup>d</sup>
X	14.75 $\pm$ 0.077 <sup>b</sup>	8.50 $\pm$ 0.064 <sup>cd</sup>	23.25 $\pm$ 0.222 <sup>b</sup>	1.85 $\pm$ 0.067 <sup>ab</sup>	23.35 $\pm$ 0.061 <sup>bc</sup>	19.05 $\pm$ 0.095 <sup>bc</sup>	14.70 $\pm$ 0.085 <sup>cd</sup>	12.80 $\pm$ 0.186 <sup>d</sup>	2.20 $\pm$ 0.06 <sup>c</sup>	69.45 $\pm$ 0.136 <sup>c</sup>
W	10.30 $\pm$ 0.066 <sup>ab</sup>	3.60 $\pm$ 1.00 <sup>a</sup>	13.90 $\pm$ 0.107 <sup>a</sup>	3.60 $\pm$ 0.086 <sup>d</sup>	28.35 $\pm$ 0.207 <sup>cd</sup>	18.65 $\pm$ 0.095 <sup>b</sup>	10.85 $\pm$ 0.179 <sup>b</sup>	7.30 $\pm$ 0.058 <sup>b</sup>	1.70 $\pm$ 0.707 <sup>b</sup>	55.05 $\pm$ 0.074 <sup>c</sup>
V	88.75 $\pm$ 1.00 <sup>c</sup>	39.05 $\pm$ 1.00 <sup>h</sup>	128.20 $\pm$ 1.00 <sup>f</sup>	4.00 $\pm$ 0.086 <sup>dc</sup>	0.00 $\pm$ 1.00 <sup>a</sup>	17.45 $\pm$ 0.095 <sup>b</sup>	20.95 $\pm$ 1.00 <sup>f</sup>	17.70 $\pm$ 1.000 <sup>f</sup>	2.35 $\pm$ 0.06 <sup>c</sup>	86.50 $\pm$ 1.0 <sup>h</sup>
U	8.05 $\pm$ 0.066 <sup>a</sup>	6.45 $\pm$ 0.174 <sup>bc</sup>	14.50 $\pm$ 0.107 <sup>a</sup>	5.85 $\pm$ 1.00 <sup>f</sup>	12.10 $\pm$ 0.061 <sup>b</sup>	31.35 $\pm$ 1.0 <sup>h</sup>	8.40 $\pm$ 1.00 <sup>a</sup>	10.40 $\pm$ 0.496 <sup>c</sup>	2.85 $\pm$ 1.00 <sup>d</sup>	48.45 $\pm$ 1.0 <sup>b</sup>
T	7.80 $\pm$ 0.066 <sup>a</sup>	6.0 $\pm$ 0.174 <sup>b</sup>	13.80 $\pm$ 0.107 <sup>a</sup>	4.45 $\pm$ 0.22 <sup>c</sup>	37.70 $\pm$ 0.100 <sup>dc</sup>	28.35 $\pm$ 0.26 <sup>g</sup>	11.25 $\pm$ 0.179 <sup>b</sup>	15.65 $\pm$ 0.068 <sup>c</sup>	3.95 $\pm$ 1.00 <sup>e</sup>	71.90 $\pm$ 0.136 <sup>cf</sup>
S	12.45 $\pm$ 0.066 <sup>ab</sup>	7.45 $\pm$ 0.174 <sup>bc</sup>	19.85 $\pm$ 0.107 <sup>ab</sup>	2.50 $\pm$ 0.086 <sup>bc</sup>	27.30 $\pm$ 0.207 <sup>cd</sup>	21.75 $\pm$ 0.058 <sup>cd</sup>	14.65 $\pm$ 0.085 <sup>cd</sup>	15.05 $\pm$ 0.068 <sup>c</sup>	2.40 $\pm$ 0.06 <sup>c</sup>	75.0 $\pm$ 0.067 <sup>f</sup>
M	12.15 $\pm$ 0.066 <sup>ab</sup>	7.30 $\pm$ 0.174 <sup>bc</sup>	19.45 $\pm$ 0.107 <sup>ab</sup>	2.65 $\pm$ 0.404 <sup>c</sup>	31.10 $\pm$ 0.207 <sup>cd</sup>	23.10 $\pm$ 0.292 <sup>dc</sup>	15.25 $\pm$ 0.395 <sup>d</sup>	14.90 $\pm$ 0.068 <sup>c</sup>	2.35 $\pm$ 0.06 <sup>c</sup>	78.35 $\pm$ 0.05 <sup>g</sup>
R	13.10 $\pm$ 0.077 <sup>b</sup>	10.75 $\pm$ 0.638 <sup>e</sup>	23.85 $\pm$ 0.222 <sup>b</sup>	4.25 $\pm$ 0.086 <sup>dc</sup>	45.60 $\pm$ 0.170 <sup>cf</sup>	26.90 $\pm$ 0.099 <sup>fg</sup>	13.45 $\pm$ 0.085 <sup>c</sup>	10.15 $\pm$ 0.496 <sup>c</sup>	1.75 $\pm$ 0.707 <sup>b</sup>	58.20 $\pm$ 0.074 <sup>cd</sup>
P	39.00 $\pm$ 1.00 <sup>d</sup>	30.85 $\pm$ 1.00 <sup>g</sup>	69.85 $\pm$ 1.00 <sup>e</sup>	8.00 $\pm$ 1.00 <sup>g</sup>	56.70 $\pm$ 0.074 <sup>f</sup>	19.85 $\pm$ 0.095 <sup>bc</sup>	11.80 $\pm$ 0.179 <sup>b</sup>	6.55 $\pm$ 0.074 <sup>ab</sup>	2.10 $\pm$ 0.06 <sup>c</sup>	55.90 $\pm$ 0.074 <sup>cd</sup>

Values are mean  $\pm$  standard deviation and different superscripts across a column represent statistically significant difference ( $P < 0.05$ ).

**Table 4.8. Review results for *flavour compounds* in beer**

Flavour compounds in beer	No. of beer brands	Threshold value	No. of brands below the threshold value	No. of brands above the threshold value	Concentration range
Total VDK	11	(20-40) µg/l	09	02	(13.8-128.2) µg/l
Acetaldehyde	11	(5-20) mg/l	09	02	(1.6 -8.0) mg/l
DMS	11	(25-50) µg/l	08	03	(0.0-76.35) µg/l
Ethylacetate	11	(25-30)mg/l	10	01	(11.75-28.35) mg/l
Isoamylacetate	11	(1.0-1.6)mg/l	01	10	(0.10-3.95) mg/l
n-Propanol	11	800mg/l	11	0	(8.40-20.95) mg/l
Isobutanol	11	200mg/l	11	0	(5.85-17.7) mg/l
Amylalcohol	11	65mg/l	06	05	(38.05-86.50) mg/l

As summarized in Table 4.8 the majorities of alcoholic beers have value less than the threshold value but in case of *Iso-amyleacetate* the majorities of alcoholic beers have values greater than the threshold value and for amyralcohol almost half of brands have the value above the threshold value. All brands have the value of n-propanol and Isobutanol below the threshold value.

#### **Vicinal Diketones**

When comparing by brand difference of Ethiopian alcoholic beer as shown in the scatter diagrams Figure 4.3 (a). and Table 4.7., for total *VDK* value only three brands have values greater than the threshold value of 40µg/L (Barnes, 2013). This is according to Europeans standard. When comparing with Ethiopian standard the maximum threshold value for total *VDK* is 60µg/L, only two brands have values greater than 60µg/L. Among the eleven brands, brands T, W and U have the lowest value and brand V has the highest value of *VDK*. Brand P has also the highest value next to brand V. The beer is considered mature when the *VDK* levels have fallen under a certain level. This shows that brands with the highest value was not have enough maturation time during fermentation. As maturation time decreases the quality of beer also decreases.

#### **Acetaldehyde**

According to European standard only two brands have *Acetaldehyde* value greater than the threshold value > 5mg/L; see Figure 4.3 (b) and Table 4.7 .When comparing the eleven brands, brand Z and X have the lowest value of acetaldehyde and brand P and U has the highest value. This shows that for brand P and U the fermentation were less than optimal as a result the conversion of acetaldehyde to alcohol remains incomplete (Educ'alcool, 2012). Or it could be

due to brewing process of brand P and U the use of poor-quality yeast, a too high temperature during fermentation, a too fast addition of the yeast or a too high pitching rate, and a too high level of wort oxygenation could be the reasons. In addition, these brands might have hangover effect when consumed in large amount.

### **Dimethyle Sulphide**

As shown in scatter diagram Figure 4.3(c) and, Table 4.7 in one brand *DMS* is not detected and three brands have values greater than threshold value of 50µg/L (Barnes, 2013). This means for brand V *DMS* were not detected. Brand U also has the lowest value next to brand V. Brand Z has the highest value and next to this brand, brand P and Y have the highest value, this could be because of the malt, as a result of the short or weak boil of the wort, slow wort chilling .Or these brands with high value of *DMS*, could not have enough maturation time during fermentation.

### **Higher Alcohols**

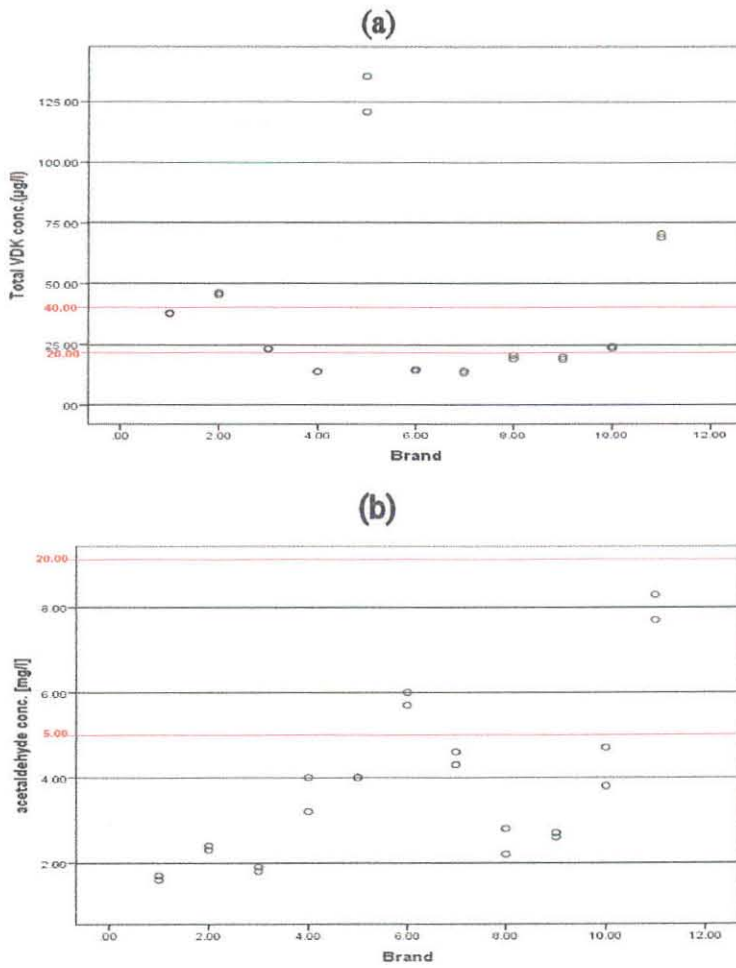
When comparing the value of n-Propanol and Iso-butanol ,see Figure 4.3 (e) and (f) respectively and Table 4.7, all brands have values less than the threshold value of < 80 (Kobayashi et al., 2006) mg/L and < 200mg/L (Kobayashi et al., 2007) respectively. For Amyl alcohol as shown in Figure 4.3 (h) and, Table 4.7 five brands have values greater than the threshold value of 65mg/L (Kobayashi et al., 2007). Brand V has the highest value of Amyl alcohol and next to this brand, brand M, S, and T have highest values of Amyl alcohol. Conversely, brand Z has the lowest value, next to this brand, brand U also has the lowest value of Amyl alcohol.

### **Esters**

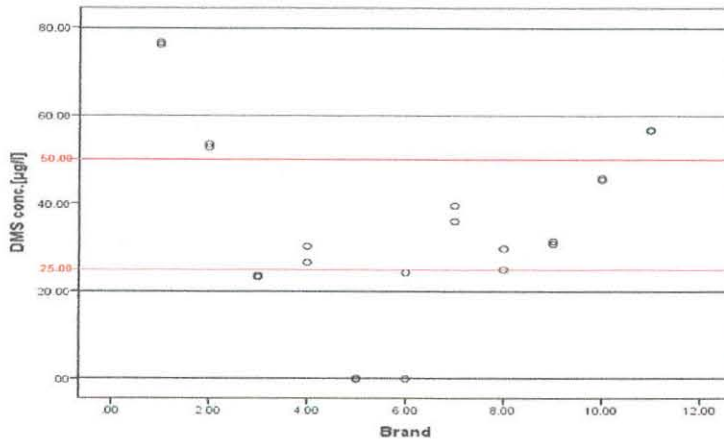
As shown in Figure 4.3 (d),(g) and Table 4.7, only one brand has value of ethyle acetate greater than the threshold value 30mg/L (Barnes, 2013). Brand Z has the lowest value of ethyl acetate but brand U has the highest value and next to this brand, brand T has the highest value of ethyle acetate. For Iso-amaylacetate only one brand has value which is less than the threshold value of 1.6 mg/L (Brányik et al, 2008) but the rest ten brands have the value greater than 1.6mg/L. Brand T has the highest value and next to this brand , brand U has also highest value. Brand Z has the lowest value of Iso-amaylacetate. In general brand Z has lowest value of ester and brand U and T have the highest value of ester. A sizeable quantity of esters can be produced as a result of yeast metabolism .High value of ester in these two brands might be because of the use of low quality of yeast during the brewing process.

Figure 4.3. Scatter diagram for Volatile flavour compounds level as compared to the threshold value

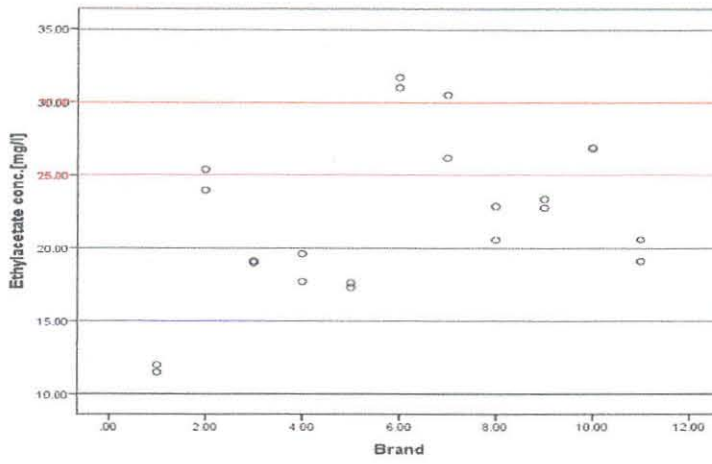
**KEY**  
 — Concentration  
 — Cut off value  
 ○ Beer brands



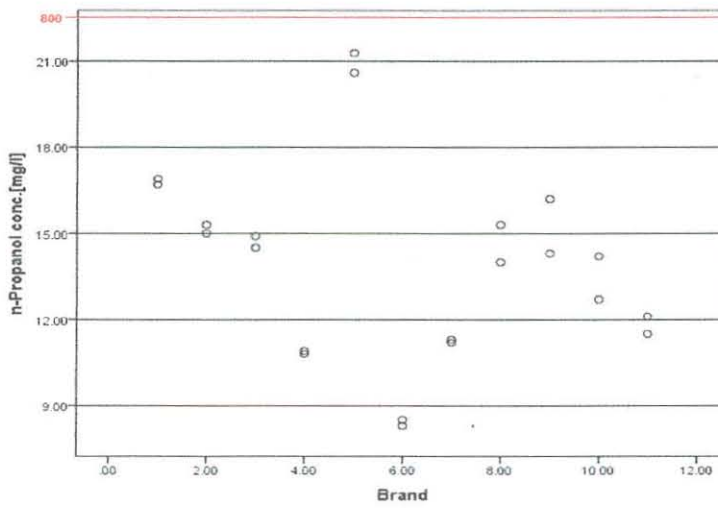
(c)



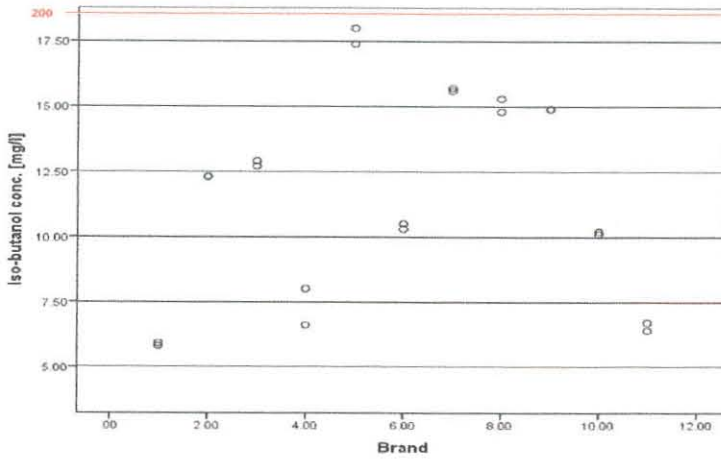
(d)



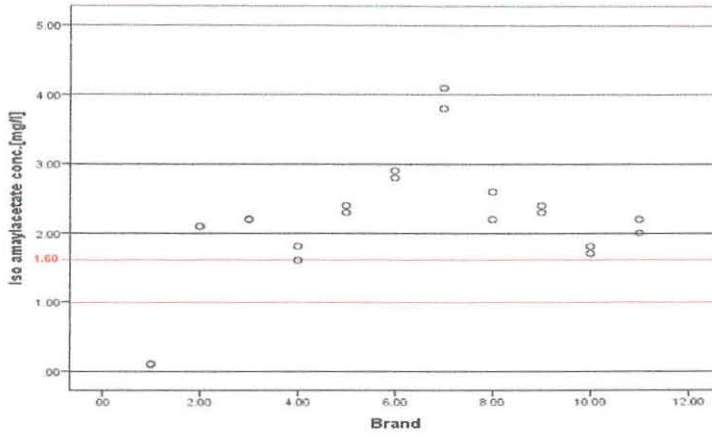
(e)



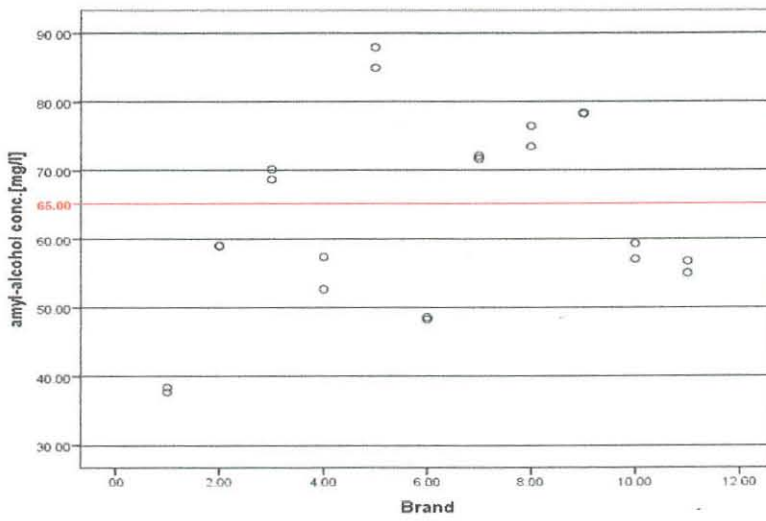
(f)



(g)



(h)



#### 4.4. KAP study

A semi-structured questionnaire results shows KAP (Knowledge, attitude and practice) assessments related to quality and food safety issues of the breweries based on a purposive sampling. On this survey 30 professional employees working in breweries participated and the response summarized in three parts.

##### 4.4.1. General information about the breweries and respondents

Part one questionnaire contains a general information about the breweries and the respondents and as shown in Table 4.9

**Table 4.9. General information about the breweries and respondents**

No.	Questions/ area covered	Summarized response
1	Qualification of the respondents	Chemist (graduate of applied and industrial chemistry), Biologist (graduate of applied biology and microbiology), Food technologist and Chemical engineers.
2	Respondents position in the factory	Quality analyst, Microbiologist, brewing operators, shift team leaders, quality manager.
4	Brewing capacity in hectoliter per year	The eight breweries brewed around 10 million hectoliter per year
5	Existing brands	A total of 16 brands beer brewed
6	Export status	There is no breweries that started to export their products

As shown in Table 4.9. 9 chemists, 7 biologist, 8 food technologist and 6 chemical engineers a total of 30 professional respondents participated on this survey. The breweries brew around 10 million hectoliter per year.

##### 4.4.2. General information about quality standard and certification

Part two questionnaire contains information about quality standards and certification of the breweries. The question and response of the respondents is illustrated in Table 4.10. The issue of safety and quality control in beverage is the main concern for manufacturers. By referring Table 4.10., 65% of the breweries have employee hygiene program. Regarding to Quality standards and Certifications, 45% of the breweries complies and attained ISO international certification and the rest work on it and all breweries audited periodically. All breweries monitor quality throughout process/production and perform microbiological as well as physico-chemical testing.

**Table 4.10. General information about quality standards and certification of the breweries**

No.	Questions/ area covered	Summarized response in percent
1	Quality standards and Certifications like ISO 9001 (QMS), 14001 (EMS) or 22000 (FSM and HACCP)	45% of the breweries comply and attained ISO international certification and the rest are working on it.
2	Periodic audit system by authorities like FMHACA, ISO inspecting bodies etc	All breweries auditing periodically by FMHACA and few are inspected by ISO systems
3	Sanitation standard procedure	65% of the breweries have sanitation standard procedure
4	Employee hygiene program	65% of the breweries have employee hygiene program
5	Quality monitored throughout processing/production	All breweries monitor quality throughout process/production
6	Performing microbiological product testing	All breweries perform microbiological product testing
7	Performing physico -chemical product testing	All breweries perform physico – chemical product testing

**4.4.3. KAP related to Vicinal diketons in beer**

Part three questionnaire contains information about KAP related to *Vicinal diketons* in beer industries. The question and response of the respondents are illustrated in Table 4.11.

**Table 4.11. KAP related to *Vicinal diketons* in beer industries**

No.	Questions/area covered	Summarized response in percent
1	Expectance of <i>VDK</i> in bottled beer	10% of respondents not expect <i>VDK</i> in bottled beer.
2	Knowledge about sources of <i>VDK</i>	40% of respondents has knowledge about the sources of <i>VDK</i> .
3	Knowledge about how <i>VDK</i> affect beer	60% of respondents knows how <i>VDK</i> affect beer
4	The system for temperature control during fermentation	All breweries have a system for temprature control during fermentation.
5	Knowledge about how <i>VDK</i> is reduced during process.	25% of respondents know how <i>VDK</i> is reduced during process
6	Product testing for level of <i>VDK</i>	100% cheking level of <i>VDK</i>

With regard to KAP related to *VDK* all breweries check the level of *VDK* in their laboratories but the majority of respondents had gap on Knowledge about source and reduction of *VDK* during processing of beer.

# Chapter Five

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*Conclusions and  
recommendations*

## 5. Conclusions and Recommendations

### 5.1 Conclusion

Flavour compounds in beer significantly influence the taste, and the rich variety of volatile compounds in beer contributes to their sensory properties. In relation to the objectives of the study, majority of the alcoholic beers contain acceptable level of volatile flavour compounds except for their *Iso-amyl acetate* and *Amyl alcohol* content. In addition, level of *Vicinal diketones*, *Acetaldehyde*, and *DMS* in some Ethiopian alcoholic beers is significant. This shows that the maturation time during fermentation process might not be enough to reduce the level of these compounds. Brands with significant amount of *acetaldehyde* might have hangover effect when consumed in large amount. The knowledge about selected flavour compound *VDK* is not satisfactory since the participants in this survey were professional employees. All breweries check the level of *VDK* in their laboratories but the majority of respondents had gap on knowledge about source and reduction of *VDK* during the process. Therefore, knowledge attitude and practice among beer industries towards *VDK* should be improved.

### 5.2. Recommendation

- By considering customer satisfaction, brewers have to take actions and assess the root causes of those flavour compounds which were detected above the threshold value specially *Iso-amyl acetate* and *Amyl alcohol*.
- Further studies are needed to develop a way to control *volatile flavour compounds* in alcoholic beers.
- Further KAP evaluation of the breweries related to all *volatile flavour compounds* should be done.
- Previous studies on the presence of *VDK* above flavour threshold in beer results in off flavour but has no side effects on human health. This study therefore recommends further investigations to be made whether the presence of *Vicinal diketones* in fermented foods especially in beer could affect human health.
- Because of financial limitation the designed sample size may not be enough so, further plans that would provide representative beer samples could be done to overcome the variability of *volatile flavour compounds* and reducing sampling error.

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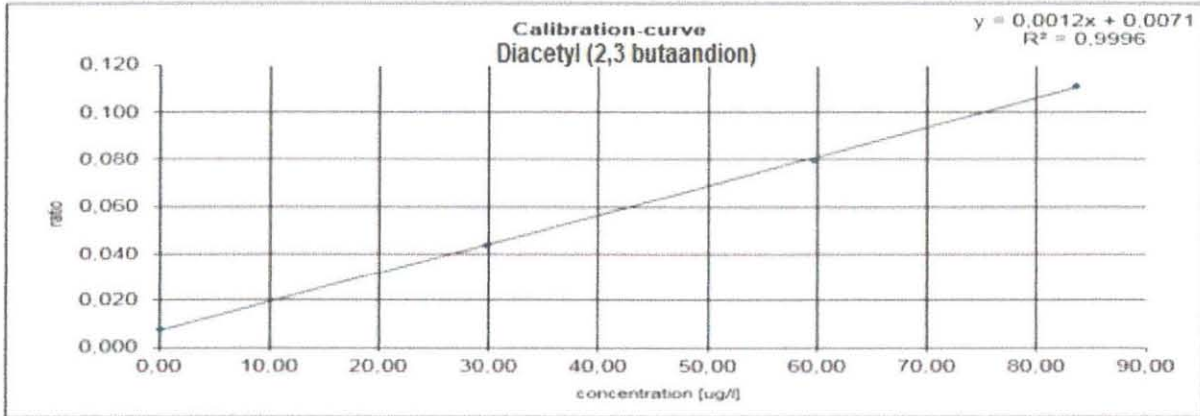
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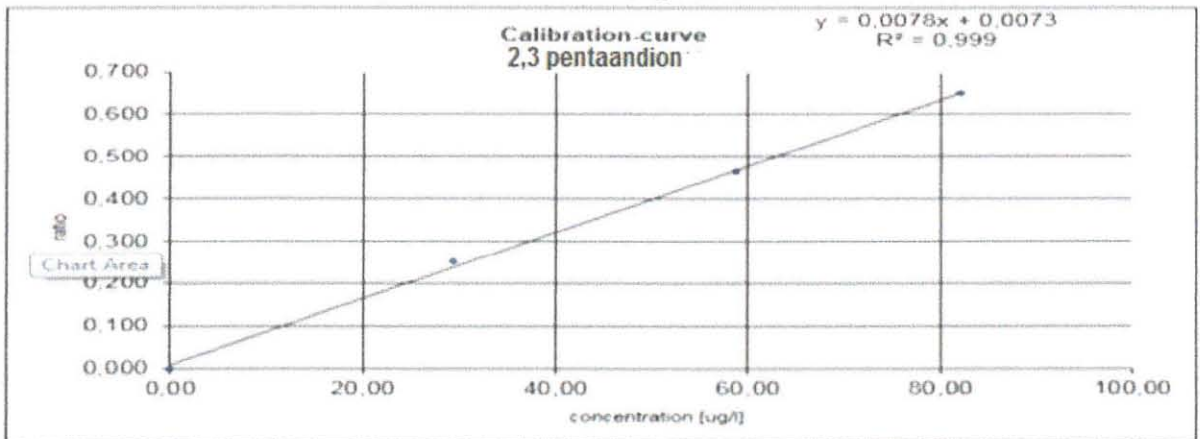
## 7. List of Appendices

### Appendix I Calibration Curves of volatile flavour compounds

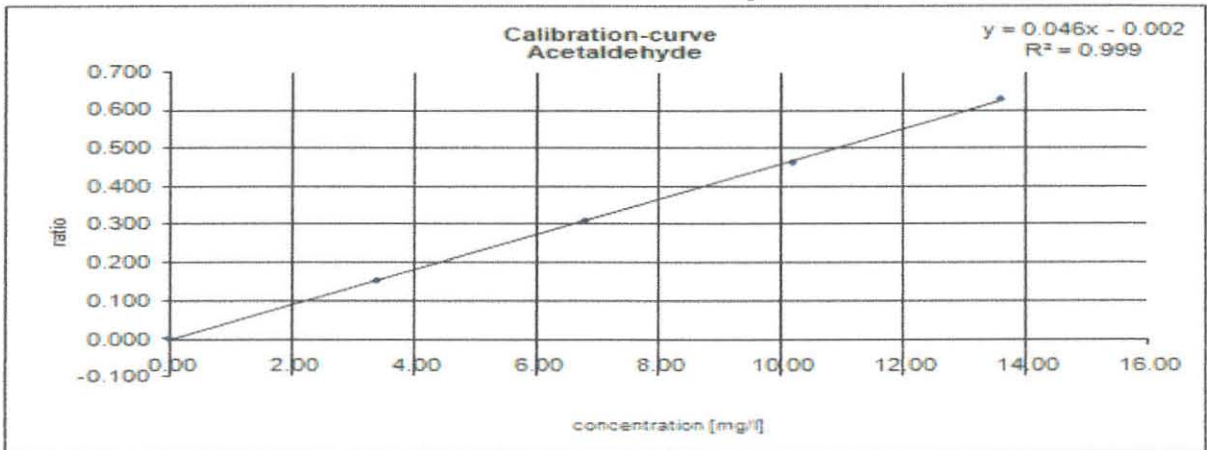
Calibration curve for Diacetylyle



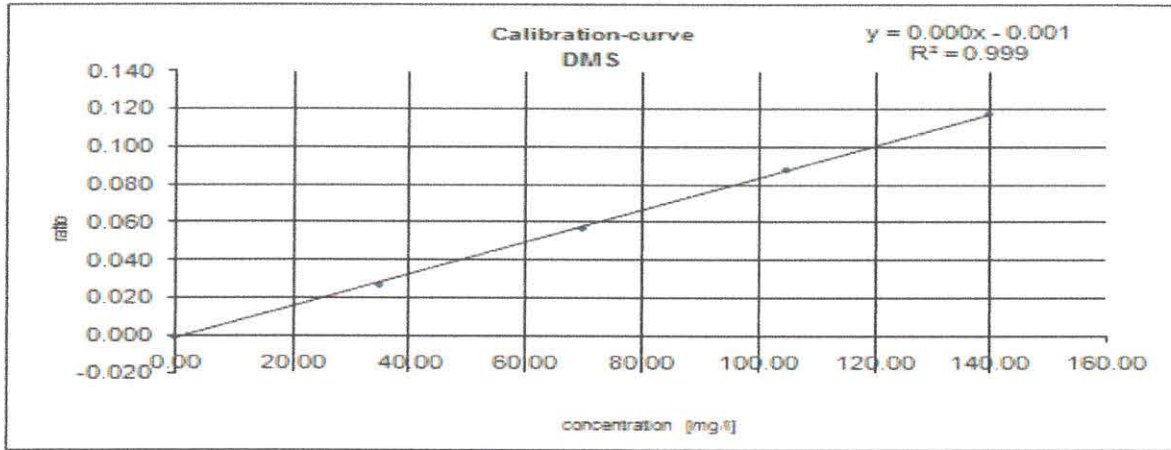
Calibration curve for 2,3 pentanedion



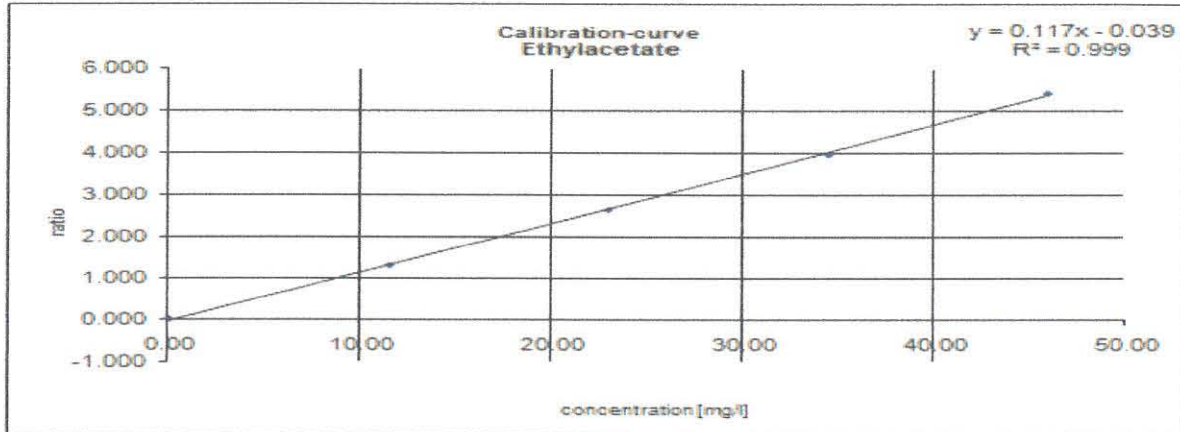
Calibration curve for Acetaldehyde



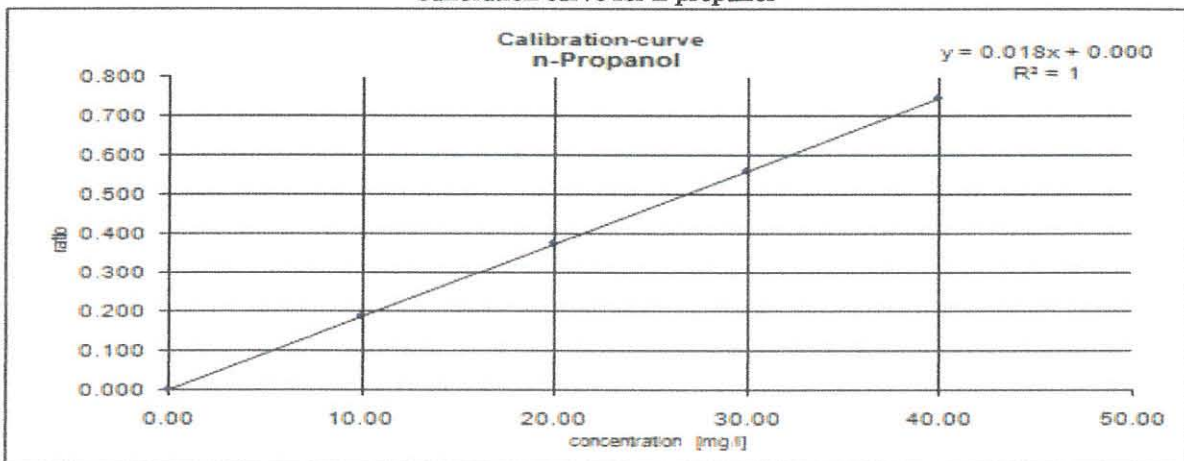
Calibration curve for DMS



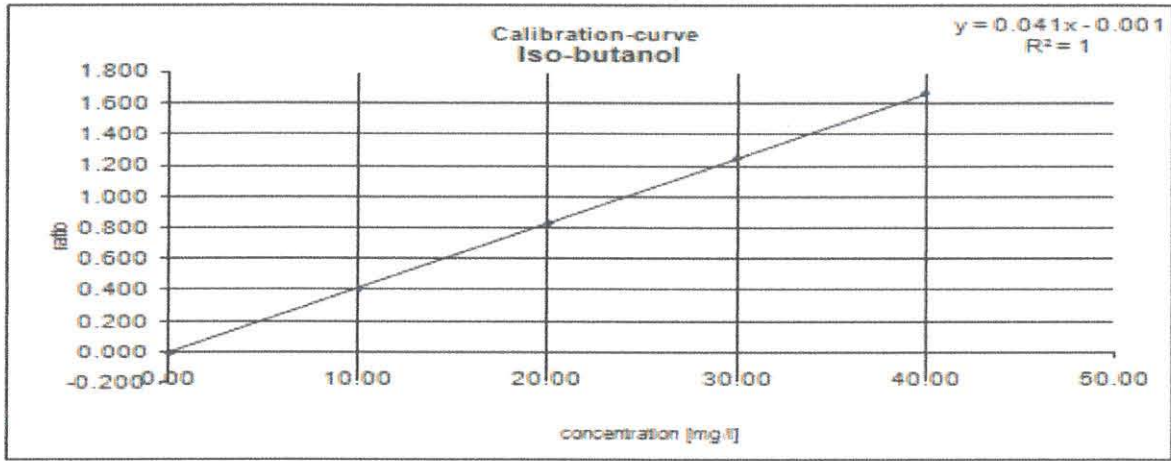
Calibration curve for Ethyleacetate



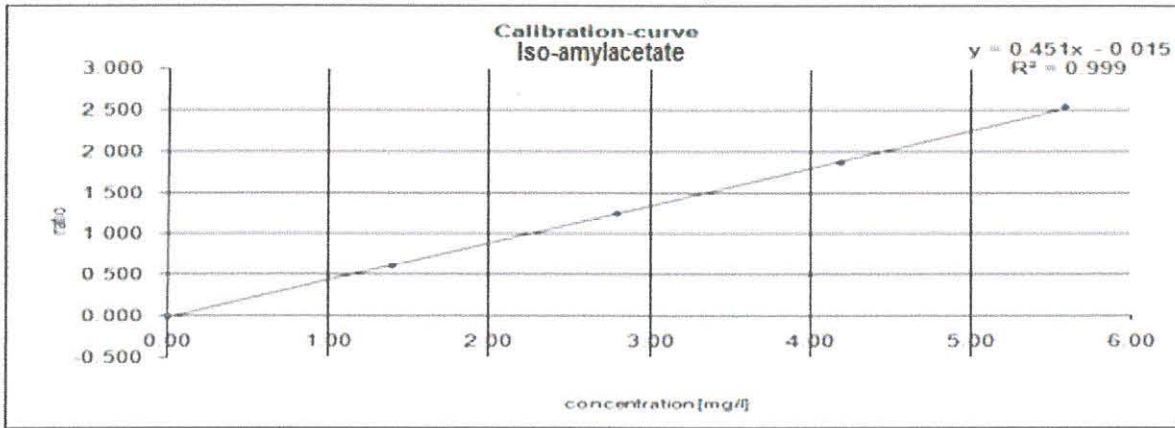
Calibration curve for n-propanol



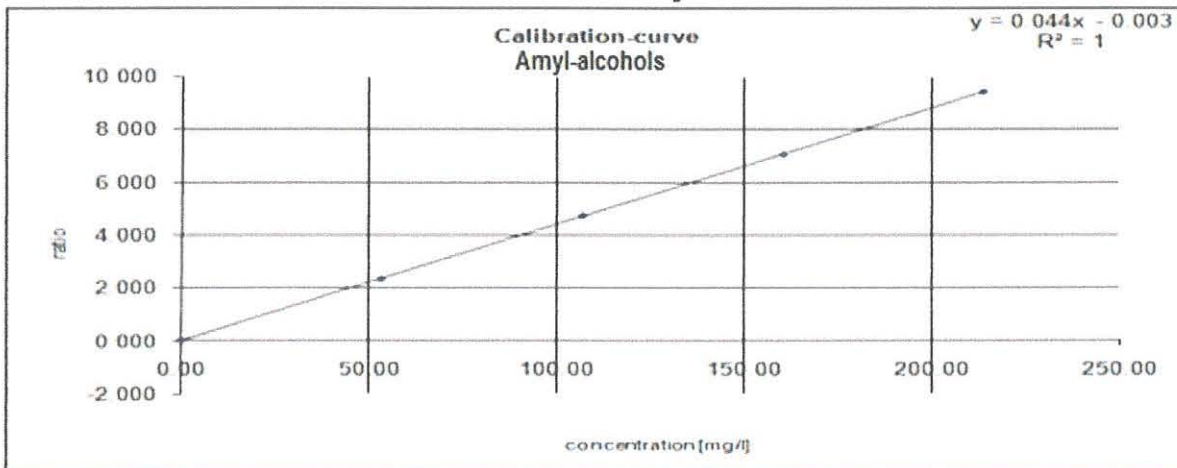
Calibration curve for Iso-butanol



Calibration curve for Iso-amylacetate



Calibration curve for Amyl-alcohols



Appendix II Questionnaire for VDK Study

**Dear respondent**

Good morning/Good afternoon. Thank you for your interest to talk with me today. I am EMNET TEGENE who is a post graduate student of Addis Ababa University center for food science and nutrition, conducting a study to determine the level of *Vicinal diketone* in Ethiopian alcoholic beer types. The purpose of my visit today is to take information from you on the aforementioned issue. If you are willing to participate in the study, I will ask you few questions for 20-30 minutes. Your honest answers to these questions will help me for a better understanding of the topic, and will eventually help in designing and implementing appropriate interventions to alleviate related problems.

Are you willing to participate in the study? Yes No

Date: \_\_\_\_\_

Qualification: \_\_\_\_\_

Position in the Company: \_\_\_\_\_

**1. Company Information**

Name of company:	
------------------	--

Address:	
----------	--

Post code:	
------------	--

Telephone number:	
-------------------	--

**2. General Information**

How much beer you brew per day in Hectoliters?	
--	--

How many products (brand beers) do you have? Could you list them?	
--	--

Do your brewery factories export its product to foreign country?	Yes <input type="checkbox"/>	No <input type="checkbox"/>
--	------------------------------	-----------------------------

### 3. Quality Standards Certifications

Questions	Yes	No	Comment
Do you hold certification(s) against any recognized quality standards by accredited third party body e.g. ISO 9001(QMS), 14001 (EMS) or 22000 (FSM and HACCP)? If yes list which you certify?			
Has the company been subject to periodic audit by competent authorities e.g. FMHACA, ISO inspection body etc?			
Do you have Sanitation Standard Operating procedures (SSOP's)?			
Do you have an employee hygiene program?			
Do you quality monitored throughout processing/production?			
Do you perform microbiological product testing?			
Do you perform physic-chemical product testing?			

### 4. Quations related to Vicinal Diketons

Are you familiar with VDK?	Yes <input type="checkbox"/>	No <input type="checkbox"/>
If it is 'YES'what are they?		
Would you think that VDKs are present in bottled beer?	Yes <input type="checkbox"/>	No <input type="checkbox"/>
If it is 'YES',Do you think it is desirable?	Yes <input type="checkbox"/>	No <input type="checkbox"/>
How VDKs affect the beer?		
What are Sources of vicinal diketones in beer?		
Which conditions are favors VDK formation in beer?		
Do you have a system for temperature control during Fermentation?		

Do you think VDK can reduce during process?	Yes <input type="checkbox"/> No <input type="checkbox"/>
If it is 'YES' how?	
Have you ever determined the level of vicinal diketones either in your or other certified laboratory?	Yes <input type="checkbox"/> No <input type="checkbox"/>
If 'Yes', What kind of analytical techniques do you use?	
If 'Yes', how much amount do you get?	

Do you have any comments or suggestions for this research? \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

**THANKYOU!**

Appendix III Addition of ethanol to lower alcohol beers  
[< 4.0 % (V/V)]

<b>Ethanol % (V/V) in the beer sample</b>	<b>Ethanol addition per 250 ml beer (ml)</b>
0	12.5
0.5	11.2
1.0	10
1.5	8.8
2.0	7.7
2.5	6.3
3.0	5
3.5	3.8
4	2.5

Appendix IV Dilution with water of higher alcohol beers

[> 6.0 % (v/v)]

<b>Ethanol % (V/V) in the beer sample</b>	<b>ml of beer to be diluted with water to 250 m</b>	<b>Dilution factor</b>
6	210	1.19
6.5	190	1.32
7	180	1.38
7.5	165	1.52
8	155	1.61
8.5	145	1.72
9	140	1.79

# Appendix V Heineken Reference Sample Certificate of Analysis



## Certificate of Analysis

### Quality Assurance Laboratories

Burtonkroonster Smeetsweg 1  
2562 MH Zoeterwoude, The Netherlands  
phone: +31 (0)79 545 90 45  
e-mail: SampleServiceCentre@heineken.com

**Kilinto Brewery**  
Akaki Kaliti  
Ethiopia

Product : Heineken lager beer  
Sample code : RB-04  
Sample date : 22-04-2017  
Bottle date : 13-04-2017  
Arrival at QAL : 01-05-2017

Sample quantity : 24 Bottles(s)

Your reference :  
**RB-04**

Our reference :  
**B1700550**

Component analysed sample	Result	Unit	Product specification
<b>Original extract, alcohol and extract</b>			
Q Original extract	11.43	%m/m	+ 11.2 - 11.6
Q Alcohol	4.06	%m/m	
Q Real extract	3.49	%m/m	
Q Apparent extract	1.60	%m/m	
Q Specific gravity 20°C/20°C	1.002		
Q Alcohol by volume	5.20	%V/V	+ 4.80 - 5.20
<b>Apparent extract after final attenuation</b>			
Q Apparent extract after final attenuation	1.31	%m/m	# 1.4 - 2.0
Q Delta apparent extract	0.29	%m/m	+ 0.0 - 0.3
<b>Carbon dioxide and foreign gas</b>			
Q Dissolved carbon dioxide (TPA)	0.49	%m/V	+ 0.48 - 0.52
Q Dissolved carbon dioxide (TPA)	0.49	%m/V	+ 0.48 - 0.52
Q Total foreign gas	0.4	ml/l	+ 0.2 - 0.6
Q Total foreign gas	0.3	ml/l	+ 0.2 - 0.2
Q Turbidity (90°, 0°C, 650 nm)	0.4	EBC	+ 0.0 - 0.6
Q Turbidity (90°, 0°C, 650 nm)	0.4	EBC	+ 0.0 - 0.6
Q Turbidity 7 days test (90°, 0°C, 650 nm)	1.0	EBC	+ 0.0 - 2.5
Q Turbidity 7 days test (90°, 0°C, 650 nm)	1.0	EBC	+ 0.0 - 2.5
Q Colour	7.6	EBC	+ 6.0 - 8.0
Q Bitterness	18.9	SU	+ 17.5 - 20.5
Q Foam stability	239	s	# 250 - 350
Q pH	4.78		# 4.45 - 4.65
Q Total sulphur dioxide	5.4	mg/l	# 5 - 8
Q Free amino nitrogen (FAN)	125	mg/l	# 0 - 80

Budget code : S.2343.008.03  
Comment :

Date : 15-05-2017  
Version : 1  
Page 1 of 2

Written Specification  
Out of Specification  
Out of Reference

Approved by : Eric Innemee (Sample Coordinator)

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Disclaimer: The interpretation of the analysis results against the product specifications is not a part of the ISO 17025 / 7050 compliance.



Your reference

RB-04

Dial reference

B1700550

Component analysed sample	Result	Unit	Product specification
<b>Esters, alcohols and dimethylsulphide</b>			
Q Acetaldehyde	2.6	mg/l	< 0.4
Q Dimethyl sulphide (DMS)	38.4	µg/l	< 55 - 55
Acetone (propanone)	< 0.30	mg/l	
Ethylformiate	< 0.1	mg/l	
Q Ethylacetate	25.9	mg/l	* 20.0 - 25.0
Methanol	< 5.90	mg/l	< 0.00 - 6.00
Ethylpropionate	< 0.1	mg/l	
Q Propanol	8.7	mg/l	
Q Isobutanol	13.4	mg/l	
Q Isoamylacetate	3.68	mg/l	< 3.25 - 4.25
Q Amyl alcohols	62.5	mg/l	
Ethylcapronate (ethylhexanoate)	0.1	mg/l	
Q Total higher alcohols	14.6	mg/l	< 0 - 30
<b>Diacetyl (2,3-butanedione) and 2,3-pentanedione</b>			
Q Diacetyl (2,3-butanedione)	8.1	µg/l	< 0 - 15
Q 2,3-Pentanedione	4.1	µg/l	< 0 - 25
<b>Standard taste test</b>			
Q See Certificate Sensory Evaluation	Tasted on		
	10/05/2017		

Remarks: