DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF VOLATILE ORGANIC COMPOUNDS ASSOCIATED WITH BEER AGING

by

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Abstract

A method for the extraction, identification and quantification of eleven specific volatile organic compounds (VOCs) associated with off-flavours and off-odours in two different types of beers were developed. For this purpose, purge and trap extraction in combination with thermal desorption gas chromatography coupled to a mass spectrometric detection (TD-GC-MS) were used. This method was used to determine: furfural, ethyl nicotinate, (E)-2-nonenal, ethyl lactate, isoamyl acetate, 2-methyl-1-propanol, dimethyl trisulphide, 2, 3-butanedione, limonene, (+) (-) α pinene, and 2, 3- pentanedione in "pilsner" and "India Pale Ale" (IPA) beer. The samples were collected in the local brewery and analysed for the presence of eleven VOCs using the developed method. The VOC measurements after accelerated ageing of the beer were compared. The mean concentrations of eleven VOCs were measured over a range of 1.8 ng/L – 214 ng/L. An increase in the concentration of VOCs was observed during the ageing treatment for most of the VOCs. All the VOC measurements were below the acceptable flavour threshold limits: except (E)-2 nonenal and DMTS in IPA, their acceptable flavour threshold values correspond to 30 ng/L and 10 ng/L, respectively. The forced ageing at 40°C/21 days was a better stability test compared to 60°C/1 day as all of eleven VOCs were identified and quantified at 40°C/21 days in both beers. Very few samples in pilsner were detected above LOD for few compounds; but they might not be present initially in the pilsner. The developed method was demonstrated to be useful for the determination aldehyde, vicinal diketones, sulphur compounds, hop compounds, esters and furfurals simultaneously in beer.

List of Abbreviations Used

ASBC	American Society of Brewing Chemist
Cu	Copper
Co-A	Coenzyme A
CSLR	Calibration Solution Rig
DMS	Dimethyl sulphide
DMTS	Dimethyl trisulphide
DMDS	Dimethyl disulphide
ECD	Electron Capture Detector
EI	Electron Impact
Fe	Iron
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-O	Gas Chromatography Olfactometry
HMF	Hydroxy-methyl-furfural
HPLC	High Performance Liquid Chromatography
HS-SPME	Head Space- Solid Phase Microextraction
IPA	India Pale Ale
IAA	Iso amyl acetate
ISQ	Single Quadrapole
LOD	Limit of Detection
LCMS	Liquid Chromatography Mass Spectrometry
m/z	Mass/Charge

MS	Mass Spectrometry
NPD	Nitrogen Phosphorus Detector
NIST	National Institute of Standard and Technology
NIH	National Institute of Health
P&T	Purge and Trap
ROS	Reactive Oxygen Species
RSI	Reverse Search Matching
SI	Direct Matching
SPME	Solid Phase Microextraction
SBSE	Stir Bar Sorptive Extraction
TA	Trapping Agent
TDT	Thermal Desorption Tube
TD	Thermal Desorption
USEPA	United States Environmental Protection Agency
U-5	Ultrapure Grade-5
USDOE	United States Department of Energy
USDOD	United States Department of Defence
VDKs	Vicinal Diketones
VOCs	Volatile Organic Compounds

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Chapter1. Introduction

Brewing is Canada's oldest industry with domestic brewers holding 85% share in domestic beer market. Over the last twenty years, beer brewing grew from an age old biotechnology into a 1.97 billion hectoliters industry, where only ten companies are responsible for the major portion of world's production (Statistica ., 2014). Figure 1 indicates the global beer production in different regions in 2013.

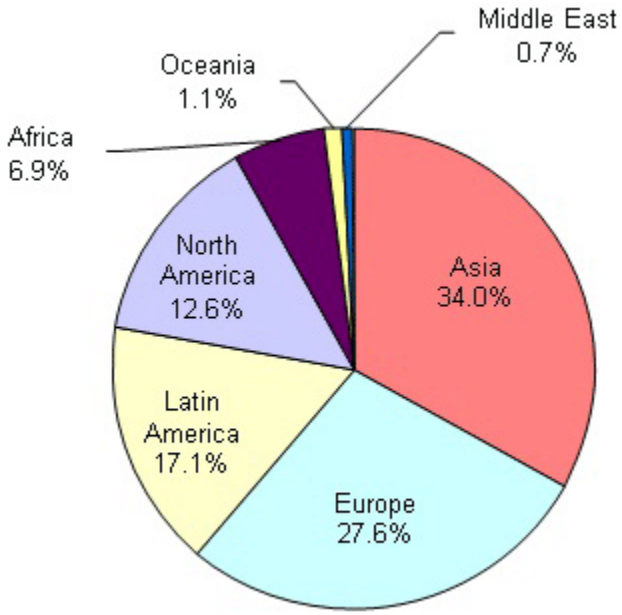

Figure 1. Depicts the global beer production by region in 2013 (Kirin, 2014).

Beer flavour stability has proven to be a difficult problem to solve, as the flavour of beer is affected by factors such as oxygen, temperature, exposure to light and storage time (Bamforth, 2002). Oxygen plays a pivotal role in staling of beer; the ingress of oxygen early in the brewing process can initiate staling, even in the malt house (Tressl et al., 1997). The natural ingredients in beer are susceptible to oxidation. For example, malt contains diverse precursors that contribute to staling, such as lipooxygenase, thiol oxidase (Bamforth et al., 2009) and oxalate oxidase

(Kanauchi et al., 2010) both of which will absorb oxygen. However, the polyphenol oxidase is not as relevant as it is lost during malting and mashing (Clarkson et al., 1991). Oxygen may react with the wort and beer components through the action of reactive oxygen species or through enzyme catalyzed reactions. Oxygen can react with thiol groups non-enzymatically (Muller, 1997) and via thiol oxidase to produce hydrogen peroxide, which in turn is a substrate for peroxidases (Clarkson et al., 1992), through the action of which polyphenols become oxidized. Therefore, the protection against oxidation is important at each and every step of beer processing.

According to Arrhenius, the rate of chemical reaction (e.g. oxidation, Strecker degradation etc.) in beer increases by two to three folds with every 10ºC increase in temperature. A beer that develops a clear stale character after around 100 days at 20ºC may retain its flavor for a month at 30^0C or a day at 60ºC (Bamforth, 2004). The shelf life of beer under refrigeration temperature is increased enormously. In order to estimate the shelf life of beer and the effect of these parameters, a thermal deterioration protocol can be used to mimic natural aging (Saison et al., 2009)

The volatile organic compound (VOC) profile of beer consists of a wide range of compounds comprised of organic acids, vicinal diketones, fermentation by-products, esters, hop oil derivatives, sulphur compounds, Maillard reaction products and Strecker degradation products. The eleven VOCs i.e. furfural ethyl nicotinate, (E)-2-nonenal, ethyl lactate, isoamyl acetate, 2-methyl-1-propanol, dimethyl trisulphide, 2, 3 butanedione, limonene, (+) (-) α pinene, 2, 3 pentanedione, were identified and quantified in this study. These compounds have different degrees of polarity, volatility and are present over a wide range of concentrations in beer, varying from ng/L (ppm) to mg/L (ppb) (Rodrigues, Caldeira, & Câmara, 2008). These

compounds play an important role in the organoleptic characteristics of beer and consumer acceptability (Shale et al., 2012). Research has been conducted in the past to understand the formation of these compounds as well as their reduction in packaged beer. However, the analytical techniques used for the detection of these compounds generally require laborious sample preparation, skilled personnel for sample preparation and analysis, and often result in poor reproducibility.

The isolation of flavour-active compounds from the beer matrix is important in order to determine the compounds responsible for the aroma of beer. Static headspace, solid phase extraction, ultrasound extraction, liquid-liquid extraction, simultaneous-distillation extraction and solvent extraction are frequently used techniques for the extraction of VOCs from beer (Rodrigues et al., 2008). The purge and trap method is an extraction protocol used for analysing environmental samples, and has advantages over the more commonly used methods for beer as it is a simpler procedure. In this thesis, the purge and trap method was used for VOCs extraction from the beer matrix along with gas chromatography/mass spectrometry (TD-GC-MS), to analyse the VOC profile of beer after different stability test conditions, to identify and measure the major VOCs known to impart off odours and flavours to ageing beer.

1.1 Objectives

There were two objectives of this study:

1) To develop a method for determination of VOCs, (i.e. Furfural, ethyl nicotinate, (E)-2-nonenal, ethyl lactate, isoamyl acetate, 2-methyl-1-propanol, dimethyl trisulphide, 2, 3 butanedione, limonene, (+) (-) α pinene, 2, 3 pentanedione), in beer using purge and trap extraction followed by TD-GC-MS analysis.

2) To use the developed method to determine the concentration of eleven VOCs in India pale ale (IPA) and pilsner beer and relate them to the characteristics of ageing beer.

Chapter 2. Literature Review

2.1 Overview of Brewing Process

Beer is an alcoholic beverage brewed from barley by addition of hops, yeast and water. The other natural ingredients may be added to create different styles and flavors of beer. The main stages involved in brewing process are wort production, alcoholic fermentation and maturation, processing and stabilization of the beer. Within the wort production are malting, mashing and lautering stages (Bamforth, 2009)

In the malting process raw barley is converted into a product with a high level of enzymes and with better chemical and physical properties appropriate for brewing. The barley is steeped in cool water and drained occasionally, once the grain is thoroughly wet it is spread for germination to proceed. The maltsters stop germination to prevent the growth of a barley plant by the process called kilning. Kilning involves the introduction of hot air in order to dry malt and imparts color and flavor to the malt. The grains are milled and adjuncts such as rye, sorghum, oats and rice are seldom added to produce more stable product, add flavours and to produce beer at a lower cost (Parcunev et al., 2012).

Mashing is the process of mixing of crushed malt and adjuncts (if used in the brewing process) with the hot water and allows it to stand to yield more soluble sugars in the wort. The three most common processes of mashing are: infusion mashing, decoction mashing and double mashing. In infusion mashing, the mash tun is used without agitation and at a single temperature, whereas in decoction mashing, different mashing conditions are used in order to achieve varying degrees of protein degradation and starch hydrolysis. In decoction mashing, the optimal temperature for protein denaturation is between 40-50^{0}C, whereas the starch hydrolysis temperature is 54-65^{0}C

and the wort separation temperature is around 71^0C. Double mashing is the type of mashing used when cereal adjuncts require pre-cooking (Priest & Stewart, 2006).

The wort is separated by a process called lautering in the lauter tun. Lautering takes approximately 2 hrs followed by mash filtration (90 min) for the separation of wort from the spent grains. Lautering is followed by wort boiling in the wort kettle where hops are mixed with the wort. Wort boiling assists in sterilization, extraction of bittering compounds from the hops, color and aroma and coagulation of proteins and tannins. The coagulated proteins and tannins are known as trub, which are removed later in whirlpool by the centripetal and centrifugal forces on the tangentially rotating wort (Bamforth, 2006).

The wort is then cooled by a plate heat exchanger, using cold water as a cooling medium. The wort is pitched with yeast for fermentation and two different strains of yeast are usually introduced: ale yeast (*Saccharomyces cerevisiae*) or lager *(S. pastorianus).* Generally ale fermentations occur at higher temperature of 18-22^0 C, where the yeast is very active at this temperature and attenuate wort quickly to final gravity (Bamforth, 2003). The lager fermentations are carried at lower temperatures of 6-15^0 C and usually last for a few months (Bamforth, 2003). The yeast is removed after fermentation and the beer is transferred to the bright beer tank for storage. The beer is then injected with carbon dioxide and bottled in the bottling plant (Priest & Stewart, 2006).

2.2 The Volatile Organic Compounds (VOCs) in Beer

Beer flavour is a result of the interaction of between more than 800 chemical compounds. The compounds that impart taste can be sensed directly on the tongue, whereas the volatile compounds imparting aroma can be perceived through the nose (Saison et al., 2009). Many chemical reactions occur during the storage of beer that result in deterioration of fresh flavour notes and the appearance of typical aged flavour (Saison et al., 2009). The lack of flavour stability in the beer is a major problem for the brewers, as it is important to maintain the consistency of the beer in order to satisfy consumers. The various compounds that impart flavour to the beer and their sources are discussed following sections.

2.2.1 Vicinal Diketones (VDKs)

The compounds 2, 3 butanedione (diacetyl) and 2, 3- pentanedione, are formed in every brewery fermentation as they are the by-products of yeast metabolism. When grouped, these two compounds are also termed vicinal diketones (VDKs) (Bamforth, 2014). They affect the aroma and flavor of beer drastically (Krogerus & Gibson, 2013). The flavor threshold of diacetyl in lager beer is 0.01-0.2 ppm and in ale is around 0.1-0.4 ppm (Wainwright, 1973). The flavor threshold of 2, 3- pentanedione is sometimes ten times higher than diacetyl and varies from 0.9-1 ppm (Wainwright, 1973). Diacetyl has a characteristic butterscotch and popcorn aroma, whereas pentanedione has a honey, butter, caramel and just plain sweet aroma (Bamforth, 2014). The desired concentration of VDKs depends upon the particular flavor desired in different types of beer. Flavor defects can be caused by excessive levels of VDKs in the final beer and many brewers may prefer to have no diacetyl present in beer (Wainwright, 1973). Table 1 indicates the properties of VDKs present in the beer.

Table 1. Chemical properties of 2, 3 Butanedione and 2, 3-Pentanedione.

Property	Compound	
	2,3 Butanedione	**2,3- Pentanedione**
Molecular formula	$CH_3COCOCH_3$	$CH_3CH_2COCOCH_3$
Molecular weight	86.09 g/mol	100.12 g/mol
Boiling Point	88^0C	$110\text{-}112^0C$
Precursor	α-acetolactate	α-acetohydroxybutyrate
Flavor	Butterscotch, Popcorn	Honey ,Butter, Caramel
Flavour threshold	0.01-0.04 ppm	0.9-1.0 ppm

*American Society of Brewing Society, Beer Flavour Database, 2005.

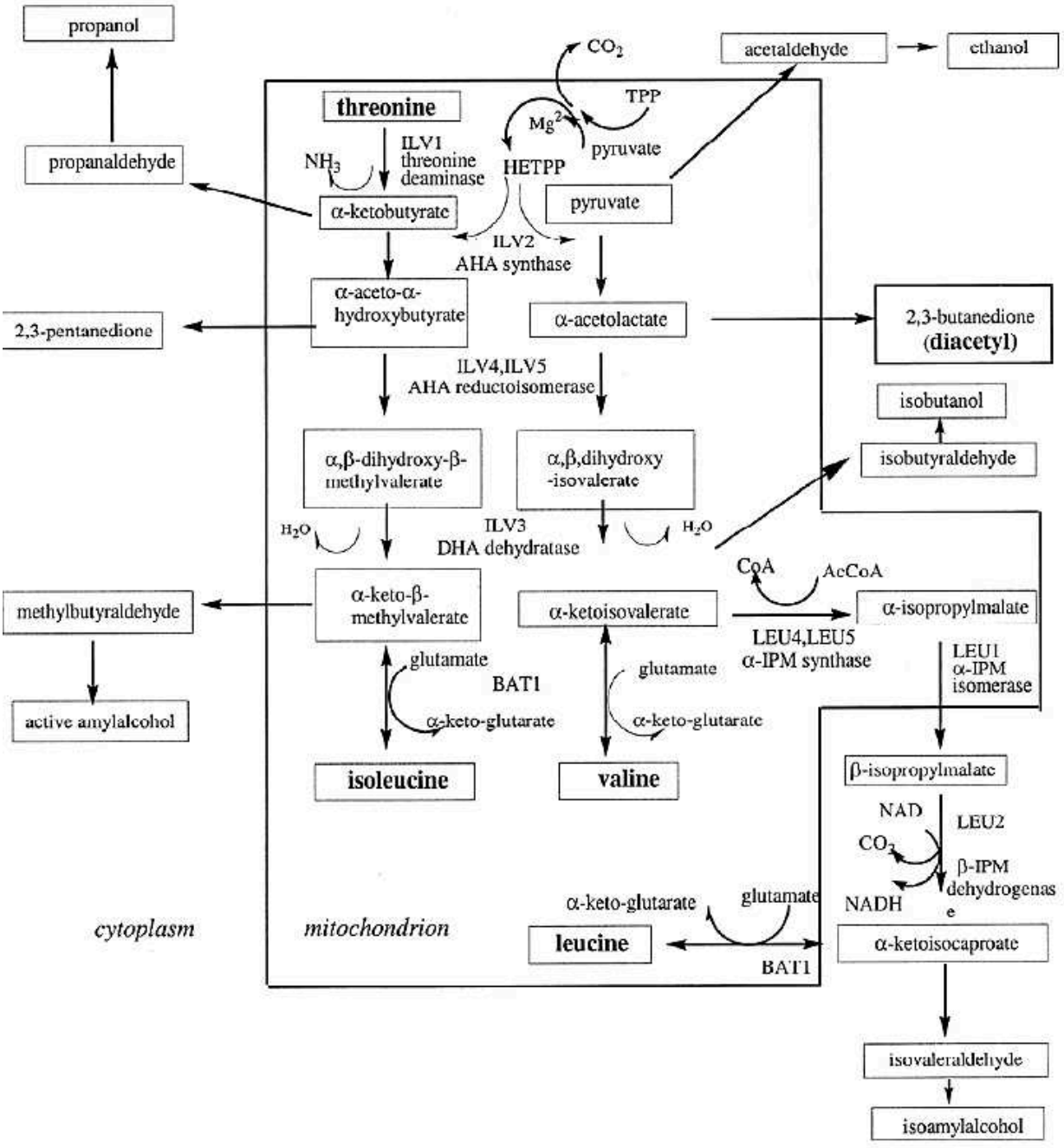

Figure 2. The pathway of production and reduction of VDKs in Yeast (*Saccharomyces spp)* (Peterson et al., 2004)

The production of diacetyl and 2, 3- pentanedione in wort is due to the non- enzymatic oxidative decarboxylation of α-acetohydroxy acids that are intermediates of valine and isoleucine biosynthesis pathways (Krogerus & Gibson, 2013). Figure 2 summarizes the pathway of production and reduction of VDKs in yeast. The precursors for production of diacetyl and 2, 3-pentanedione are α-acetolactate and α-acetohydroxybutyrate, respectively. The VDKs produced in the wort are re-consumed by the yeast, where they are enzymatically reduced to a product that does not affect the flavor of the beer. As shown in figure 2 the diacetyl is reduced to acetoin and finally to 2, 3-butanediol and 2,3 pentanedione is reduced to 3-hydroxy-2-pentanone and then to 2,3- pentanediol (Krogerus & Gibson, 2013). If the acetolactate and acetohydroxybutyrate are not completely converted during fermentation and maturation, then they will survive in the beer and will progressively degrade in the packaged beer (Bamforth & Lentini, 2002).

2.2.2 Esters

An ester molecule is made up of carboxylic acid and alcohol moieties. Isoamyl acetate is a key ester that is present in beer and is largely responsible for the fruity note of beer. The isoamyl acetate is made from a reaction between isoamyl alcohol and acetic acid (Bamforth, 2014), and the flavor threshold of isoamyl acetate is 0.6-1.2 mg/L (Bamforth, 2014). The esters are also a product of yeast metabolism. Figure 3 depicts the synthesis of esters from fusel alcohols and Acetyl Co-A. The ester synthase gene helps in the formation of ester synthase enzyme that helps in the production of the esters by yeast. The fusel alcohol and Acetyl-Co-A are important substrates in the synthesis of ester (Verstrepen et al., 2003).Table 2 indicates the chemical properties of some important esters.

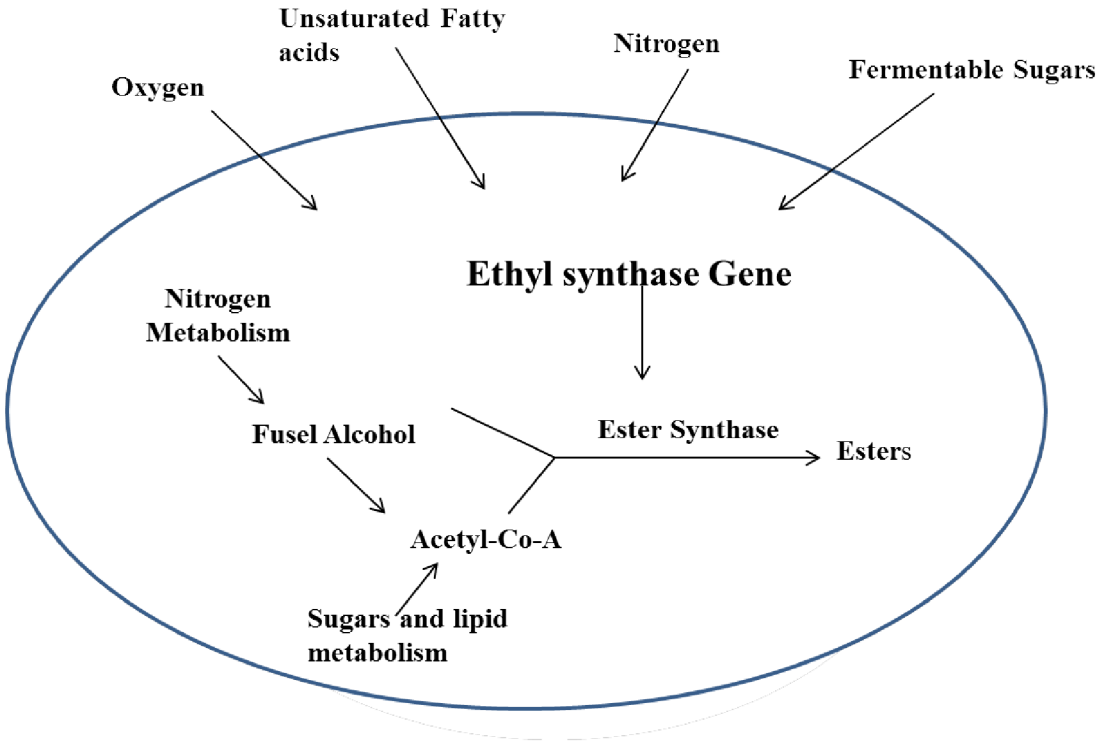

Figure 3. Schematic of ester production from yeast (Verstrepen et al., 2003).

Table 2. Chemical properties of selected esters

Compounds	Ethyl Nicotinate	Ethyl Lactate	Isoamyl acetate
Molecular Formula	$C_8H_9NO_2$	$C_5H_{10}O_3$	$C_7H_{14}O_2$
Molecular Weight	151.16 g/mol	118.13 g/mol	130.18
Boiling point	225^{0}C	155^{0}C	254^{0}C
Precursor	Ester synthase	Ester Synthase	Ester synthase
Flavour	solvent, anis, grainy	Perfumed, fruity	Banana, pear, apple
Flavour Threshold	6 mg/L	25 mg/L	0.6-1.2 mg/L

*American Society of Brewing Society, Beer Flavour Database, 2005

Some volatile esters (such as isoamyl acetate) have a positive impact on the beer and are responsible for imparting a fruity flavor to the beer, however their concentration decreases below their flavor threshold with the ageing of beer (Vanderhaegen et al., 2006b). In contrast, other esters such as ethyl-3-methylbutyrate, ethyl-2-methylbutyrate, ethyl-2-methyl propionate, ethyl nicotinate, diethyl succinate, ethyl lactate, ethyl phenylactetate, ethyl formate and ethyl

cinnamate have a negative effect on beer flavor and the concentration of these esters increases during the aging of beer (Vanderhaegen et al., 2006).

2.2.3 Carbonyl compounds

Carbonyl compounds are related to the stale flavor of beer. Acetaldehyde is an important member of this group, having the aroma characteristic of green apple, and is produced by yeast in the later stages of sugar degradation (Bamforth, 2014). Acetaldehyde is an immediate precursor of ethanol and if present in significant amounts in beer, is an indication of poorly performing yeast (Bamforth, 2014). Acetaldehyde is also produced by spoilage bacteria *Zymomonas spp* (Bamforth, 2014). In addition to acetaldehyde, other carbonyl compounds that are detected in aged beer are the alkanals and alkenals (Vanderhaegen, Neven, Verachtert, & Derdelinckx, 2006).

Hashimoto in 1977 was the first to study the composition and pathway of formation of stale aldehydes in bottled beer. (E)-2-nonenal is considered an important off-flavor of beer, related to the flavor of beer staling (Scherer, Wagner, & Godoy, 2010). The cardboard flavor in the beer, which is induced by E-(2)-nonenal, was first detected by Jameison and Van Gheluwe in 1970. The pathway of formation of (E)-2 -nonenal in beer is unclear, but some reaction mechanisms such as Strecker degradation of amino acids, oxidative degradation of iso-humulones, oxidation of fatty acids and aldol condensation are thought to result in (E)-2-nonenal formation (Scherer et al., 2010).Table 3 indicates the chemical properties of acetaldehyde and trans-2-nonenal.

Table 3. Chemical properties of selected carbonyl compounds.

Compounds	Acetaldehyde	(E)-2-nonenal
Molecular formula	C_2H_4O	C_9H_6O
Molecular weight	44.05 g/mol	140.22 g/mol
Boiling point	20.2^0C	60^0C
Flavour	Grassy, sweet, pungent	Papery, cardboard, waxy
Flavour threshold	1.1 mg/L	0.00003 mg/L

*American Society of Brewing Society, Beer Flavour Database, 2005

2.2.4 Sulphur compounds

Some of the characteristic aroma of beer is due to sulphur compounds. Many types of ale contain high amounts of hydrogen sulphide, where the levels are above the flavour threshold, resulting in a not unpleasant, distinctive flavour of some ales (Baxter& Hughes, 2001). During wort boiling dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS) have been identified as being present in the wort (Lermusieau & Collin, 2003). Some of the sulphur volatiles are removed during fermentation, but a few can survive maturation and final packaging (Lermusieau & Collin, 2003). The DMTS can reappear in bottled beer from methanesulfenic acid or methional (Lermusieau & Collin, 2003). Generally sulphur compounds have a very low flavour threshold and even small changes in concentration can be noticeable (Vanderhaegen et al., 2006). The flavour threshold of DMTS is 0.00001 mg/L (ASBC, flavor database). During beer storage, DMTS is produced by a reaction between methanesulfenic acid and hydrogen sulphide (Vanderhaegen et al., 2006). The ß-elimination of S-methylcysteine introduced in beer from hop produces methanesulfenic acid (Vanderhaegen et al., 2006).

2.2.5 Hops-related compounds

The essential hop oil contributes tremendously to the flavor and aroma of beer (Bamforth, 2014). The hops are added in the wort kettle and boiled for at least 4 hours in order to extract aroma compounds from hops. The majority of aroma compounds in the hops are derived from terpenes

and are made up of 10-40 carbons containing isoprene units. Monoterpenes are the product of two isoprene units and include α-pinene, ß-pinene, myrcene, cymene and limonene. Sesquiterpenes and oxygen containing sesquiterpenes are made up of 3 isoprene units and include humulene, farnesene, caryophyllene, humulenol and humelol (Yakima & Lefervre, 2012). Very limited literature is available on the state of pinene and limonene in aged beer but they contribute to the "hoppy" flavour of beer.

2.2.6 Heterocyclic compounds

The concentration of heterocyclic compounds changes with the ageing of beer. Furfural, 5 methyl-furfural, 5-hydroxymethyl-furfural (HMF), 2-acetyl-furan, 2-acetyl-5-methyl-furan, 2-propionylfuran, furan and furfuryl alcohol are the few furans formed during the ageing of beer (Vanderhaegen et al., 2006). The concentration of furfural and HMF increases with storage temperature and can be used as an indicator of temperature-induced flavour deterioration (Vanderhaegen et al., 2006). These heterocyclic compounds are the intermediates of Maillard reactions between amino acids and sugars (Vanderhaegen, Even, Oghe, & Erstrepen, 2003). Table 4 shows the chemical properties of some heterocyclic compounds that are present in aged beer.

Table 4. **Chemical properties of selected heterocyclic compounds**

Compounds	Furfural	HMF	5-methyl-furfural
Molecular formula	$C_5H_4O_2$	$C_6H_6O_3$	$C_6H_6O_2$
Molecular weight	96.08 g/mol	126.11 g/mol	110.11 g/mol
Boiling point	161.7^0C	116^0C	187^0C
Flavour	Caramel, bready, husky	Stale, vegetable oil	Almond, burnt, spicy
Flavour threshold	0.01-1.8 mg/L	1000 mg/L	1.2 mg/L

*American Society of Brewing Society, Beer Flavour Database, 2005

2.2.6 Alcohols in beer

Ethanol is the most abundant alcohol in beer, with a flavour threshold of 14,000-2,000 mg/L, which gives very strong solvent-like notes to the beer (Baxter & Hughes, 2001). The yeast produces higher alcohols as secondary metabolites of amino acid metabolism, as indicated in

Figure 4 depicts the yeast can also produce higher alcohols by assimilating nitrogen available from wort (Baxter& Hughes, 2001). Table 5 summarizes some of the chemical properties of selected higher alcohols.

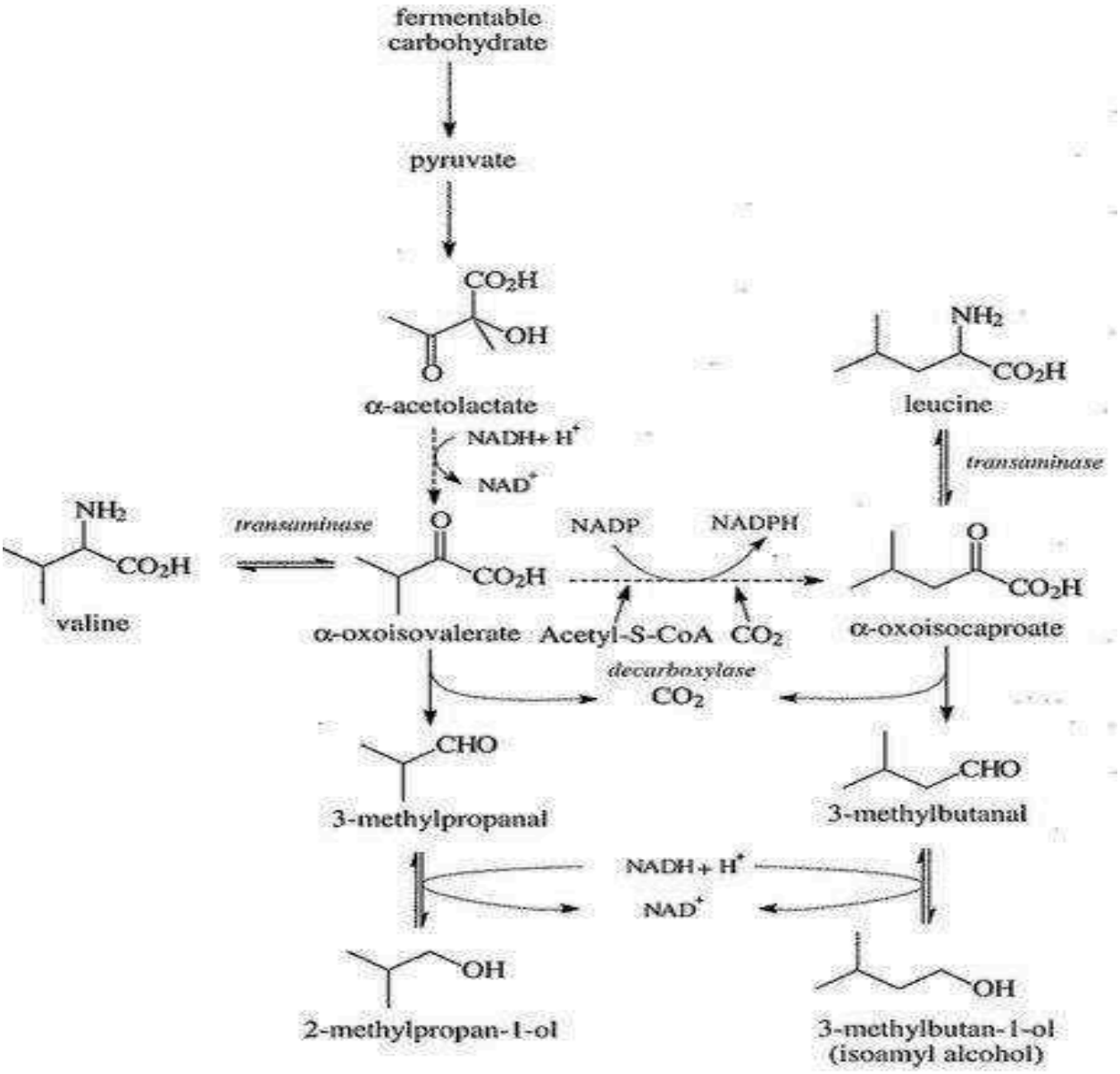

Figure 4. Production of higher alcohol by yeast in beer (Baxter & Hughes, 2001)

Table 5. **Chemical properties of selected higher alcohols**

Compounds	Propane-1-ol	2-Methyl-1-propanol	2-Methylbutanol	2-Phenyl ethanol
Molecular formula	C_3H_8O	C_4H_8O	$C_5H_{12}O$	$C_8H_{10}O$
Molecular weight	60.10 g/mol	72.11 g/mol	88.15 g/mol	120.15 g/mol
Boiling point	97^0C	108^0C	127.5^0C	221^0C
Flavour	Alcoholic	Fruity, banana	Malty, sweet	Alcohol, roses
Flavour threshold	600 mg/L	0.05 mg/L	0.045 mg/L	0.018 mg/L

*American Society of Brewing Society, Beer Flavour Database, 2005

2.3 Mechanism of the ageing process for beer

2.3.1 Impact of oxygen on stability of beer

Oxygen comprises about 1/5th of the gases in the atmosphere and can react with the wort and beer. The wort is oxygenated before pitching with the yeast and this oxygen is utilized by the yeast, however, some of the oxygen remains in the system (During malting, fermentation and packaging) which can cause spoilage. Throughout the whole brewing process there is substantial amount of oxygen present, which can create problems (Bamforth & Lentini, 2002).

The importance of reactive oxygen species (ROS) was first discovered by Bamforth and Parson in 1985. Oxygen in its ground state is very stable and it does not react with any organic molecules. Figure 5 shows a schematic of the mechanisms introduced in production of ROS species. The ferrous ions act as a catalyst in beer, and in presence of these ions, oxygen captures electrons, and forms super oxides as it is electronegative in nature. Copper ions behave similarly to ferrous ions. It is believed that Cu +/Cu^{2+} and Fe^{2+} /Fe^{3+} are the part of heterogeneous oxidation system in which polyphenols, sugars, iso-9 humulones and alcohols might act as electron donors (Kaneda et al., 1992). The superoxide anion can accept electron and is converted to perhydroxyl (OOH•) which is more reactive (Bamforth, 2009)

In the beer, the superoxide anions are readily converted to hydrogen peroxide (H_2O_2) or the superoxide anion (O_2^-), by the metal induced reactions such as Fenton and Haber-Weiss

reaction. The reactivity of oxygen species follows usually this trend: hydroxyl radical>per hydroxyl radical> superoxide radical. The concentration of free radicals during the aging of beer increases due to increases in oxygen concentration (Bamforth, 2009).

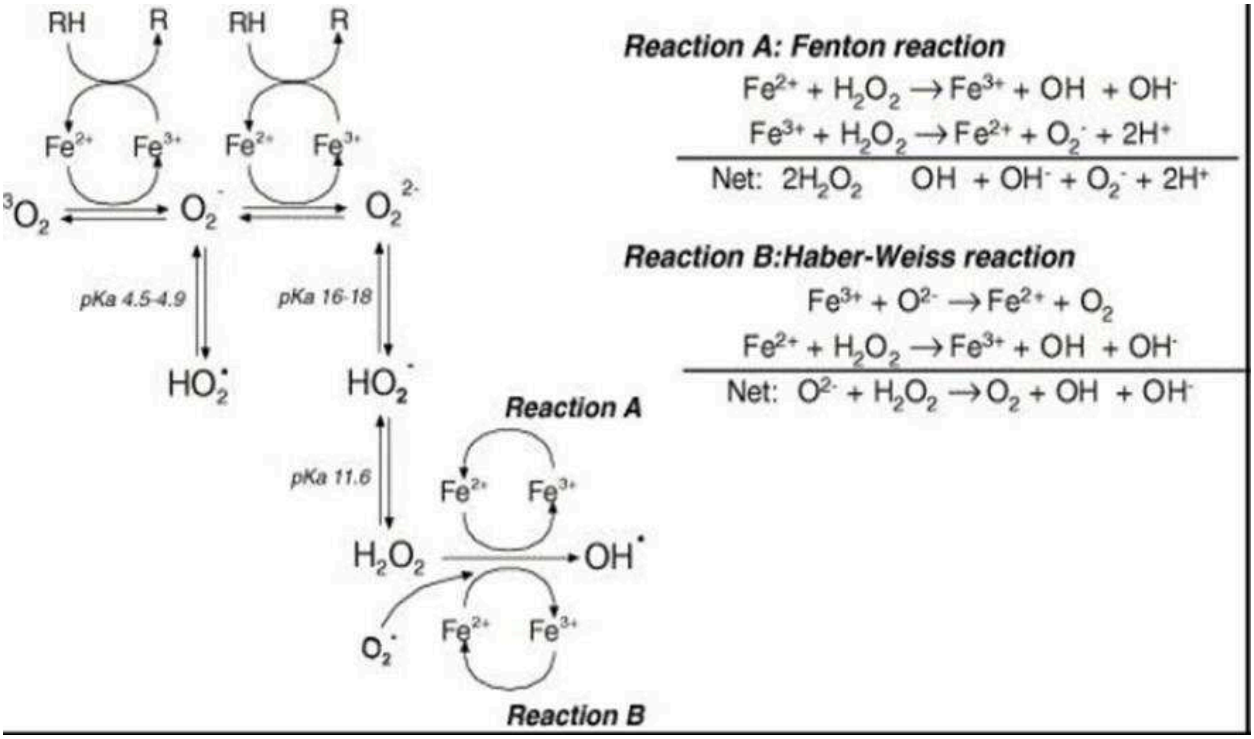

Figure 5. Reactions producing reactive oxygen species (ROS) in beer (Vanderhaegen et al., 2006)

2.3.2 Oxidation of higher alcohols in beer

The increased formation of aldehydes due to exposure with oxygen and high storage temperature was studied by Hashimoto (1972). The most important alcohols in beer are ethanol, 1, 2-methyl-propanol, 2-methyl-butanol and 2-phenyl-ethanol (Hashimoto and Eshima, 1977). High temperature, low pH and the availability of higher alcohols in the beer led to higher concentrations of aldehydes. Andersen & Skibsted, (1998) reported that 1-hydroxyethyl radical is quantitatively the most important radical because of its reaction with ethanol.

2.3.3 Oxidation of unsaturated fatty acids in beer

The oxidation of polyunsaturated fatty acids (mainly as linoleic acid) is catalyzed by lipooxygenase. The end products of linoleic acid oxidation are the hydroperoxides, which act as a substrate for hydroperoxide isomerase. The hydroperoxides that are produced upstream in malting and brewing persist in the finished beer and decay to release a stale character in the finished beer. The lipooxygenase is produced in the barley embryo during germination, and during this time period hydroperoxides are also produced. Lipooxygenase is a heat sensitive enzyme and is destroyed during kilning (Bamforth, 2009).

Linoleic acid also is susceptible to oxidation even in the absence of enzymes. This is due to an autocatalytic degradation, where the process is triggered by the presence of hydroxyl and perhydroxyl radicals. The small amount of linoleic acid, oxygen and metal ions such as iron in the beer has a potential to cause oxidation of beer and result in off-flavours (Bamforth, 2009).

2.3.4 Strecker degradation of amino acids in beer

Amino acids in stored beer (such as proline, tyramine, histamine etc.) may be a source of aldehydes (Gorinstein et al., 1999). An increased formation of 2-methyl-propanal and 3-methyl-butanal was reported when either valine or leucine was added to beer in the presence of oxygen (Blockmans, Devreux, and Masschelein., 1975), where the reaction was catalyzed by Fe and Cu ions. The Strecker reaction is between amino acids and α-dicarbonyl compounds. This reaction involves transamination, followed by decarboxylation of subsequent α-ketoacid resulting in one carbon atom less than the amino acid. The Maillard reaction also results in formation of α-dicarbonyl compounds in beer (Bamforth & Lentini, 2002).

2.3.5 Role of temperature in deterioration of beer flavour

According to Arrhenius, the rate of chemical reaction in beer increases by two to three folds with every 10ºC increase in temperature. A beer that develops a clear stale character after around 100 days at 20ºC may retain its flavor for a month at 30^0C or a day at 60ºC (Bamforth, 2004). The storage temperature greatly affects the formation of ageing compounds in beer. The shelf life of beer under refrigeration temperature is increased enormously. In order to detect the shelf life of beer and estimate the effect of these parameters on shelf life of beer, thermal deterioration protocols are used to mimic natural aging.

2.3.6 Maillard reaction

The reaction between amino acids and reducing sugar is classified as the Maillard reaction. Many heterocyclic compounds found in aged beer are the products of Maillard reactions (Vanderhaegen et al., 2006). Maillard reaction products are furfural, HMF, 5-methyl furfural, which are typically present below their threshold in beer (Bamforth & Lentini, 2002). The Maillard reaction is responsible for producing bready, malty and burnt flavour notes in beer (Vanderhaegen et al., 2006). Figure 6 explains the production of melanoidins.

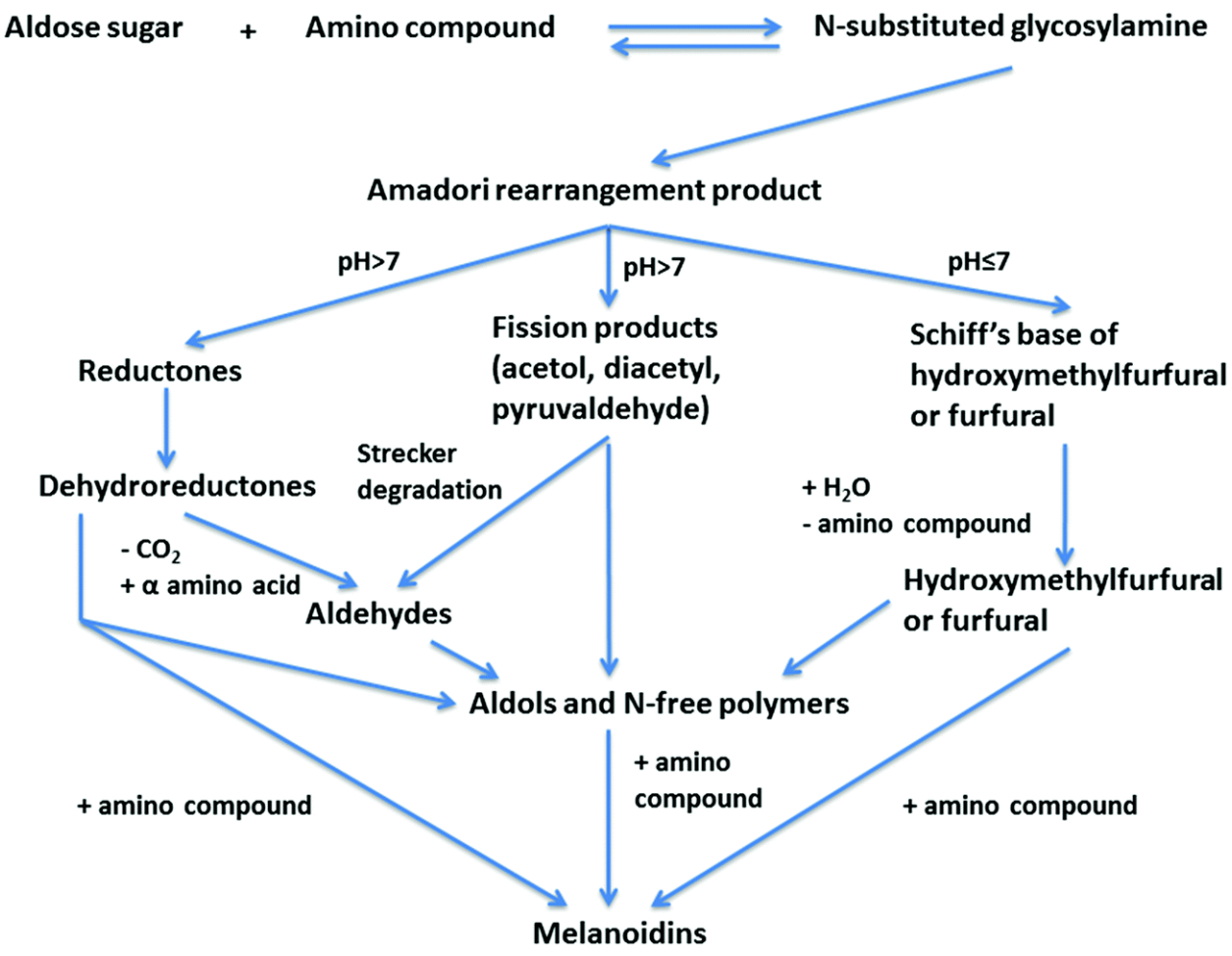

Figure 6. Maillard Reaction resulting in formation of Melanoidins (Zhang, Tao, Wang, Chen, & Wang, 2015)

2.4 VOCs Detection and Quantification

Gas chromatography (GC) is a widely-used technique for the analysis of VOCs in beer. However, the VOCs must be extracted from the beer matrix prior to the analysis. Static headspace, solid phase extraction, ultrasound extraction, liquid-liquid extraction, simultaneous-distillation extraction and solvent extraction are frequently used techniques for the extraction of VOCs from the beer (Rodrigues et al., 2008). The headspace gas chromatographic method helps

in the detection of individual VOCs in the beer. In this technique, the beer sample is heated in a closed container at 55^0C for 15 min and then the sample is injected from the head space to the GC system, coupled with, a flame-ionization detector (ASBC, 2010). Most of these techniques have disadvantages, such as laborious sample preparation, high cost, use of organic solvents and requiring multiple handling steps.

The most extensively-used extraction techniques used in industry for analysis of VOCs and semi-volatiles in alcoholic beverages are solid phase microextraction and headspace analysis (Rodrigues et al., 2008). However, the purge and trap method has several advantages over other methods, as it does not require extensive labour and also gives better reproducibility ("Bulletin 916 Purge-and-Trap System Guide," 1997). The purge and trap technique is extensively used for the analysis of VOCs in environmental samples (RestekTechnichal Guide, 2003). However there are certain limitations of purge and trap method such as oversaturation of adsorbent, breakthrough of desorption tubes need to be monitored, purge gas volume and dry purge need to be monitored.

2.4.1 Analytical method

In this section the purge and trap method for extraction of VOCs from liquid or solid matrices will be described. In addition, thermal desorption, which is required for introducing the VOCs into the GC, will be outlined, followed by a description of GC.

2.4.1.1 Purge and trap method

The purge and trap method has been used to extract VOCs from liquid or solid matrices prior to their introduction to a GC for separation and identification. The purge and trap method is used as a standard protocol for analysing environmental samples, with the United States

Environmental Protection Agency (USEPA), United States Department of Energy (USDOE) and United States Department of Defence (USDOD), using applications based on purge and trap methodology (Lewis & Sensel, 2004). The typical components used in the purge and trap method are shown in Figure 7.

In the purge and trap method, samples containing VOCs are first introduced to the purge and trap vessel. During the sparging process, inert gas, usually U-5 (Ultra-pure) helium is passed through at a typical flow rate of 30-50 mL/min for between 10-20 min depending upon the recovery of the system. The purge gas passes through the solid or liquid matrix and removes the volatile onto the adsorbent trap (Edmund et al., 2004). This is followed by a dry purge process. Here, the flow rate of the U-5 helium is typically 80-100 mL/min for 10 mins, depending upon the VOCs of interest. During this stage, the VOCs are trapped on a sorbent. The purpose of the dry purge is to remove excess moisture from the sorbent tube. The purge gas is directed to the trap tube and only the sorbent tubes containing hydrophobic sorbent can be dry-purged (Hollis & Prest, 2012).

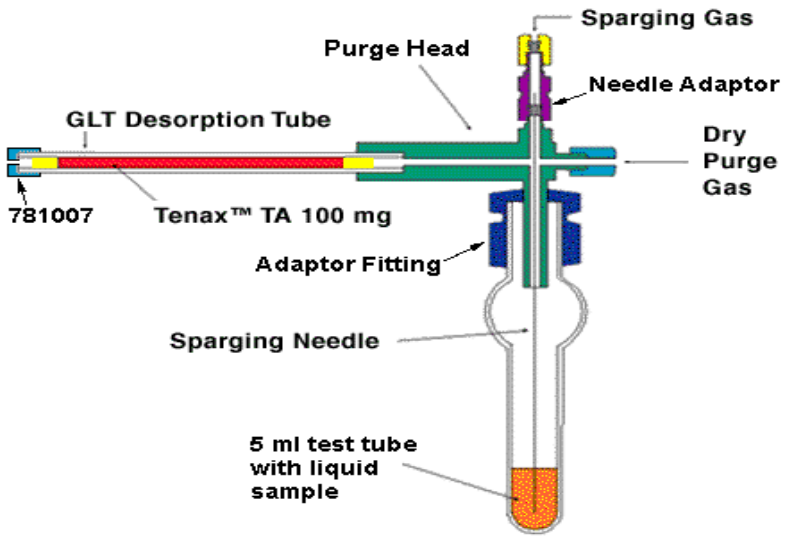

Figure 7 Purge and Trap components (www.Sisweb.com /reference/applnote/app-30.htm)

In the purge and trap system, two valves are needed for the inert gas during sparing and dry purge stages, as during the dry purge, the inert gas is directed to the sorbent tube. Within the purge vessel, the type of sparge is an important consideration. Depending on the application, frit spargers and needle spargers can be used. Frit spargers produce bubbles and help in increasing the purge efficiency. They are used for water samples whereas the fritless spargers are used in samples with high particulate matter such as waste water. Needle spargers are usually used for solid samples (Hollis & Prest, 2012).

The effectiveness of the purge and trap method is dependent on the properties of the sorbent material used to trap VOCs from the sample matrix and subsequently release them during the desorption stage in the thermal desorption unit. The choice of adsorbent is dependent on the volatility of the compounds; compounds with higher volatility are trapped on stronger adsorbents and vice versa. Tenax trapping agent (TA), charcoal, silica gel, carbopack, coconut charcoal and carboxen-1000 are common types of adsorbent used in the environmental and food industries. Tenax TA is an excellent sorbent for non-polar compounds and is hydrophobic in nature. Tenax is appropriate for aliphatic hydrocarbons, aromatic compounds, esters, ketones and some higher boiling point compounds. Activated carbon, Carboxen and Chromosorb® 106 are used as sorbents for very volatile compounds (Plant & Keen, 2007).

2.4.1.2 Thermal desorption

Thermal desorption utilizes heat and a flow of carrier gas to remove volatiles from a sorbent matrix. It is a sample introduction technology for GC. Thermal desorption offers various advantages over solvent extraction such as higher recoveries, increased sensitivity, and is compatible with solid liquid and gaseous samples (Markes, 2011). The key factor in thermal desorption is cold-trap, focussing where the analytes released from the sorbent tubes are trapped

on the cold-trap. The focussing step improves the peak shape and compound resolution. The cold-trap is then heated rapidly to inject analytes to the capillary GC column (Plant and Keen, 2007).

A Markes Unity-2 system was used for this study and the system was equipped with a sophisticated Markes software program to run desorption. Figure 8 depicts the interior of a Markes Unity-2 TD unit.

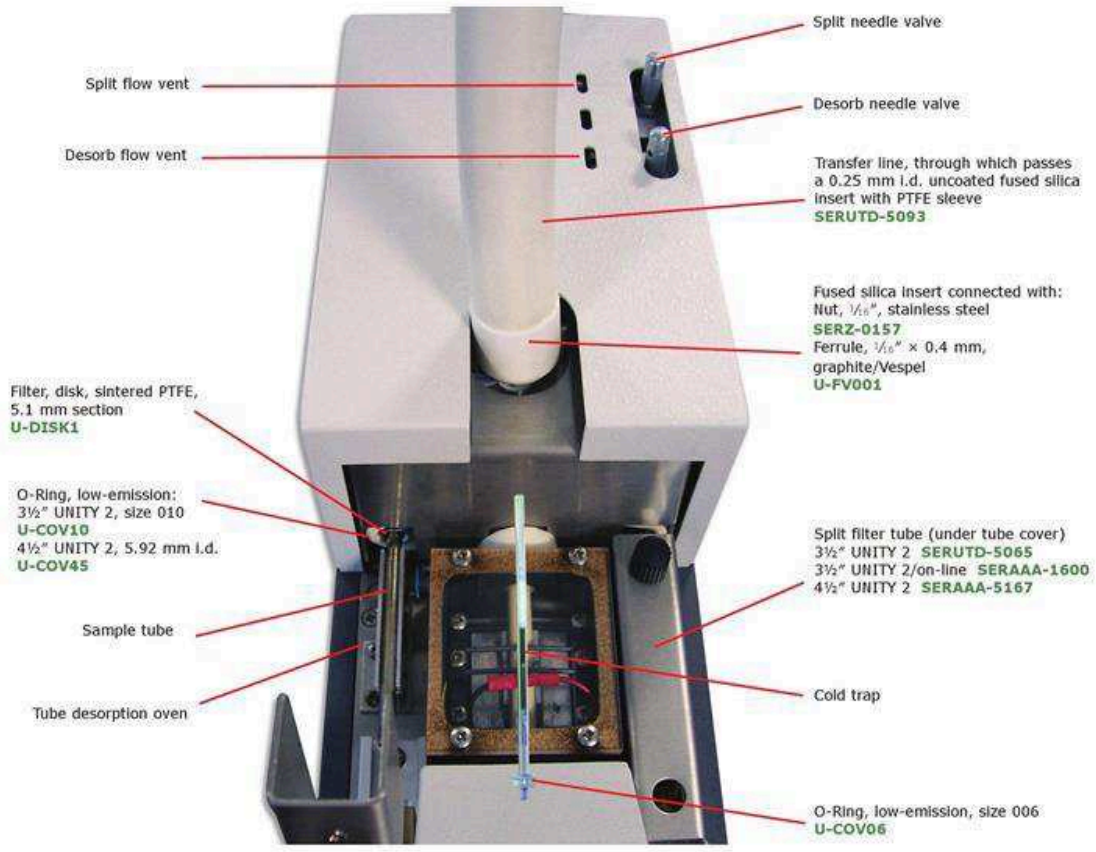

Figure 8. Interior of Markes Unity-2 Thermal desorption unit (Markes Manual, TD unit, 2011)

Currently TD coupled with GC-MS is used for air monitoring application, food, flavour, material emissions and fragrance (Markes Application Guide, 2011).

2.4.1.3 Gas chromatography (GC)

GC has been used for the separation of volatile compounds in brewing industry for more than 20 years now. In 1955 the first gas-liquid chromatography appeared on the market and growth of this technique has been phenomenal, especially in the food industry. Vicinal diketones, sulphur compounds, esters, higher alcohol and any volatile compound in beer can be identified using GC (Bamforth, 2014). These compounds are important for the organoleptic property of beer. Certain breweries identify and quantify VDKs on regular basis in their brew i.e. SABMiller, Tsingtao, Molson Coors etc.

2.4.1.3.1 Principles of gas chromatography

Gas chromatography is based on separation of analyte between the gaseous mobile phase and a liquid stationary phase on the surface of an inert solid packing on the walls of column (Skoog, 2014).

2.4.1.3.2 Components of gas chromatography instrument

The basic components of gas chromatography instrument are explained below:

- Carrier gas: The carrier gas in the gas chromatography is the mobile phase and must be chemically inert. Helium is most commonly used mobile phase although hydrogen, argon and nitrogen are also used. These gases are available in pressurised tanks and gauges are required to control the flow rate of the gas (Skoog, 2014).
- Sample injection system: In order to introduce the sample in to the GC instrument a sample injection port is required. The suitable sample size is introduced as a stream of vapour for high column efficiency. There are various injectors such as split/split less injector, programmable temperature vaporising injector, and programmable temperature

vaporising injector for backflush applications are available. Newer gas chromatographs use auto injectors and auto samplers for most reproducible sample injection. Injection volumes can vary from 0.1 µL with a 10 µL syringe to a 200 µL syringe (Skoog, 2014)

- Column and column oven: There are two types of columns available: such as packed and capillary. The column requires temperature programming where the column temperature increases automatically or in steps for separation. The analytical column is installed in the oven to perform sample separation into its components. Analytes with limited volatility can sometimes be determined by forming derivatives that are more volatile (Skoog, 2014).
- Detector: The separated sample compounds are detected with the help of detectors. Various detectors can be coupled with GC such as flame ionisation detector (FID), electron capture detector (ECD), nitrogen phosphorus detector (NPD), thermal conductivity detector and mass spectrometers (MS).

2.4.1.4 Mass spectrometry (MS)

The mass spectrometer produces ions that are separated on the basis of their mass/ charge values to be finally detected. The mass spectrum is a plot of relative abundance of ions produced in the MS chamber as a function of their m/z ratio (Skoog, 2014). The MS detectors used in this study consist of single quadrapole mass analyzer with an electron impact ion source.

2.4.1.4.1 Electron impact source

The most widely used source is an electron impact source; the molecules in this source are bombarded with a high-energy beam of electrons that produces positive, neutral and negative ions. Due to electrostatic repulsion the positive ions are directed towards the analyzer.

The electron beam results in many fragments that are useful in identifying the molecular species entering the mass spectrometer (Skoog, 2014).

2.4.1.4.2 Single quadrapole detection

Quadrapole analysers are the mass filters that allow only selected m/z ratio ions to pass. The system consists of cylindrical rods that are placed parallel in a radial array. Opposite rods are charged either by direct current or radio frequency. A stable path for the ions of certain m/z ratio is created with the proper adjustments of voltage so that they can pass through the analyzer to the transducer. The mass spectrum is obtained by scanning the voltages applied to the rods (Skoog, 2014)

2.4.1.4.3 Transducer

Electron multiplier is the most common transducer available for mass spectrometry. The secondary electrons are emitted when the ions strike the Cu-Be cathode and are attracted to the dynode which is kept at a higher voltage. The device can multiply signal strength by a factor of up to 10^7 (Skoog, 2014)

Chapter 3 Materials and Methods

3.1 Sample Collection and Storage

The Propeller Brewing Company (Dartmouth, Nova Scotia, Canada) provided samples of beer for analysis. Two brands of beer, India Pale ale (IPA) and pilsner were chosen for this study. Packaged samples were collected directly from the brewery after 2 hours of packaging and analysed as fresh samples. The beer samples were also subjected to heated storage conditions at different temperatures to simulate different storage periods (accelerated ageing). Samples were stored at 60^0C for a day (to simulate 21 days at room temperature storage), 40^0C for three weeks (to simulate 150 days of storage at room temperature) (Bamforth & Lentini, 2002). An oven (Despatch, MN, US) was used for the accelerated storage conditions.

3.2 Experimental supplies

3.2.1 Reagents

The following chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) with highest purity available. Gas chromatography grade chemicals included: furfural (≥ 98%), ethyl nicotinate (≥ 99.0%), (E)-2-nonenal (≥ 97%), ethyl lactate (≥98%), isoamyl acetate (≥ 97%), 2-methyl-1-propanol (≥ 99.8%), dimethyl trisulphide (≥ 98%), 2,3 butanedione (≥99.0%), limonene (≥ 99%), (+)(-) α pinene (98.5%), 2,3 pentanedione and purge and trap grade methanol (≥ 99.8%) purchased from Perkin-Elmer (Guelph, ON, Canada)

3.2.2 Thermal Desorption Tubes

Stainless steel 3.5” x ¼”, sorbent tubes, prepacked with Tenax TA (Perkin-Elmer, Guelph, ON, Canada) were used to capture (by the purge & trap method) VOCs of interest from the beer samples investigated. Tenax TA is a porous polymer with a 60/80 mesh and particle size of 0.18-0.25 mm. Tenax TA has the ability to adsorb non polar VOCs between n-C7 to C26

(Plant& Keen, 2007). Figure 9 depicts Tenax TA thermal desorption tubes (TDT) with brass Swagelok end cap fittings and two CapLok tools.

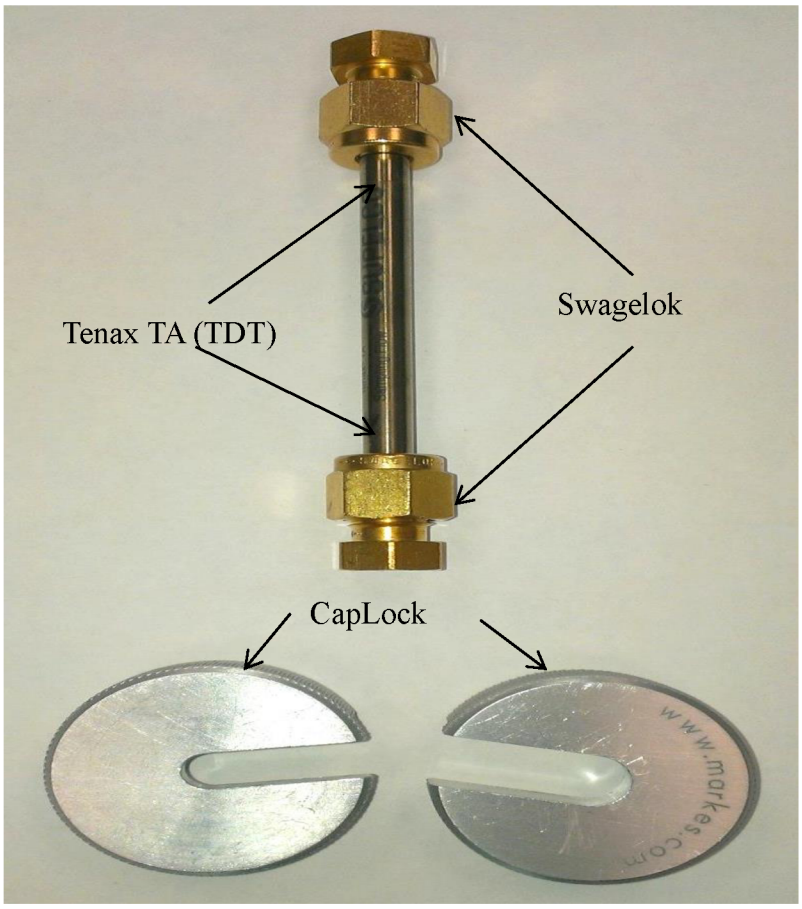

Figure 9 Tenax TA Desorption Tubes with Swagelok Fittings and CapLock Tools

Tenax TA tubes were conditioned in a Markes TC-20 (Llantrisant, UK) multi-tube conditioner and dry-purge unit before use. Figure 10 depicts a Markes TC-20 multi-tube conditioner, where twenty TDTs can be conditioned at a time. Conditioning removes any sample artefacts or contamination prior to re-use. The TDTs can be reused up to approximately 300 times. The temperature for conditioning Tenax TA was set to 320^{0}C for 4 hours and used U-5, scrubbed, nitrogen gas. When the tubes were not in use they were sealed with brass Swagelok caps

containing polytetrafluoroethylene (PTFE) ferrules (Markes, Instruction Manual, 2009). As shown in Figure 9 a CapLock tool (Markes, Llantrisant, UK) was used to tighten the caps (Markes, Instruction Manual, 2009). The tubes after sampling were capped and stored in the refrigerator at 4^0C. The refrigerated tubes before analysis were removed from the refrigerator and kept at laboratory temperature in order to prevent humidity from the air condensing inside the cold tube.

Figure 10 Markes TC-20 multi-tube conditioner and dry-purge Unit (Figure retrieved from http://www.markes.com/Products/Instrumentation/TC-20.aspx)

3.3 Sample Analysis

3.3.1 Purge and trap method for extraction of volatile organic compounds

The equipment used for the purge and trap (P&T) was SIS Purge and Trap equipment (Scientific instrument services, NJ, USA). The purge and trap technique has been used extensively in the literature to extract VOCs from solid or liquid matrixes prior to introduction in gas chromatography for separation and identification (Technical Guide, Restek, 2003). During the purge mode, the VOCs were extracted from the sample matrix using U-5 helium (Air liquide Canada Inc, Dartmouth, NS, Canada) that was first passed through a triple gas scrubber

(Supelco, Model# 27600U, Sigma Aldrich, ON, Canada) to remove organic contaminants, moisture and oxygen. Beer samples were diluted in a 4:1 ratio before purging to help reduce foam formation and also to prevent over-loading of the sorbent with sample VOCs and other contaminants. A volume of 40 mL of the diluted beer was purged at a flow rate of 25 mL/min for 10 min to allow the capture of volatiles onto the preconditioned Tenax TA TDTs. After sampling, the TDT containing the sample VOC analytes was subjected to a dry purge for 10 mins at a flow rate of 25 mL/min. In order to prevent contamination, the purge and trap equipment was cleaned first with methanol and three times with nanopure water before reuse. Figure 11 shows the purge and trap apparatus used to sample VOCs from the beer samples.

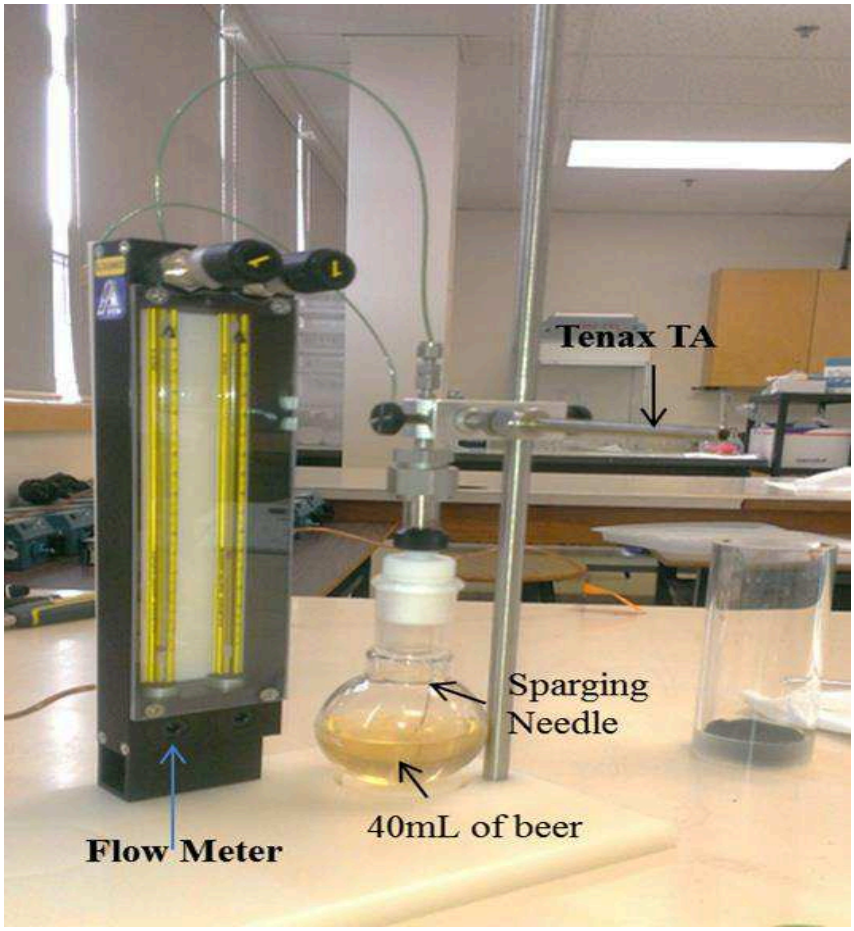

Figure 11 Purge and Trap apparatus used for extracting VOCs from the beer

3.3.2 Markes Unity 2 Thermal Desorption Unit (TDU)

The thermal desorption technique has been extensively used for environmental pollution, material emission and flavour fragrance profiling. This technique permits the transfer of VOCs and semi-VOCs from different sample matrix, directly into GC-MS equipment. A three step thermal desorption sequence was used, consisting of the following steps: primary sample desorption, followed by sample trapping and focussing, and finally trap thermal desorption. The first step in the three step thermal desorption process was pre-desorption for the removal of analytes and moisture from the TDTs. Here, each tube was pre-purged before the heat was

applied to remove air, to prevent analyte and sorbent oxidation which would result in artifacts and compromise data quality. The prepurge was set to 2 min prior to undergoing desorption at 290^0C for 5 min. The second step was to heat the TDT in order to free the analytes from the sample TDT tube into the pure helium stream to be collected onto a focusing cold trap (quartz tube containing a 2 mm diameter x 60 mm long, environmental™ sorbent bed). The cold trap was set at 25^0C and was heated rapidly to 290^0C for 5 min and the analytes were injected as a stream of vapor to the GC via a heated transfer line (175^0C). A zero air source (Sabio, model 2020, Texas, and USA) provided pressurised clean air to remove water or any residual air from the chamber surrounding the cold trap prior to trap fitting. The cooling and heating of the cold trap was regulated by a 2 stage peltier cell. Once the sample analytes were collected and focused on the cold trap, the trap oven was heated up rapidly at a rate of 20^0C/sec to 290^0C and held at this temperature for 5 mins. This rapid thermal desorption allowed the liberation of the trapped VOCs into a stream of pure helium for transfer directly into the GC via the heated transfer line. The transfer line temperature was 175^0C.

To prevent the over-flooding of the column and saturation of the detector, higher split flows were selected. The column flow was set to 1 mL/min, desorb flow was set to 50 mL/min, split flow during tube desorb was set to 50 mL/min and split flow during trap desorb was set to 0. The resulting split flow ratios used were 2:0:1 for inlet, 1:0:1 for outlet and 2:0:1 for total. Following the VOC sample transfer from the thermal desorption instrument, the samples were separated using capillary GC. The column flow used was 25.0 mL/min. Table 6 depicts the parameters used for the Markes unity-2 TD unit.

Table 6 Markes unity 2 series thermal desorber unit parameters

Steps	Time(min)	Temp(^{o}C)	Flow (mL/min)
Leak Check	1		
Pre-Purge	2		
Primary Desorption	5	290	25
Pre-Trap Fire Purge	2	25	25
SecondaryTrapDesorption	5	290	25

3.3.3 Gas chromatography set up

A Thermo Trace 1300 gas chromatograph (GC) (Thermo Fisher Scientific, Austin, Texas, U.S) was used for the separation of analytes of interest prior to identification and quantification by mass spectrometry (MS). A Restek-Rxi-1ms cross bond® 100% dimethyl polysiloxane, 1.0 µm df, 60 m x 0.25 mm ID non-polar capillary column was used for chromatographic separation (Restek, Column Brochure, 2014 cat# 13356). Ultrapure helium was used as a carrier gas at a flow rate of 1.1 mL/min. Three separate helium gas scrubber cartridges mounted in series were used to remove moisture, organics and oxygen (Thermo Fisher Scientific, Austin, Texas, US).

Table 7. GC oven program used for the separation of the VOCs of interest prior to MS detection.

Sequence	Rate (^{0}C/min)	Temp (^{0}C)	Hold Time (min)
0		35	5
1	7	220	1.70

The GC oven temperatures were set up as follows: an initial temperature of 35^{0}C was held for 5 min followed by temperature ramp of 7^{0}C/min to 220^{0}C which was held for 1.70 min. The maximum temperature was set to 350.0 ^{0}C, whereas the prep-run timeout was set to 999.00 min

and equilibration time was set to 0.5 min. The total run time was 33.13 min. Table 7 indicates the GC-oven program for Trace 1300.

3.3.4 Mass spectrometry

The GC was coupled to a Thermo Scientific ISQ Single Quadrapole MS (Thermo Fisher Scientific, Austin, Texas, US). Detection was achieved using electron impact (EI) with a mass to charge (m/z) scan range between 35 – 259.98 amu. Detection of VOC species was achieved, m/z 35-260 amu and were recorded in full scan mode on Thermo ISQ MS connected to GC operating at 70 eV. The temperature of GC-MS transfer line and ion source was set to 300^{0}C for the current analysis. Figure 12 shows the entire thermal desorption-gas chromatography-mass spectrometer instrument used for the analysis of the VOCs associated with off-flavours and off-odours in the beer samples.

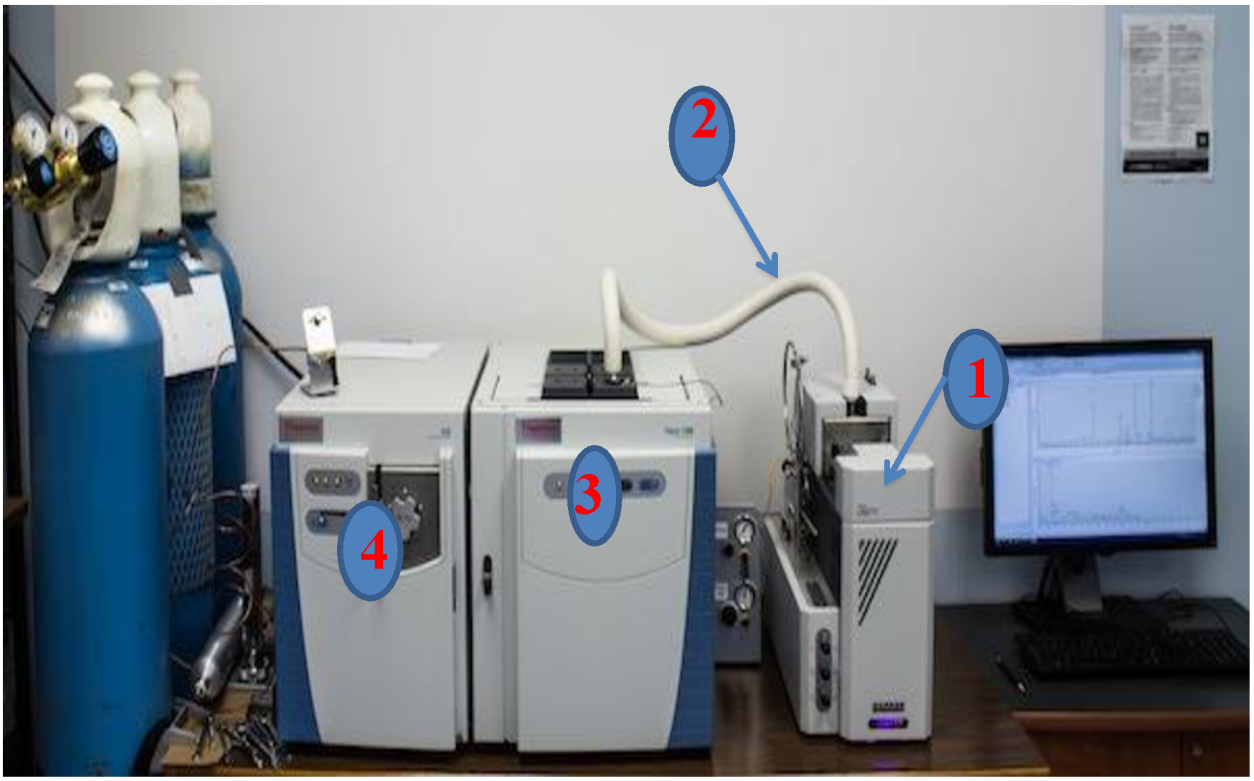

Figure 12 1-Markes Unity 2 Thermal Desorption Unit-, 2- Transfer Line Thermo, 3- 1300 Trace GC, 4-Thermo ISQ MS system used for determination of VOCs in beer

3.4 Quantification

3.4.1 Preparation of working standards

As with all the instrumental methods of analysis, it was necessary to calibrate the instrument. On the day of analysis, working standards were prepared fresh every morning. The primary stock calibration standard comprised of a mixture of furfural, ethyl nicotinate, ethyl lactate, isoamyl acetate, 2-methyl-1-propanol, dimethyl trisulphide, 2,3 butanedione, limonene, (+)(-) α pinene, 2,3 pentanedione, and (E)-2 nonenal, diluted in purge and trap grade methanol at a concentration of 5000 ng/µL. The final concentration of 10ng/µL of VOC-mix was used- serial dilutions of 50 ng/µL, 100 ng/µL, 200 ng/µL, 300 ng/µL and 500 ng/µL were prepared from the primary stock calibration standard solution.

Cleaned glass syringes (Hamilton, Nevada, US cat# 14815256 and 14815250) of 5 µL and 100 µL volumes were used to load the standard onto the Tenax TA tubes. The syringes were cleaned three times with purge and trap grade methanol in order to prevent contamination. The microliter syringes had a stainless steel plunger that was hand-fitted to the glass barrel and designed for liquid or gas sampling. This design of the syringe serves to prevent vaporisation of VOCs in the glass. A Markes International calibration solution loading rig (CSLR) is shown in Figure 13 and was used to load a known amount of mixed VOC standard onto TDTs. Ultra-pure helium was used as a carrier gas for the CSLR at a flow rate of 25 mL/min. The CSLR helium carrier gas flow was adjusted manually using needle valve. The flow rate was measured at the exit of the tube using a digital flow meter (Perkin Elmer, PE-1000). After the calibration standard was injected into the CSLR, the TDT was left in the stream of helium gas for 5 min to purge the calibration mixture methanol matrix from the tube (Dohoo, Read Guernsey, Gibson, & VanLeeuwen, 2013). Tenax TA TDT containing the calibration standard VOC mixture were

analysed on the same day as sample analysis. The mass of analytes in the sample was calculated internally using the calibration curve generated for each VOC species. The calibration curve intensities were checked every week by running a known volume of standard tubes.

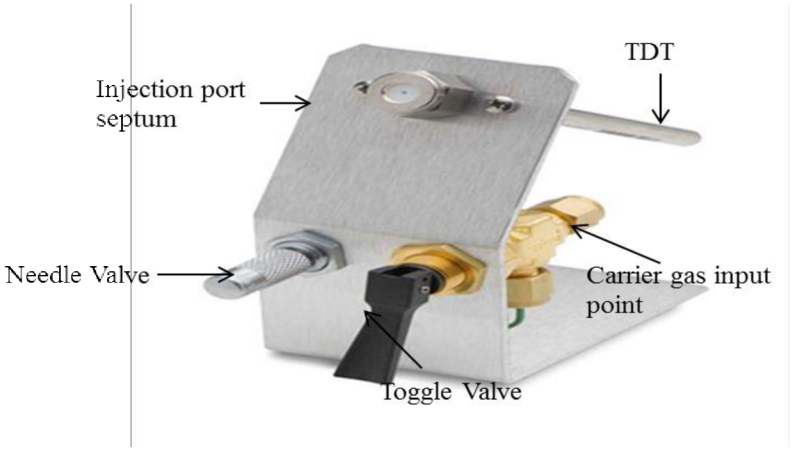

Figure 13. Calibration solution loading rig (http://www.markes.com/Resources/Application-notes/Technical-support.aspx).

3.4.2 Data Processing

Chromatograms and mass spectra were processed using Thermo Fisher Scientific, Xcalibur software, version 2.1. The compounds were identified based on their fragmentation spectra compared with the built-in National Institute of Standard and Technology (NIST/US EPA), US National Institute of Health (NIH) Mass Spectral Library (Xcalibur, Instruction Manual, 2010). The data processing method was developed by first confirming chromatographic peak using the NIST library followed by 5-point calibration. Figure 14 shows the chromatogram of 100 ng/µL of the standard VOC mixture with the retention time of the compounds present in the VOC mixture. The samples were quantified internally using the Excalibur software, where they were matched to the spectra in the library and direct matching (SI) and reverse search matching (RSI) factors of greater than 800 were accepted. The probability match above 25% was considered as a

good acceptance guide (Xcalibur, Quantitative Analysis Guide, 2012). Table 8 explains the SI/RSI and matching probability % of the VOCs of interest.

Table 8. The SI and RSI ratio and probability of matching of compounds with the National Institute of Standard and Technology library

Compounds	SI	RSI	Probability (%)
2,3- Butanedione	907	956	74.92
2,3 Pentanedione	887	901	77.74
Furfural	960	961	86.63
Limonene	946	949	50.31
pinene	942	943	29.35
Ethyl Lactate	919	933	54.70
Ethyl Nicotinate	914	928	89.32
Isoamyl acetate	944	946	86.21
(E)-2-nonenal	921	925	39.35
2-methyl-1-propanol	841	886	77.74
DMTS	918	923	80.93

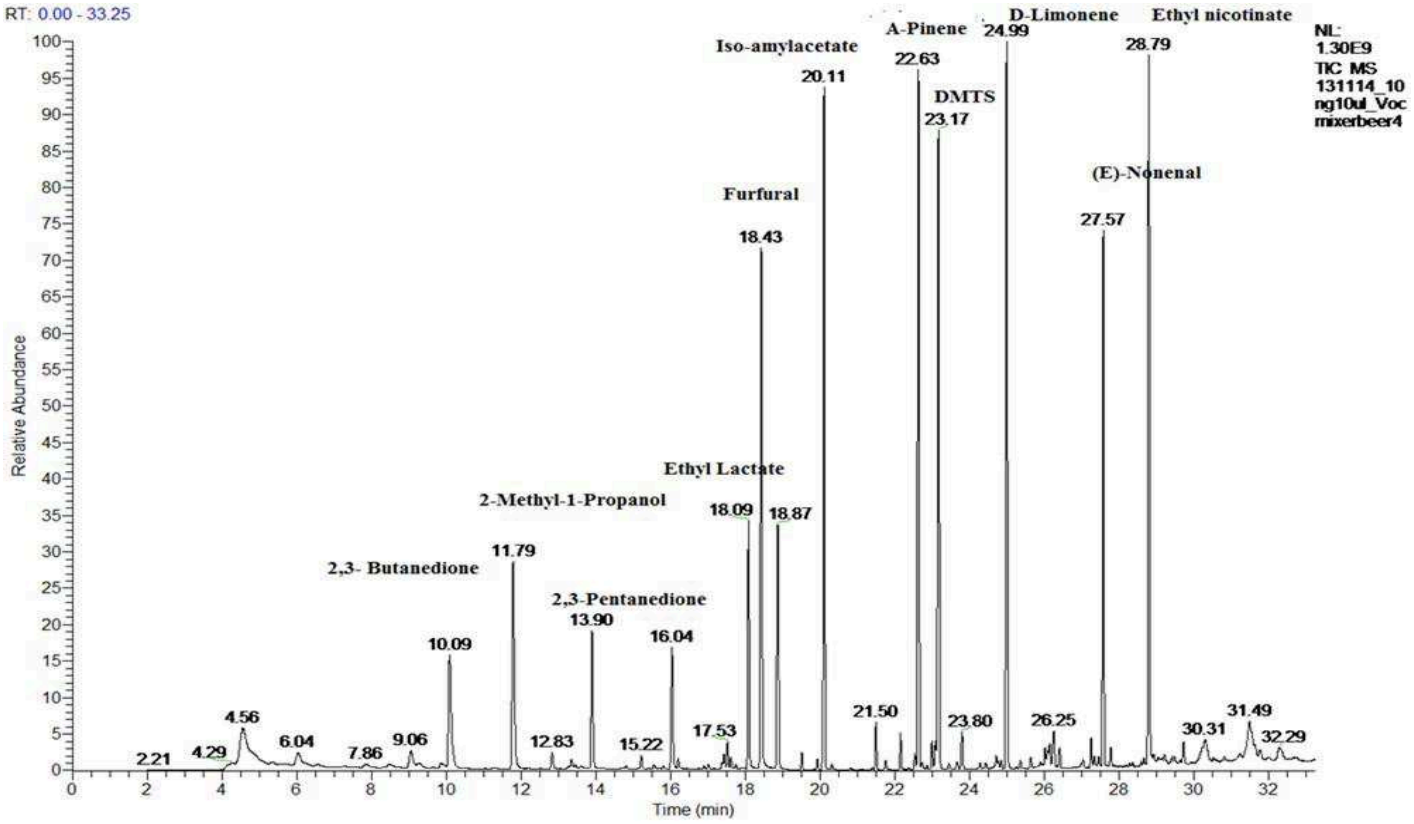

Figure 14. Chromatogram of 100ng/µL of standard VOC mix with the retention time of the compounds present in the VOC mix

3.4.3 Data handling and statistical analysis

The results of all the above data processing were exported to Microsoft Excel. Statistical analysis of the VOCs in the two beers was performed using Minitab 17. A comparison between the VOCs in the two different types of beer, and at different storage periods was conducted using Mann-Whitney U test. The graphs were generated using Microsoft Excel.

3.4.4 Detection Limits

The limit of detection (LOD) was determined by measuring seven replicates of the lowest detectable mixed VOC standard (100ng/µL). For each of the eleven VOC analytes, the n-1 value obtained from the t-distribution (one-tail, 0.01 level of confidence) was multiplied by the standard deviation of the seven replicates to obtain the LOD. This follows the USEPA TO-17 method for the determination of VOCs by active sampling onto packed bed tubes (McClenny & Holdren, 1999)

Chapter 4. Results and Discussion

To obtain better insight into the presence of chemical compounds associated with aged beer flavour, TD-GC-MS analysis was used for the detection and quantification of eleven VOCs linked to ageing in locally brewed IPA and pilsner beers. In this section the VOCs detection limits from the GC-MS in this study are presented. Then a summary of the data is presented in tabular form for the three different conditions (i.e. fresh and forced aging at 60°C for 1 day and 40° C for 21 days) for the two beer types. Overall trends are then discussed, followed by a more detailed analysis of VOC groups where Mann Whitney non-parametric statistical comparison tests are included. The statistical analysis results are summarized in Appendix C, with the parameters used to determine suitability of this statistical test.

4.1 VOCs Detection and Calibration

The practical method for determining the limit of detection (LOD) is to analyse seven samples of concentration near the expected limit of detection. The standard deviation is determined and multiplied by the one- sided t distribution value at a 99% level of confidence (McClenny & Holdren, 1999)

Table 9 shows the detection limits for all 11 VOCs in the standards mixture. The t test value for 99% level of confidence was 3.14 using T table (USEPA, 1999). Detection of 2, 3-butanedione, 2, 3-pentanedione, furfural, ethyl lactate, 2-methyl-1-propanal was achieved down to 500 ng/µL, whereas for limonene, (+) (-) α pinene, ethyl nicotinate, (E)-2 nonenal, DMTS, Iso amyl acetate was achieved down to 700 ng/µL. The signal response was linear for all the VOCs up to

500 ng/µL and 700 ng/µL. A calibration curve (Appendix A) was performed with aqueous standards containing the following concentrations for each of the VOCs: 50, 100, 200, 300, 500, and 700 ng/µL.

Table 9 Detection limits for 11 VOCs in the standard mixture

VOC mix (100ng)	Standard deviation (ng)	Limit of Detection (LOD) (ng)
2,3-Butanedione	43.1	135.4
2,3-Pentanedione	69.6	218.5
Ethyl Lactate	24.10	75.8
Ethyl Nicotinate	28.3	88.9
Furfural	23.07	72.4
α-Pinene	15.30	48.02
Limonene	18.24	57.3
DMTS	38.2	119.9
(E)-nonenal	21.07	66.2
Iso amyl acetate	15.97	50.2
2-Methyl-1propanal	32.7	102.6

4.2 Summary of results

The following section will discuss the results and the samples that were detected above the LOD for eleven VOCs by GC-MS under fresh and aging conditions. Table 10 and Table 11 shows the number of samples above LOD for IPA and pilsner. Table 12-17 summarize the data for the mean, standard deviation (SD) and number of samples with VOCs above the LOD, for IPA and pilsner samples under fresh and aged conditions.

Table 10 Number of IPA samples that were above the limit of detection (LOD) for VOCs

Compounds	**Number of samples above LOD in fresh IPA (14 samples were tested)**	**Number of samples above LOD in IPA at 60°C/1 day (17 samples were tested)**	**Number of samples above LOD in IPA at 40°C/21 day (7 samples were tested)**
2,3-Butanedione	3	2	2
2,3Pentanedione	4	5	2
α-Pinene	8	12	7
Limonene	6	13	7
Furfural	2	10	5
DMTS	7	7	3
(E)-2nonenal	11	10	5
IAA	12	13	6
Ethyl lactate	0	2	3
Ethyl nicotinate	0	3	2
2-methyl-1-propanol	13	17	6

Table 11 Number of pilsner samples that were above the limit of detection (LOD) for the VOCs, as detected by GC-MS

Compounds	**Number of samples above LOD in fresh pilsner (7 samples were tested)**	**Number of samples above LOD in pilsner at 60^0C/1 day (4 samples were tested)**	**Number of samples above LOD in pilsner at 40^0C/21days (6 samples were tested)**
2,3-Butanedione	1	1	2
2,3-pentanedione	0	1	1
α-Pinene	2	4	4
Limonene	3	1	3
Furfural	3	3	4
DMTS	0	1	2
(E)-2nonenal	3	1	6
IAA	7	4	6
Ethyl lactate	1	1	1
Ethyl nicotinate	1	1	2
2-methyl-1-propanol	7	3	5

Table 12. Mean, standard deviation and number of samples above LOD for fresh IPA

Compounds	Mean (ng/L)	Standard deviation (ng/L)	Samples above LOD
2,3-Butanedione	6.2	(+/-) 1.066	3
2,3Pentanedione	29.6	(+/-) 23.7	4
α-Pinene	13	(+/-) 17.63	8
Limonene	3.6	(+/-) 0.632	6
Furfural	3.465	(+-) 0.72	2
DMTS	16.05	(+/-) 26.42	7
(E)-2nonenal	7.28	(+/-) 7.28	11
IAA	31.82	(+/-) 33.9	12
Ethyl lactate	Not detected	*	0
Ethyl nicotinate	Not detected	*	0
2-methyl-1-propanol	38.2ng/L	(+/-) 39.6	13

Table 13. Mean, standard deviation and number of samples above LOD for IPA stored at 60°C/1 day

Compounds	Mean (ng/L)	Standard deviation (ng/L)	Samples above LOD
2,3-Butanedione	8.7	(+/-) 1.75	2
2,3Pentanedione	32.8	(+/-) 43.2	5
α-Pinene	25.5	(+/-) 57.8	12
Limonene	4.6	(+/-) 2.781	13
Furfural	6.28	(+/-)5.89	10
DMTS	24.8	(+/-)31.8	7
(E)-2nonenal	6.66	(+/-)6.36	10
IAA	94.7	(+/-)71.3	13
Ethyl lactate	4.6	(+/-)0.032	2
Ethyl nicotinate	13.02	(+/-)5.17	3
2-methyl-1-propanol	139.9	(+/-)144.9	17

Table 14. Mean, standard deviation and number of samples above LOD for IPA stored at 40°C/21 days

Compounds	Mean (ng/L)	Standard deviation (ng/L)	Samples above LOD
2,3-Butanedione	27.5	(+/-) 12.36	2
2,3Pentanedione	159.9	(+/-) 211	2
α-Pinene	36.4	(+/-) 53	7
Limonene	6.0	(+/-) 3.89	7
Furfural	18.6	(+/-) 12.01	5
DMTS	65.6	(+/-) 38.9	3
(E)-2nonenal	30.33	(+/-) 32.6	5
IAA	26.51	(+/-) 18.37	6
Ethyl lactate	47.2	(+/-) 73.8	3
Ethyl nicotinate	20	(+/-) 18.2	2
2-methyl-1-propanol	210.5	(+/-) 145	6

Table 15. Mean, standard deviation and number of samples above LOD for fresh pilsner

Compounds	Mean (ng/L)	Standard deviation (ng/L)	Samples above LOD
2,3-Butanedione	9	*	1
2,3Pentanedione	Not found	*	0
α-Pinene	7	(+/-) 0.113	2
Limonene	1.8	(+/-) 0.923	3
Furfural	4.282	(+/-) 0.287	3
DMTS	Not found	*	0
(E)-2nonenal	3.0	(+/-) 0.2	3
IAA	22.36	(+/-) 12.01	7
Ethyl lactate	Not found		1
Ethyl nicotinate	3.1	*	1
2-methyl-1-propanol	10	(+/-) 7.25	7

Table 16. Mean, standard deviation and number of samples above LOD for pilsner stored at 60^0C/1 day

Compounds	Mean (ng/L)	Standard deviation (ng/L)	Samples above LOD
2,3-Butanedione	15.4	*	1
2,3Pentanedione	12	*	1
α-Pinene	5.26	(+/-) 2.84	4
Limonene	3.0	*	1
Furfural	4.4	(+/-) 1.637	3
DMTS	3.6	*	1
(E)-2nonenal	3.0	*	1
IAA	74.5	(+/-) 123.8	4
Ethyl lactate	10.7	*	1
Ethyl nicotinate	2.28	*	1
2-methyl-1-propanol	214	(+/-) 344	3

Table 17. Mean, standard deviation and number of samples above LOD for pilsner stored at 40^0C/21 days

Compounds	Mean (ng/L)	Standard deviation (ng/L)	Samples above LOD
2,3-Butanedione	142	(+/-) 193	2
2,3Pentanedione	8	*	1
α-Pinene	10.5	(+/-)7.07	4
Limonene	4.2	(+/-)2.31	3
Furfural	12.96	(+-/)10.62	4
DMTS	8.25	(+/-)1.019	2
(E)-2nonenal	3.5	(+/-) 1.6	6
IAA	117.9	(+/-) 202.4	6
Ethyl lactate	49.66	*	1
Ethyl nicotinate	3.1	(+/-) 0.0203	2
2-methyl-1-propanol	311	(+-) 542	5

4.3 Quantitative analysis of VOCs in beer samples

This section includes graphical and statistical analysis of eleven VOCs in pilsner and IPA. The first section explains the overall analysis of VOCs; the second section will analyse the results in more detail by grouping the VOCs into six categories of flavour compounds that are found in beer.

4.3.1 Overall analysis of VOCs in beer

The mean of concentration of eleven VOC compounds (2, 3-butanedione, 2, 3-pentanedione, ethyl lactate, ethyl nicotinate, nonenal, furfural, iso amyl acetate (IAA), 2-methyl-1-propanal, α-pinene and limonene) in IPA and pilsner are shown graphically in Figure 15-Figure 16 . From analysing the plots there are some trends are apparent, particularly the increase in overall concentrations of VOCs after aging in comparison to the fresh samples, as exhibited by both beers. In addition, IAA is observed in both IPA and pilsner beers, as is expected as it imparts a fruity flavour to beer and is generally present in all fresh beer. Also observed is a significant increase in the 2-methyl-1-propanol concentration for the aged beer in comparison to the fresh samples, such that it is the highest concentration VOC out of all the VOCs measured for aged beers.

Figure 15indicates the concentration of all eleven VOC compounds in IPA. The compounds were mostly detected in fresh IPA with the exception of the esters, ethyl lactate ethyl nicotinate. However, after incubation at 60^0C/1 day, increases in the overall concentrations of all VOCs were observed. In second treatment of beer at 40^0C/21 days, the overall concentration of VOCs was higher than fresh beer and aged beer at 60^0C/1 day, suggesting the ageing of beer and the beer had developed a stale flavour and that the beer was not suitable for consumption.

Figure 16 depicts the mean concentration of all VOCs in pilsner. Most of the VOCs were not detected in the fresh pilsner beer, in contrast to IPA. As pilsner is considered to be a fully flavoured wheat lager, with a more complex flavour profile than the IPA, there would be many more VOC compounds present in the headspace, and this may have affected the adsorption capacity of VOCs on the Tenax TA tube. From the chromatograms, it was generally observed that there were other VOCs present (possibly other semi-volatiles) and these could have interfered with the quantification of the 11 VOCs studied here. Similarly, aged beer at 60^0C/1 day and 40^0C/21 days showed higher overall VOC concentrations.

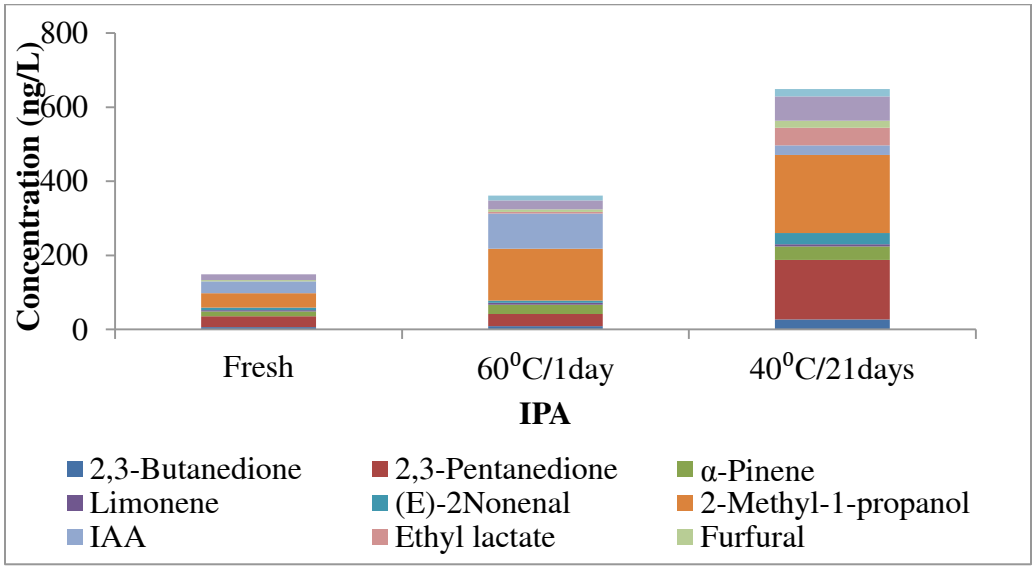

Figure 15 The mean concentration of eleven VOCs in IPA

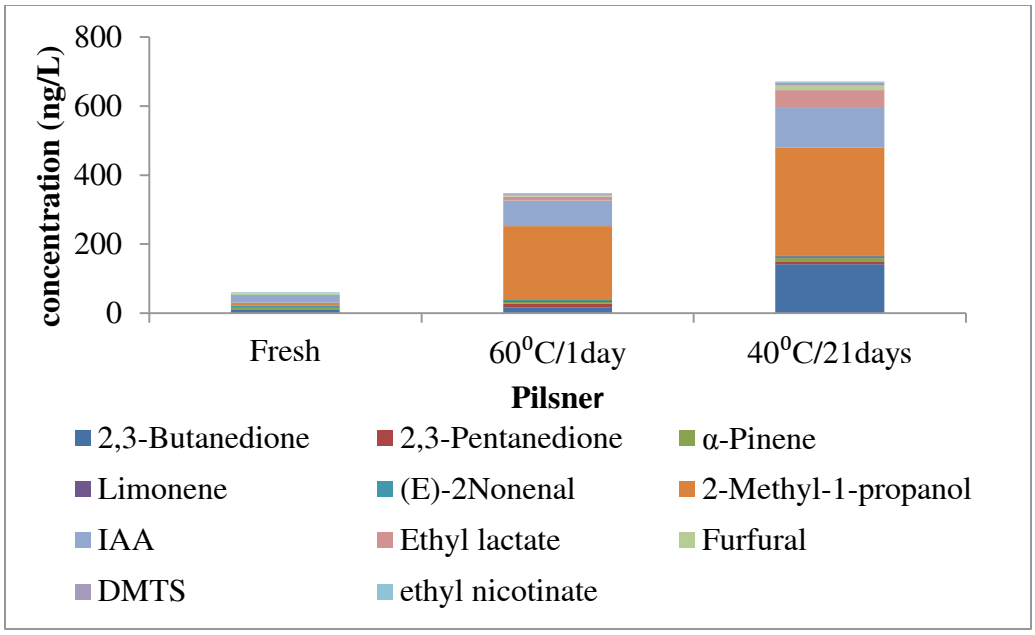

Figure 16 the mean concentration of eleven VOCs in pilsner

4.3.2 Analysis of VOC groups in IPA and Pilsner

In this section, the compounds are divided into six groups and the results from the analysis of eleven VOCs are reported in IPA and pilsner. The focus will be on the following groups: vicinal diketones and carbonyl, hop, heterocyclic and sulphur compounds. Results from Mann-Whitney statistical analysis are presented in the Appendix C and discussed in this section.

4.3.2.1 Vicinal Diketones in beer

There are two significant vicinal diketones (VDKs) in beer: 2, 3-butanedione (or diacetyl) and 2, 3-pentanedione. The level to which they are detected depends upon the type of beer. The flavour threshold is the minimum concentration that is detectable by a sensory panel. For diacetyl in fully flavoured lager the flavour threshold is 0.1 ppm (10,000 ng/L), whereas in less intensely flavored lager it is 0.2 ppm (20,000 ng/L) (Bamforth, 2014). The flavor threshold of 2, 3-pentanedione is sometimes ten times higher than diacetyl and can vary from 0.9-1 ppm (90,000-100,000 ng/L) (Wainwright, 1973).

Figures 17 and 18 show the results for IPA and pilsner beer samples with VDKs detected. From Figure 17 (a), the concentration of vicinal diketones appears to increase with storage time in IPA. The mean concentration of 2, 3- butanedione in fresh IPA was reported to be 6.2 ng/L. However the slight increase in the concentration of 2, 3- butanedione was observed at 60^0C/1 day of 8.7 ng/L and the highest concentration was reported to be 27.5 ng/L at 40^0C/21 days. The initial concentration of 2-3 pentanedione in fresh IPA was reported to be 29.6 ng/L, whereas the mean concentration at 60°C/1 day was reported to be 32.8 ng/L. The mean concentration of 2, 3-pentanedione at 40°C/21 days was reported to be 159.9 ng/L. Thus for IPA, all beer samples were well under the flavour threshold for both 2, 3- butanedione and 2-3 pentanedione. The graphs indicate that there may be development of these off-flavours, particularly under the aging conditions of 40^0C/21 days.

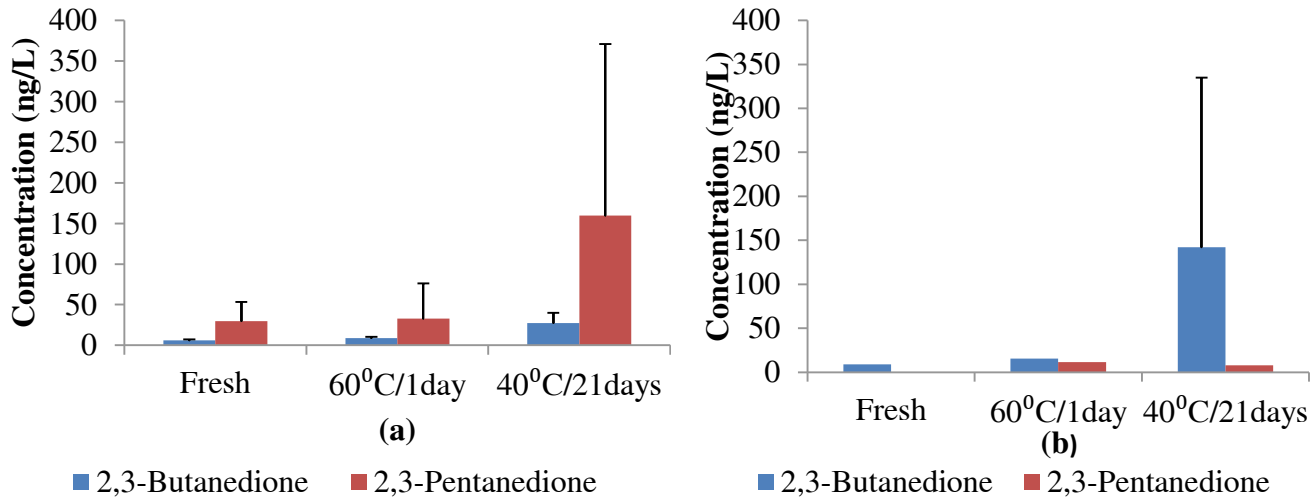

Figure 17. Comparison of 2, 3-butanedione and 2, 3-pentanedione concentration in (a) IPA and (b) pilsner. Where error bars represent standard deviation

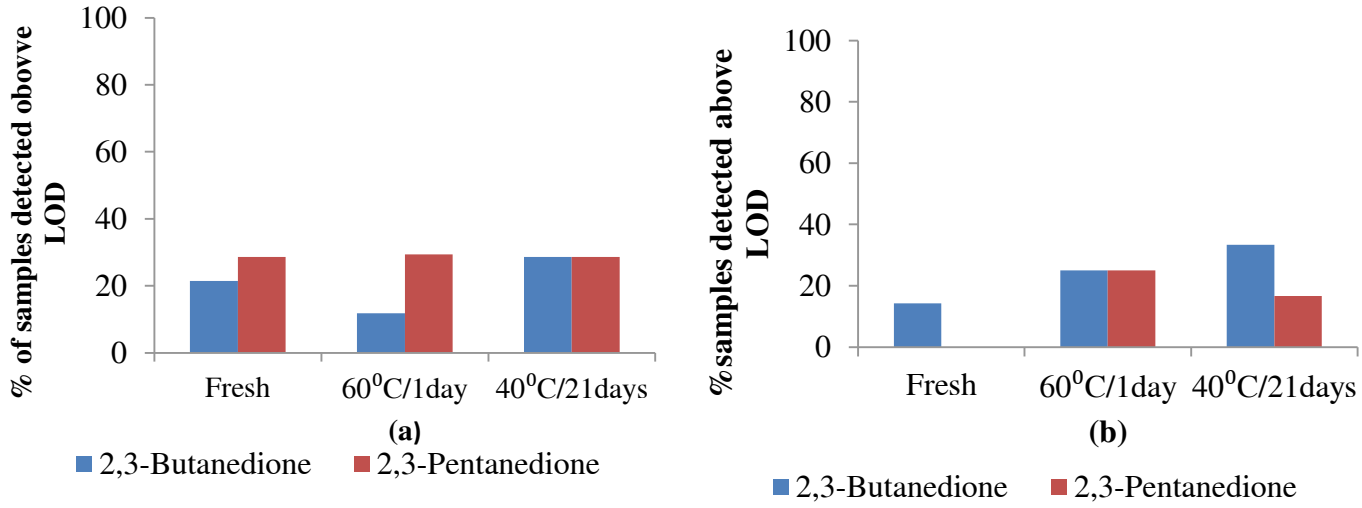

Figure18. The percentage of samples where 2, 3-butanedione and 2,3-pentanedione concentrations were above the GC-MS detection limit in (a) IPA and (b) pilsner beer

However, the results from Mann-Whitney statistical analysis using α=0.05 (Appendix C), resulted in p-values that were all above 0.05. This suggests that there is no significant difference between the fresh and aged IPA beers or between the results from aging at 60^0C/1 day and 40^0C/21days for both 2, 3- butanedione and 2-3 pentanedione.

Figure 17 (b) depicts the presence of 2, 3-butanedione and 2, 3-pentanedione in pilsner. The mean concentration of 2, 3-butanedione in fresh pilsner was reported to be 9 ng/L in fresh pilsner. The mean concentration in pilsner at 60°C/1 day was reported to be 15.4 ng/L; whereas the highest concentration of 142 ng/L was reported at 40°C/21 days. The mean concentration of 2, 3-pentanedione in pilsner at 60°C/1 day was reported to be 12 ng/L, and this was lower at 40°C/21 days. The 2, 3-pentanedione was absent in all the seven samples for fresh pilsner. Only one sample was detected for the presence of 2, 3-pentanedione in pilsner at 60^0C/1 day and 40^0C/21 days. Both VDK compounds in pilsner were below the flavour threshold, as was the case for IPA. It was not possible to conduct Mann-Whitney statistical analysis for these VDK compounds in pilsner beer, as there were only 1 or fewer samples for each condition where the compounds were above the LOD, as indicated in Tables 15-17.

Figure 18 depicts the percentage of samples detected above LOD in IPA and pilsner. It is evident that the majority of the samples were below the LOD, indicating that for most samples, either the compounds were not present in the beer or in insufficient concentrations to be detected by the GC-MS. Since diacetyl and 2, 3-pentanedione are usually present in very low concentration in fresh beer, their presence even in low concentrations suggests the start of off-flavour development in beer. If VDKs are present initially in the fresh beer their concentrations increases with storage time and temperature increase. VDKs are the indicators of deterioration of

organoleptic properties of beer. However it is not necessary that all the beers should test positive for the presence of VDKs. The concentration of VDKs in beer depends upon the processing conditions, storage and ingress of oxygen during ageing. In addition, the temperature and incubation time plays an important role in the ageing of beer (Vanderhaegen et al., 2006). As discussed, the results from statistical analysis of IPA samples indicate no significant difference in the VDK concentrations. However, the graph shows that there may be an increase after aging at 40^0C/21 days – further testing on more samples would be needed to determine if there is a statistically significant difference.

The application of GC in analysis of brewery samples is aided by high resolution capillary, column technology and multi-detector GC systems that are now available. However, most of the methods developed for detection of vicinal diketones require derivatisation of diacetyl and pentanedione. (Pejin et al. 2002) For example, in recent studies by Silva et al., (2015), a GC-Electron- capture detector and HS-SPME GC-MS method was developed for the detection and quantification of vicinal diketones and off flavour esters in 15 different brands of Brazilian pilsner. The concentration of diacetyl was reported to be 0.10 ng/L and IAA was 3.88 ng/L, both the concentrations were lower than the tasters' threshold detection limit (da Silva et al., 2015) and also lower than what was reported for the present study. The major drawback of their method was the time needed, as the samples before analysis were kept for conditioning for one hour in thermostatic bath. In comparison, the method used in this study provides rapid determination of vicinal diketones is less time consuming and does not require derivatisation of diacetyl and pentanedione. An alternative method was developed using liquid chromatography/ mass spectrometry method for the determination of total vicinal diketones in beer (Blanchette et al., 2007). However this method includes derivatisation of diacetyl and pentanedione with o-

phenylenediamine (OPDA) to quinoxaline compounds. Disadvantages of this method were that it was very labour intensive as it involved different steps and was rather complex method for industrial application. In their study, the method was developed with spiked beer samples; however quantitative data for VDKs in finished beer samples were not reported.

4.3.2.2 Hop compounds in beer

This section will focus on the hop oil compounds which are present in beer due to the addition of hops during processing of beer. Figure 19 and 20 depicts results for IPA and pilsner beer samples for α-pinene and limonene compounds.

Figure 19 (a) indicates the mean concentration of hop compounds in IPA. IPA is known to have a strong antiseptic hoppy flavour (Bamforth, 2009). The mean concentration of α-Pinene in fresh IPA was reported to be 13 ng/L. An increase in the concentration was observed at 60°C/1 day and was reported to be 25.5 ng/L. However the maximum concentration of α-pinene was reported to be 36.4 ng/L for IPA at 40^0C/21 days. Very limited literature is available on the state of pinene and limonene in aged beer but they contribute to the hoppy flavour of beer. The mean concentration of limonene was reported to be 3.6 ng/L in fresh IPA. The increase in the mean concentration of limonene was observed after incubation at 60°C/1 day and was reported to be 4.6 ng/L. The mean concentration of limonene at 40°C/21 days was reported to be 6.0 ng/L. The graph indicates that there may be an increase in the concentration of α-pinene in both the treatments of IPA at 60°C/1day and 40°C/21 days. However the results from Mann-Whitney statistical analysis using α=0.05 (Appendix C), indicates that there is no significant difference between the concentration of α-pinene in fresh and aged IPA and between the two aged conditions (p values were higher than 0.05). Figure19 (a) indicates little change in limonene

concentration between fresh and aged beer and agrees with the statistical analysis that there was no significant difference.

Figure 19 (b) depicts the mean concentration of α-pinene and limonene in pilsner. The mean concentration α-pinene in fresh pilsner was reported to be 7 ng/L and the mean concentration was slightly reduced to 5.26 ng/L at 60°C/1 day. However the highest mean concentration of α-pinene was reported to be 10.5ng/L for pilsner at 40°C/1 day. The mean concentration of limonene in fresh pilsner was reported to be 1.8 ng/L and 3.0 ng/L in pilsner at 60^0C/1day. The mean concentration of limonene at 40^0C/21 days was reported to be 4.2 ng/L. Graphically there was not much difference between limonene and pinene in fresh pilsner and after 60°C/1 day, though an apparent increase in the mean concentration of α-pinene was observed after 40°C/21 days in comparison to fresh pilsner. Mann-Whitney statistical analysis suggests that there is no significant difference in α-pinene concentration between the fresh and aged pilsner and between the two treatments at 60°C/1 day and 40°C/21 days. However, as the p value for limonene for fresh and aged pilsner at 40°C/21 days was below 0.05, this suggests that there is a significant difference between limonene concentration in fresh and aged pilsner. Statistical analysis could not be conducted between pilsner at 40°C/21 days and 60°C/1 day, as there was only one sample where limonene was detected after 60°C/1 day. Further testing on more samples would be needed to determine if there is a statistically significant difference.

Figure 20 depicts the percentage of samples detected above LOD for IPA and pilsner. The majority of samples for limonene and α-pinene were above the LOD in IPA, particularly after aging at 40°C/21 days. However for pilsner, there was less consistent behaviour.

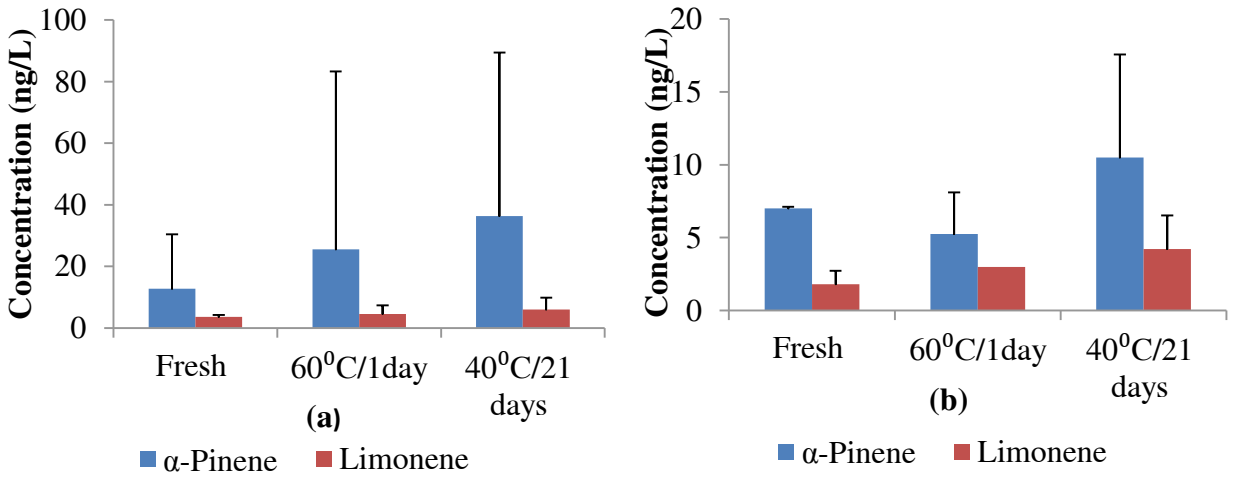

Figure 19. Comparison of α-pinene and limonene concentration in (a) IPA and (b) pilsner. Where error bars represent standard deviation.

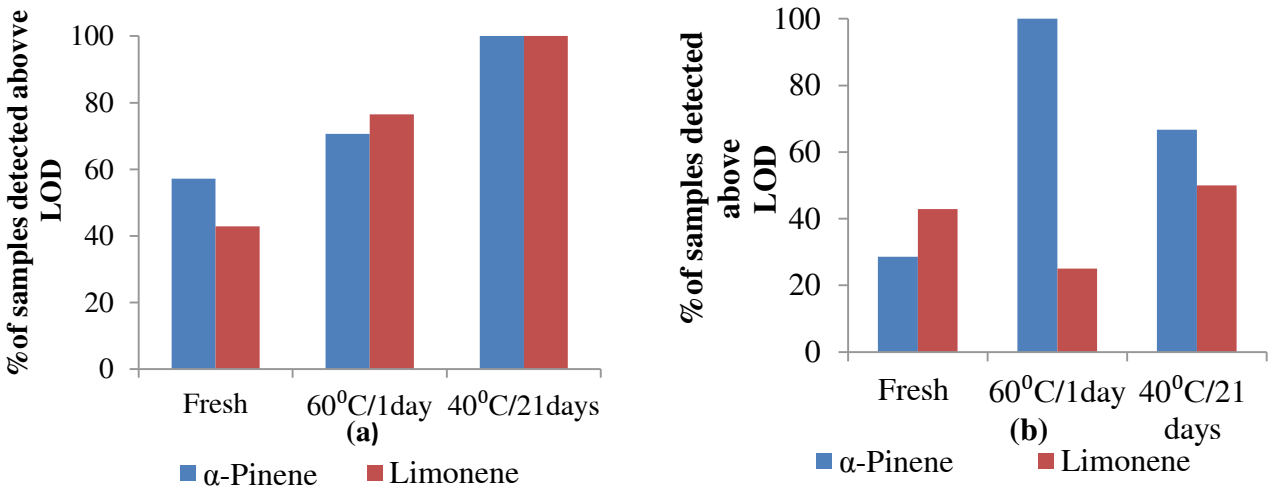

Figure 20. The percentage of samples where α-pinene and limonene were above GC-MS detection limit in (a) IPA and (b) pilsner.

4.3.2.3 Carbonyl compounds and higher alcohols in beer

This section will focus on the carbonyl compounds and higher alcohols in beer. The mean concentration of (E)-2-nonenal (or trans-2-nonenal) in beer as reported in ASBC flavour database is 0.001 mg/L (1000 ng/L) and minimum concentration is 0.00001 mg/L (10 ng/L). The average concentration of 2-methyl-1-propanol was reported to be 36.50 ppm and the flavour threshold of 10-200 ppm (10,000,000 ng/L- 2,000,0000 ng/L) in wheat beer (Bryant, 2011). Figures 21 and 22 depicts the results for IPA and pilsner beer samples with (E)-2-nonenal and 2-methyl-1-propanol.

Figure 21 (a) shows the results for IPA. The mean concentration of (E)-2-nonenal in fresh IPA, IPA after 60°C/1 day and after 40°C/21 days was 7.28 ng/L, 6.66 ng/L and 30.33 ng/L, respectively. The mean concentration in IPA at 40°C/21 days is above the flavour threshold of 10ng/Land thus would impact the flavour of beer. The mean concentration of 2-methyl-1-propanol in fresh IPA was reported to be 38.2 ng/L. However an increase in the concentration of 139.9 ng/L was observed at 60^0C/1 day and the highest concentration was reported to be 210.5ng/L at 40^0C/21 days. The graph indicates there may be an increase in the concentration of (E)-2nonenal and 2-methyl-1-propanl in both the treatments of IPA at 60°C/1day and 40°C/21 days as compared to the fresh IPA. However the Mann-Whitney statistical analysis (Appendix C) for (E)-2nonenal results in p values higher than 0.05 suggesting that there is no difference between fresh IPA and either of the aged conditions and no difference between the two aged treatments. The statistical analysis (Appendix C) for 2-methyl-1-propanol in fresh IPA and 2-methyl-1-propanol in IPA at 60°C/1 day results in p value lower than 0.05 suggesting a significant difference between the two treatments. The Mann-Whitney statistical analysis for 2-methyl-1-propanol in fresh IPA and IPA at 40°C/21 days also resulted in p value lower than 0.05

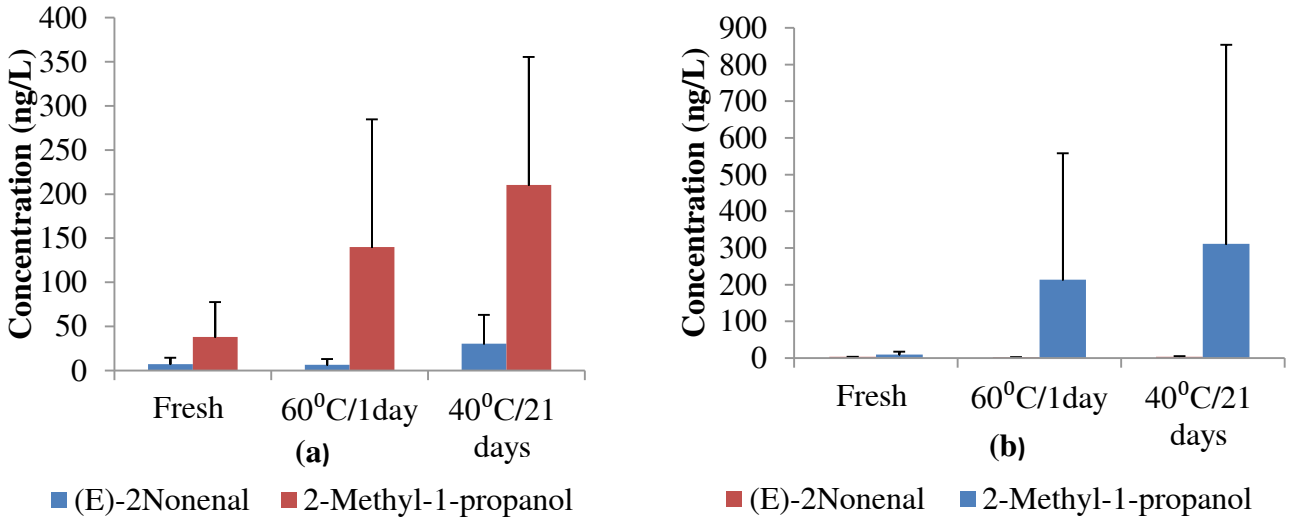

Figure 21. Comparison of nonenal and 2-methyl-1-propanol concentration in (a) IPA and (b) pilsner. Where error bars represent standard deviation.

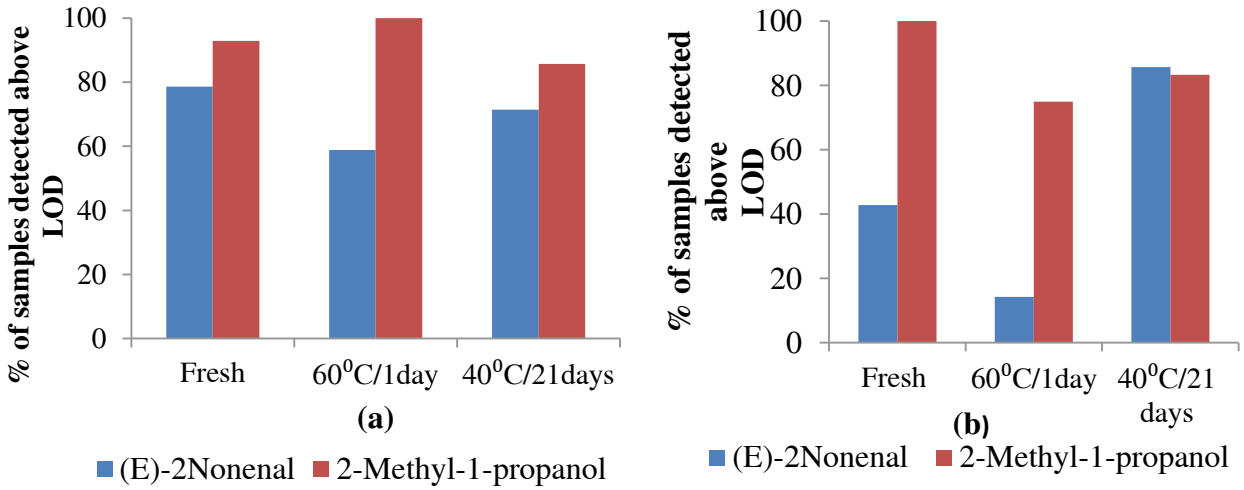

Figure 22. The percentage of samples where (E)-2nonenal and 2-methyl-1-propanol were above GC-MS detection limit in (a) IPA and (b) pilsner

(Appendix C) suggesting a significant difference between two treatments. However the p value for Mann-Whitney analysis (Appendix C) is higher than 0.05 for IPA at 60°C/1 day and IPA40°

/21 days. Thus this suggests there is no significant difference between the two treatments. All the samples for 2-methyl-1-propanol were lower than the flavour threshold of beer (10,000,000 ng/L- 20,000,000 ng/L).

Figure 21 (b) indicates the mean concentration of 2-nonenal and 2-methyl-1-propanol in pilsner. The mean concentration of nonenal in fresh pilsner was reported to be 3.0 ng/L and the mean concentration was reported to be 3.0 ng/L in pilsner at 60°C/1 day. The mean concentration of nonenal for pilsner at 60°C/1 day was reported to be 3.5 ng/L. The mean concentration of 2-methyl-1-propanol in fresh pilsner was reported to be 10 ng/L. The mean concentration of 2-methyl-1-propanol at 60°C/1 day was reported to be 214 ng/L and the highest mean concentration of 311 ng/L was reported in pilsner at 40°C/21 days. It is apparent from the graph that the concentration of the higher alcohol increases with storage time as compared to the fresh pilsner. However from the graph indicates that there may be no difference between fresh pilsner and other two treatments for (E)-2nonenal. The statistical analysis (Appendix C) suggests that there is no significant difference (p values greater than 0.05) between the fresh and aged pilsner beers or between the results from aging at 60^0C/1 day and 40^0C/21 days for (E)-2nonenal. However, statistical analysis (Appendix C) suggests a significant difference between the 2-methyl-1-propanol concentration in fresh pilsner and pilsner after 40°C/21 days (p value lower than 0.05). Also, there was no significance difference found between the concentration of 2-methyl-1-propanol in fresh pilsner and pilsner at 60°C/21 days and between pilsner for both the aging treatments. All the pilsner samples detected (E)-2nonenal and 2-methyl-1-propanol concentrations that were much lower than the flavour threshold of 2-methyl-1-propanol in beer (10,000,000 ng/L- 20,000,000 ng/L)

Figure depicts the percentage of samples with concentrations above the LOD for both IPA and pilsner. It became apparent from the graph that the majority of the samples were above LOD for (E)-2nonenal and 2-methyl-1-propanol in IPA. However in pilsner there were very few samples above LOD in fresh pilsner and pilsner at 60^0C/1 day. The number of samples below the LOD suggests that more samples should be tested but due to limited sample supply this was not possible.

In the literature, an increase in carbonyl compounds has been linked to the ageing of beer. When added to beer, (E)-2 nonenal was the compounds impart a cardboard flavour to the beer, and a similar note has also been observed in the aged beer (Palamand & Hardwick, 1969). Saison et al. (2009) analysed trans-2-nonenal in lager at 40°C/21 days and the fresh lager using headspace GC-MS; the concentration was reported to be 0.09 ppb (90 ng/L) for fresh lager and 0.15 ppb (150 ng/L) 40°C/21 days. Saison et al. (2009) also analysed 2-methyl-1-propanol using GC-MS in beer at 30^0C/21 day and the concentration was reported to be 37.7ppb (37,700 ng/L) and initial concentration was reported to be 790 ng/L.

Due to growing importance of trans-2-nonenal in beer, many methods have been proposed to measure their concentration in beer, with head space solid phase microextraction and static headspace extraction being common methods for extraction of higher alcohols in beer. However the P&T method has advantages as it can extract alcohols even at room temperature and does not require any pre-treatment of beer.

An alternative HPLC method was developed by Wang & Siebert in 1974 to follow the increase in the concentration of trans-2-nonenal in beer stored at 38°C for six days. Here, the extraction of beer with dichloromethane was followed by derivatisation of (E)-2 nonenal with 2, 4 -

dinitrophenylhydrazine (DNPH) under acidic conditions. The derivatives were than subjected to separation by thin layer chromatography and finally analysed by HPLC. However methods involving derivatisation are laborious and time consuming. In comparison, P&T-TD-GC-MS methods allow the analysis of samples containing nonenal in just 1 hour and if the system is automated, 20 samples can be analysed in the same time.

4.3.2.4 Esters in beer

This section will include analysis of the important esters in beer that are known to impart fruity flavours to the beer. The average concentration of IAA in wheat beer is reported to be 3-4 ppm (3,000,000- 4,000,000 ng/L) (Bryant, 2011) and the flavour threshold of 1.0-1.6 mg/L (1,000,000-1,600,000 ng/L) (ASBC, database). For ethyl nicotinate the flavour threshold of 6 mg/L (6,000,000 ng/L) and for ethyl lactate 25mg/L (25,000,000 ng/L). A figure 23 and 24 depicts the results for IPA and pilsner beer samples with ethyl lactate, ethyl nicotinate and IAA.

Figure23 (a) depicts the mean concentrations of ethyl lactate, ethyl nicotinate and IAA during different treatments of IPA. Since ethyl lactate and ethyl nicotinate are indicators of ageing of beer, they were not observed in fresh beer, as expected. The mean concentration of ethyl lactate after 60^0C/1 day and 40^0C/21 days was 4.6 ng/L and 47.2 ng/L, respectively. There was a noticeable increase in the concentration of ethyl lactate. The mean concentration of IAA in fresh IPA was reported to be 38.2 ng/L and a higher concentration of 94.7 ng/L was observed in beer kept at 60^0C/1 day. However the concentration of IAA was lower at 26.51ng/L after 40^0C/21 days in comparison to fresh beer. This was expected, as during ageing the fruitiness of IAA reduces. The mean concentration of ethyl nicotinate at 60^0C/1day was reported to be 13.02 ng/L and at 40^0C/21 days was 20 ng/L.

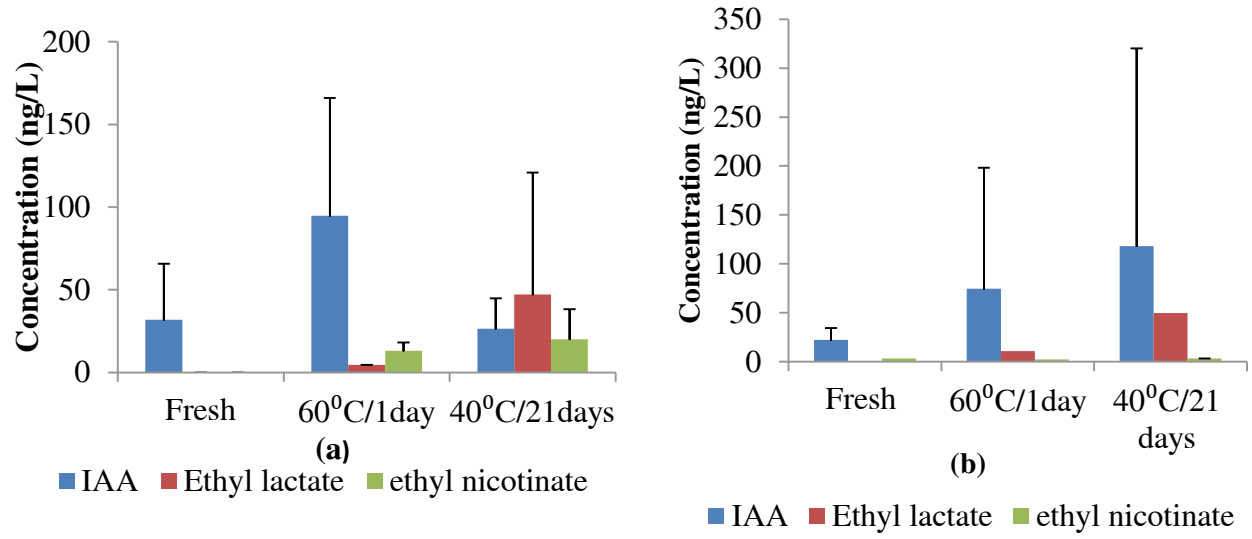

Figure 23. Comparison of ethyl lactate, ethyl nicotinate and IAA concentration in (a) IPA and (b) pilsner. Where error bars represent standard deviation

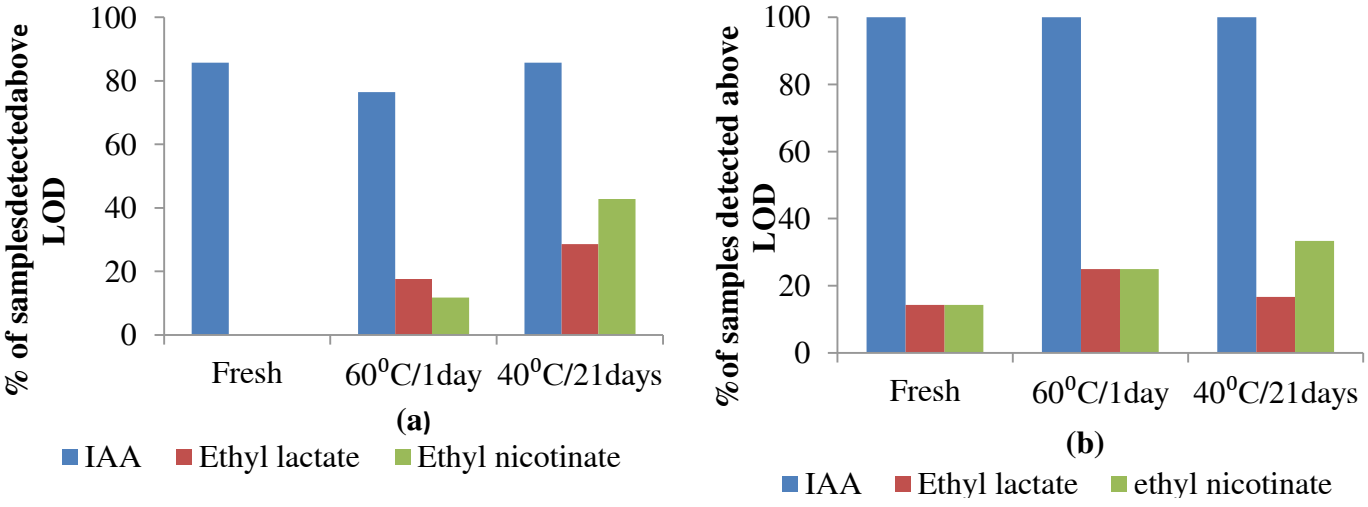

Figure 24. The graph indicating the percentage of samples detected with ethyl lactate, ethyl nicotinate and IAA above detection limit of GC-MS for (a) IPA and (b) pilsner.

The graph indicates that there may be a development of these off- flavours particularly under the ageing condition of 40^0C/21 days. However the statistical analysis for ethyl lactate and ethyl nicotinate in fresh IPA and other two treatments could not be conducted due to the small sample size. The p values (Appendix C) for ethyl lactate at 40^0C/21 days and 60^0/1 day was above 0.05; suggesting there was no significant difference between the IPA in two treatments. The Mann-Whitney statistical analysis (Appendix C) using α=0.05 resulted in p value lower than 0.05 for IAA in fresh IPA and IPA 60^0C/1 day and between the two treatments suggesting there is a significant difference between fresh IPA and IPA at 60^0C/1 day and IPA60^0/1 day and IPA40^0/21 days. However the p value for IAA in fresh IPA and IPA at 40^0C/21 days was above 0.05 suggesting there was no difference between the two beers. This can be due to decrease in the concentration of IAA during ageing. The concentration of all ester measured were well below the flavour threshold.

Figure 23(b) depicts the mean concentration of ethyl lactate, ethyl nicotinate and IAA in pilsner. Ethyl lactate was not identified in the fresh beer. The mean concentration of ethyl lactate was reported to be 10.7 ng/L at IPA at 60°C/1 day and at 40^0C/21 days was reported to be 49.66 ng/L. However ethyl lactates in pilsner are of less importance (Bryant, 2011). The mean concentration of ethyl nicotinate in fresh pilsner was reported to be 3.1 ng/L, which was slightly higher than the 2.8 ng/L measured after 60^0C/1 day and was no different to the concentration after 40^0C/21 days. The results showed that the mean concentration of ethyl nicotinate remained unchanged after the temperature treatments for beer. The mean concentration of IAA in fresh pilsner was reported to be 22.36 ng/L and in pilsner at 60^0C/1 day to be 74.5 ng/L. The highest mean concentration of IAA was reported to be 117.9 ng/L in pilsner at 40^0C/21 days. However a slight change was observed in IAA concentration as after ageing the concentration of IAA

usually decreases. The graph indicates that there may be a development of off flavours particularly at an ageing condition of 40°C/21 days for pilsner. However statistical analysis could not be conducted for ethyl lactate and ethyl nicotinate as very few samples were detected above the LOD for pilsner. Statistical analysis for IAA concentrations for pilsner samples generally did not result in significant differences, though there was a significant difference between fresh pilsner and pilsner after 40°C/21 days.

Figure 24 depicts the percentage of samples detected with ethyl lactate, ethyl nicotinate and IAA in pilsner and IPA above the LOD. There were very few samples where ethyl lactate and ethyl nicotinate were detected. All samples of pilsner had IAA present.

SPME & SBSE followed by GC analysis is commonly used for the extraction and analysis of volatile esters in beer (Horák et al., 2010). For example, Siason et al. (2007) measured the concentration of ethyl nicotinate and IAA in fresh lager beer after 40°C/21 days using GC-MS. The concentration of ethyl nicotinate in fresh beer was reported to be 6.5 ppb (6,500 ng/L) and in lager at 40°C/21 days as 15.3 ppb (15,300 ng/L). The concentration of IAA in fresh beer was reported to be 473ppb (47,300 ng/L) and in lager at 40°C/21 days as 427ng/L (42,700 ng/L) (Siason et. al, 2007). The concentration of IAA in pilsner was also determined using HS-SPME-GC and HS-GC-ECD and the concentration of IAA was reported to be 3.88ng/ml (3880 ng/L) (da Silva et al., 2015). The concentrations reported in current work are much lower than the results in these studies and well below the flavour threshold of these compounds in beer.

4.3.2.5 Furfurals in beer

Furfurals are the Maillard reaction products present in the beer. During the boiling the amines react with free carbonyl group and produce heterocyclic compounds such as furfurals. During ageing of beer various intermediates of Maillard reaction between sugar and amino acids were identified. The increase in the concentration of furfural is also oxygen dependent. Reduction of furfural by yeast may affect the initial concentration of furfural in the fresh beer (Vanderhaegen et al., 2003). The maximum concentration measured for furfural as published in ASBC database for beer is 1.8 mg/L (18,000,000 ng/L). Figures 25 and 26 depict results for IPA and pilsner beer samples with furfural present.

Figure 25 (a) indicates the mean concentration of furfural in IPA. The mean concentration of furfural in fresh IPA was 3.465 ng/L and appeared to increase slightly after storage at 60^0C/1 day to 6.28 ng/L. However the maximum concentration of furfural in IPA was observed after 40^0C/21 days at 18.26 ng/L. The graph indicates that there may be a development of furfural in aged IPA as compared to fresh IPA. However, statistical analysis using α=0.05 (Appendix C) suggests that there is no significant difference between the fresh and aged IPA and or between the results from ageing at 60°C/1 day and 40°C/21 days for furfural in IPA (as p values were all above 0.05).

Figure 25 (b) indicates the mean concentration of furfural in pilsner. The mean concentration of furfural in fresh pilsner was 4.3 ng/L, similar to the mean concentration of 4.4 ng/L observed in after 60°C/1 day. The mean concentration of furfural in pilsner at 40°C/21 days was higher at 12.96 ng/L. The graph indicates that there may be a development of furfural particularly under

the ageing conditions of 40°C/21 days. However, statistical analysis (Appendix C) suggests that there is no significant difference between the fresh and aged pilsner and or between the results from ageing at 60°C/1 day and 40°C/21 days for furfural in pilsner. Figure depicts the percentage of samples identified above the LOD for furfural in pilsner and IPA. It is evident that for all conditions, some of the samples were below the detection limit.

Increases in the storage temperature can significantly increase the level of heterocyclic compounds and furfural can act as an indicator of flavour deterioration in beer even though the concentration may be lower than the flavor threshold in beer (Vanderhaegen et al., 2006). The presence of furfurals can also indicate temperature related deterioration in the beer (Bamforth & Lentini, 2002).

In another study, Siason et al. (2007) measured the concentration of furfural in fresh lager beer at lager at 40°C/21 days using GC-MS. The concentration of furfural in fresh beer was reported to be 25 ppb (25000 ng/L) and in lager at 40°C/21 days as 457ppb (45,7000 ng/L). In comparison to this study, the concentrations in the current work are very low.

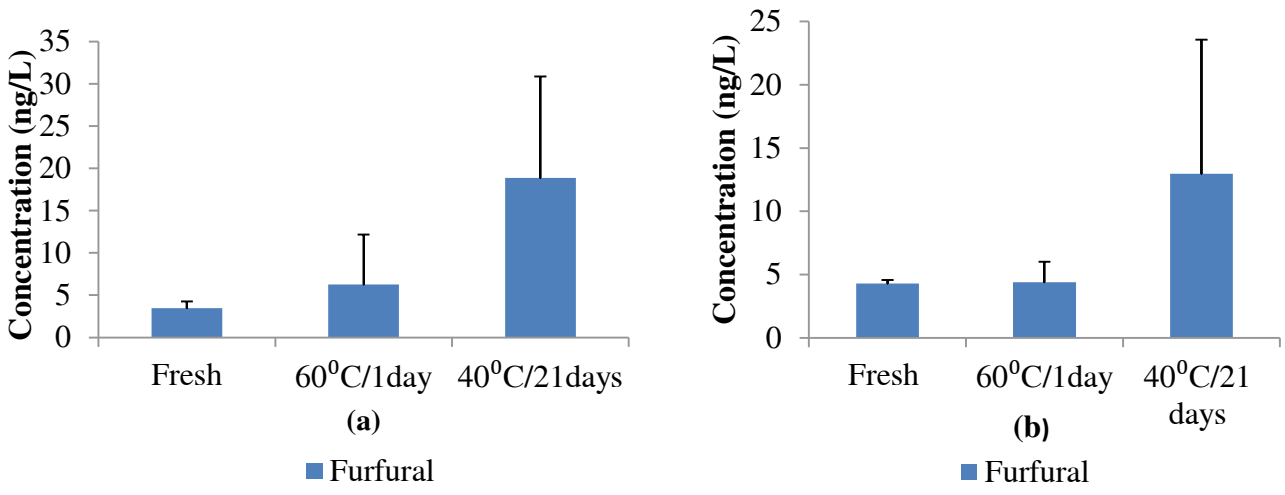

Figure25. Comparison of furfural mean concentration in (a) IPA and (b) pilsner. Where error bars represent standard deviation

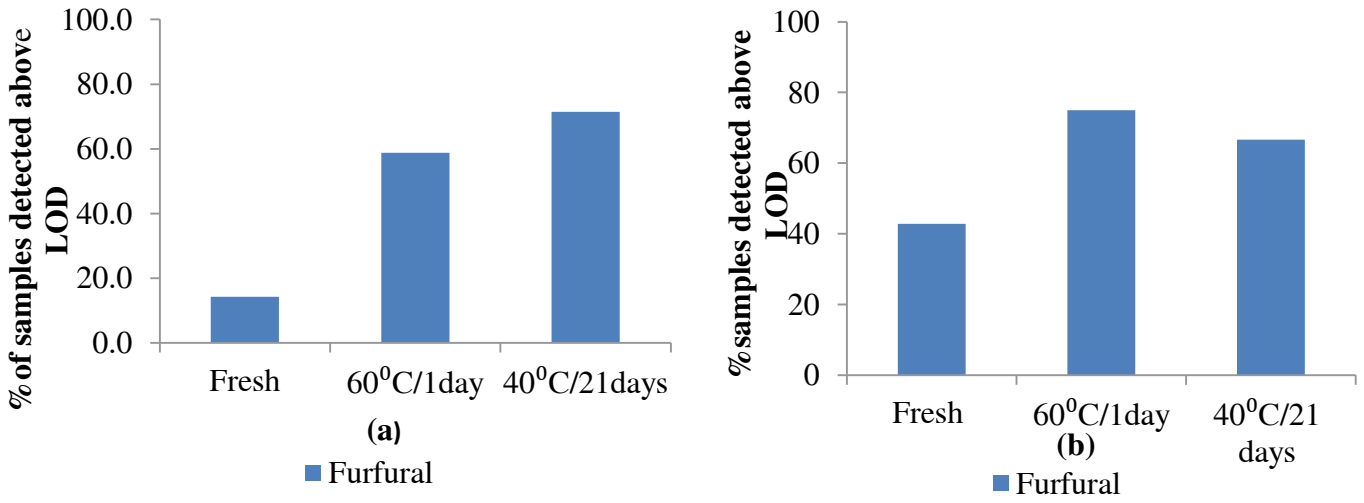

Figure26. The percentage of samples detected in furfural were above GC-MS detection limit in (a) IPA and (b) pilsner

4.3.2.6 Sulphur compounds in beer

Some of the most potent compound in beer contains the sulphur atom. The sulphur in beer can be from two sources such as inorganic sulphur in the water supply, antioxidants (e.g. metabisuplphite and isinglass finings) and organic sulphur from amino acids such as cysteine and methionine (Bamforth, 2014). There are various sulphur compounds in beer such as dimethyl sulphides, DMTS and MBT. In the current study we will focus on DMTS in IPA and Pilsner. Sulphur compounds have generally a low flavour threshold and even present in small quantities can alter the organoleptic properties of beer (Vanderhaegen et al., 2006). The mean concentration of DMTS in beer according to the ASBC database is 0.00001 mg/L (10 ng/L) and flavour threshold of 0.000027 mg/L (27 ng/L). Usually sulphur compounds have very low flavour threshold and small quantities of sulphur compounds can deteriorate beer flavor (Vanderhaegen et al., 2006). Figures 27 and 28 depict the results for IPA and pilsner beer samples with DMTS.

Figure 27(a) indicates the mean concentration of DMTS in IPA. The mean concentration of DMTS in fresh beer is reported to be 16.05 ng/L and it increased in IPA stored at 60^0C/1 day to 24.8 ng/L. The mean concentration of DMTS in IPA at 40^0C/21 day was reported to be 65.6ng/L which was above the flavour threshold of beer (27 ng/L) and hence considered an aged beer. The graph indicates that there may be a development of DMTS particularly under the ageing conditions of 40^0C/21 days. However, statistical analysis (Appendix C) showed no significant difference between fresh IPA and after 60^0C/1day or between ageing at 60^0C/1 day and 40^0C/21 days for DMTS in IPA. The statistical analysis did show a significant difference in DMTS between fresh IPA and IPA after 40^0C/21days.

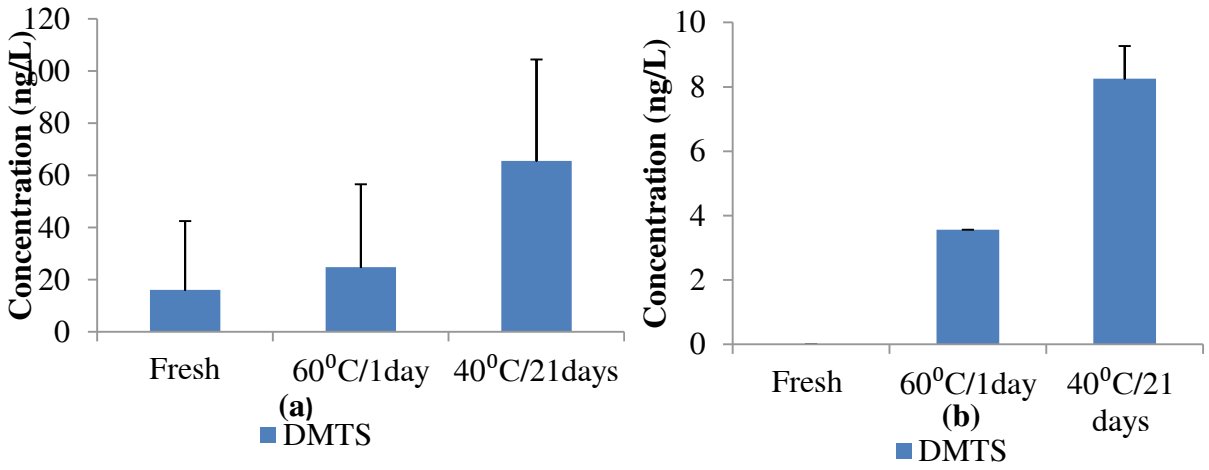

Figure27. Comparison of DMTS concentration in (a) IPA and (b) pilsner. Where error bars represent standard deviation

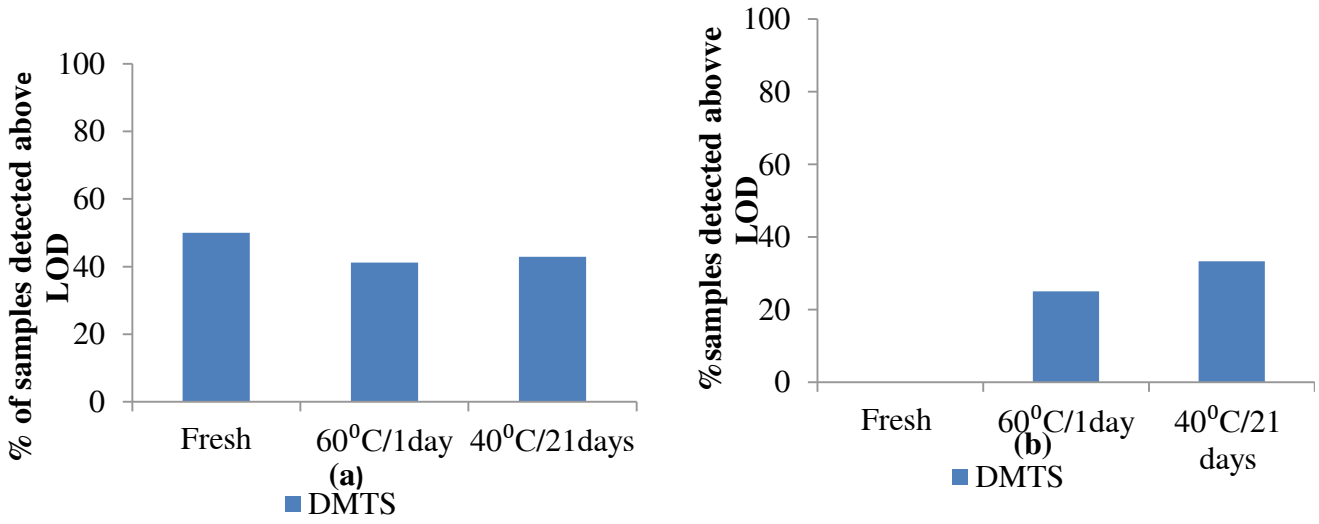

Figure 28. The percentage of samples where DMTS were above GC-MS detection limit in (a) IPA and (b) pilsner.

Figure27 (b) depicts the mean concentration of DMTS in pilsner. The DMTS was not identified in fresh and the mean concentration was 3.56 ng/L in pilsner at 60^0C/1 day. However the mean concentration of DMTS in pilsner at 40^0C/21 days was reported to be 8.25 ng/L. This was near the flavour threshold concentration 30 ng/L and can impact the beer flavour. The graph indicates that there may be a development of DMTS particularly under the ageing conditions of 40^0C/21 days. However, statistical analysis could not be performed due to the lack of samples with DMTS detected in pilsner. Figure 28depicts the percentage of samples identified above the LOD with DMTS in pilsner and IPA. The majority of samples were detected below LOD for IPA and pilsner, indicating that the levels were very low or absent from the samples.

Other methods for detecting sulphur compounds include those by Hill and Smith (2000), who determined sulphur compounds in beer using HSPME and gas chromatography analysis with pulsed flame photometric detector. The use of purge and trap gas chromatography and microwave induced plasma atomic emission spectrometry was used to determine VOCs in water beer and coffee samples. The detection limits range from 0.4-0.9 ng/L for dimethyl disulphides. However only DMS was detected using this methodology in beer (Gerbersmann, Lobinski, & Adams, 1995).

4.4 General Discussion

The limonene in pilsner at 40^0C/21days was significantly different from fresh pilsner. The limonene in beer imparts citrus flavour to the beer; however this characteristic flavour of hop oils but sourness in beer in unacceptable (Bamforth, 2014). There was a significant difference between IAA in fresh pilsner and pilsner at 40^0C/21 days. However the concentration of IAA decreases with the ageing as the fruity flavour of beer reduces (Vanderhaegen et al., 2006). 2-methyl-1-propanol in fresh IPA and IPA at 40^0C/21 was significantly different. 2methyl-1-propanol in fresh IPA and IPA at 40^0C/21 days was also significantly different. The higher concentration of alcohol also indicates the ageing of beer as they impart solvent flavour to beer. The concentration of (E)-2nonenal in IPA at 40^0C/21 days was 30.33 ng/L which was above the flavour threshold of 10 ng/L and can be detected easily detected by sensory panel. (E)-2nonenal impart cardboard flavour to the beer and increase in concentration above flavour threshold is considered unacceptable for consumption. The mean concentration of DMTS in IPA at 40^0C/21 days was reported to be 65.6 ng/L which was above the flavour threshold of beer (27 ng/L) and hence considered an aged beer. There was a significant difference in DMTS between fresh IPA and IPA after 40^0C/21 days. The sulphur compounds are detectable even at very low concentration and thus effect the organoleptic property of beer as they impart rotting vegetables and onion flavour to beer (Bamforth, 2014).

Beer is generally divided into two types: ales and lagers. They both vary in colour, bitterness, flavour and aroma. Indian pale ale was designed to have a longer shelf life as compared to pilsner. IPA has a high alcohol content (6.8% alc), low levels of residual sugars, plenty of antiseptic hop bitterness and are top fermenting beers. The pilsner is bottom fermenting lager, with 4.8-5% alcohol level and are pale gold in colour (Bamforth, 2009).

The method used in this study determined VOC concentration in ng/L, and in order to prevent the over flow of the column small concentration of standards were used. This resulted in final concentration of ng/l. The beers used in this study were craft beers which are considered to be fresh, requiring minimum additives and fewer preservatives. The calibration curves were prepared, which presented concentration intervals that encompass the off flavours concentration usually found in beers which are proper for consumption. This method is also suitable for determining nonenal and sulphur compounds which are usually present in lower concentration.

SPME is often used for aroma compounds analysis. The conventional SPME has some drawbacks over other methods such as fibre fragility, low sorption and step by step recalibration is required during extraction /desorption (Hriv, Šmogrovi, Lakatošová, Nádaský, & Brew, 2010). Simultaneous distillation extraction is one of the oldest techniques used to separate volatile compounds from non-volatile compounds. It is a simple fast aroma extraction technique but however the use of high distillation temperature can result in production of artificial compounds (Engel et al., 1999). The purge and trap method of extraction offers higher recovery, lower detection levels and are less time consuming as compared to other extraction methods (Edmund et al., 2004).

Chapter 5. Conclusion and Recommendations

5.1 Conclusion

There were two objectives of this study:

1) To develop a method for determination of VOCs i.e. Furfural, ethyl nicotinate, (E)-2-nonenal, ethyl lactate, isoamyl acetate, 2-methyl-1-propanol, dimethyl trisulphide, 2, 3 butanedione, limonene, (+) (-) α pinene, 2, 3 pentanedione in beer using purge and trap extraction followed by TD-GC-MS analysis.
2) To use the developed method to determine eleven VOCs in India pale ale and pilsner and relate them to the characteristics of ageing beer.

The single VOC mixture was prepared containing eleven VOCs having different volatility; this is not a common practice in brewery to mix aldehyde and ketones together as they are seldom stable in the mixture. The identification and quantification of the eleven compounds was achieved using TD-GC-MS method. Usually in the breweries that lack analytical instruments, sensory analysis is the only method used to identify the off –flavours and off odours in beer. The tasting panels consist of brewers with experience in the type of test and regular consumers that are trained for analysis. Because of health concerns regarding sensory analysis of beer and possible toxicity issues, the number of tests to be performed must be limited. This fact is important in order to develop of an analytical methodology as an alternative to identify and quantify off-flavours in beer. The proposed method has several advantages such as simple extraction, low sample volumes and high reproducibility. The automation of this method is also possible.

The relationship between VOCs concentration and signal abundance was equivalent for beer sample and aqueous standards; allowing calibration to be performed by aqueous standards. The

detection limits obtained were satisfactory. The 11 VOCs were identified and quantified in fresh and forced aged IPA and pilsner. The following compounds were significantly different in fresh and aged IPA at 40°C/21days: 2-methyl-1-propanol, E-2nonenal, DMTS and IAA. The concentration of (E)-2nonenal and DMTS were above the flavour threshold of beer and hence considered unacceptable for consumption. In pilsner, the following compounds were significantly different in fresh and aged beer at 40°C/21 days: limonene and IAA. There was no significant difference in the VOC compounds between fresh beer and after aging at 60°C/1 day.

According to the results obtained it can be concluded that the forced ageing test at 40°C/21 days is more efficient than forced ageing at 60°C/21 days as all of the VOCs were identified and quantified at forced ageing at 40°C/21 days for IPA and pilsner. However more samples would be required to determine if there is a significant difference between the fresh beer and the two force ageing treatments.

The forced ageing at 40°C/21 days resulted in higher concentration of the off flavour compounds as compared to the fresh beer sample. Initial concentration of these compounds were very low as compared to the concentration at 40°C/21 days therefore after the forced ageing an increase in the concentration of 11 VOCs was reported. This can be said that the off flavour development was initiated with the help of forced ageing and further comparison should be done between the natural aging of beer after six months of incubation at 10 °C and forced ageing of beer at higher temperatures.

5.2 Recommendations

Continuous sampling starting from the wort to the finished beer would have been more important as the precursors of ageing in the beer can be easily identified. Further research is required to analyse the VOCs and semi-volatile compounds during the ageing process of beer to gather better understanding of development of these compounds.

Due to limited sampling and limited access of the TD-GC-MS instrument the day to day increase of the concentration of VOCs was not performed. This would have been a great tool to measure the effect of storage time and temperature on the ageing of beer. Sensory evaluation of the aged beer can help to yield the flavour threshold of the flavour compounds in beer.

The column capacity, size, column length etc. was also an important part to be considered as an increase in the concentration was resulting in overflow of the column. There were very few industrial samples that were analysed due to the limited supply from the brewery. In order to develop a statistical difference the larger sample size was required.

Further studies for ageing of the beer and other test such as Thiobarbituric acid test for the oxidation of beer, haze analysis, Total packed oxygen analysis and dissolved oxygen analysis should be required to develop a relationship between development of the compounds that caused aging of beer and the factors affecting aging.

However this method can be used in the brewing industry to identify and quantify a range of the eleven VOCs present in the beer and is faster and less time consuming as compared to other methods. The instrument is portable so on-line sample collection for continuous sampling is possible.

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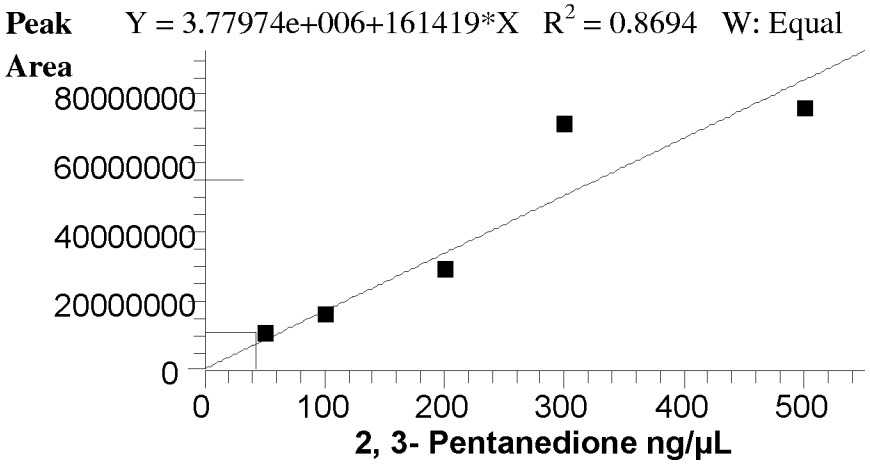
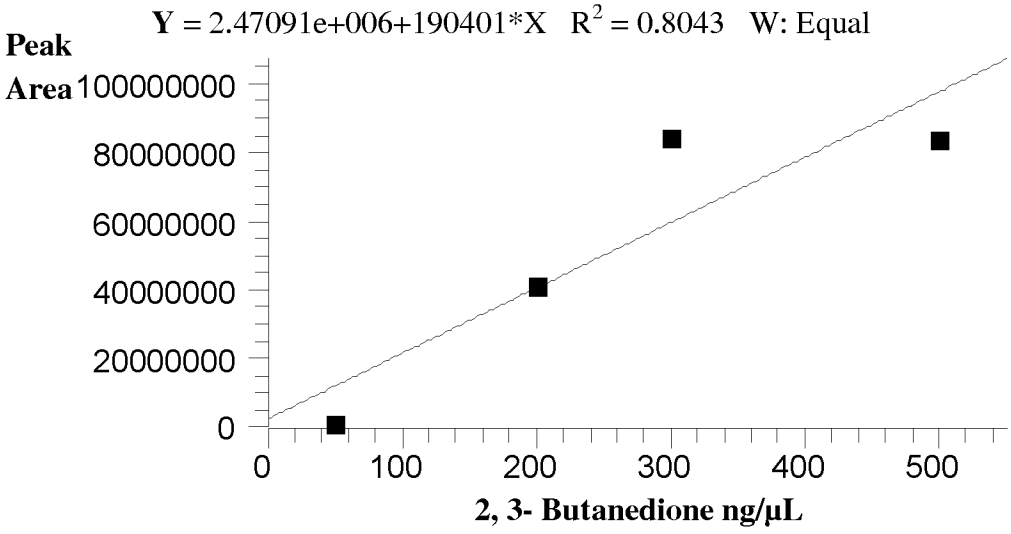
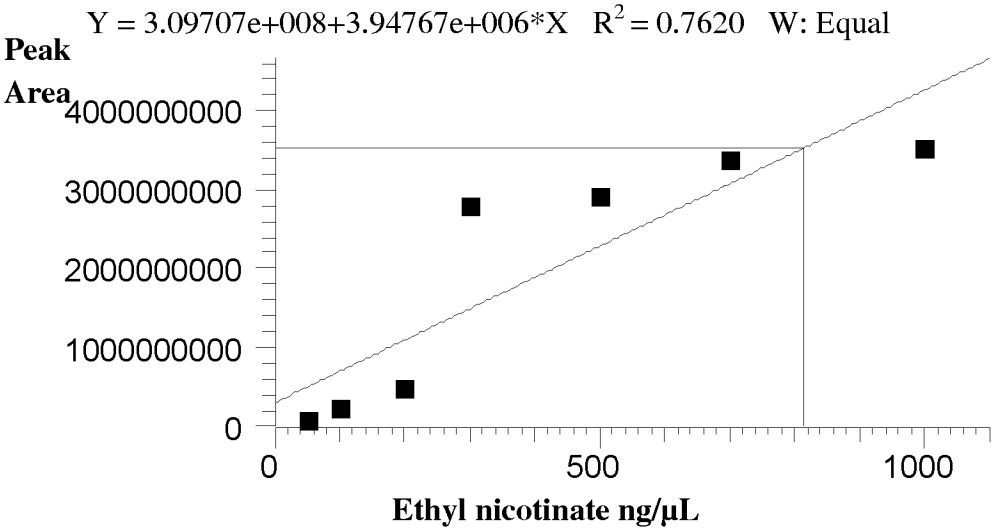
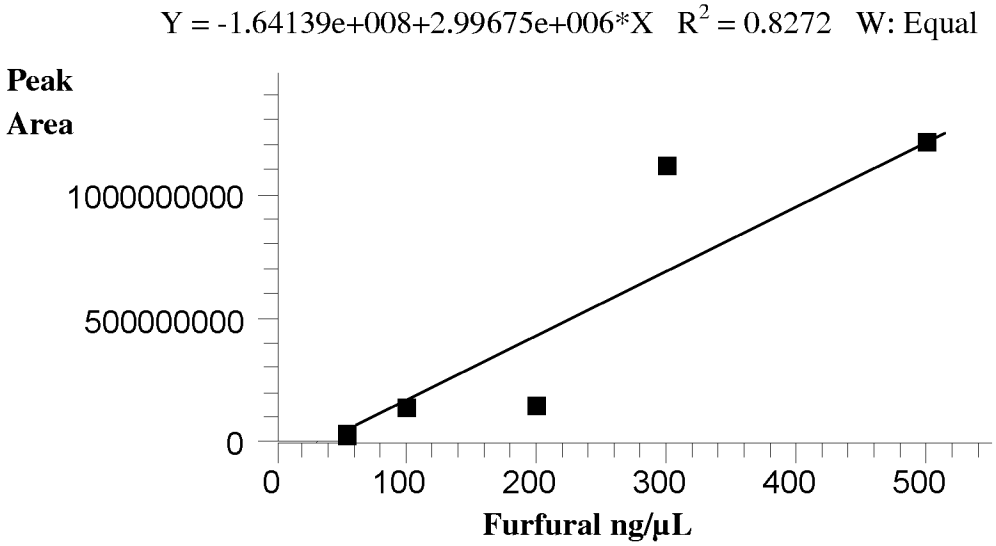
Appendix A

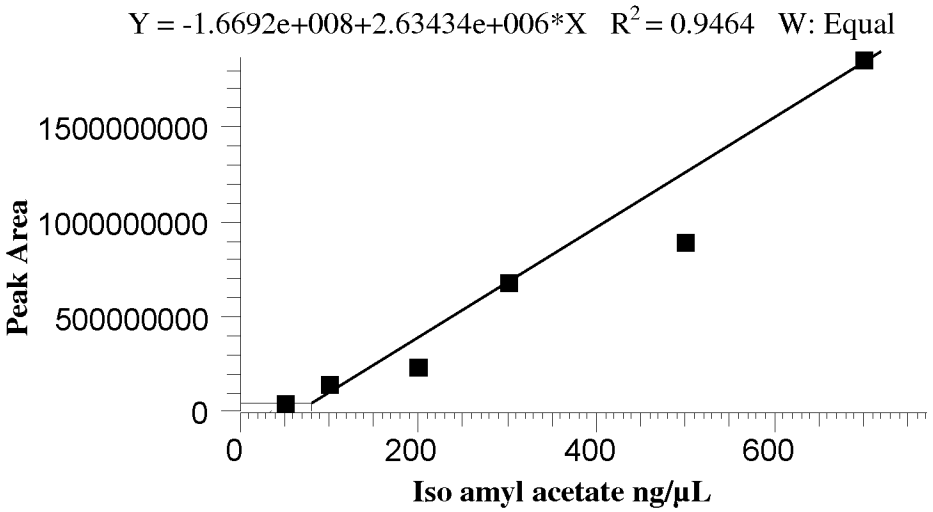
Calibration Curve for all the eleven VOCs

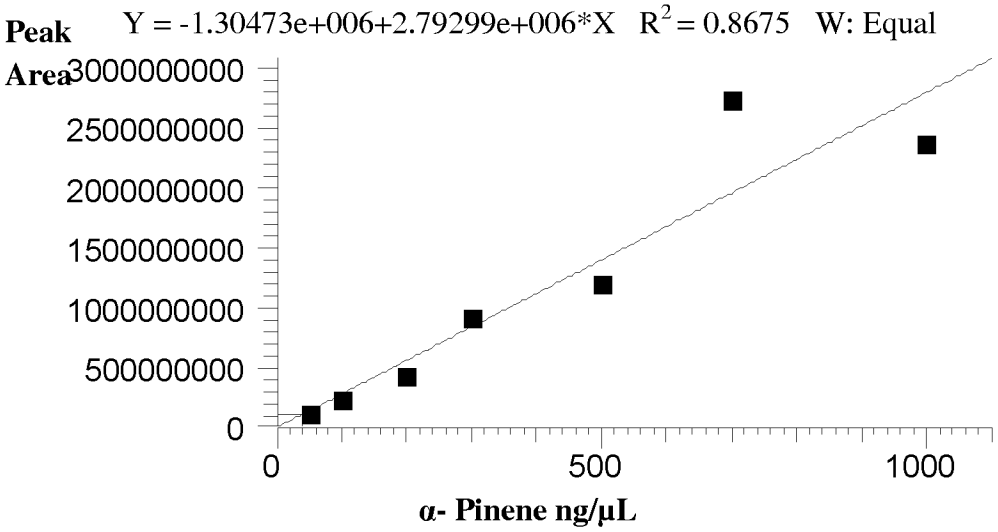


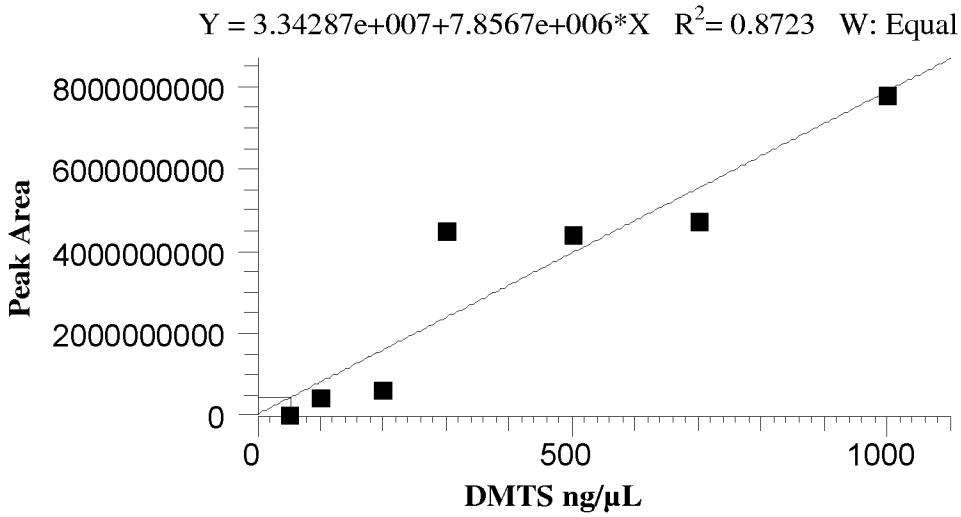


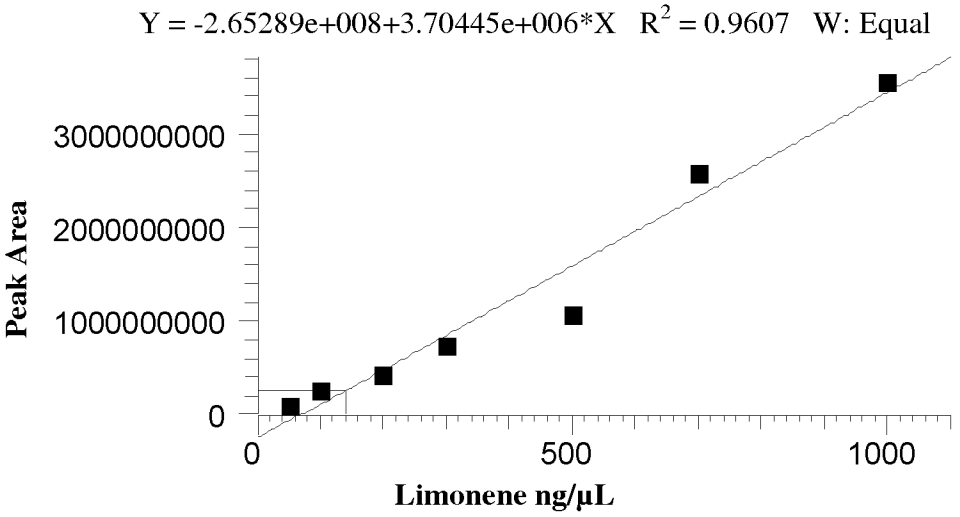

Y = 3.09707e+008+3.94767e+006*X R² = 0.7620 W: Equal
Peak Area
4000000000
3000000000
2000000000
1000000000
0
0
500
1000
Ethyl nicotinate ng/µL

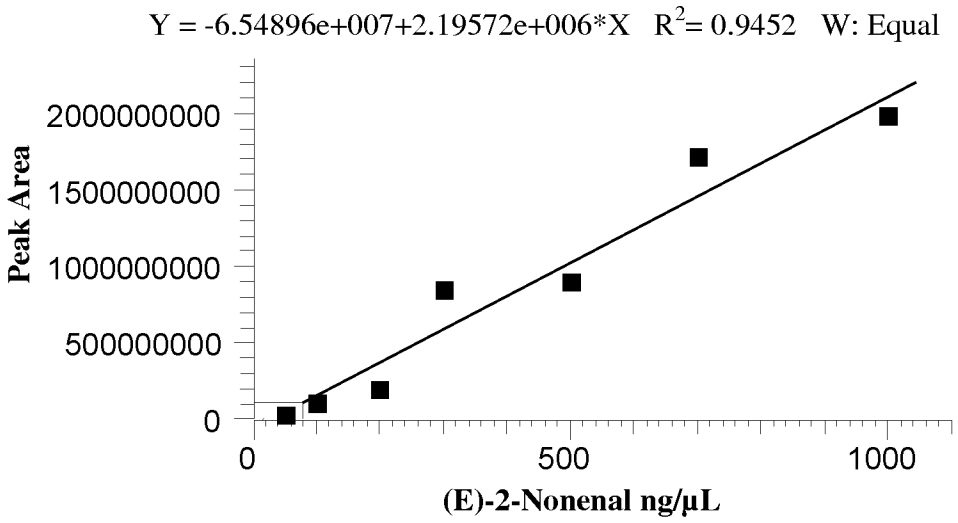

Y = -1.64139e+008+2.99675e+006*X R² = 0.8272 W: Equal
Peak Area
1000000000
500000000
0
0
100
200
300
400
500
Furfural ng/µL

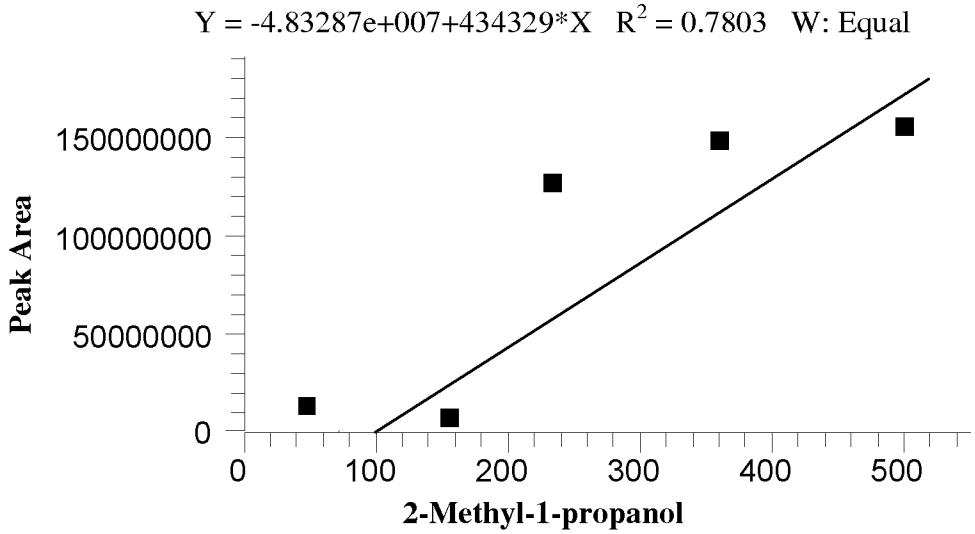

Y = -1.6692e+008+2.63434e+006*X R^2 = 0.9464 W: Equal
Peak Area
1500000000
1000000000
500000000
0
0
200
400
600
Iso amyl acetate ng/µL


Y = -1.30473e+006+2.79299e+006*X R^2 = 0.8675 W: Equal
Peak Area
3000000000
2500000000
2000000000
1500000000
1000000000
500000000
0
0
500
1000
α- Pinene ng/µL


Y = 3.34287e+007+7.8567e+006*X R²= 0.8723 W: Equal
Peak Area
8000000000
6000000000
4000000000
2000000000
0
0
500
1000
DMTS ng/µL


Y = -2.65289e+008+3.70445e+006*X R² = 0.9607 W: Equal
Peak Area
3000000000
2000000000
1000000000
0
0
500
1000
Limonene ng/µL


Y = -6.54896e+007+2.19572e+006*X R²= 0.9452 W: Equal
Peak Area
2000000000
1500000000
1000000000
500000000
0
0
500
1000
(E)-2-Nonenal ng/µL


Y = -4.83287e+007+434329*X R² = 0.7803 W: Equal
Peak Area
150000000
100000000
50000000
0
0
100
200
300
400
500
2-Methyl-1-propanol

Appendix B

1.Descriptive statistics IPA

1.1 Descriptive Statistics: 2,3-Pentanedione Fresh

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR
2,3-Pentanedione Fresh	4	0	29.6	23.7	9.2	12.3	22.8	53.9	63.9	41.6

Variable	Skewness	Kurtosis
2,3-Pentanedione Fresh	1.54	2.86

Descriptive Statistics: 2,3 -pentanedione-60/1

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR
2,3 -pentanedione-60/1	5	0	32.8	43.2	5.4	7.1	9.7	70.2	107.7	63.1

Variable	Skewness	Kurtosis
2,3 -pentanedione-60/1	1.92	3.67

Descriptive Statistics: 2,3-PENT 40/21

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness	K
2,3,PENT 40/21	2	0	160	211	11	*	160	*	309	*	*	*

1.2 Descriptive Statistics: 2,3 Butanedione Fresh

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR
2,3 Butanedione Fresh	3	0	6.220	1.066	5.434	5.434	5.794	7.434	7.434	2.000

Variable	Skewness	Kurtosis
2,3 Butanedione Fresh	1.51	*

1.3Descriptive Statistics: 2,3-b-60/1

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness	Kurt
2,3-b-60/1	2	0	8.72	1.75	7.49	*	8.72	*	9.96	*	*	*

1.4 Descriptive Statistics: 2,3-B-40/21

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness	kurt
2,3-B-40/21	2	0	21.22	12.36	12.48	*	21.22	*	29.96	*	*	*

1.5 Descriptive Statistics: pinene

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
pinene	8	0	12.78	17.63	4.21	5.81	6.42	9.49	56.22	3.69	2.78

Pinene Fresh	Kurtosis
	7.77

1.6 Descriptive Statistics: alpha pinene at 60/1

```
Variable     N  N*  Mean  StDev  Minimum   Q1  Median   Q3  Maximum  IQR  Skewness  K
alphaP 60/1  12   0  25.4   57.8      2.1  4.8     5.5  6.8    204.2  1.9      3.19

                   Kurtosis
                    10.40
```

1.7 Descriptive Statistics: Alpha pinene 40/21

```
Variable            N  N*  Mean  StDev  Minimum   Q1  Median    Q3  Maximum   IQR
Skewness
Alpha pinene 40/21  7   0  36.4   53.0      6.5  6.6    13.1  60.2    148.5  53.6
2.06

Variable            Kurtosis
Alpha pinene 40/21      4.10
```

1.8 Descriptive Statistics: Limonene Fresh

```
Variable        N  N*   Mean  StDev  Minimum     Q1  Median     Q3  Maximum    IQR
Skewness
Limonene Fresh  7   0  3.575  0.632    2.865  3.094   3.575  3.965    4.694  0.872
0.82

Variable        Kurtosis
Limonene Fresh      0.31
```

1.9 Descriptive Statistics: Limonene 60/1

```
Variable        N  N*   Mean  StDev  Minimum     Q1  Median     Q3  Maximum    IQR
Skewness
Limonene 60/1  13   0  4.569  2.781    2.867  2.877   3.034  5.227   10.714  2.350
1.78

Variable       Kurtosis
Limonene 60/1      2.00
```

1.10 Descriptive Statistics: Limone 40/21

```
Variable      N  N*  Mean  StDev  Minimum    Q1  Median     Q3  Maximum   IQR
Skewness
Limone 40/21  7   0  6.00   3.89     2.79  2.87    4.97  11.52    11.52  8.64
0.98

Variable      Kurtosis
Limone 40/21     -1.08
```

1.11 Descriptive Statistics: NON-fresh

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
NON-fresh	11	0	7.20	7.28	2.76	3.15	4.18	8.07	22.23	4.92	1.77

Variable	Kurtosis
NON-fresh	1.65

1.12 Descriptive Statistics: nonenal 60/1

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
nonenal 60/1	10	0	6.66	6.36	2.78	2.89	3.32	8.30	21.13	5.41	1.83

Variable	Kurtosis
nonenal 60/1	2.38

1.13 Descriptive Statistics: Nonenal40/21

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
Nonenal40/21	5	0	30.3	32.6	3.3	3.3	26.7	59.1	82.9	55.8	1.27

Variable	Kurtosis
Nonenal40/21	1.60

1.14 Descriptive Statistics: Furfural fresh

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
Furfural fresh	2	8	3.465	0.792	2.905	*	3.465	*	4.025	*	*

Variable	Kurtosis
Furfural fresh	*

1.15 Descriptive Statistics: furfural 60/1

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
furfural 60/1	10	0	6.28	5.89	3.22	3.91	4.39	5.60	22.92	1.69	3.07

Variable	Kurtosis
furfural 60/1	9.58

1.16 Descriptive Statistics: furfural 40/21

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
furfural 40/21	4	0	18.86	12.01	4.20	6.70	20.28	29.59	30.67	22.90	-0.45

Variable	Kurtosis
furfural 40/21	-2.39

1.17 Descriptive Statistics: Ethyl Nicotinate 60/1

```
Variable              N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum
IQR
Ethyl Nicotinate 60/1  3   0  13.02   5.17     7.13  7.13   15.11  16.81    16.81
9.67

Variable              Skewness  Kurtosis
Ethyl Nicotinate 60/1    -1.52         *
```

1.18 Descriptive Statistics: EthylNicotinate 40/21

```
Variable              N  N*  Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
EthylNicotinate 40/21  2   0  20.0   18.2      7.1   *    20.0   *     32.9    *
*

Variable              Kurtosis
EthylNicotinate 40/21         *
```

1.19 Descriptive Statistics: Ethyl lactate 60/1

```
Variable            N  N*    Mean   StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
Ethyl lactate 60/1  2   0  4.6062  0.0382   4.5792   *  4.6062   *   4.6331    *
*

Variable            Kurtosis
Ethyl lactate 60/1         *
```

1.20Descriptive Statistics: ethyl lactate 40/21

```
Variable             N  N*  Mean  StDev  Minimum   Q1  Median     Q3  Maximum    IQR
ethyl lactate 40/21  3   0  47.2   73.8      4.4  4.4     4.8  132.4    132.4  128.0

Variable             Skewness  Kurtosis
ethyl lactate 40/21      1.73         *
```

1.21 Descriptive Statistics: DMTS fresh

```
Variable    N  N*   Mean  StDev  Minimum    Q1  Median    Q3  Maximum   IQR  Skewness
DMTS fresh  7   0  16.05  26.42     4.07  4.76    5.27  9.30    75.82  4.54      2.62

Variable    Kurtosis
DMTS fresh      6.89
```

1.22 Descriptive Statistics: DMTS60/1

```
Variable  N  N*  Mean  StDev  Minimum   Q1  Median    Q3  Maximum   IQR  Skewness
DMTS60/1  7   0  24.8   31.8      4.5  5.2     8.8  47.3     88.6  42.0      1.76
                      Kurtosis
                      2.44
```

1.23 Descriptive Statistics: IAA fresh

```
Variable    N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR
Skewness
IAA fresh  12   0  31.82  33.90     2.46  5.60   27.24  37.74   126.57  32.14
2.19

Variable   Kurtosis
IAA fresh      5.88
```

1.24 Descriptive Statistics: IAA 60/1

```
Variable   N  N*  Mean  StDev  Minimum    Q1  Median     Q3  Maximum   IQR  Skewness
IAA 60/1  12   0  77.5   42.3      6.0  42.2    91.7  107.9    133.6  65.6     -0.25

Variable  Kurtosis
IAA 60/1     -1.20
```

1.25 Descriptive Statistics: IAA 40/21

```
Variable   N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR  Skewness
IAA 40/21  6   0  26.51  18.37     2.65  3.11   36.19  39.87    41.43  36.77     -0.91

Variable   Kurtosis
IAA 40/21     -1.87
```

1.26 Descriptive Statistics: 2-methyl1-prop Fresh

```
Variable               N  N*  Mean  StDev  Minimum    Q1  Median    Q3  Maximum   IQR
2-methyl1-prop Fresh  13   0  38.2   39.6     13.6  21.0    21.6  49.2    162.9  28.1

Variable              Skewness  Kurtosis
2-methyl1-prop Fresh      3.00      9.74
```

1.27 Descriptive Statistics: 2-MP-60/1

```
Variable    N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR
Skewness
2-MP-60/1  17   0  139.9  144.9     22.2  23.0    62.9  254.4    427.5  231.4
0.89

Variable   Kurtosis
2-MP-60/1     -0.74
```

1.28 Descriptive Statistics: 2-MP-40/21

```
Variable    N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR
Skewness
2-MP-40/21  6   0  210.5  145.4     30.4  55.1   234.5  350.2    350.4  295.1     -
0.27

Variable    Kurtosis
2-MP-40/21     -2.51
```

2. Descriptive statistics Pilsner

2.1 Descriptive Statistics: 2,3-Butanedione Fresh

```
Variable               N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
2,3-Butanedione Fresh  1   0  8.9900      *   8.9900   *  8.9900   *   8.9900    *
*

Variable               Kurtosis
2,3-Butanedione Fresh         *
```

2.2 Descriptive Statistics: 2,3-Butanedione 60/1

```
Variable              N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
2,3-Butanedione 60/1  1   0  15.390      *   15.390   *  15.390   *   15.390    *
*

Variable              Kurtosis
2,3-Butanedione 60/1         *
```

2.3 Descriptive Statistics: 2,3-BUTANEDIONE 40/21pils

```
Variable                  N  N*  Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
2,3-BUTANEDIONE 40/21pil  2   0   142    193        5   *     142   *      279    *
*

Variable                  Kurtosis
2,3-BUTANEDIONE 40/21pil         *
```

2.4 Descriptive Statistics: 2-METHYL 1-PROP fRESH

```
Variable               N  N*  Mean  StDev  Minimum    Q1  Median     Q3  Maximum   IQR
2-METHYL 1-PROP fRESH  7   0  9.88   7.25     4.38  4.46    6.50  13.37    24.69  8.91

Variable               Skewness  Kurtosis
2-METHYL 1-PROP fRESH      1.76      3.08
```

2.5 Descriptive Statistics: 2mp-60/1

```
Variable  N  N*  Mean  StDev  Minimum  Q1  Median   Q3  Maximum  IQR  Skewness
Kurtosis
2mp-60/1  3   0   214    344       15  15      17  611      611  597      1.73
```

2.6 Descriptive Statistics: IAA40/21

```
Variable  N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR  Skewness
IAA40/21  6   0  117.9  202.4     20.8  24.7    35.1  177.5    530.1  152.9      2.43

Variable  Kurtosis
IAA40/21      5.90
```

2.7 Descriptive Statistics: Ethyl nicotinate FRESH

```
Variable                 N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Ethyl nicotinate  FRESH  1   0  3.1294      *   3.1294   *  3.1294   *   3.1294    *

Variable                 Skewness  Kurtosis
Ethyl nicotinate  FRESH         *         *
```

2.8 Descriptive Statistics: Ethyl nicotinate 60/1

```
Variable               N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
Ethyl nicotinate 60/1  1   0  2.2800      *   2.2800   *  2.2800   *   2.2800    *
*

Variable               Kurtosis
Ethyl nicotinate 60/1         *
```

2.9 Descriptive Statistics: ethyl nicotinate 40/21

```
Variable                N  N*    Mean   StDev  Minimum  Q1  Median  Q3  Maximum  IQR
ethyl nicotinate 40/21  2   0  3.1216  0.0203   3.1073   *  3.1216   *   3.1360    *

Variable                Skewness  Kurtosis
ethyl nicotinate 40/21         *         *
```

2.10 Descriptive Statistics: Ethyl lactate Fresh

```
Variable             N  N*      Mean  StDev   Minimum  Q1    Median  Q3   Maximum  IQR
Ethyl lactate Fresh  1   0  0.000000      *  0.000000   *  0.000000   *  0.000000    *

Variable             Skewness  Kurtosis
Ethyl lactate Fresh         *         *
```

2.11 Descriptive Statistics: Ethyl lactate 60/1day

```
Variable               N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
Ethyl lactate 60/1day  1   0  10.700      *   10.700   *  10.700   *   10.700    *
*

Variable               Kurtosis
Ethyl lactate 60/1day         *
```

2.12 Descriptive Statistics: Ethyl lactate 40/21

```
Variable             N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
Ethyl lactate 40/21  1   0  49.662      *   49.662   *  49.662   *   49.662    *
*

Variable             Kurtosis
Ethyl lactate 40/21         *
```

2.13 Descriptive Statistics: Alpha Pinene-Fresh

```
Variable            N  N*    Mean   StDev  Minimum  Q1  Median  Q3  Maximum  IQR
Skewness
Alpha Pinene-Fresh  2   0  6.9939  0.1133   6.9138   *  6.9939   *   7.0740    *
*

Variable            Kurtosis
Alpha Pinene-Fresh         *
```

2.14 Descriptive Statistics: AlphaPinene-60/1

```
Variable          N  N*  Mean  StDev  Minimum    Q1  Median    Q3  Maximum   IQR
Skewness
AlphaPinene-60/1  4   0  5.26   2.84     1.05  2.31    6.49  6.98     7.02  4.67     -
1.88

Variable          Kurtosis
AlphaPinene-60/1      3.57
```

2.15 Descriptive Statistics: Alpha pinene-40/21

```
Variable            N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR
Alpha pinene-40/21  4   0  10.50   7.07     6.46  6.46    7.25  17.79    21.05  11.33

Variable            Skewness  Kurtosis
Alpha pinene-40/21      1.94      3.77
```

2.16 Descriptive Statistics: Limonen Fresh

```
Variable       N  N*   Mean  StDev  Minimum     Q1  Median     Q3  Maximum    IQR
Skewness
Limonen Fresh  3   0  1.807  0.923    1.198  1.198   1.354  2.869    2.869  1.671
1.68

Variable       Kurtosis
Limonen Fresh         *
```

2.17 Descriptive Statistics: LIMO 60/1

```
Variable   N  N*    Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR  Skewness
Kurtosis
LIMO 60/1  1   0  2.8873      *   2.8873   *  2.8873   *   2.8873    *         *
*
```

2.18 Descriptive Statistics: LIMO 40/21

```
Variable    N  N*  Mean  StDev  Minimum    Q1  Median    Q3  Maximum   IQR  Skewness
LIMO 40/21  3   0  4.21   2.31     2.87  2.87    2.87  6.88     6.88  4.01      1.73

Variable    Kurtosis
LIMO 40/21         *
```

2.19 Descriptive Statistics: DMTS40/21

```
Variable   N  N*   Mean  StDev  Minimum  Q1  Median  Q3  Maximum  IQR  Skewness
Kurtosis
DMTS40/21  2   0  8.250  1.019    7.529   *   8.250   *    8.970    *         *
*
```

2.20 Descriptive Statistics: FURFURAL Fresh

```
Variable        N  N*   Mean  StDev  Minimum     Q1  Median     Q3  Maximum    IQR
Skewness
FURFURAL Fresh  3   0  4.282  0.287    3.951  3.951   4.442  4.453    4.453  0.502
-1.73

Variable        Kurtosis
FURFURAL Fresh         *
```

2.21 Descriptive Statistics: Furfural 60/1 day

```
Variable           N  N*   Mean  StDev  Minimum     Q1  Median     Q3  Maximum    IQR
Furfural 60/1 day  3   0  4.382  1.637    3.063  3.063   3.870  6.214    6.214  3.151

Variable           Skewness  Kurtosis
Furfural 60/1 day      1.27         *
```

2.22 Descriptive Statistics: Furfural 40/21 days

```
Variable  N  N*   Mean  StDev  Minimum    Q1  Median     Q3  Maximum    IQR  Skewness
F3        4   0  12.96  10.62     3.72  3.77   12.26  22.86    23.61  19.09      0.07

Variable  Kurtosis
F3           -5.60
```

2.23 Descriptive Statistics: 2mpfresh

```
Variable  N  N*  Mean  StDev  Minimum    Q1  Median     Q3  Maximum   IQR  Skewness
Kurtosis
2mpfresh  7   0  9.88   7.25     4.38  4.46    6.50  13.37    24.69  8.91      1.76
3.08
```

2.24 Descriptive Statistics: 2mp-60/1

```
Variable  N  N*  Mean  StDev  Minimum  Q1  Median   Q3  Maximum  IQR  Skewness
Kurtosis
2mp-60/1  3   0   214    344       15  15      17  611      611  597      1.73
```

2.25 Descriptive Statistics: 2-MP40/21

```
Variable   N  N*  Mean  StDev  Minimum  Q1  Median   Q3  Maximum  IQR  Skewness
Kurtosis
2-MP40/21  5   0   311    542       13  17      86  716     1274  699      2.17
4.76
```

2.26 Descriptive Statistics: Nonenal fresh

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
Nonenal fresh	3	0	2.974	0.191	2.856	2.856	2.872	3.194	3.194	0.338	1.72

Variable	Kurtosis
Nonenal fresh	*

2.27 Descriptive Statistics: Nonenal 60/1

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness	Ku
Nonenal 60/1	2	0	1.34	1.89	0.00	*	1.34	*	2.68	*	*	*

2.28Descriptive Statistics: Nonenal 60/1

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness	Ku
Nonenal 60/1	2	0	1.34	1.89	0.00	*	1.34	*	2.68	*	*	*

2.29 Descriptive Statistics: nonenal 40/21

Variable	N	N*	Mean	StDev	Minimum	Q1	Median	Q3	Maximum	IQR	Skewness
nonenal 40/21	6	0	3.510	1.589	2.737	2.779	2.798	4.081	6.734	1.302	2.39

Variable	Kurtosis
nonenal 40/21	5.76

Appendix C

3. IPA

3.1 Mann-Whitney Test and CI: 2,3-Pentanedione Fresh, 2,3 -pentanedione-60/1

```
                        N  Median
2,3-Pentanedione Fresh  4   22.76
2,3 -pentanedione-60/1  5    9.67


Point estimate for η1 - η2 is 7.87
96.3 Percent CI for η1 - η2 is (-85.99,55.09)
W = 22.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.7133
```

3.2 Mann-Whitney Test and CI: 2,3 -pentanedione-60/1, 2,3-PENT 40/21

```
                        N  Median
2,3 -pentanedione-60/1  5     9.7
2,3-PENT 40/21          2   159.9


Point estimate for η1 - η2 is -103.4
91.9 Percent CI for η1 - η2 is (-303.6,96.9)
W = 17.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.3329
```

3.3 Mann-Whitney Test and CI: 2,3-Pentanedione Fresh, 2,3-PENT 40/21

```
                        N  Median
2,3-Pentanedione Fresh  4    22.8
2,3-PENT 40/21          2   159.9


Point estimate for η1 - η2 is -123.4
89.5 Percent CI for η1 - η2 is (-299.9,53.1)
W = 13.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.8170
```

3.4 Mann-Whitney Test and CI: 2,3 Butanedione Fresh, 2,3-b-60/1

```
                       N  Median
2,3 Butanedione Fresh  3   5.794
2,3-b-60/1             2   8.725


Point estimate for η1 - η2 is -2.291
85.1 Percent CI for η1 - η2 is (-4.527,-0.055)
W = 6.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1489
```

3.5 Mann-Whitney Test and CI: 2,3 Butanedione Fresh, 2,3-B-40/21

```
                        N  Median
2,3 Butanedione Fresh   3    5.79
2,3-B-40/21             2   21.22


Point estimate for η1 - η2 is -14.79
85.1 Percent CI for η1 - η2 is (-24.53,-5.05)
W = 6.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1489
```

3.6 Mann-Whitney Test and CI: 2,3-b-60/1, 2,3-B-40/21

```
              N  Median
2,3-b-60/1    2    8.72
2,3-B-40/21   2   21.22


Point estimate for η1 - η2 is -12.50
75.5 Percent CI for η1 - η2 is (-22.48,-2.51)
W = 3.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.2453
```

3.7 Mann-Whitney Test and CI: pinene, alpha 60/1

```
              N  Median
pinene        8    6.42
alpha 60/1   12    5.53


Point estimate for η1 - η2 is 0.94
95.1 Percent CI for η1 - η2 is (-0.95,4.10)
W = 97.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.3348
The test is significant at 0.3343 (adjusted for ties)
```

3.8 Mann-Whitney Test and CI: alpha 60/1, Alpha pinene 40/21

```
                     N  Median
alpha 60/1          12     5.5
Alpha pinene 40/21   7    13.1


Point estimate for η1 - η2 is -5.4
95.3 Percent CI for η1 - η2 is (-53.6,0.0)
W = 99.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0832
The test is significant at 0.0826 (adjusted for ties)
```

3.9 Mann-Whitney Test and CI: Limonene Fresh, Limonene 60/1

```
                  N  Median
Limonene Fresh    7   3.575
Limonene 60/1    13   3.034


Point estimate for η1 - η2 is 0.059
95.2 Percent CI for η1 - η2 is (-1.655,0.781)
W = 75.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.9368
The test is significant at 0.9368 (adjusted for ties)
```

3.10 Mann-Whitney Test and CI: Limonene Fresh, Limone 40/21

```
                N  Median
Limonene Fresh  7   3.575
Limone 40/21    7   4.973


Point estimate for η1 - η2 is -1.229
95.9 Percent CI for η1 - η2 is (-7.778,0.779)
W = 45.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.3711
The test is significant at 0.3700 (adjusted for ties)
```

3.11 Mann-Whitney Test and CI: Limonene 60/1, Limone 40/21

```
                N  Median
Limonene 60/1  13   3.034
Limone 40/21    7   4.973


Point estimate for η1 - η2 is -0.276
95.2 Percent CI for η1 - η2 is (-6.285,0.347)
W = 129.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.5791
The test is significant at 0.5787 (adjusted for ties)
```

3.12 Mann-Whitney Test and CI: NON-fresh, nonenal 60/1

```
               N  Median
NON-fresh     11    4.18
nonenal 60/1  10    3.32


Point estimate for η1 - η2 is 0.25
95.5 Percent CI for η1 - η2 is (-2.81,1.40)
W = 127.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.6985
The test is significant at 0.6977 (adjusted for ties)
```

3.13 Mann-Whitney Test and CI: NON, Nonenal2

```
           N  Median
NON       11    4.18
Nonenal2   5   26.65


Point estimate for η1 - η2 is -22.48
95.9 Percent CI for η1 - η2 is (-61.84,0.87)
W = 78.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0893
```

3.14 Mann-Whitney Test and CI: nonenal 1, Nonenal2

```
            N  Median
nonenal 1  10    3.32
Nonenal2    5   26.65


Point estimate for η1 - η2 is -20.70
95.7 Percent CI for η1 - η2 is (-67.54,0.16)
W = 65.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0758
The test is significant at 0.0755 (adjusted for ties)

MTB > Mann-Whitney 95.0 'Furfural 1' 'Furfural 2';
SUBC>   Alternative 0.
```

3.15 Mann-Whitney Test and CI: Furfural 1, Furfural 2

```
             N  Median
Furfural 1   2   3.465
Furfural 2  10   4.388


Point estimate for η1 - η2 is -1.174
95.9 Percent CI for η1 - η2 is (-20.011,0.800)
W = 6.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1626
The test is significant at 0.1619 (adjusted for ties)

MTB > Mann-Whitney 95.0 'Furfural 1' 'furfural 3';
SUBC>   Alternative 0.
```

3.16 Mann-Whitney Test and CI: Furfural 1, furfural 3

```
            N  Median
Furfural 1  2    3.47
furfural 3  4   20.28


Point estimate for η1 - η2 is -16.82
89.5 Percent CI for η1 - η2 is (-27.76,-0.18)
W = 3.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1052

MTB > Mann-Whitney 95.0 'furfural 3' 'Furfural 2';
SUBC>   Alternative 0.
```

3.17 Mann-Whitney Test and CI: furfural 3, Furfural 2

```
             N  Median
furfural 3   4   20.28
Furfural 2  10    4.39


Point estimate for η1 - η2 is 10.29
96.0 Percent CI for η1 - η2 is (-0.25,26.22)
W = 42.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1039
The test is significant at 0.1035 (adjusted for ties)
```

```
MTB > Mann-Whitney 95.0 'EN1' 'EN3';
SUBC>   Alternative 0.
* ERROR * Not enough data in column.

MTB > Mann-Whitney 95.0 'EN2' 'EN3';
SUBC>   Alternative 0.
```

3.18 Mann-Whitney Test and CI: EN2, EN3

```
     N  Median
EN2  3   15.11
EN3  2   20.00


Point estimate for η1 - η2 is -8.04
85.1 Percent CI for η1 - η2 is (-25.77,9.71)
W = 9.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 1.0000
```

3.19 Mann-Whitney Test and CI: EL2, EL3

```
     N  Median
EL2  2     4.6
EL3  3     4.8


Point estimate for η1 - η2 is -0.2
85.1 Percent CI for η1 - η2 is (-127.9,0.2)
W = 5.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.7728

MTB > Mann-Whitney 95.0 'DMTS1' 'DMTS2';
SUBC>   Alternative 0.
```

3.20 Mann-Whitney Test and CI: DMTS1, DMTS2

```
       N  Median
DMTS1  7    5.27
DMTS2  7    8.80


Point estimate for η1 - η2 is -3.19
95.9 Percent CI for η1 - η2 is (-42.00,3.30)
W = 45.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.3711
The test is significant at 0.3706 (adjusted for ties)

MTB > Mann-Whitney 95.0 'DMTS2' 'DMTS 3';
SUBC>   Alternative 0.
```

3.21 Mann-Whitney Test and CI: DMTS2, DMTS 3

```
        N  Median
DMTS2   7    8.80
DMTS 3  3   84.97


Point estimate for η1 - η2 is -43.61
```

```
96.0 Percent CI for η1 - η2 is (-85.63,26.47)
W = 31.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1106

MTB > Mann-Whitney 95.0 'DMTS1' 'DMTS 3';
SUBC>   Alternative 0.
```

3.22 Mann-Whitney Test and CI: DMTS1, DMTS 3

```
        N  Median
DMTS1   7    5.27
DMTS 3  3   84.97


Point estimate for η1 - η2 is -77.14
96.0 Percent CI for η1 - η2 is (-86.15,-9.17)
W = 29.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0402
The test is significant at 0.0396 (adjusted for ties)
```

3.23 Mann-Whitney Test and CI: IAA IPA, IPA IAA2

```
           N  Median
IAA IPA   12   27.24
IPA IAA2  12   91.66


Point estimate for η1 - η2 is -52.14
95.4 Percent CI for η1 - η2 is (-85.42,-9.68)
W = 103.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0073
The test is significant at 0.0072 (adjusted for ties)

MTB > Mann-Whitney 95.0 'IPA IAA2' 'IPA IAA3';
SUBC>   Alternative 0.
```

3.24 Mann-Whitney Test and CI: IPA IAA2, IPA IAA3

```
           N  Median
IPA IAA2  12   91.66
IPA IAA3   6   36.19


Point estimate for η1 - η2 is 54.75
95.6 Percent CI for η1 - η2 is (5.72,92.63)
W = 141.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0131
The test is significant at 0.0130 (adjusted for ties)

MTB > Mann-Whitney 95.0 'IAA IPA' 'IPA IAA3';
SUBC>   Alternative 0.
```

3.25 Mann-Whitney Test and CI: IAA IPA, IPA IAA3

```
           N  Median
IAA IPA   12   27.24
IPA IAA3   6   36.19
```

```
Point estimate for η1 - η2 is -1.11
95.6 Percent CI for η1 - η2 is (-27.45,24.60)
W = 110.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.7431
The test is significant at 0.7429 (adjusted for ties)
```

4 Pilsner

4.1Mann-Whitney Test and CI: N, NON2

```
      N  Median
N     3   2.872
NON2  2   1.340


Point estimate for η1 - η2 is 1.685
85.1 Percent CI for η1 - η2 is (0.175,3.195)
W = 12.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.1489
```

4.2 Mann-Whitney Test and CI: NON2, NON3

```
      N  Median
NON2  2   1.340
NON3  6   2.798


Point estimate for η1 - η2 is -2.765
93.3 Percent CI for η1 - η2 is (-6.735,-0.058)
W = 3.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0668
```

4.3 Mann-Whitney Test and CI: N, NON3

```
      N  Median
N     3   2.872
NON3  6   2.798


Point estimate for η1 - η2 is 0.068
97.2 Percent CI for η1 - η2 is (-3.878,0.457)
W = 18.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.5186
```

4.4 Mann-Whitney Test and CI: 2-METHYL 1-PROP fRESH, 2Mp-1

```
                       N  Median
2-METHYL 1-PROP fRESH  7     6.5
2Mp-1                  3    16.6


Point estimate for η1 - η2 is -10.2
96.0 Percent CI for η1 - η2 is (-607.2,8.0)
W = 30.0
```

```
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0682
```

4.5 Mann-Whitney Test and CI: 2-METHYL 1-PROP fRESH, 2-MPP3

```
                        N  Median
2-METHYL 1-PROP fRESH   7     6.5
2-MPP3                  5    86.1


Point estimate for η1 - η2 is -79.6
96.5 Percent CI for η1 - η2 is (-1264.3,-6.4)
W = 31.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0230

MTB > Mann-Whitney 95.0 '2Mp-1' '2-MPP3';
SUBC>   Alternative 0.
```

4.6 Mann-Whitney Test and CI: 2Mp-1, 2-MPP3

```
        N  Median
2Mp-1   3    16.6
2-MPP3  5    86.1


Point estimate for η1 - η2 is -6.2
96.3 Percent CI for η1 - η2 is (-1259.4,598.5)
W = 12.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.7656
```

4.7Mann-Whitney Test and CI: IAA Fresh, IAA40/21

```
           N  Median
IAA Fresh  7    16.6
IAA40/21   6    35.1


Point estimate for η1 - η2 is -16.6
96.2 Percent CI for η1 - η2 is (-484.3,-1.3)
W = 34.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0383

MTB > Mann-Whitney 95.0 'IAA Fresh' 'IAA 60/1';
SUBC>   Alternative 0.
```

4.8 Mann-Whitney Test and CI: IAA Fresh, IAA 60/1

```
           N  Median
IAA Fresh  7    16.6
IAA 60/1   4    18.1


Point estimate for η1 - η2 is 2.8
95.3 Percent CI for η1 - η2 is (-243.2,22.4)
W = 44.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.7768

MTB > Mann-Whitney 95.0 'IAA40/21' 'IAA 60/1';
```

```
SUBC>   Alternative 0.
```

4.9 Mann-Whitney Test and CI: IAA40/21, IAA 60/1

```
          N  Median
IAA40/21  6    35.1
IAA 60/1  4    18.1


Point estimate for η1 - η2 is 19.4
95.7 Percent CI for η1 - η2 is (-226.8,506.7)
W = 39.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.2410
```

4.10 Mann-Whitney Test and CI: Alpha Pinene-Fresh, AlphaPinene-60/1

```
                    N  Median
Alpha Pinene-Fresh  2   6.994
AlphaPinene-60/1    4   6.489


Point estimate for η1 - η2 is 0.505
89.5 Percent CI for η1 - η2 is (-0.108,6.027)
W = 10.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.2472
```

4.11 Mann-Whitney Test and CI: Alpha pinene-40/21, AlphaPinene-60/1

```
                    N  Median
Alpha pinene-40/21  4    7.25
AlphaPinene-60/1    4    6.49


Point estimate for η1 - η2 is 1.53
97.0 Percent CI for η1 - η2 is (-0.56,20.00)
W = 22.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.3123
```

4.12 Mann-Whitney Test and CI: Alpha Pinene-Fresh, Alpha pinene-40/21

```
                    N  Median
Alpha Pinene-Fresh  2    6.99
Alpha pinene-40/21  4    7.25


Point estimate for η1 - η2 is -0.26
89.5 Percent CI for η1 - η2 is (-14.13,0.61)
W = 7.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 1.0000
```

4.13 Mann-Whitney Test and CI: FURFURAL Fresh, Furfural 60/1 day

```
                   N  Median
FURFURAL Fresh     3   4.442
Furfural 60/1 day  3   3.870
```

```
Point estimate for η1 - η2 is 0.571
91.9 Percent CI for η1 - η2 is (-2.264,1.391)
W = 12.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.6625
```

4.14 Mann-Whitney Test and CI: F3, Furfural 60/1 day

```
                  N  Median
F3                4   12.26
Furfural 60/1 day 3    3.87


Point estimate for η1 - η2 is 7.62
94.8 Percent CI for η1 - η2 is (-2.50,20.54)
W = 19.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.3768
```

4.15 Mann-Whitney Test and CI: FURFURAL Fresh, F3

```
               N  Median
FURFURAL Fresh  3    4.44
F3              4   12.26


Point estimate for η1 - η2 is -8.06
94.8 Percent CI for η1 - η2 is (-19.66,0.73)
W = 12.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 1.0000
```

4.16 Mann-Whitney Test and CI: 2mpfresh, 2mp-60/1

```
          N  Median
2mpfresh  7     6.5
2mp-60/1  3    16.6


Point estimate for η1 - η2 is -10.2
96.0 Percent CI for η1 - η2 is (-607.2,8.0)
W = 30.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0682
```

4.17 Mann-Whitney Test and CI: 2-MP40/21, 2mpfresh

```
           N  Median
2-MP40/21  5    86.1
2mpfresh   7     6.5


Point estimate for η1 - η2 is 79.6
96.5 Percent CI for η1 - η2 is (6.4,1264.3)
W = 47.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.0230
```

4.18 Mann-Whitney Test and CI: 2mp-60/1, 2-MP40/21

```
           N  Median
2mp-60/1   3    16.6
2-MP40/21  5    86.1


Point estimate for η1 - η2 is -6.2
96.3 Percent CI for η1 - η2 is (-1259.4,598.5)
W = 12.0
Test of η1 = η2 vs η1 ≠ η2 is significant at 0.7656
```