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ANALYSIS OF ACETALDEHYDE CONCENTRATION IN AGED BEER USING DERIVATIZATION AND LIQUID-LIQUID EXTRACTION METHODS

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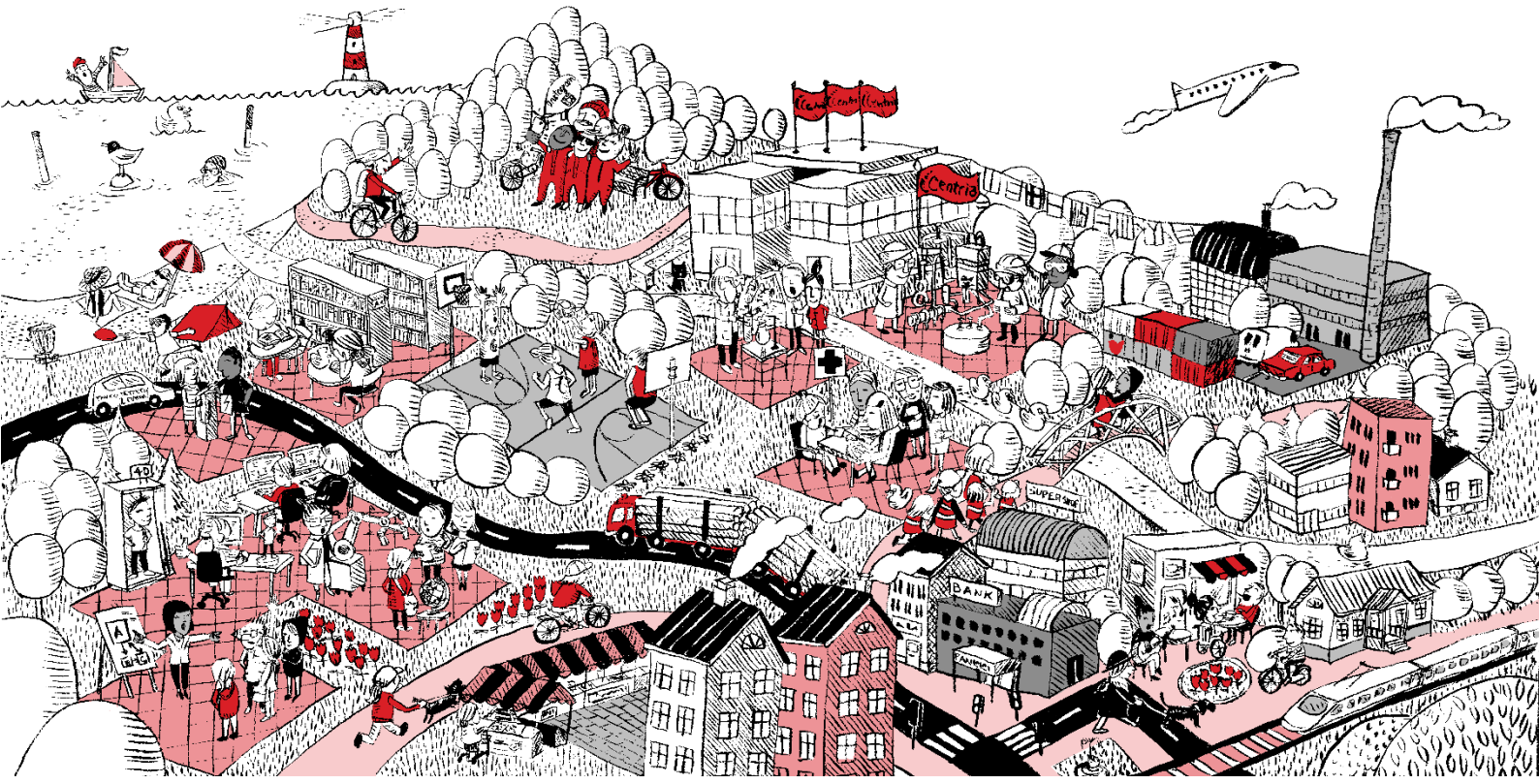

ABSTRACT

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The flavour of beer is a significant component for beer producers. It is important to consistently produce quality beer with the expected flavour and aroma. However, after packaging, flavour compounds in beer are not in a state of chemical equilibrium but are involved in chemical reactions that occur until the beer is consumed. These reactions typically result in a decrease of the expected fresh flavour and an increase in compounds that correspond to what is known as aged flavours. These aged flavours are unpleasant and include cardboard and green apple flavour. One of the compounds that contributes to aged flavour in beer is acetaldehyde.

The aim of this thesis was to analyse the concentration of acetaldehyde in the lager beer called Helles produced by Kahakka Breweries in Kokkola, Finland. Three different batches of Helles produced approximately four months apart were analysed using GC-MS. Due to the volatile nature of acetaldehyde, the beer was derivatized first using O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride to form more stable acetaldehyde oxime isomers and extracted via liquid-liquid extraction before GC-MS analysis.

The results showed that the levels of acetaldehyde in the beer were below the flavour threshold of approximately 10 mg/L, with the maximum detection at 7.98 mg/L and the lowest at 1.40 mg/L. The method used proved that it could be used for the detection of acetaldehyde in beer. However, the accuracy and repeatability of the method could not be proved as the experiment was conducted once. Furthermore, the method used was extremely time consuming (approximately 6 hours) and had many steps which increased the potential for errors and inaccurate results. It is proposed that a different method such as headspace, solid phase microextraction or stir-bar sorptive analysis is employed which are less time consuming and have less potential for errors. These methods could then be repeated to determine their accuracy and repeatability before conducting the experiment for more accurate results.

Key words
Acetaldehyde, beer, brewing, flavour, taste threshold, GC-MS

CONCEPT DEFINITIONS

BU	Bitterness Units
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
IS	Internal standard
LLE	Liquid-liquid extraction
MSD	Mass Selective Detector
PFBHA	O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine
RF	Response factor
RPM	Rounds per minute
ROS	Reactivity oxygen series
RRF	Relative response factor
SIM	Selected ion monitoring
SRM	Selected reaction monitoring

Formulas of chemical compounds

$C_6F_5CH_2ONH_2$	O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride
CH_3CHO	Acetaldehyde
CH_3CH_2OH	Ethanol
$C_6H_{12}O_6$	Sugar
$C_7H_2F_4O$	2,3,5,6 – Tetrafluorobenzaldehyde
$C_9H_6F_5NO$	Acetaldehyde-O-pfbo
CO_2	Carbon dioxide
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid

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1 INTRODUCTION

Beer flavour occurs due to the complex interactions of hundreds of chemical compounds. Taste-compounds are sensed directly on the tongue, while aroma is based on volatized compounds that are sensed nasally or retro-nasally through the back of the mouth. However, these flavour-inducing compounds are not in chemical equilibrium in freshly packaged beer and are subject to chemical reactions during storage. Some of these chemical reactions include Strecker degradation, aldol condensation, furanic ether formation, degradation of hop bitter acids, Maillard reactions, and acetate esters. These reactions result in a decrease of fresh flavour notes and a rise in some typical aged flavours, such as cardboard flavour. Some of the volatile compounds that are formed during storage of the beer include linear aldehydes, ketones, ethyl esters, and lactones. (Vanderhaegen, Neven, Verachtert & Derdelinckx 2006, 358-363.) One common aged flavour is acetaldehyde. This compound gives the flavour of green apples or cut grass in beer which is often undesirable. (Briggs et al. 2004, 456.) This instability of beer flavour during aging is of great concern to brewers, especially since customers expect to recognize the flavour of a particular brand of beer that they typically drink, even after the beer has been stored for a particular amount of time. Thus, beer flavour is thought to be the most important quality parameter of the product. (Saison, De Scutter, Uyttenhove, Delvaux & Delvaux 2009a, 1206.)

The changes in beer compounds during storage were first analysed in the 1960's due to the introduction of gas chromatography (GC) (Vandehaegen et al. 2006, 359). GC is a method of separating components in a mixture using two phases; the stationary phase which is a stationary bed having a large surface area, and the mobile phase, which is an inert gas that percolates through the stationary bed. The sample is vaporized and carried with the mobile gas phase through the stationary phase. The components of the sample are separated based on their relative vapor pressures and affinity for the stationary bed. (McNair & Miller 2009, 3.)

The aim of this thesis was to find out the concentrations of acetaldehyde in the lager 'Helles' from Kahakka Brewery in Kokkola, Finland. This thesis planned to compare the concentrations of acetaldehyde from three different batches with different brew dates that span a year, to note if there are any significant differences between the batches due to age, and find out if any of the batches exceed the flavour threshold of acetaldehyde which is approximately 10 mg/L. The analysis was done using gas chromatography mass spectrometry (GC-MS). The results are limited to the beer produced in Kahakka and cannot be generalized to other lagers.

2 THE THEORY OF BEER

In this chapter, the theory of beer is discussed. Firstly, the basics of how beer is brewed is outlined, followed by a discussion on the chemistry of beer flavours (which also reviews some of the most common beer flavours including acetaldehyde), then a brief discussion on beer aging and the aging reactions that results in acetaldehyde concentrations rising in beer.

2.1 Basics of beer brewing

Beer is a popular alcoholic beverage whose four main ingredients are water, malt (barley), hops and yeast. Occasional additional ingredients, known as adjuncts, may be used. Adjuncts are materials used to supplement the malt by adding starch or sugar. (De Keukeleire 2000, 108.) To better understand the different flavours of beer, it is important to know the brewing processes to see which reactions and ingredients contribute to the flavours.

There are a multitude of methods for brewing beer throughout the world. To limit the scope of this thesis, a summary of the most common brewing method in Europe will be discussed. In the brewing process, carbohydrates from malted barley are extracted and broken down to make a sugar solution known as wort. This solution contains the necessary nutrients for yeast growth. In yeast fermentation, the simple sugars in the wort are consumed, releasing energy and making ethanol and flavourful metabolic byproducts. (Willaert 2007, 444.)

Before beginning the brewing process, malt must be obtained from barley grains. This is done in a process known as malting. The barley is first steeped in water, and the water seeps into the barley grains through a small opening known as the micropyle. This triggers the production of enzymes. These enzymes breakdown the starchy endosperm of the grains, which is the part that makes the grains hard and difficult to mill. This process of softening the grain takes about four to six days. During this time, the grains begin to germinate. When the germination stage is believed to have progressed for a long enough period of time, the grains are heated up in a process known as kilning, so that the germination can cease. The aim of this process is also to dehydrate the grains until the moisture level in them is below 5 %. This heating process must be done carefully, as the enzyme amylase, in the grain, that was produced during the germination, is needed in the brewing process. Enzymes are susceptible to heat and high levels of

moisture, and so the kilning begins at a relatively low temperature (approximately 50 °C) and is slowly and steadily increased. After the kilning, the grains are now called finished malt. (Bamforth 2003, 51-52.)

Malt used for ale production are kilned at higher temperatures, which results in darker malts. During kilning, the sugars and amino acids that were released from the proteins and carbohydrates during germination combine to form melanoidins, which are coloured. The higher the temperature, the darker the color, though this depends on how many sugars and amino acids are present in the first place. The higher kilning temperatures also results in the development of complex flavours in the malt. Malt that is kilned to particularly high temperatures develop flavours described as “smoky” and “burnt”. (Bamforth 2003, 52-53.)

The brewing process begins with milling and mashing. The aim of these processes is to convert the starch in the malt to sugar. The malt grains are crushed using a mill in order to increase their surface area. The crushed grains are known as grist. The grist is then mixed with hot water in the second stage of brewing (mashing). The mashing is done in a mash tun (a tun is a type of tank used for holding beer and wines). The starch in the grist is converted to sugars via the amylase that was in the grains. As much of the starch as possible should be converted into sugars. The sugary solution formed is known as wort. (Barth 2013, 132 – 133.)

After milling and mashing, when no more starch worth recovering is still in the grist, the wort must be separated from the spent grains in a process known as wort separation. This is a filtration process that can be performed in the mash tun. Many mash tuns, especially those used in craft breweries, have a false bottom. This false bottom has some holes that keep back the grain while the wort flows through. The spent grain settles and forms the filtration layer. The wort is pumped through the grain bed and recirculated until it runs clear. (Barth 2013, 149 – 150.)

After wort separation, comes wort boiling. This is a complex process in which a wide range of reactions occur including chemical, physical, physio-chemical, and biochemical. It is also the most energy-intensive stage of the brewing process. The wort is typically boiled for about an hour. Wort boiling is done to kill off bacteria and wild yeast, deactivate the enzymes, dissolve the hops (which are added during boiling) and cause the hop resins to change molecular form. Bad flavour compounds go off with the steam (they are volatized), and grain proteins clump together making it easier to remove them. The set-up of the boiling process is to bring all parts of the boiling wort to the surface where it can come in

contact with steam. (Barth 2013, 152 -153.) The hops added during the wort boiling can be whole or milled and pelletized. The hops added at the beginning of the boiling will lose nearly all their oils. Therefore, some more hops are added towards the end of the boiling, which allows essential oils to survive and provide essential and distinctive aroma notes. (Bamford 2003, 55 -56.) When the wort boiling is done, the coagulated proteins (known as trub) and other solids are removed. This can be done via a whirlpool or through settling. A whirlpool is a tank where the wort flows in a circular motion, causing the solids to collect at the bottom centre of the tank. (Barth 2013, 157.)

The next step is fermentation, but before this can occur the hot wort should be chilled from approximately 100 °C to between 22 °C – 7 °C. The chilling occurs rapidly to avoid the formation of off-flavour compounds. This is done using a counter current heat exchanger. The hot wort flows through channels while cold water or other coolants flow in channels in the opposite direction. Heat is transferred from the wort to the cold water. After the wort is chilled to the desired temperature, a small amount of oxygen is bubbled through the wort before the yeast is added. The fermentation process is anaerobic but the yeast does require some oxygen to help it make parts of its cell membrane and grow. (Bamforth 2003, 57.)

Fermentation occurs when yeast is added (an action known as pitching) because yeast feeds on the sugars in the wort and produces ethanol, carbon-dioxide and energy. This conversion of sugars to alcohol is depicted in equation 1 (Barth 2013):

$$C_6H_{12}O_6 \text{ (sugar)} \rightarrow 2CH_3CH_2OH \text{ (ethanol)} + 2CO_2 \text{ (carbon-dioxide)} + \text{energy} \quad (1)$$

The rate at which fermentation occurs is directly proportional to temperature and the amount of yeast added. Ale fermentations can take two to three days, while traditional lager can take up to a fortnight to ferment. This process also involves the production of a mixture of subtle flavour compounds that gives beer certain characteristics. The balance of these flavours depends on the yeast strain used. (Bamforth 2003, 57.) Many different yeast strains exist for use in distilling and brewing. Having alternative yeast strains allows for more efficient fermentations and diverse flavour characteristics in beers. (Stewart 2017, 7.)

After fermentation, the beer is chilled to stabilize it. It is chilled to as low a temperature as possible without freezing the beer, which is approximately -1 °C, for about three days. Cold-sensitive proteins are filtered out, and materials can be added to the beer to improve its stability. Proteins and polyphenols

are removed by some of the added materials, while others act as antioxidants which prevents beer from staling. The beer is then filtered into beer tanks to await packaging. The packaging should be done such that there is no oxygen added in the beer as this results in rapid staling. (Bamforth 2003, 58.) A summary of the beer-making process is depicted in Figure 1.

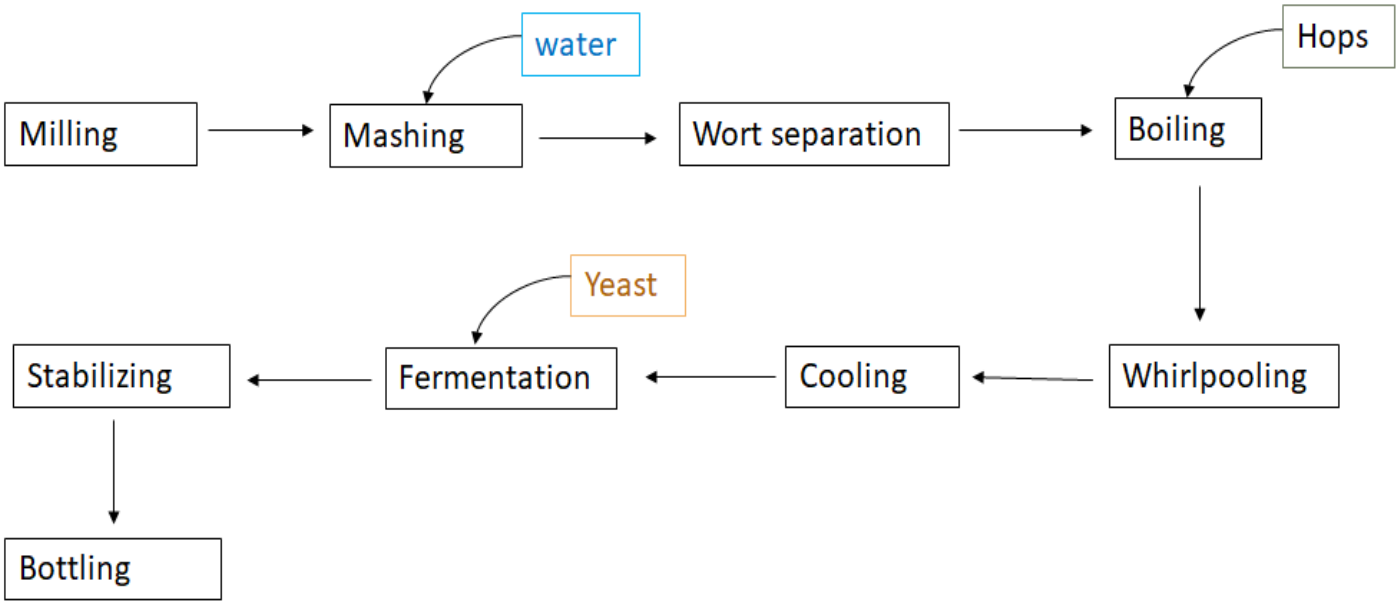


FIGURE 1. A general process flow diagram of beer production (adapted from Lordan, O'Keefe, Tsoupras & Zabetakis 2019, 5)

2.2 The chemistry of beer flavour

The flavours in beer vary widely depending on how much flavour compounds are present and how strong the flavour is. For example, the amount of ethanol in beer is approximately 40 grams per litre. Ethanol can be detected at a level of 14 grams per litre, which is its flavour threshold. When the total weight per litre is divided by the flavour threshold, the result is known as the flavour units (thus ethanol has approximately 2.9 flavour units in beer). (Barth 2013, 201.)

Additionally, a large aspect of beer flavour is attributed to the beer aroma. There are in fact relatively few compounds that contribute to the true taste of beer (bitter, sweet, salt, and sour). In contrast, multiple

different compounds influence the aroma. Many of these are produced during fermentation such as aldehydes, esters, and fusel alcohols. (Bamforth 2003, 74.) A few of the compounds contributing to the overall taste and aroma of beer will be discussed below.

2.2.1 Hops flavour

Hops in beer, account, not only for the bitter taste, but also a distinct, delicate hoppy flavour. The hoppy aroma in beer is due to a complex of sensory impressions which comes from many different volatile compounds, many of which act in synergism. Throughout the multi-staged brewing process, hop-derived components arise, that may contribute to the overall sensory characteristics of the beer. (De Keukeleire 2000, 109.)

Hop flowers produce a yellow sticky powder known as lupulin. Lupulin contains many components, but the most significant is a family of compounds known as alpha acids (or α-acids). In organic chemistry, alpha denotes the first carbon atom that is attached to a functional group. During the wort boiling, the alpha acids are converted to iso-alpha acids in a reaction known as isomerization. In isomerization, a compound is converted to another form having the same molecular formula but different structure. Iso-alpha acids (or iso-α acids) are the source of the bitterness taste in beers. (Barth 2013, 153.)

Alpha acids and iso-alpha acids can have multiple functional groups at the same time, including ketones, alcohols and alkenes. Under the conditions of boiling, approximately 40 % of the alpha acids are converted to iso-alpha acids. In the end, there are only trace amounts of alpha acids left in the beer since the unconverted acids are nearly insoluble at the cold temperature that is used for conditioning (stabilizing). The most abundant alpha acid is known as humulone. (Barth 2013, 153.)

The bitterness of beer is measured on a scale known as bitterness units (BU), or sometimes called International Bitterness Units (IBU). This is roughly equal to one milligram of iso-humulone per litre. However, there are often other compounds that contribute to the overall bitterness of the beer, and so the perceived bitterness may be higher than the BU value. (Barth 2013, 202.)

2.2.2 Esters and fusel alcohols

During fermentation, yeast cells produce various minor products that can have a significant effect on the beer's flavour. The production of these products depends on the strain of yeast used and the fermentation conditions. One group of these minor products is esters. (Barth 2013, 174.) Esters are organic compounds that are derived (usually) from a carboxylic acid and an alcohol in a reaction known as a condensation reaction. The functional group of a carboxylic acid is -COOH, and in an ester, the hydrogen is replaced by a hydrocarbon group such as alkyl, phenyl or benzyl from the alcohol. During the reaction, water is produced from the hydrogen in the carboxylic acid and the -OH group of the alcohol. An example of such a reaction is the reaction between acetic acid and ethanol to form the ester ethyl acetate (Clark 2019.) This reaction is depicted in Figure 2.

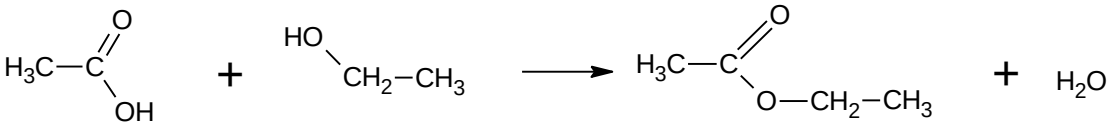


FIGURE 2. Reaction between acetic acid and ethanol to form ethyl acetate and water. (Adapted from Clark, 2019)

The flavours from esters are fruity and flowery. These are considered good flavours for most ale type beers but not for lagers. In beer, the most common esters are formed from acetic acid (CH_3COOH). The alcohol reacting with the acetic acid can be the ethanol or fusel alcohols, which are higher alcohols produced in trace amounts during fermentation. When acetic acid and ethanol react, they produce ethyl acetate, which is the most prevalent ester in beer. At low concentrations, ethyl acetate has a flowery aroma, but at higher concentrations the flavour becomes solvent-like. (Barth 2013, 174.)

Fusel alcohols are alcohols with more than two carbon atoms. Their concentration increases with increased fermentation temperature, though they are also affected by the yeast strain and wort composition. Fusel alcohols contributing to beer flavour include n-propanol, iso-butanol, 2-methylbutanol and 3-methylbutanol. Other than being precursors of esters, they also contribute to the overall flavour of beer by intensifying the ethanol flavour. (Briggs, Boulton, Brookes & Stevens 2004, 460.) Fusel alcohols are formed when the yeast absorbs amino acids from the wort so that it can incorporate the amino group into its own structure. The remainder of the amino acid (α-keto acid) is then involved in an irreversible chain reaction, ultimately resulting in fusel alcohols as a by-product. (Pires, Teixeira, Brányik & Vicente 2014, 1938.)

2.2.3 Acetaldehyde

An aldehyde is an organic compound having a carbonyl group (a carbon atom double bonded to an oxygen atom) at the end of the chain (Hill & Holman 2011, 306). Up to 200 carbonyl compounds contribute to the flavours of fresh beer and other alcoholic beverages. These carbonyl compounds are mostly aldehydes and vicinal diketones. Carbonyl compounds significantly influence beer flavour stability. In excess concentrations, they can cause stale flavour in beer. (Stewart 2017, 11.) Some of the significant aldehydes found in beer are described in Table 1.

TABLE 1. Significant aldehydes in beer (adapted from Buiatti 2009, 221)

Aldehyde	**Concentration (mg/L)**	**Descriptors**
Acetaldehyde	2-20	Green apple, paint, cut grass
Propionaldehyde	0.01-0.3	Green apple, fruity
Butyraldehyde	0.003-0.02	Melon, varnish
Hexanaldehyde	0.003-0.07	Bitter, vinous

Acetaldehyde (the structure is shown in Figure 3) is the most common aldehyde in beer and imparts the flavour of green apples which is undesirable in most beers. It is produced by yeast as the product of the decarboxylation of pyruvate, and its flavour threshold is approximately 10 mg/L. It is formed in the early and mid-stages of fermentation as a precursor to ethanol. The extent of its accumulation is determined by the yeast strain used and the fermentation conditions. (Stewart 2017, 11 – 12.)

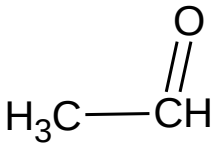


FIGURE 3. Structure of acetaldehyde (Adapted from Pubchem 2019)

There are three main sources of acetaldehyde in beer after fermentation: spoilage from the bacteria Zymomonas and Acetobacter, oxidation and spillover from fermentation. The acetaldehyde concentration is at its maximum during the peak of the logarithmic growth phase of the yeast, which is typically within

the first four days of fermentation. This concentration then gradually decreases during the secondary stages as it is reduced to ethanol. Spillover levels of acetaldehyde occurs if the yeast is removed prematurely, that is before the reduction to ethanol is complete. (Bickhamb 2001.) Other factors that causes high concentrations of acetaldehyde in packaged beer are excessively high fermentation temperatures, poor quality pitching yeast, excessive wort oxygenation and excessive pitching rates (Briggs et al. 2004, 456). Some of the chemical and physical properties of acetaldehyde are depicted in Table 2.

TABLE 2. Chemical and physical properties of acetaldehyde (Adapted from Pubchem 2019)

Property	**Value**
Molecular weight	44.05 g/mol
Molecular formula	CH_3CHO
Boiling point	21.1 °C
Melting point	-123.0 °C
Density at 25 °C	0.785 g/mL
Solubility in water	miscible

2.3 Aging reactions producing acetaldehyde in beer

There are several sensory changes that occur in beer with aging. This is because the varying components in beer are not in equilibrium after packaging. In terms of thermodynamics, a bottled beer is a closed system that strives to reach a state of minimal energy and maximum entropy. As a result, many chemical reactions occur during storage, leading to characteristics of aged beer. One reported change is a constant decrease in bitterness. There is also an increase in a sweet taste which is partly responsible for the decrease in bitterness as it masks it. The increased sweet taste coincides with an increase of caramel, burnt sugar and toffee-like aromas. Aging flavours vary between beer types. Factors causing aging flavours include oxygen in the beer after packaging, storage temperature, and beer type. (Vanderhaegen et al. 2006, 357 – 358, 363.)

Hundreds of different compounds from various chemical groups are attributed to the flavours associated with aged beer. Molecules form at concentrations above their flavour thresholds which leads to notice-

able sensory effects. Meanwhile, other molecules degrade to concentrations below their flavour thresholds therefore decreasing the expected fresh beer flavours. Furthermore, there are interactions between different aroma volatiles which can either enhance or suppress the impacts of flavour molecules. (Vanderhaegen et al. 2006, 358.) The chemical reactions attributed to flavour deterioration include Maillard reaction, ester degradation, degradation of hop bittering acids, and formation of linear aldehydes. These reactions may occur due to the type of beer, the raw materials used, and excess exposure to oxygen and light, temperature, and time. (Aron 2014.)

When research first began on staling compounds in beer, carbonyl compounds attracted the most attention. These compounds were already known to create flavour changes in many food products such as milk, butter, and vegetables. One of the first compounds observed to increase in concentration in aged beer was acetaldehyde. (Vanderhaegen et al. 2006, 356.)

One of the possible aging reactions that results in acetaldehyde is oxidation. Dissolved oxygen molecules in beer has been attributed to the rise of many staling compounds including acetaldehyde. The pathway from the dissolved oxygen molecule to oxygen-containing organic compounds such as aldehydes, involves intermediates known as reactive oxygen series (ROS). The majority of these are free radicals (they have unpaired electrons) such as the hydroxyl radical. The formation of ROS can also be significantly accelerated by certain metals which are often present in the beer at low concentrations. These metals can form two stable ions such as iron (Fe^{2+} and Fe^{3+}) and copper (Cu^{+} and Cu^{2+}). These metals can reduce oxygen to the superoxide ion as shown in equation 2. (Barth 2013, 255 – 257.)

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^- \quad (2)$$

In an acidic solution such as beer, the superoxide ion exists in the acid form as hydroperoxyl radical, $HO_2\bullet$. Two hydroperoxyl radicals can then react to form hydrogen peroxide (H_2O_2) and oxygen (O_2). (Barth 2013, 257.) The hydrogen peroxide then takes part in a free radical mechanism (the Fenton reaction) in which ethanol is oxidized to acetaldehyde. The mechanism proceeds as shown in equations 3 - 6 (Baert, Clippeleer, Hughes, Cooman & Aerts 2012, 11459):

Initiation: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ (Fenton Reaction) (3)

Propagation: $\bullet OH + CH_3CH_2OH \rightarrow CH_3\bullet CHOH + H_2O$ (4)

$CH_3\bullet CHOH + Fe^{3+} \rightarrow CH_3CHO$ (acetaldehyde) $+ Fe^{2+} + H^+$ (5)

Termination: $Fe^{2+} + \bullet OH \rightarrow Fe^{3+} + OH^-$ (6)

In order to minimize the effects of oxidation, brewers try to minimize the amount of oxygen in packaged beer. Controlling the oxygen content in beer can be difficult as 20 % of air already consists of oxygen and it is not typically the practice to package beer in an oxygen-free environment. Brewers prevent oxygen from mixing with beer during packaging by using equipment that fills the packaging without splashing. Also, prior to filling the packaging with beer, it is often filled with carbon dioxide thus preventing air from coming in when it is filled with beer. Finally, just before the packaging is closed, the beer is made to foam which results in driving off air. However, for glass bottles, the plastic liners inside the crown caps that seals the bottles let in a small amount of oxygen dissolve in the plastic which then migrates into the bottle via a process known as permeation. For aluminium cans, the seals are all metal therefore permeation is near zero. (Barth 2013, 260-261.)

Amino acids in stored beer may also be a possible source of acetaldehydes due to a reaction known as Strecker degradation (Vanderhaegen et al. 2006, 364). Strecker degradation is a reaction discovered by the German chemist Strecker when he observed the production of acetaldehyde after heating a solution of alloxan [2,4,5,6-(1H,3H)-pyrimidinetetraone) and the amino acid alanine. Carbon dioxide and ammonia were also produced in this reaction. The feature in alloxan that is essential for the production of carbon dioxide and acetaldehyde is its α-dicarbonyl structure (with carbonyl compounds, α denotes the carbon adjacent to the carbonyl functional group) which is why the reaction also works with other α-dicarbonyl compounds. An aldehyde produced via Strecker degradation is known as a "Strecker aldehyde". (Cerny 2009, 241-242.) Figure 4 depicts the formation of acetaldehyde via Strecker degradation.

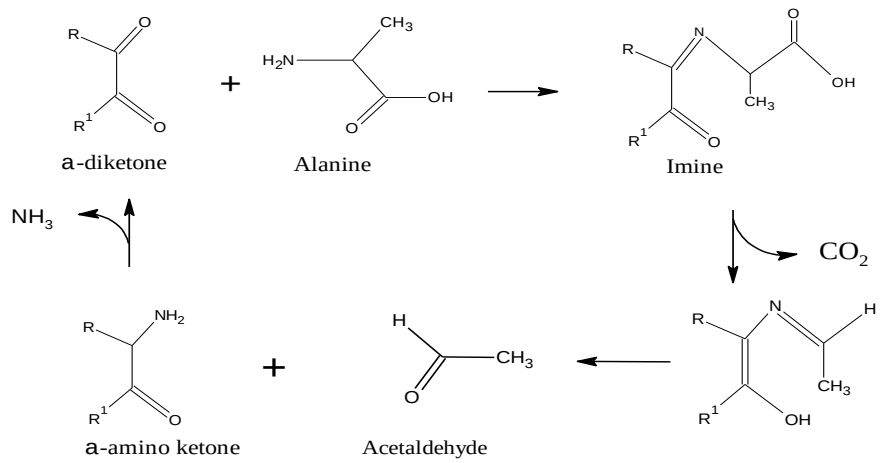


FIGURE 4. Strecker degradation mechanism (Adapted from Croguennec 2016, 141)

3 GAS CHROMATOGRAPHY

GC is a laboratory technique used in analytical chemistry to separate mixtures into individual components. These individual components can then be identified, and their concentrations determined. (Agilent Technologies 2002, 9.) GC works by separating vaporized components in a sample by distributing the sample between a stationary phase and a mobile phase. The mobile phase is an inert gas that carries the components of the analyte through the heated column. The stationary phase is either one of two things: a solid adsorbant (gas-solid chromatography) or a liquid situated on an inert support such as silica (gas-liquid chromatography). The devices used in GC are typically a gas source, an injection port, a column, heaters to maintain the temperatures of the injection port and the column, and a detector. The process of gas chromatography can be seen in Figure 5 (Thet & Woo 2019.)

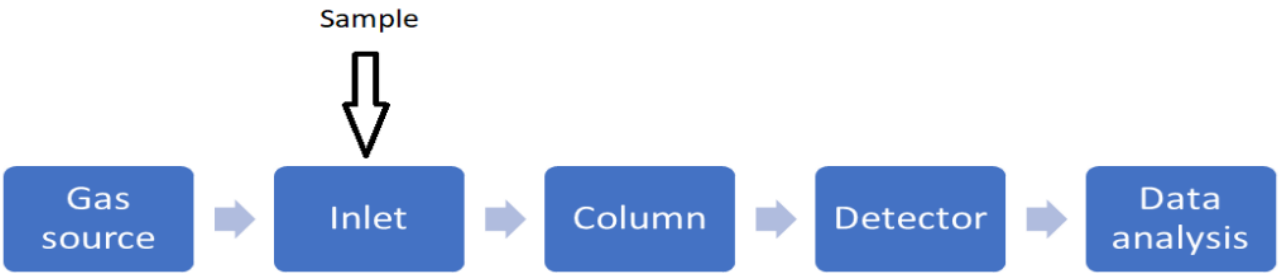


FIGURE 5. Gas chromatography process scheme (Adapted from Agilent technologies 2002, 11)

3.1 Function of gas chromatography

Most of the samples used in GC are liquids and must be vaporized before entering the column. The vaporization is typically done with a heated injection port that is combined with a liquid syringe or a liquid sampling valve. (Agilent Technologies 2002, 17.) A calibrated microsyringe is used to extract a sample volume (a few microliters) and injects it through a rubber septum to a vaporization chamber. Typically, only a small fraction of the initial sample volume is needed for analysis. The rest is separated using a sample splitter and then directed to waste. Modern injection ports are heated such that the sample is injected and vaporized almost simultaneously (Thet & Woo 2019.)

The inert carrier gas used varies depending on the GC used. The gas must be pure as contaminants can react with the samples and cause inaccurate readings. The gas typically used is helium as it is safer, has a wide range of flow rates and is compatible with numerous detectors. Other gases used include nitrogen, argon, and hydrogen. The carrier gas flows through the injection port, carrying the vaporized sample to the column. (Thet & Woo 2019.)

In the column is where the separation of a mixture into separate components occurs. There are different columns that exist for separating mixtures, such as capillary and packed columns, and are chosen according to the nature of the mixture and the type of information needed. However, columns generally perform using the same basic function. The separation of the sample in the column is based on the relative attraction of the sample to the two phases. This attraction can be based on solubility, volatility, and polarity among others. Different components in the sample will have different "levels of attraction" (or interaction with the stationary phase) and will therefore separate with time as they travel in the mobile phase. The components with weaker levels of attraction will exit (elute from) the column first. Those with stronger levels of attraction will exit the column last. The purpose of the column is to ensure that the detection system produces narrow and well-separated peaks. Highly efficient columns produce narrow peaks. The efficiency of a column is based on the column construction (a column with a small tubing diameter and thin stationary phase layer is best) and the carrier gas flow rate. (Agilent Technologies 2002, 27-30.)

The columns are mounted in a temperature-controlled oven since the separations of the components in the sample is highly temperature-dependent (Agilent Technologies 2002, 36). The thermostatted oven controls the temperature of the column to within a few tenths of a degree for precise results. There are two operation modes of the oven: isothermal and programmed. With isothermal, the temperature of the column is constant throughout the separation. The optimum column temperature for this operation is about the middle point of the boiling range of the sample. Isothermal works best only when the boiling point range of the sample is small. If the boiling point range of the sample is wide and a low isothermal temperature is used, then the components with high boiling points will be slow to elute and will be displayed with extensive band broadening in the detector. If the temperature is increased such that it is closer to the boiling point of the higher boiling components, then they elute as sharp peaks, but the low boiling components elute so quickly that there is no separation. (Thet & Woo 2019.)

With the programmed oven, the column temperature typically changes upwards, either continuously or in increments as the separation progresses. This is the best operating mode for the oven when there is a

wide boiling range in the sample. Rates of about 5-7 °C/min are typical used in the programmed oven. (Thet & Woo 2019.) One advantage of using temperature programming is that the analysis time is reduced. Also, the peak shapes are constant throughout the analysis making it easier for detection and measurement. (Agilent Technologies 2002, 37.)

At the end of the column is a detector. This device gives a quantitative measurement of the components of the sample as they elute together with the carrier gas. Each detector has two main parts that work together to serve as transducers that converts the detected property changes (analogue signals) to an electrical signal that is recorded in the form of a chromatogram. The first part of the detector is the sensor which is put as close to the column exit as possible to optimize detection. The second part is the electric equipment that digitizes the analogue signal so a computer can analyse the acquired chromatogram. The analogue signal is easily susceptible to many types of interferences (noise) therefore the sooner the analogue signal is converted into a digital signal the better, as this reduces the chances of interferences influencing the results. There are many different types of detectors including mass spectrometer (MS), flame ionization (FID), electron-capture (ECD) and thermal conductivity (TCD). (Thet & Woo 2019.) The detector used during the experiment for this thesis is a mass spectrometer.

3.2 Gas Chromatography – Mass Spectrometry (GC – MS)

In Gas Chromatography – Mass Spectrometry, the mass spectrometer detects the eluate by ionizing it as it enters from the GC column. The eluate contains the mobile phase (or carrier gas) analyte molecules, volatile matrix components (matrix components are components other than the one of interest) that may have eluted with the analyte, and molecules that came off of the stationary phase of the GC column decomposing (typically cyclic siloxanes). (Sparkman, Penton & Kitson 2011, 89.) After exiting the GC column, the eluate passes through a transfer line and is directed into the inlet of the mass spectrometer. The sample is then ionized and fragmented. This is typically done by an electron-impact ion source. In this process, the sample is bombarded by energetic electrons causing the molecule to lose an electron from electrostatic repulsion thereby being ionized. The ions fragment with further bombardment. The ions then pass into a mass analyser and here the ions are sorted according to their m/z value or mass-to-charge ratio. Most ions are singly charged. (Thet & Woo 2019.)

The results of mass spectroscopy are displayed as a mass spectrum in a vertical bar graph where each bar represents an ion with a specific m/z value (x-axis) while the length of the bar depicts the relative

abundance of the ion (y-axis). The relative abundance is the percentage of the ion in relation to the base peak. The base peak is the most intense ion and is assigned a relative abundance of 100. Since most ions formed in the mass spectrometer are singly charged, the m/z value is then equivalent to the mass itself. The ion with the highest mass in the spectrum is usually considered to be the molecular ion while lower-mass ions are the fragmentations of the molecular ion, presuming the sample is a pure single compound. (Reusch, 2013.)

GC-MS can also display the results in the form of a chromatogram. In the chromatogram, the x-axis is the retention time, or the amount of time taken for the analytes to travel through the GC column and reach the mass spectrometer, while the y-axis (as well as the area under the peaks) is the concentration or intensity counts, which gives the amount of analyte present. The retention time is largely impacted by the type of GC column used as well as GC parameters (oven temperature, flow rate and injection temperature) therefore, when comparing the retention times from different analyses or other laboratories, it is critical that the parameters are the same to ensure accuracy. Furthermore, it is significant to note that some compounds produce peaks that appear larger than their real concentration would be compared to other peaks on the chromatogram, and this occurs due to the compounds having a better affinity with the detector . This typically happens with compounds that ionize readily. To overcome this challenge, standards having known concentrations of compounds can be used to ensure accurate count. (Matthias 2018.)

In quantitative analysis, the concentration in a sample (target analyte) is determined. This can be accomplished by using the GC alone, but there are advantages to using GC-MS over GC. When quantitating with GC, it is significant to have a good separation of components of interest. When a mass spectrometer is used, this is not crucial as unique ions can be used for identification and quantitation can be done based on mass. (Sparkman et al. 2011, 208.)

Analytes in a quantitative analysis are typically referred to as target analytes, since their identities are already known, and it is just their concentration being determined. In GC-MS, the quantity of a target analyte can be determined using the continuous monitoring of full-spectrum, selected reaction monitoring (SRM) or selected ion monitoring (SIM). When using the continuous monitoring of full-spectrum mode to quantitate, a quantitation ion (more simply a quant ion) is selected. The quant ion is a uniquely characteristic ion of the target analyte. It is often represented by the base peak in the mass spectrum as long as its m/z value is not the same as a coeluting compound. A mass chromatogram of the quant ion gives a chromatographic peak area that is then used in quantitation. (Sparkman et al. 2011, 209.)

There are two main methods of quantitation that can be used based on the chromatogram: the external standard method and the internal standard method. With the external standard method, a comparison is made between the peak areas of the target analytes in the sample and the peak areas of the same analytes in a standard solution. The calibration standards contain the compound(s) of interest at known concentrations which should be in the expected range of the unknown samples that are to be analysed. Once the calibrations standards and samples have been analysed using the GC-MS, the response factor (RF) of the calibration standards should be calculated. A response factor is the ratio between the concentration of the analyte and the detector's response to that analyte (peak area). The formula for calculating the RF is shown in equation 8: (Sparkman et al 2011, 213.)

$$RF = \frac{Area_{std}}{C_{std}} \tag{8}$$

where $Area_{std}$ is the peak area of the calibration standard, and C_{std} is the concentration of the calibration standard. The mean of the RFs is then calculated, and this mean is used to calculate the concentration of target analytes in the samples, as shown in equation 9: (Sparkman et al 2011, 213.)

$$C_x = \frac{Area_x}{RF_{mean}} \tag{9}$$

where C_x is the concentration of the sample analyte, $Area_x$ is the peak area of the analyte, and RF_{mean} is the mean response factor from the calibration standards. Most modern software can do this calculation. The data system should also plot a calibration curve of the concentration of each analyte against the peak area and do a linear regression analysis which shows the correlation coefficient to a straight line. (Sparkman et al 2011, 213.)

However, to get good quantitative results from the external standard method, the sample preparation procedure should not be complicated. Steps such as solvent extraction, derivatization, and evaporation in the sample preparation procedure, can result in unreliable results in the external standard method. Instead, the internal standard method should be used. The internal standard method produces the most accurate quantitative results in GC-MS. The use of an internal standard corrects for any losses during any separation and concentration steps in the sample preparation procedure, as well as any variation in the amount of sample injected into the GC. In this method, a known quantity of a compound, (the internal standard or IS), is added to an accurately measured aliquot of the sample to be analysed. The IS should be chemically similar to the target analyte but should elute in an empty space in the chromatogram.

(Sparkman et al 2011, 214.) The IS should be a compound that cannot be found in the sample as this can give false results and should be sufficiently stable, such that it does not easily degrade during the sample preparation. It should also give a well-resolved peak in the chromatogram. Finally, the IS should have similar extraction characteristics, stability, detector response and retention times as the target analyte. Often, the IS used in analytical chemistry is the target analyte but with specially synthesized modifications so that it does not have same retention time as the main target analyte. Such an IS includes deuterated compounds, which are compounds that have had one or more of the hydrogen atoms replaced by deuterium atoms. (Dolan 2012.)

Just as in the external standard method, a series of calibration standards should be prepared in the range of the target analyte for the internal standard method. There should be approximately five or six calibration levels. Next, a known weight of the IS is dissolved in a solvent and an equal amount is added to each of the calibration standards and unknown samples. The concentration of the IS should be approximately in the mid-range of the expected concentration of the target analyte. The volumes of the calibration standards and unknown samples should also be equal. (Sparkman et al 2011, 214.)

After the GC-MS analysis, the quantitation begins by the relative response factor (RRF). This is based on the response factors of the calibration standard and the internal standard and is calculated as shown in equation 10: (Sparkman et al 2011, 215.)

$$RRF = \frac{Area_{std} * C_{IS}}{C_{std} * Area_{IS}} \tag{10}$$

where $Area_{std}$ is the peak area of the calibration standard, C_{IS} is the concentration of the internal standard, C_{std} is the concentration of the calibration standard and $Area_{IS}$ is the peak area of the internal standard. The data system can then calculate a calibration curve and linear regression data as well as a mean RRF for all the calibration standards. This mean RRF is then used to calculate the concentration of the target analyte in the unknown sample using equation 11: (Sparkman et al 2011, 216.)

$$C_x = \frac{Area_x * C_{is}}{RRF_{mean} * Area_{is}} \tag{11}$$

where C_x is the concentration of the target analyte in the unknown sample, $Area_x$ is the peak area of the target analyte in the unknown sample, C_{is} is the concentration of the internal standard, RRF_{mean} is the mean relative response factor and $Area_{is}$ is the peak area of the internal standard.

4 QUANTITATIVE ANALYSIS OF ACETALDEHYDE IN AGED BEER

This chapter discusses the motivation behind assessing the levels of acetaldehyde in aged beer as well as the research methods used in analysing acetaldehyde in beer. The research methods discussed are the ones used for this thesis and are not the only methods currently available for the analysis of acetaldehyde in beer.

4.1 Motivation for the analysis of acetaldehyde in aged beer

One of the most challenging quality problems for brewers is the achievement of flavour stability. There are many negative influences that affect flavour stability. Some of these influences include; higher storage temperatures in shops, constant movement and exposure to light (this is why beer bottles are tinted though green tinted bottles do not protect the beer sufficiently and so long exposure to sun- and daylight and even artificial light can result in an unpleasant flavour in the beer). (Kunze 2014, 536-537.) However, the debate of flavour stability is often centred on whether this issue is for the consumer or for the brewer. Brand identity tends to have a major impact on beer selection, and this rises above any perceived aged beer characteristics. Furthermore, aromas that may be regarded as unacceptable by professional drinkers may be preferred, or otherwise ignored, by regular drinkers. (Bamforth 2011, 491.)

Broadly speaking, beer tastes best when it is fresh. Thus, the sell-by date, which is required in many countries to be on the label, says nothing about the quality of the beer, especially since beer is practically limitlessly bright due to stabilization. The more important date, for both the brewer and the customer, is the bottling date which ensures fresh and good-tasting beer. However, most customers are unaware of the connection between the bottling date and the freshness of the beer flavour. (Kunze 2014, 537.)

If the goal for every brewer is to minimize flavour instability, the challenge is evident. It can be argued that any change in aroma or taste is a representation of flavour instability, and there are hundreds of flavour molecules in beer that might change in concentration at or above threshold with time. This is rendered even more complex as the flavour thresholds for some molecules are incredibly low (0.1parts per billion for E-2-nonenal). As a result, it is more logical to adopt procedures minimizing changes in the level of all flavour active molecules such as reducing or eliminating oxygen and its reactive variants,

reducing temperature, and including (if applicable) antioxidants and binding agents. (Bamforth 2011, 491.)

As mentioned in subchapter 2.2.3, carbonyl compounds in beer have a significant influence on beer flavour stability (Stewart 2017, 11.) Acetaldehyde is one of several compounds that are key contributors to aged beer flavour and hence flavour instability (Bamforth 2011, 491). Analysing the concentrations of acetaldehyde in an old batch of beer compared to a new batch indicates the extent of flavour instability. Large levels of acetaldehyde could be an indication of issues in quality control in the brewing process.

4.2 Analysis of acetaldehyde

Direct analysis of acetaldehyde in untreated beer using gas chromatography is not applicable because there are other more abundant volatiles that cover the acetaldehyde peaks (Vanderhaegen et al. 2006, 359). Furthermore, carbonyl compounds in beer are in extremely low concentrations, have high volatility and are highly reactive due to the polar carbonyl group. Therefore, it has always been challenging to develop methods that have high extraction recoveries so that adequate sensitivity is obtained. One of the easiest and most successful techniques is to use a derivatization reagent. (Saison, De Schutter, Delvaux, & Delvaux 2009b, 5061.) This agent will react with the carbonyl compounds to form compounds that are less volatile. The oximes can then be extracted via liquid-liquid extraction and the sample is then analysed via gas chromatography. (Simkus, Hilts, Herd, Aponte & Elsila 2016, 1; Rodigast, Mutzel, Iinuma, Haferkorn & Herrmann 2015, 2410.)

4.2.1 Derivatization

Derivatization is a reaction that modifies an analyte's functionality so that it is enabled for chromatographic separations. The modified analyte is referred to as the derivative. The volatility of a sample is a significant factor in GC analysis. Derivatization renders highly polar compounds to be sufficiently volatile in order to elute at reasonable temperatures without thermal decomposition or molecular re-arrangement. Derivatization will either increase or decrease the volatility of an analyte, improve detector response, reduce adsorption of the analyte in the GC system, and improve peak separation as well as peak symmetry. (Orata 2012, 83.)

The derivative reagent is the substance that causes the derivatization. A suitable reagent should produce more than 95 % of derivatives, not contribute to the loss of sample when reacting, produce derivatives that do not react with the GC column, produce stable derivatives, and should not cause any structural alterations or rearrangements of the analyte when forming the derivative. (Orata 2012, 84.) The typical reagents used for the derivatization of acetaldehyde for GC are pentafluorophenylhydrazine (PFPH) and o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBHA). PFBHA is more commonly used and has been shown to produce better results in aqueous samples. (Saison et al. 2009b, 5061.) Acetaldehyde reacts with PFBHA to form pentfluorobenzyl oxime derivatives. These derivatives can then be extracted using organic solvents such as n-hexane and dichloromethane. When acetaldehyde (as well as most of the other carbonyl compounds) reacts with PFBHA it forms (E) and (Z) isomers. (Simkus et al. 2016, 2; Munch 1998.) This reaction is depicted in Figure 6.

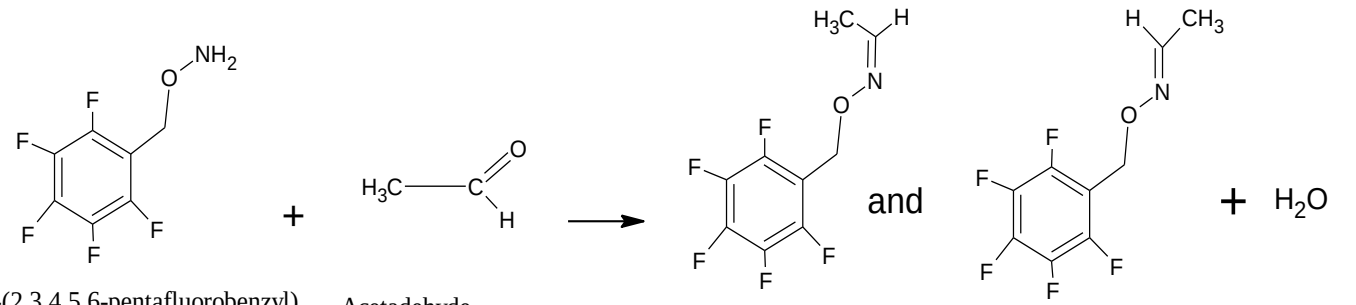


FIGURE 6. The reaction of PFBHA with acetaldehyde to form (E) and (Z) derivative isomers and water. (adapted from Simkus et al., 2016)

Some of the properties of acetaldehyde oxime isomers (acetaldehyde-O-pfbo) are displayed in Table 3. After these oxime isomers are formed, they can be extracted using either liquid-liquid extraction (LLE), solid-phase micro extraction or stir-bar sorptive extraction (Saison et al. 2009b, 5061.) The extraction method used for this thesis is liquid-liquid extraction.

TABLE 3. Properties of acetaldehyde-O-pfbo (adapted from guidechem 2020 and scbt 2020)

Property	**Value**
Molecular weight	239.14 g/mol
Molecular formula	$C_9H_6F_5NO$
Boiling point	213.3 °C
Density at 25 °C	1.37 g/mL
Solubility	Soluble in hexane

4.2.2 Liquid-Liquid extraction (LLE)

The International Union of Pure and Applied Chemistry (IUPAC) defines liquid-liquid extraction (LLE) as (Clement & Hao 2012, 51):

> The process of transferring a dissolved substance from one liquid phase to another (immiscible or partially miscible) liquid phase in contact with it.

Thus, LLE involves the movement of one or more substances from one liquid phase to another based on the solubility differences between the two liquid phases (typically an aqueous phase and an organic phase are used). The substance(s) will move to the phase where it has a higher solubility, so when the system reaches equilibrium, one phase will contain more of the substance than the other. The extracted substance is typically referred to as the solute, the liquid where it is extracted from referred to as the feed, the liquid used for the extraction is referred to as the solvent, the liquid enriched with the solute is referred to as the extract and the liquid depleted of the solute is the raffinate. (Clement & Hao 2012, 51.) In the case of extracting acetaldehyde from beer, the oximes formed from derivatization are extracted from the aqueous phase (in this case the beer which is also the feed) to an organic phase such as hexane (the solvent). The extract can be removed using an instrument such as a separation funnel or using a pipet in the case of microextraction. (Wells 2003, 57; Munch, Munch & Winslow 1998.)

The distribution coefficient, K_D, of a solute is used in LLE procedures to measure the effectiveness of the solute as it transfers from the feed to the extract. It is defined as the ratio of the concentration of the solute in the extract to the concentration of the solute in the raffinate at equilibrium. This ratio is depicted in equation 12: (Clement & Hao 2012, 51.)

$$K_D = C1/C2 \quad (12)$$

where C1 and C2 are the equilibrium concentrations of the solute in the extract and raffinate respectively. The distribution coefficient can be further defined as the relative preference of the solute for the solvents. Its value is typically greater than 1. The higher the distribution coefficient, the more solute is transferred from one phase to the other therefore the better the extraction. Thus, the choice of solvents used is significant. Solvents should be selected such that the solute has a higher solubility in it than in the feed. (Clement & Hao 2012, 51; Berk 2013, 306).

LLE primarily consists of two steps; mixing (contacting) followed by phase separation. Careful consideration should be taken on the selection of solvents and mode of operation because, while vigorous mixing favours the transfer of the solute from one solvent to the other, it may also cause an emulsion to form which impairs the ease of phase separation. Furthermore, equilibrium should ideally have been reached prior to extraction. (Berk 2013, 306.) Other parameters that can affect the results from LLE include the pH of the system, the adjustment of temperature, contact time and the nature of the extraction (for example the volume of solvent and feed used or whether it is a batch extraction or single extraction.) (Clement & Hao 2012, 54.)

LLE is typically used in analytical laboratories to extract organic compounds in aqueous phase using organic solvents. For volumes less than 10 ml, a conical vial or centrifuge tube is used while for volumes in the range of 10 – 2000 ml a separatory funnel is often used. To ensure efficient transfer of the target to the extract, the two liquid phases must be mixed thoroughly. The extent of mixing required relies on the solute's and two phases' properties. After the mixing is completed, the two phases will separate due to density differences. If emulsion occurs, salt can be added to the aqueous phase in order to increase its density. Another method is to centrifuge the liquid to accelerate the separation of the phases, though this method can exacerbate the situation if the emulsion is stable. After phase separation, the layers are easily identifiable based on their density. (Clement & Hao 2012, 57 - 58.)

5 EXPERIMENTAL DESIGN

This chapter discusses the design of the experiment and includes detailed information on the extraction of acetaldehyde from beer and the instrumentation used for analysis. The design follows the four main steps in chemical analysis: sampling, sample preparation, measuring and data analysis (Telgheder, Bader & Alshelmani 2008, 56). The experimental procedure is divided into four parts: the preparation of the internal standard, calibration standards and the beer samples, the derivatization, the extraction of acetaldehyde via liquid-liquid extraction (LLE) and the analysis via GC-MS. The chemicals and solvents used for this experimental procedure can be seen in Table 4.

TABLE 4. Solvents and chemicals used for the experimental procedure

Chemicals	**Chemical formula**	**Molecular mass (g/mol)**	**Density, ρ (g/ml)**	**Purity**	**Source**
Acetaldehyde	CH_3CHO	44.05	0.785	99.5 %	Thermo Fisher (Kandel) GmbH, Germany
2,3,5,6-Tetrafluorobenzaldehyde	$C_7H_2F_4O$	178.08	1.525	94 %	Tokyo Chemical Industry CO., LTD, Japan
n-hexane		86.18	0.659	99%	Merk KGaA, Germany
O-2,3,4,5,6-pentafluorobenzyl hydroxylamine (PFBHA)	$C_6F_5CH_2ONH_2$	249.57	1.370	99+%	Thermo Fisher (Kandel) GmbH, Germany
3 M sulfuric acid	H_2SO_4	98.08	1.180	-	Thermo Fisher (Kandel) GmbH, Germany

Table 5 depicts the glassware and other equipment used for the sampling preparation. The GC used is Agilent Technologies 7890A GC System, the MS used is the Agilent Technologies 5975C inert MSD (Mass Selective Detector) with Triple-Axis Detector and the autosampler is Agilent G4513A.

TABLE 5. Glassware and other equipment used for the sampling preparation

Equipment	**Amount**	**Where needed**
10 ml volumetric flask	3	PFBHA solution, internal standard stock solution and internal standard
20 ml volumetric flask	5	Calibration standard and IS stock solution
100 ml volumetric flask	2	Acid dilution and calibration stock solution
10 ml volumetric pipette	11	Calibration standards, blank and beer samples
22 ml glass vial	11	Derivatization, extraction and acid wash step for calibration standards and beer samples
10 ml beaker	11	Extraction
25 ml beaker	2	PFBHA and internal standard solution
100 ml beaker	3	Acid, hexane and distilled water
pH paper	11	Samples and calibration standards
100 ml Erlenmeyer flask	6	Pre – treatment (filtration of beer samples)
Micropipette	3 (20 – 1000 µl 1 – 5ml 5 – 10 ml)	Making solutions and extraction
1.5 ml GC sample vials	11	GC-MS analysis
Büchner funnel	6	Pre – treatment (filtration of beer samples)
800 ml beaker	2	Ice and water bath
42.5 mm filter paper	6	Beer samples pretreatment
Disposable pipette	2 (1 ml and 3 ml)	Distilled water and acid
Laboratory weighing scale	1	PFBHA and internal standard
Disposable weighing dish	2	PFBHA and internal standard
Stuart® orbital incubator SI500	1	Extraction

The samples are bottled beer from the brewery Kahakka in Kokkola, Finland. The beers (named Helles) are lagers. They were produced using the yeast strain Saflager™ S-23. This yeast strain originated from Berlin, Germany and is often used to produce fruity lagers (Fermentis 2017). Three different batches were used for this thesis. From each batch, two bottled beers were used as samples. The samples were labelled as shown in Table 6. Table 6 also displays the batch versions and their ages.

TABLE 6. Beer samples analysed for this thesis

Batch	**Sample**	**Age**
Helles version 5 (v.5)	HEL 1	1 year three months
	HEL 2	
Helles version 6 (v.6)	HEL 3	1 year
	HEL 4	
Mandarina Helles version 4 (v.4)	HEL 5	3 months
	HEL 6	

5.1 Preparation of solutions and samples

The experiment began by filtering 30 – 50 ml of each beer sample into separate 100 ml Erlenmeyer flasks. The filtration mostly degassed the beer samples, though it was observed that there was a little bubbling in some of the samples after filtration. Each filtration took approximately ten minutes. 10 ml of each sample was withdrawn, and each poured into a labelled 22 ml glass vial.

Approximately 90 ml of distilled water in a 100 ml volumetric flask was chilled overnight in a fridge to a temperature of approximately 4 °C. This is to ensure that the acetaldehyde added does not evaporate as it is a volatile substance. 100 µl of pure chilled acetaldehyde solution was added directly into the water to further prevent the acetaldehyde evaporating. The volumetric flask was filled to the mark with distilled water. This is the calibration stock solution. For pure substances, the mass concentration is the same as the density (Torontech 2020). The density of acetaldehyde at 25 °C is 785 mg/ml.

Five 20 ml volumetric flasks were filled with approximately 10 ml of distilled water and put in an ice bath for fifteen minutes. 20 µl (0.79 mg/L), 50 µl (1.96 mg/L), 100 µl (3.93 mg/L), 250 µl (9.81 mg/L) and 700 µl (27.48 mg/L) of the calibration stock solution was added directly into the water in each 20 ml volumetric flask respectively and each flask was filled to the mark with distilled water. Using a separate 10 ml volumetric pipette for each, 10 ml of each standard was withdrawn and put into five labelled 22 ml glass vials respectively. These are the calibration standards. A sixth 22 ml glass vial was

filled with 10 ml of distilled water, but no acetaldehyde solution was added to it. This is the blank solution.

100 ml of 0,05M sulfuric acid was prepared by putting 1.665 ml of 3M H_2SO_4 into a 100 ml volumetric flask and filled to the mark with distilled water (this dilution is based on equation 13). Two drops of 0.05M of H_2SO_4 was added to each calibration standard and blank. Their pH was tested using pH paper and was approximately 4. The pH of the beer samples was approximately 4 after the addition of one drop of 0.05M of H_2SO_4.

Finally, the PFBHA and IS solutions were made. The PFBHA solution was made by weighing 100 mg of PFBHA and dissolving it in a 10 ml volumetric flask filled to the mark with distilled water. By dividing the mass with the volume, the mass concentration of the PFBHA solution is 10 mg/ml which is 10 000 mg/L. The IS was made by first making a stock solution of 2,3,5,6-tetrafluorobenzaldehyde. 56 mg of 2,3,5,6-tetrafluorobenzaldehyde was dissolved in a 20 ml volumetric flask filled to the mark with n-hexane. By dividing the mass with the volume, the mass concentration of the IS in n-hexane is 2.8 mg/ml which is equal to 2800 mg/L. Using a micropipette, 600 µl of the IS stock solution was added to a 10 ml volumetric flask and it was filled to the mark with n-hexane. This is the internal standard, and has a concentration of 168 mg/L.

5.2 Derivatization and liquid-liquid extraction

For the derivatization, 500 µl of the PFBHA solution was added to each vial. They were then put in a water bath at 45 ± 3 °C for two hours. After the two hours they were removed from the water bath and left in room temperature for approximately 30 minutes to cool down. 5 drops of 0.05M of sulfuric acid was added to each vial. This is to prevent the extraction of excess PFBHA which can cause chromatographic interference (Munch et al. 1998, 21).

After the derivatization the LLE is carried out. 2.5 ml of n-hexane was added to each vial. The vials must be shaken vigorously for maximum possibility of transfer of the acetaldehyde oximes from the aqueous layer (sample) to the organic layer (n-hexane). During a test run, it was observed that the beer formed a stable emulsion when vortexed (or otherwise mixed vigorously) with n-hexane. Therefore, during the experimental procedure, 2.5 ml of n-hexane was added to the beer samples and they were laid horizontally and gently rolled back and forth for approximately 1 minute. This idea was based on the

research by Martineau, Acree and Henick-Kling (1994, 9). Meanwhile, the blank and calibration standards were placed in an orbital incubator horizontally and it was run at 300 rounds per minute (rpm) for 15 minutes.

After mixing, the top n-hexane layer was carefully extracted using a micropipette and each was put in a separate 10 ml beaker. An additional 2.5 ml of n-hexane was put in the vials and they were mixed in the same way again. After the mixing the top n-hexane layer was again carefully extracted using a micropipette and each was put in the same 10 ml beaker as before.

3 ml of the n-hexane layer of each vial was withdrawn from the 10 ml beakers and put into a vial containing 5 ml of 0.05M of sulfuric acid and shaken. This is to further remove any chromatographic interference. 200 µl of the internal standard was added to eleven 1.5 ml GC vials. 1.3 ml of the n-hexane layer was withdrawn and put into a labelled 1.5 ml GC vial containing the IS. The samples, standards and blanks were then transferred to the GC for analysis. The samples were arranged in the GC such that the blank sample was put first, followed by the standards in descending order and finally the beer samples in sequential order.

6 RESULTS AND DISCUSSION

This chapter will display the results from the GC-MS analysis. These results will then be further discussed in this chapter. The GC-MS settings used were developed based on those used in the article by Bao et al. 2014. The GC was equipped with a 30 m × 0.25 mm I.D., 0.25-µm film thickness, ZB-1701 capillary column (Phenomenex, Torrance, CA, USA). The GC oven temperature program was as follows: initial 50 °C, 5 °C/min to 160 °C, and held at 160 °C for one minute. The carrier gas was helium at a flow rate of 1.0 mL/min. The MS was operated in the SIM mode.

6.1 Results

The results from the GC-MS were analysed using the software Agilent 5975C Data Analysis, which is also referred to as the MSD Chemstation. The compounds identified, their retention times and confirmation ions are displayed in Table 7. These compounds are the IS and the acetaldehyde oxime isomers (acetaldehyde-O-pfbo). The chromatograms produced can be viewed in Appendices 1 to 3.

TABLE 7. Retention times and ions selected for the compounds identified by the GC-MS

Compound	**Retention time (min)**	**Quantitation ion (m/z)**	**Confirmation ion (m/z)**
2,3,5,6-Tetrafluorobenzaldehyde (IS)	8.49	178	177, 99
Acetaldehyde O-pfbo	11.19, 11.40	181	239, 209

6.1.1 Results assumptions

It is assumed that all the acetaldehyde in each sample was derivatized. This is based on the knowledge that the amount of PFBHA used for the reaction was in excess. This can be proven by first noting the balanced equation of the reaction, which can be seen in equation 16. PFBHA reacts with acetaldehyde to form acetaldehyde-O-pfbo isomers and water.

$$C_6F_5CH_2ONH_2 + CH_3CHO \rightarrow C_9H_6F_5NO + H_2O \quad (16)$$

The molarity of PFBHA reacting in the equation can be calculated using equation 17:

$$Molarity\ (M) = \frac{concentration\ \left(\frac{g}{L}\right)}{molar\ mass\ \left(\frac{g}{mol}\right)} \quad (17)$$

where the concentration is the amount of PFBHA dissolved in the distilled water and the molar mass is the mass found in one mole of PFBHA. The molar mass of PFBHA, as shown in Table 4 is 249.57 g/mol. The molar mass of acetaldehyde is 44.05 g/mol. The concentration of PFBHA used for this experiment is 10 g/L. The highest concentration that the PFBHA is reacting to in this experiment is the 700 µl calibration standards, which has a concentration of 0.027 g/L. Using equation 17, the molarity of the PFBHA is then calculated as shown in equation 18, and the molarity of the acetaldehyde in the 700 µl calibration standard is also calculated as shown in equation 19:

$$Molarity\ of\ PFBHA = \frac{10\ g/L}{249.57\ g/mol} \approx 0.04M \quad (18)$$

$$Molarity\ of\ 700\ \mu l\ \text{calibration standard} = \frac{0.027\ \text{g/L}}{44.05\ \text{g/mol}} \approx 0.00061M \quad (19)$$

Since the ratio of the molar ratio of PFBHA and acetaldehyde in equation 16 is 1:1, then the reagent in excess would be PFBHA as it has a higher molarity than the calibration standard.

It is also assumed that the derivatized acetaldehyde was from the acetaldehyde stock solution for the calibration standards, and from the beer for the unknown samples, and is not found in any other solvent used. This is proven by the fact that there were no peaks at the retention time 11.19 and 11.40 (mins), which corresponds to the acetaldehyde oxime peaks, in the blank solution. This can be seen in the chromatogram in Appendix 1. The software Agilent 5975C Data Analysis suggests that the peak at 11.94, which is a peak that can also be seen in the calibration standards chromatograms, may be from the compound 1,3-dihydroxypropanone oxime which is a derivative of 1,3-dihydroxypropanone. This may be a compound that was in the distilled water used as its peak does not appear in the beer samples. However, there is a peak at the retention time 12.13 min in the beer samples. The analysis software suggested that this peak is due to 2,3,4,5,6-tetrafluorobenzyl alcohol which may be due to the derivatization agent reacting with the ethanol in the beer samples.

6.1.2 Quantitation

The quantitation of the results was conducted by the MSD Chemstation. In order to quantitate, a calibration curve of each acetaldehyde-O-pfbo isomer had to be made. The curves resulted from a linear regression analysis performed by the software. The calibration curve for the acetaldehyde-O-pfbo isomer at retention time 11.19 minutes is shown in Figure 7.

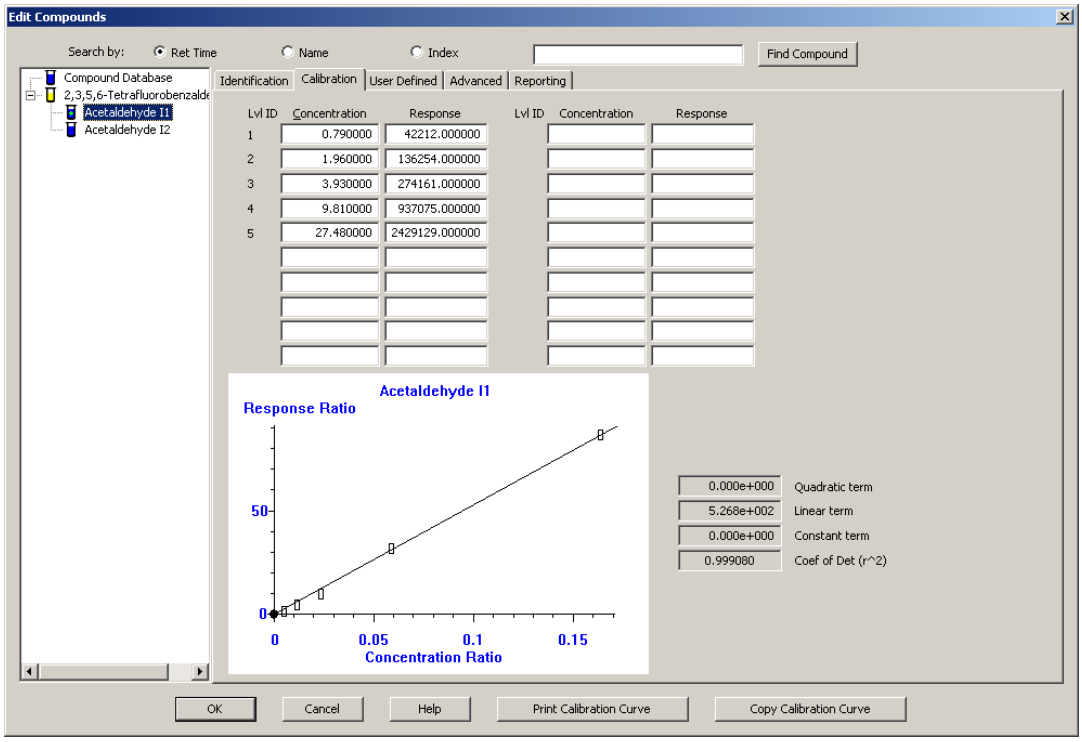


FIGURE 7. Calibration curve of the acetaldehyde isomer at retention time 11.19 mins

The calibration curve for the acetaldehyde oxime isomer at retention time 11.40 minutes is shown in Figure 8.

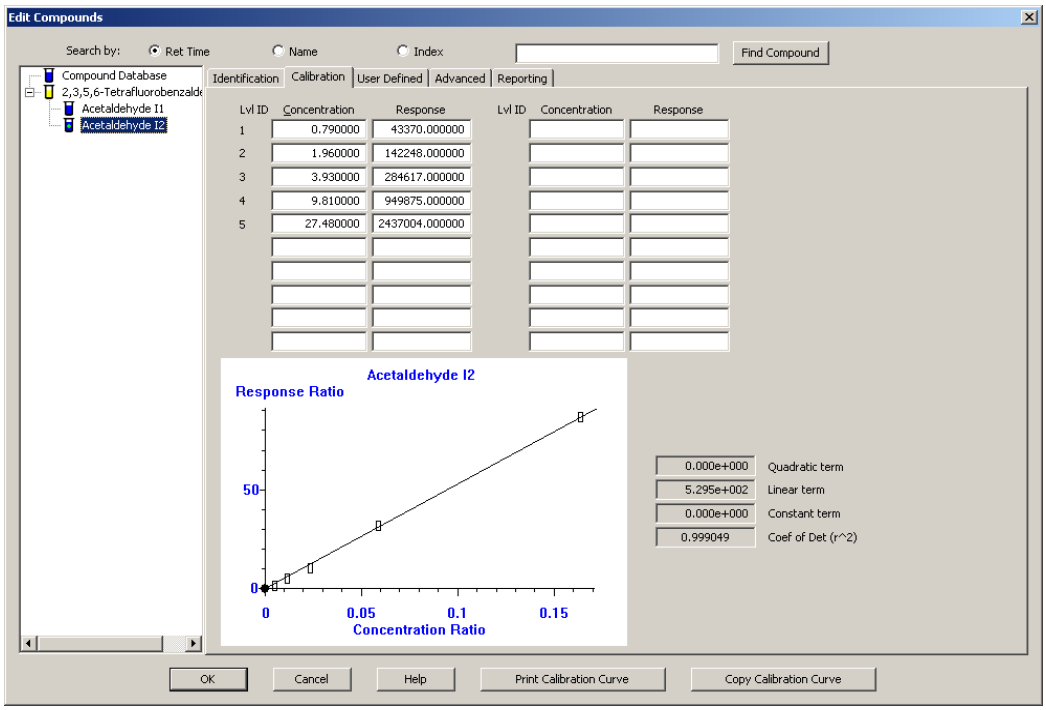


FIGURE 8. Calibration curve of the acetaldehyde isomer at retention time 11.40 mins

After the calibration curves were made, the concentration of each isomer was quantitated, and the average amount is reported as one value of the target analyte, as suggested by Munch et al. (1998, 19). These concentrations can be seen in Table 8.

TABLE 8. The concentrations of acetaldehyde in the beer samples

Batch	**Sample**	**Acetaldehyde isomer 1 concentration (mg/L)**	**Acetaldehyde isomer 2 concentration (mg/L)**	**Average acetaldehyde concentration (mg/L)**
Helles v.5	HEL 1	2.16	2.38	2.27
	HEL 2	7.66	8.30	7.98
Helles v.6	HEL 3	1.57	1.82	1.70
	HEL 4	1.33	1.47	1.40
Mandarina Helles v.4	HEL 5	3.65	4.05	3.85
	HEL 6	3.41	3.79	3.60

6.2 Discussion

This subchapter discusses the methodology used for this thesis, including its reliability and discrepancies. The results are also discussed including deductions made from them.

6.2.1 Methodology

The methodology used proves that it can be used for the detection of acetaldehyde in beer. The calibration curves for each isomer had an r^2 value of 0.999 which means that they were highly linear (Shevlyakov & Oja 2016, 17). However, while these results can be used to determine the unknown concentrations of the target analyte in the beer samples, it is difficult to conclude on the accuracy of the results, as the experiment was only conducted once. For more accurate results, it is important to determine the repeatability of the experiment. The repeatability of the experiment is determined by repeating the experiment under identical conditions for all controllable factors and assessing the outcome. The experiment is deemed repeatable if the outcome remains relatively consistent, though this depends on the analysis and the expected range of results. (Esbensen & Wagner 2016).

Furthermore, there were some discrepancies in the methodology. According to literature (Sparkman et al 2011, 214; Wang, Wang & Han 2017, 697; Cuadros-Rodríguez, Bagur-Gonzalez, Sánchez-Viñas, González-Casado & Gómez-Sáez 2007, 44), the IS should be added as early as possible in the sample preparation, as this corrects for any analyte losses that may occur during the sample preparation Unfortunately, in previous test runs where the IS was added at the same time as PFBHA, it either did not elute in the GC or appeared to react and form tetrafluorobenzanitrile, which was why it was added in the end. It was also observed that it was more soluble in water than in n-hexane as 100 mg of the IS dissolved in 10 ml of distilled water during a test run, yet less than half appeared to dissolve before achieving saturation in the same amount of n-hexane. This means that when it is added in the beginning of the sample preparation step, very little of it is extracted during the extraction step. The conditions of the derivatization step may also have affected the IS. In the research by Gabrio and Bertsch (2004) where 2,3,5,6-tetrafluorobenzaldehyde was used as the internal standard, the derivatization step was performed at room temperature and in the dark. It could be that derivatizing at 45 °C and in the presence of light may have

affected the IS. However, despite adding the IS at the end of the sample preparation, the resultant calibration curves for both acetaldehyde isomers were extremely linear.

Another potential source of error is loss through evaporation. Due to the high volatility of the n-hexane, approximately 2 ml of it evaporated from the 10 ml beaker during the extraction process which resulted in concentrating the target analyte and resulted in larger than expected area peaks. This may have occurred because the extraction process was relatively long (approximately 30 minutes) but can be avoided in the future by using a watch glass to cover the beakers.

Finally, as stated in subchapter 4.1 a stable emulsion was observed during a test run when a beer sample was vigorously mixed with n-hexane. While a different method was employed and acetaldehyde was detected, it cannot be deduced that the acetaldehyde detected is a true reflection of the concentration of acetaldehyde in beer. According to Clement and Hao (2012, 58) the two liquid phases need to contact and mix thoroughly for efficient transfer of the target analyte to the extract. To overcome the emulsion issue, Wardencki, Orlita, & Namieśnik (2001), diluted their alcoholic beverages to solutions containing 20 % ethanol and acidifying them to pH 2. They used n-heptane as the extractant and were able to obtain a satisfactory separation of the organic and aqueous layer. Additionally, Clement and Hao (2012, 54) stated that the general 'rule of thumb' in laboratory practices when extracting, is to extract three times to quantitatively recover the target analyte. Thus, the extraction results in Table 7 may not have been accurate.

6.2.2 Deductions from GC-MS results

The quantitation results of HEL 2 were approximately three times that of HEL 1. This was also observed with a different beer batch during a test run, in which a blank and 5 calibration standards were run before the beer samples. During this test run, the second beer sample (also from the same batch as the first) also had a peak in the chromatogram that was approximately three times that of the first. One possible reason for this is that there was a carryover of the target analyte from the first sample to the next. A carryover is when the injected sample is not completely removed from the analytical system, and so the subsequent analysis will contain some residual compound from the previous injection (Chromatography Today, 2013). It seems unusual that there should be a carryover between these specific samples when there does not appear to have been a carryover for the other samples. To check if a carryover is occurring, extra

blank samples could be added during the GC-MS run, perhaps one blank after every three or four samples. If a peak of the target analyte appears in the blanks, this means there is a carryover. The potential for a carryover can be eliminated by running the calibration standards separately from the unknown samples.

The results for both samples of Helles v.6 (HEL 3 and HEL 4) were similar, and the same can be said for the results of the Mandarina Helles v.4 (HEL 5 and HEL 6). These levels are significantly lower than the taste threshold of approximately 10 mg/L which implies (as discussed in subchapter 2.2.3) that the current production practices are sanitary (there is no beer spoilage), the yeast is not removed prematurely during fermentation, and little to no oxygen is introduced in the beer during bottling.

However, no clear trend could be observed between the storage time and acetaldehyde concentration, as shown in figure 9. Based on the theory discussed in subchapter 2.3, the expected results would be an increase in acetaldehyde concentration with age. Yet Mandarina Helles v.4 (three months in storage and samples HEL 5 and HEL 6) had a larger acetaldehyde concentration than Helles v.6 (one year in storage and samples HEL 3 and HEL 4).

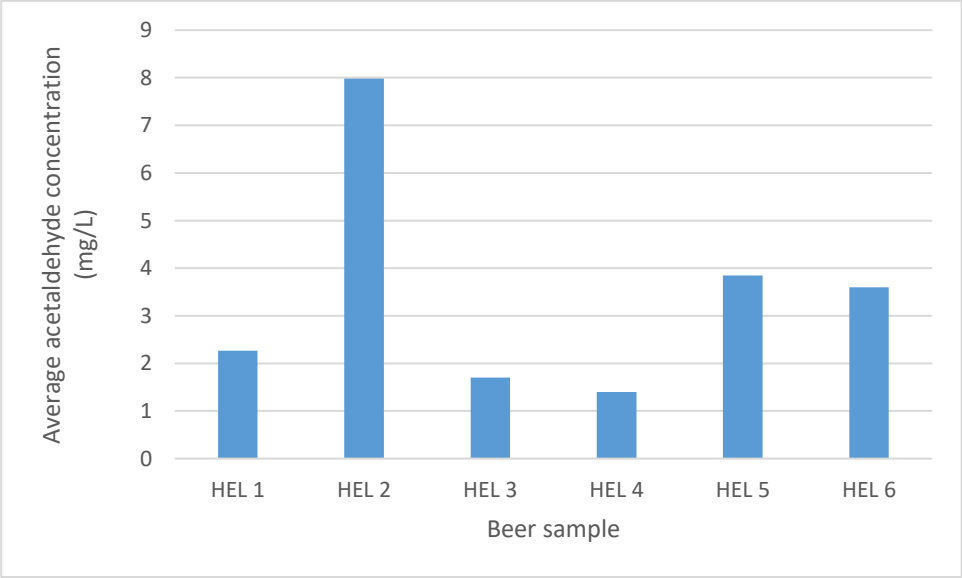


FIGURE 9. Graph of sample quantitation results

There is one proposed reason why the acetaldehyde levels in the Mandarina Helles v.4 is higher than that of Helles v.6 and that is the fermentation temperatures. The fermentation temperature for the Mandarina Helles v.4 was on average 8.5 °C which is considered a low fermentation temperature, compared

to the average fermentation temperature of Helles v.6 which was 12.5 °C. Higher than usual levels of acetaldehyde may be observed when fermentation is done at this temperature (Kucharczyk & Tuszyński 2018, 233).

7 CONCLUSION

The aim of this thesis was to find out the concentrations of acetaldehyde in three different batches of the lager ‘Helles’ from Kahakka Brewery in Kokkola, Finland in order to observe if there were any significant differences in the acetaldehyde levels between an old batch compared to a new one. The acetaldehyde in the beer samples was derivatized using the derivatization agent PFBHA and extracted with n-hexane during liquid-liquid extraction before being analysed using GC-MS.

While the method proved the detection of acetaldehyde, the methodology cannot be determined as accurate or ideal. There is need for future development of a more accurate method by using a different internal standard such as deuterated acetaldehyde (D_4 acetaldehyde) and focusing on one batch of beer to see if values are consistent. After the replicability and accuracy of the method has been determined, changes in acetaldehyde from the same batch of beer can be tested over a year, perhaps one test per month, to see if there is any change in the acetaldehyde and if there is an increase, then the rate of increase with time can be determined.

Furthermore, the overall method used in this thesis was extremely time consuming (it took approximately 6 hours to prepare the samples and calibration standards) and involved many steps, therefore there was the potential for many errors. A different method can also be employed such as headspace analysis, solid phase microextraction or stir-bar sorptive analysis which are all less time consuming and may result in less laboratory errors.

Lastly, it is difficult to conclude on how the changing concentration of acetaldehyde will affect the overall flavour of the beer as there are other compounds that attribute to the changes in flavour during aging. For the future, it may also be beneficial to also test the sensory changes in the beer, where taste experts test the sensorics of beer that has been aged for 1 year compared to a recent batch of the same beer and to give an overview on sensory changes (if any) that they detect. This experiment may also be conducted but using consumers rather than professional beer testers to conclude if the overall change in taste of the beer (if any) can be detected by them and if it is an unpleasant change to them.

REFERENCES

Agilent Technologies. 2002. Fundamentals of Gas Chromatography. First Edition. Agilent Technologies, Inc: Delaware

Aron, P. 2014. Beer Stability: A Perspective on Beer Flavour Stability. Available at https://www.new-foodmagazine.com/article/14096/beer-stability-a-perspective-on-beer-flavour-stability/. Accessed on 22 July 2019

Baert, J. J., De Clippeleer, J., Hughes, P. S., De Cooman, L. & Aerts, G. 2012. On the Origin of Free and Bound Staling Aldehydes in Beer. Journal of Agriculture and Food Chemistry 60, 11449 – 11472. dx.doi.org/10.1021/jf303670z

Bamford, C. W. 2003. Beer: Tap into the Art of Brewing. Second Edition. New York: Oxford University Press, Inc.

Bamforth, C.W. 2011. 125th Anniversary Review: The non-biological instability of beer. J. Inst. Brew. 117, 488–497

Bao, M., Joza, P. J., Masters, A. & Rickert, W. S. 2014. Analysis of Selected Carbonyl Compounds in Tobacco Samples by Using Pentafluorobenzylhydroxylamine Derivatization and Gas Chromatography-Mass Spectrometry. Contributions to Tobacco Research, 26 (3), 86 – 97. DOI: 10.2478/cttr-2014-0017

Barth, R. 2013. The Chemistry of Beer. The Science in the Suds. New Jersey: John Wiley & Sons, Inc.

Berk, Z. 2013. Food Process Engineering and Technology. Second Edition. Israel: Elsevier Science and Technology

Bickhamb, S. 2001. Hoppy and Maturation Flavours in a Nutshell. Available at https://www.more-beer.com/articles/Hoppy_Maturation_Flavors. Accessed on 14 February 2020

Briggs, D. E., Boulton, C. A., Brookes, P. A., & Stevens, R. 2004. Brewing Science and Practice. Cambridge: Woodhead Publishing Limited

Buiatti, S. 2009. Beer Composition: An Overview. In V. R. Preedy (Ed.). Beer in Health and Disease Prevention. London: Elsevier Inc. 213 - 225

Cerny, C. 2009. Thermal Generation of Aroma-Active Volatiles in Food. In A. Herrmann (Ed.). The Chemistry and Biology of Volatiles. West Sussex: John Wiley & Sons, Ltd. 231 – 254

Chen, L. 2019. Chemical potential and Gibbs free energy. MRS Bulletin 44(7), 520-523. doi:10.1557/mrs.2019.162

Chromatography Today. 2013. Why Do I have a Carryover? Available at: https://www.chromatography-today.com/article/hplc-uhplc/31/the-chromatography-helpdesk/why-do-i-have-carryover/1490. Accessed on 19 December 2020

Clark, J. 2019. Properties of Esters. Available at https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Supplemental_Modules_(Organic_Chemistry)/Esters/Properties_of_Esters. Accessed on 26 July 2019

Clement, R. E. and Hao, C. 2012. Liquid-Liquid Extraction – Basic Principles and Automation. In H. L. Lord & J. Pawliszyn (Eds.). Comprehensive Sampling and Sample Preparation: Analytical Techniques for Scientists: Volume 2. Oxford: Elsevier Inc. 51 - 63

Croguennec, T. 2016. Non-Enzymatic Browning. In R. Jeantet, T. Croguennec, P, Schuck and G, Brulé (Eds.). Handbook of Food Science and Technology 1: Food Alteration and Food Quality. London: John Wiley & Sons, Inc. 133 - 157

Cuadros-Rodríguez, L., Bagur-Gonzalez, M. G., Sánchez-Viñas, M., González-Casado, A. & Gómez-Sáez, A. M. 2007. Principles of analytical calibration/quantification for the separation sciences. Journal of Chromatography A, 1158, 33-46

De Keukeleire, D. 2000. Fundamentals of Beer and Hop Chemistry. QUÍMICA NOVA 23(1), 108-112

Dolan, J. W. 2012. When Should an Internal Standard be Used? Available at: https://www.chromatographyonline.com/view/when-should-internal-standard-be-used-0. Accessed on 17 December 2020

Esbensen, K. H. & Wagner, C. Sampling quality assessment: the replication experiment. Available at: https://www.spectroscopyeurope.com/sampling/sampling-quality-assessment-replication-experiment. Accessed on 16 December 2020

Fermentis. 2017. Saflager™ S-23. Available at: https://fermentis.com/wp-content/uploads/2018/03/SafLager-S-23.pdf. Accessed on 29 December 2020

Flowers, P., Theopold, K & Langley, R. 2017. Molarity and Dilutions. Available at: https://chem.libretexts.org/Courses/Valley_City_State_University/Chem_121/Chapter_4%3A_Solution_Chemistry/4.5%3A_Molarity_and_Dilutions. Accessed on 21 November 2020

Gabrio, T. & Bertsch, A. 2004. Determination of Carbonyl Compounds in Pool Water With *O*-(2,3,4,5,6-Pentafluorobenzyl)Hydroxyamine Hydrochloride and Gas Chromatographic–Tandem Mass Spectrometric Analysis. Journal of Chromatography A, 1046, 293 – 296

Guidechem. 2020. Acetaldehyde,O-[(2,3,4,5,6-pentafluorophenyl)methyl]oxime. Available at: https://www.guidechem.com/encyclopedia/acetaldehyde-o-2-3-4-5-6-penta-dic207088.html. Accessed on 15 December 2020

Hill, G & Holman, J. 2011. Chemistry in Context. Sixth Edition. Oxford: Oxford University Press

Kucharczyk, K. & Tuszyński, T. 2018. The effect of temperature on fermentation and beer volatiles at an industrial scale. Journal of the Institute of Brewing, 124(3), 230 - 235

Kunze, W. 2014. Technology Brewing & Malting: Fifth Edition. Berlin: VLB Berlin.

Lordan, R.; O'Keeffe, E.; Tsoupras, A.; Zabetakis, I. 2019. Total, Neutral, and Polar Lipids of Brewing Ingredients, By-Products and Beer: Evaluation of Antithrombotic Activities. Foods 8(5), 171.

Martineau, B., Acree, T. & Henick-Kling, T. 1994. A Simple and Accurate GC/MS Method for the Quantitative Analysis of Diacetyl in Beer and Wine. Biotechnology Techniques 8(1), 7 – 12

Matthias, J. 2018. A Beginner's Guide: How to Interpret Gas Chromatography Mass Spectrometry Results. Available at https://www.innovatechlabs.com/newsroom/1841/how-to-interpret-gas-chromatography-mass-spectrometry-results/. Accessed on 17 February 2020

McNair, H. M. & Miller, J. M. 2009. Basic Gas Chromatography. Second Edition. New Jersey: John Wiley & Sons Inc

Munch, J. W. 1998. A user's guide to aldehyde analysis using PFBHA derivatization and GC/ECD detection: avoiding the pitfalls. Available at: https://cfpub.epa.gov/si/si_public_record_Report.cfm?Lab=NERL&dirEntryId=95977. Accessed on 13 February 2020

Munch, J W., Munch, D. J & Winslow, S.D. 1998. Determination of Carbonyl Compounds in Drinking Water by Pentafluorbenzylhyroxylamine Derivatization and Capillary Gas Chromatography and Electron Capture Detection. Method 446. Ohio: EPA

Orata, F. 2012. Derivatization Reactions and Reagents for Gas Chromatography Analysis. In M. A. Mohd (Ed.) Advanced Gas Chromatography - Progress in Agricultural, Biomedical and Industrial Applications. InTech, 83 – 108. Available at: http://www.intechopen.com/books/advanced-gaschromatography-progress-in-agricultural-biomedical-and-industrial-applications/derivatization-reactions-andreagents-for-gas-chromatography-analysis

Pires, E. J., Teixeira, J. A., Brányik, T & Vicente, A. A. 2014. Yeast: the soul of beer's aroma—a review of flavour-active esters and higher alcohols produced by the brewing yeast. Applied Microbiology and Biotechnology 98(5), 1937 – 1949. DOI: 10.1007/s00253-013-5470-0

Pubchem. 2019. Acetaldehyde (Compound). Available at https://pubchem.ncbi.nlm.nih.gov/compound/Acetaldehyde#section=Information-Sources. Accessed on 21 October 2019

Reusch, W. 2013. Mass Spectrometry. Available at https://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/MassSpec/masspec1.htm. Accessed on 15 February 2020

Rodigast, M., Mutzel, A., Iinuma, Y., Haferkorn, S. & Herrmann. H. 2015. Characterisation and Optimisation of a Sample Preparation Method for the Detection and Quantification of Atmospherically Relevant Carbonyl Compounds in Aqueous Medium. Atmospheric Measuring Techniques 8, 2409-2416. doi:10.5194/amt-8-2409-2015

Saison, D., De Scutter, D. P., Uyttenhove, B., Delvaux, F. & Delvaux F. R. 2009a. Contribution of Staling Compounds to the Aged Flavor of Lager Beer by Studying their Flavour Thresholds. Food Chemistry 114, 1206 – 1215

Saison, D., De Scutter, D. P., Delvaux, F. & Delvaux F. R. 2009b. Determination of carbonyl compounds in beer by derivatisation and headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry. Journal of Chromatography A 1216, 5061 – 5068, doi:10.1016/j.chroma.2009.04.077

Scbt. 2020. Acetaldehyde,O-[(2,3,4,5,6-pentafluorophenyl)methyl]oxime. Available at: https://www.scbt.com/p/acetaldehyde-o-pentafluorophenylmethyl-oxime-114611-59-5. Accessed on 15 December 2020

Shevlyakov, G.L. & Oja, H. 2016. Robust Correlation. Theory and Applications. Chichester: John Wiley & Sons Ltd.

Simkus, D. N., Hilts, R. W., Herd, C. D. K., Aponte, J. C. & Elsila, J. E. 2016. First Report of Aldehydes and Ketones in the Tagish Lake Meteorite: Optimized Methodology and Preliminary Results. 47th Lunar and Planetary Science Conference

Sparkman, O. D., Penton, Z. E. & Kitson. F. G. 2011. Gas chromatography and mass spectrometry: A practical Guide. Second Edition. Oxford: Elsevier Inc

Stewart, G. G. 2017. The Production of Secondary Metabolites with Flavour Potential during Brewing and Distilling Wort Fermentations. Fermentation 3(63), doi:10.3390/fermentation3040063

Telgheder, U., Bader, N & Alshelmani, N. 2008. Stir bar soptive extraction as a sample preparation technique for chromatographic analysis: An overview. Asian Journal of Nanoscience and Materials 1(2), 56 -62

Thet, K and Woo, N. 2019. Gas Chromatography. Available at https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumental_Analysis/Chromatography/Gas_Chromatography. Accessed on 13 August 2019

Torontech. 2020. Densimeter/ Specific Gravity Tester. Available at https://www.torontech.com/densimeter-specific-gravity-tester. Accessed on 22 November 2020

Vanderhaegen, B., Neven, H., Verachtert, H & Derdelincx, G. 2006. The Chemistry of Beer Aging – A Critical Review. Food Chemistry 95, 357 – 381

Wang, M., Wang, C & Han, X. 2017. Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry-What, how and why?. Mass Spectrom Rev 36(6), 693-714

Wardencki, W., Orlita, J. & Namieśnik, J. 2001. Comparison of Extraction Techniques
For Gas Chromatographic Determination of Volatile Carbonyl Compounds in Alcohols. Journal of Analytical Chemistry, 369, 662 - 665

Wells, M.J.M. 2003. Principles of Extraction and the Extraction of Semivolatile Organics from Liquids. In J.D. Winefordner and S. Mitra (Eds.). Sample Preparation Techniques in Analytical Chemistry

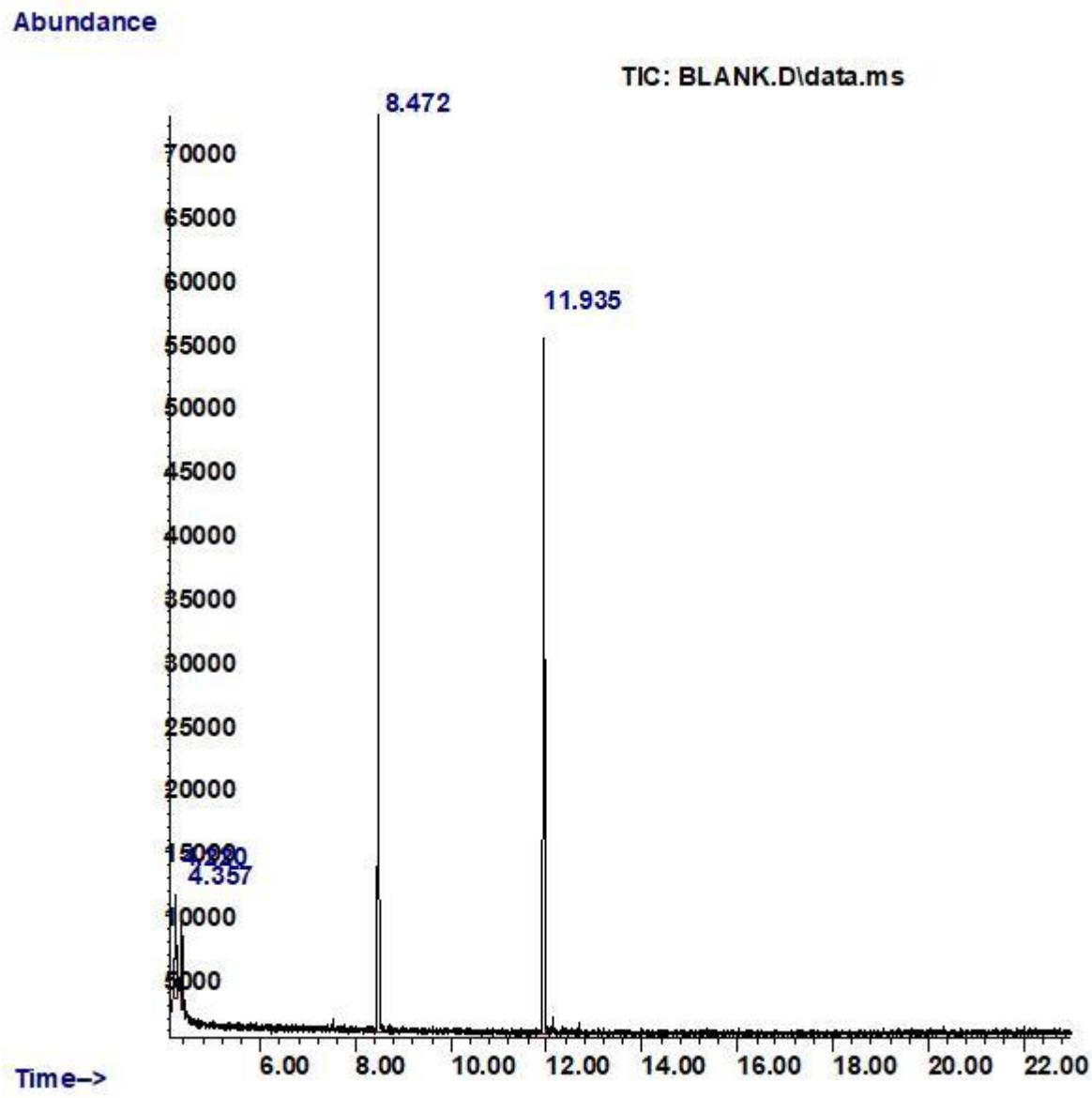
Willaert. R. 2007. The Beer Brewing Process: Wort Production and Beer Fermentation. In Y. H. Hui (Ed.). Handbook of Food Products Manufacturing. New Jersey: John Wiley and Sons, Inc. 443 - 506

APPENDICES

APPENDIX 1

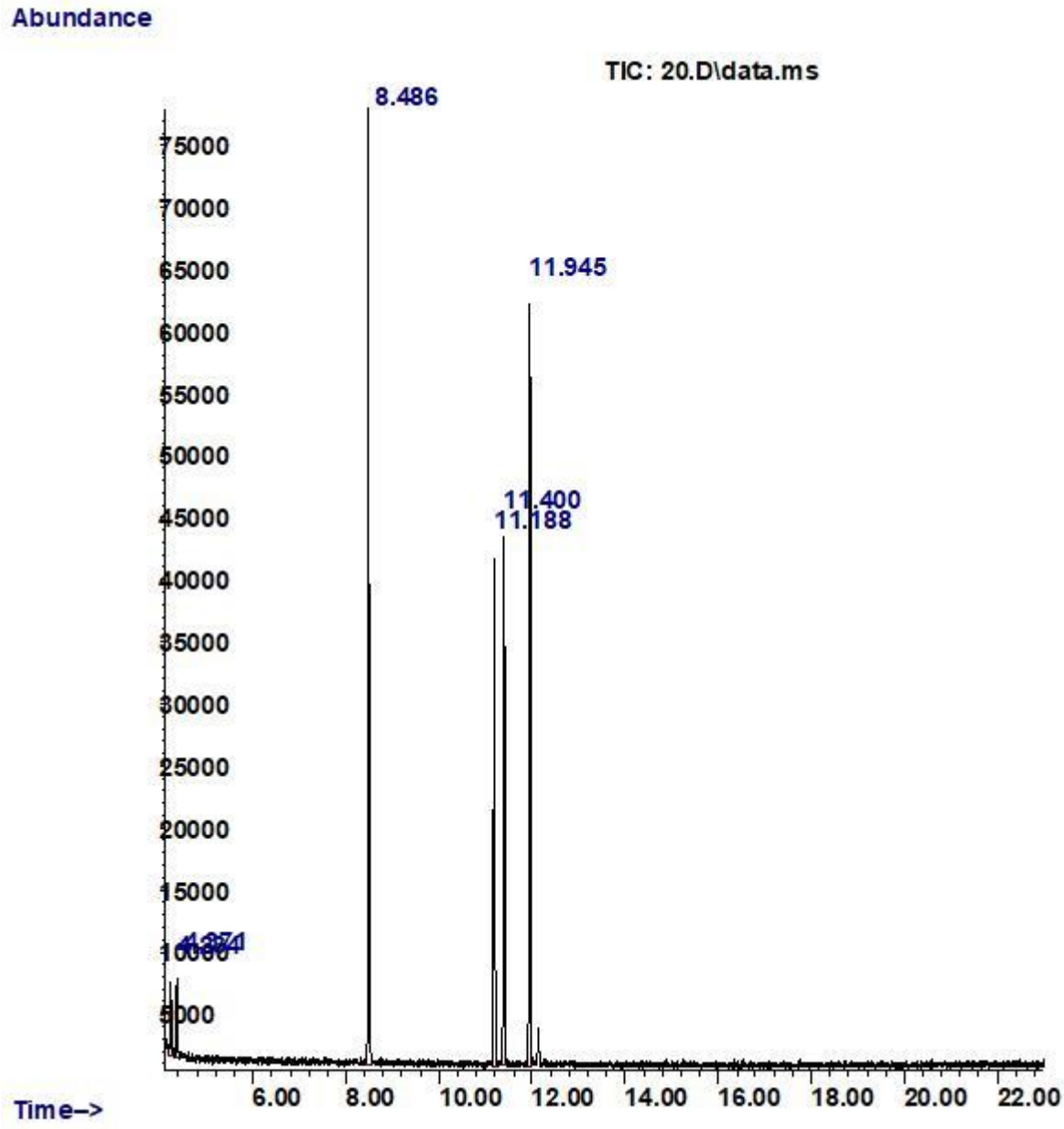
BLANK

Blank chromatogram



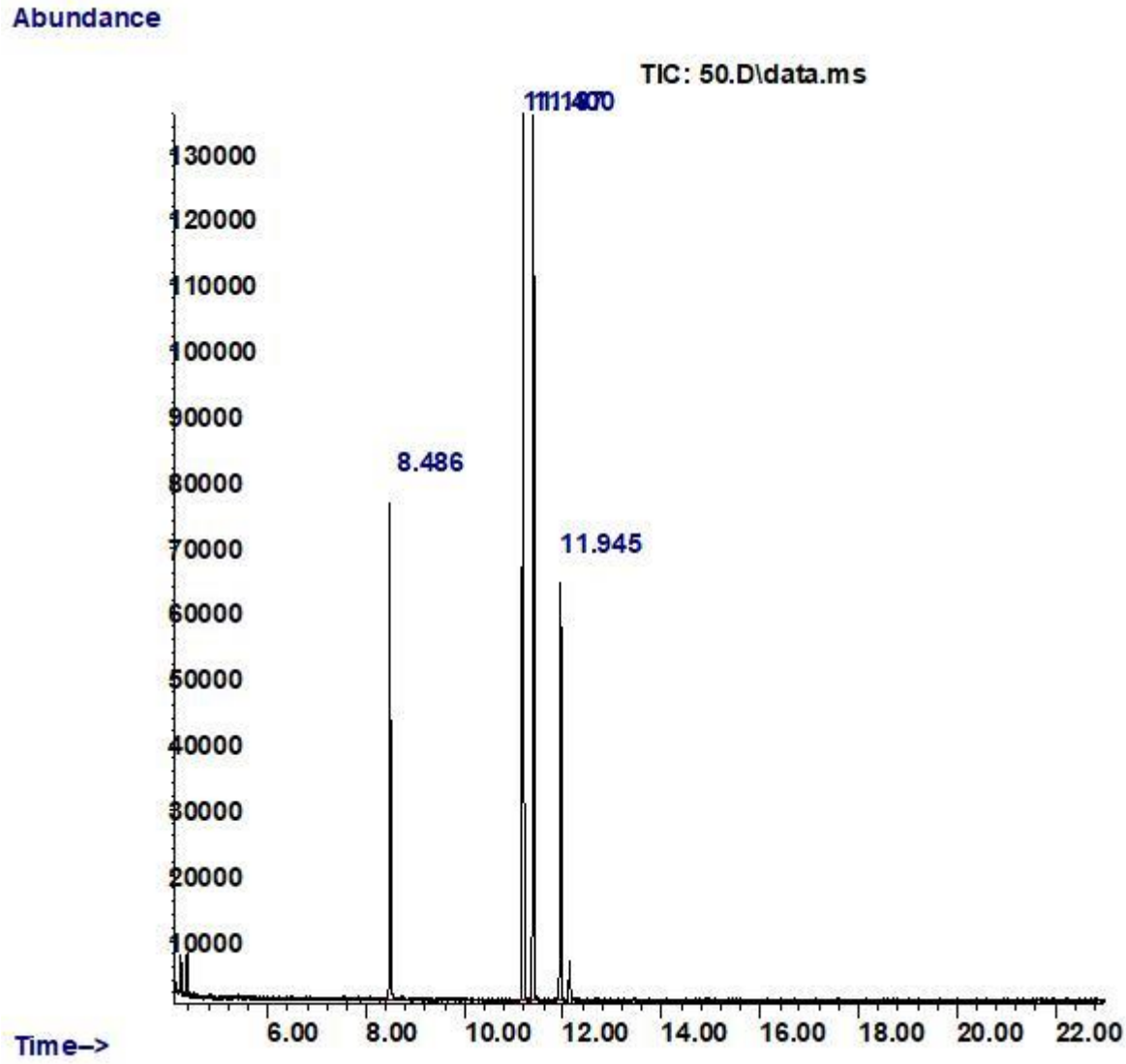
CALIBRATION STANDARD

0.78 mg/L calibration standard chromatogram



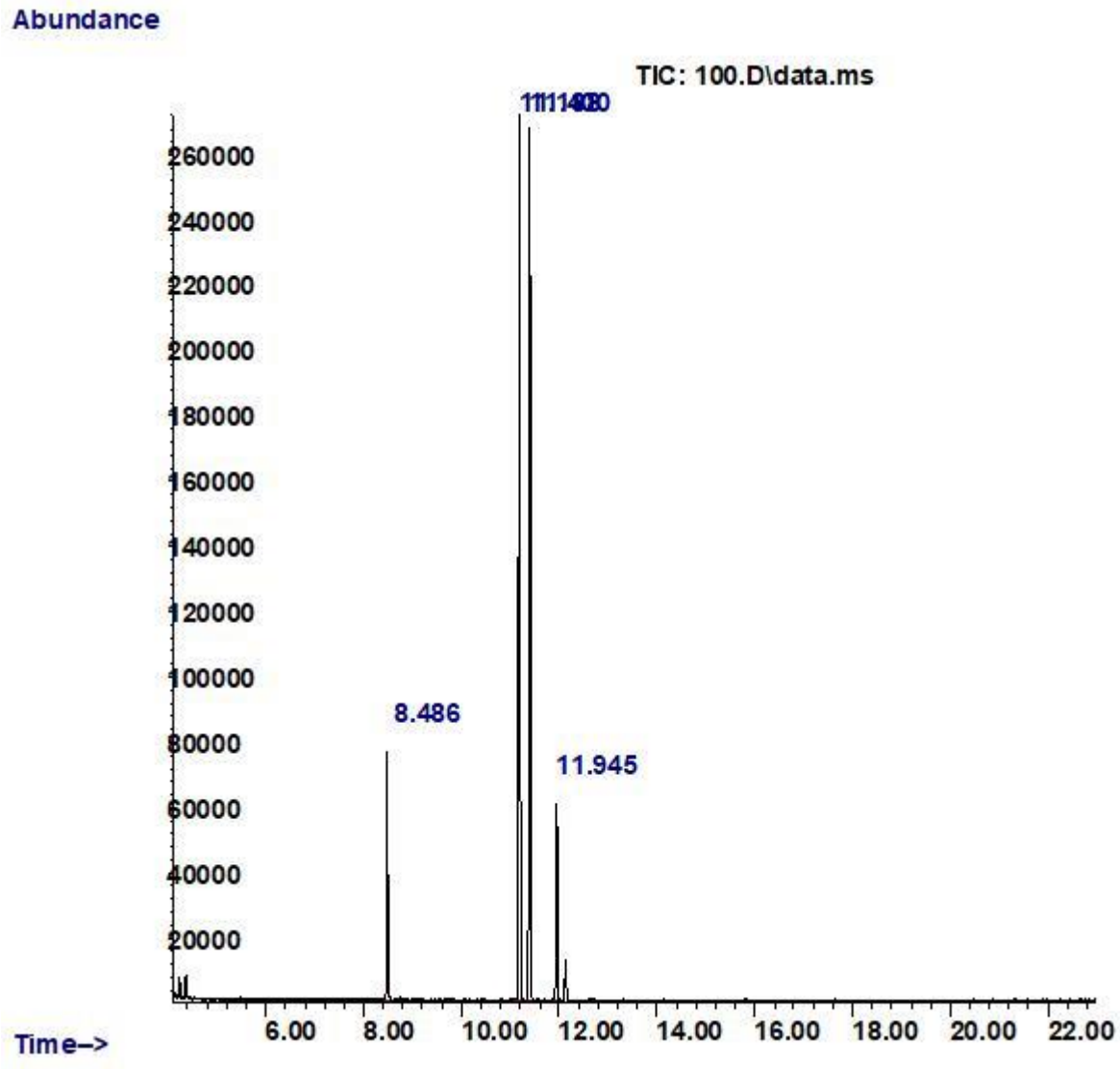
CALIBRATION STANDARD

1.96 mg/L calibration standard chromatogram



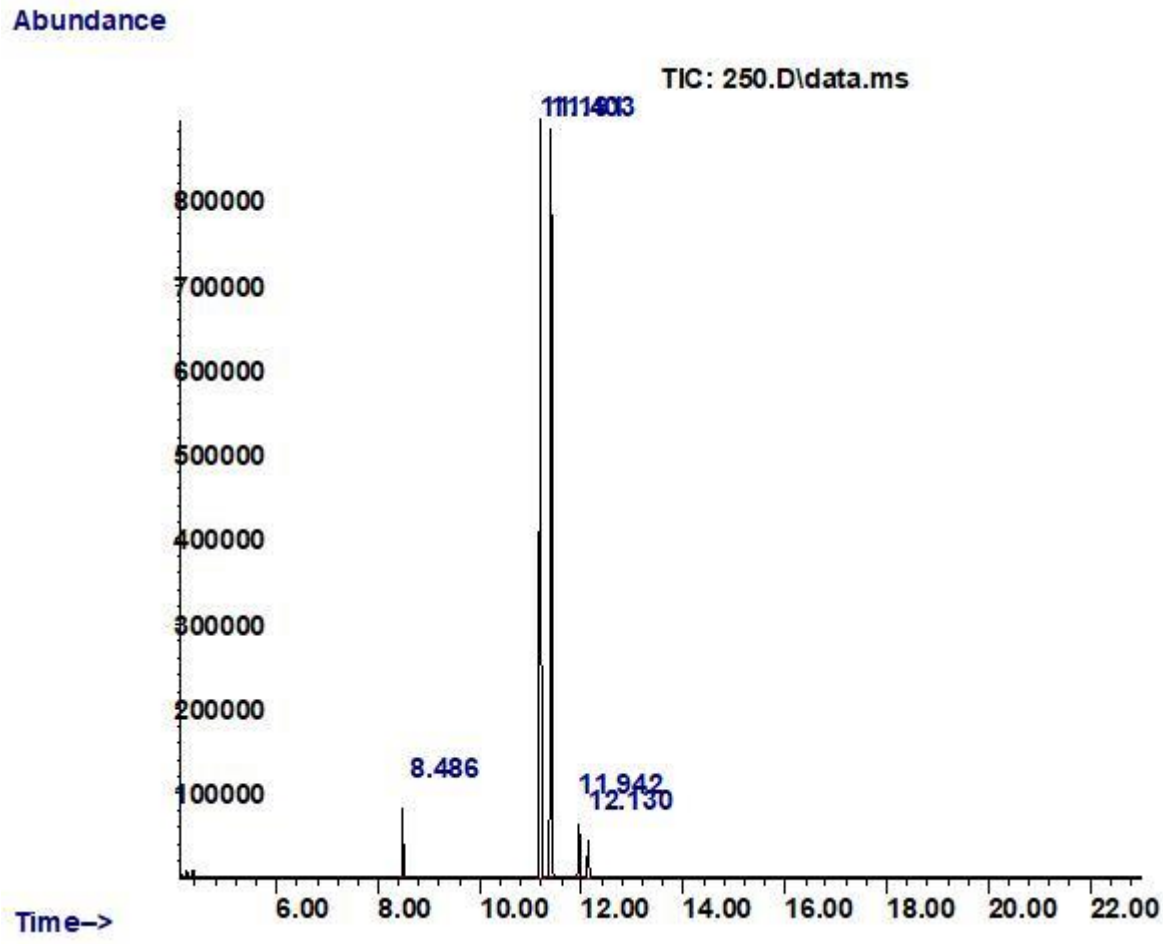
CALIBRATION STANDARD

3.92 mg/L calibration standard chromatogram



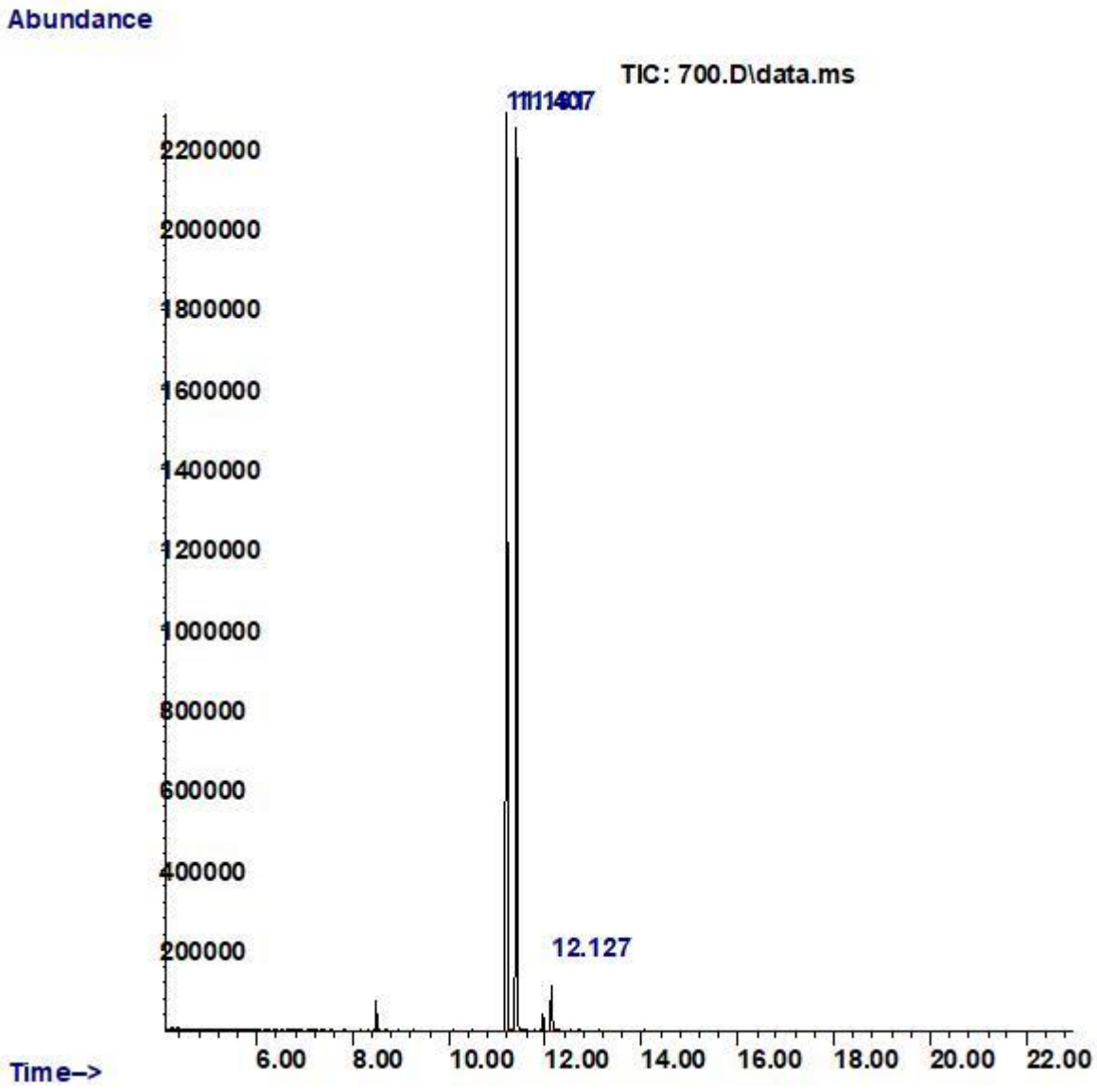
CALIBRATION STANDARD

9.80 mg/L calibration standard chromatogram



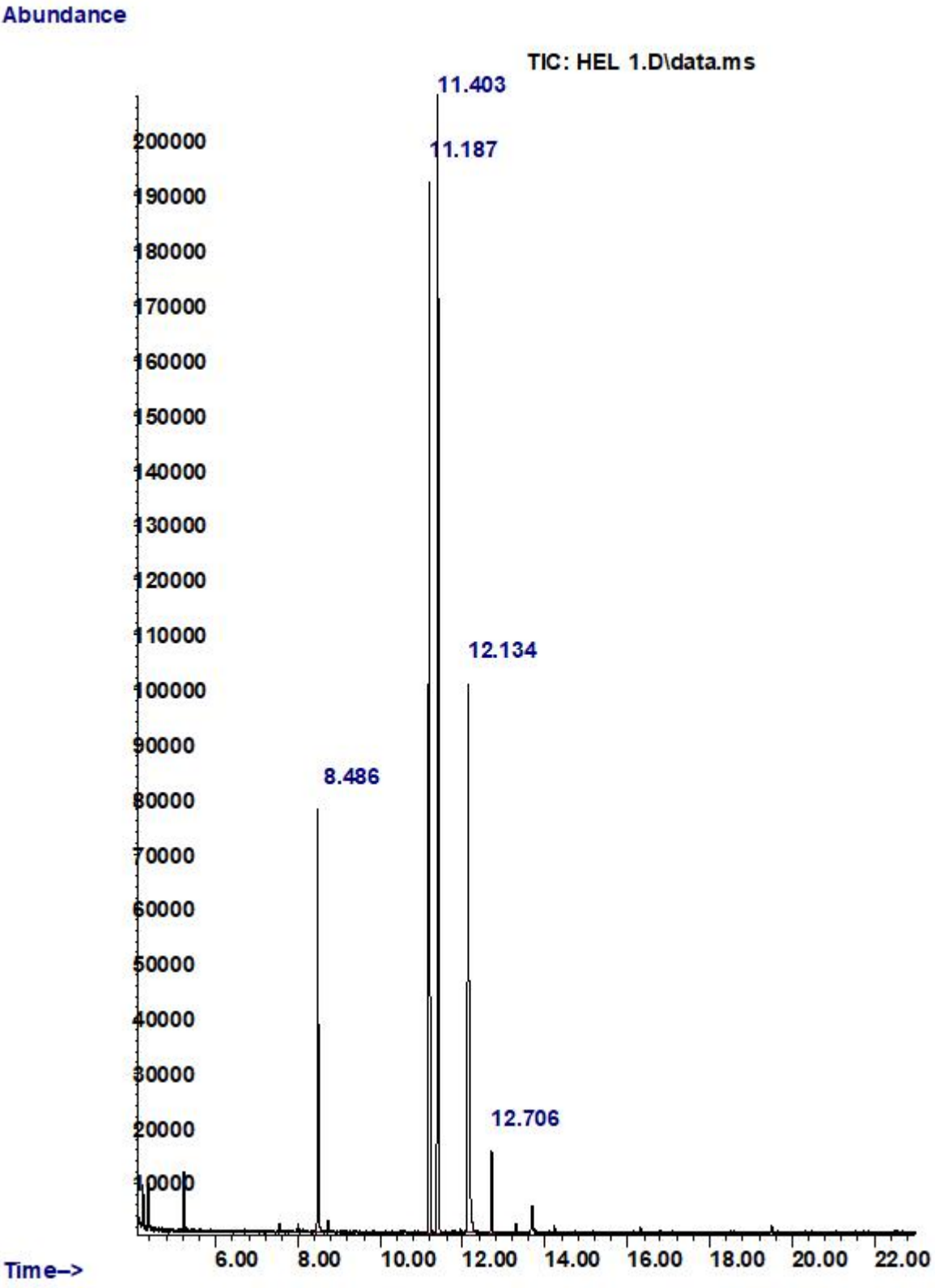
CALIBRATION STANDARD

27.44 mg/L calibration standard chromatogram



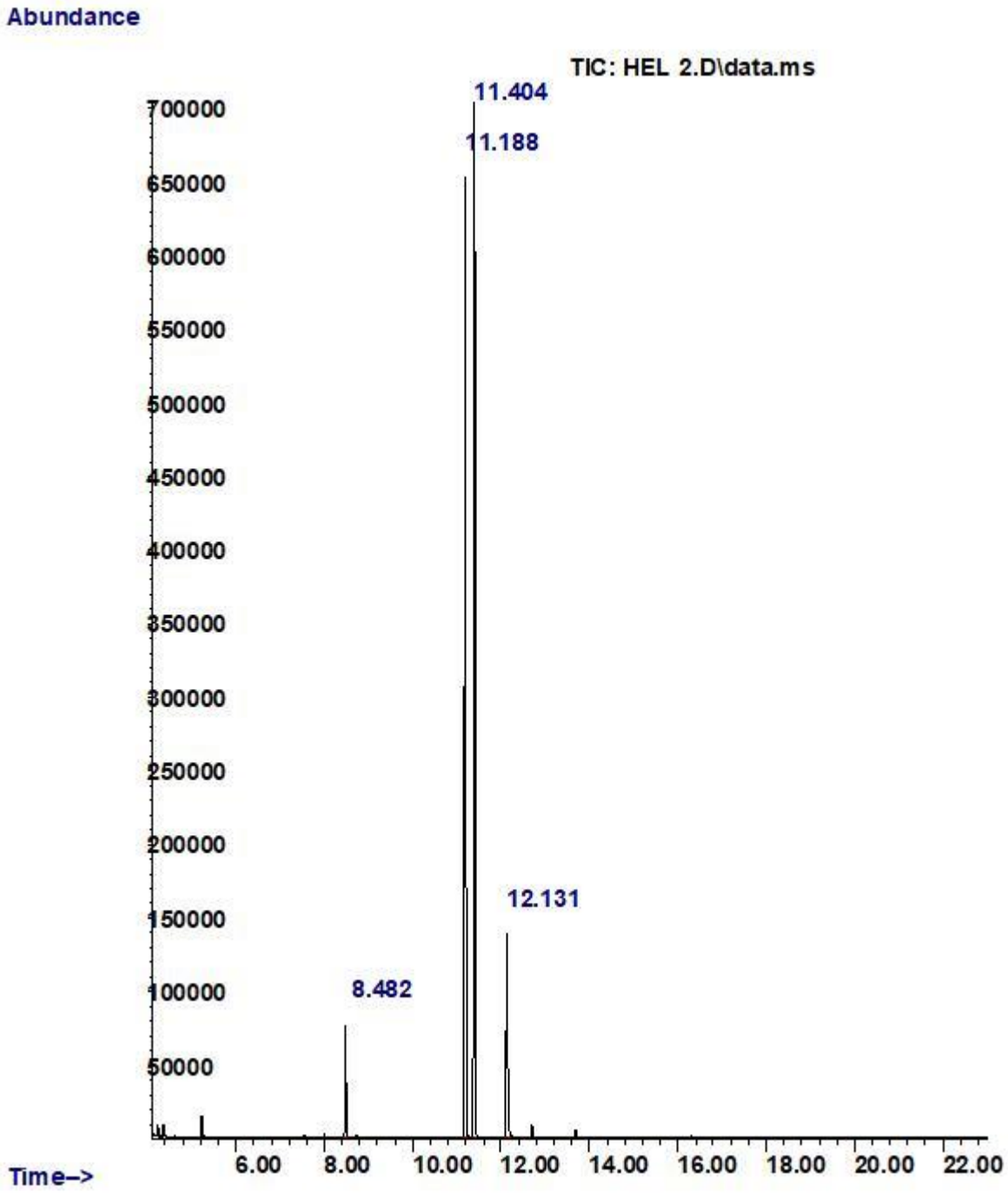
BEER SAMPLE

HEL 1 chromatogram



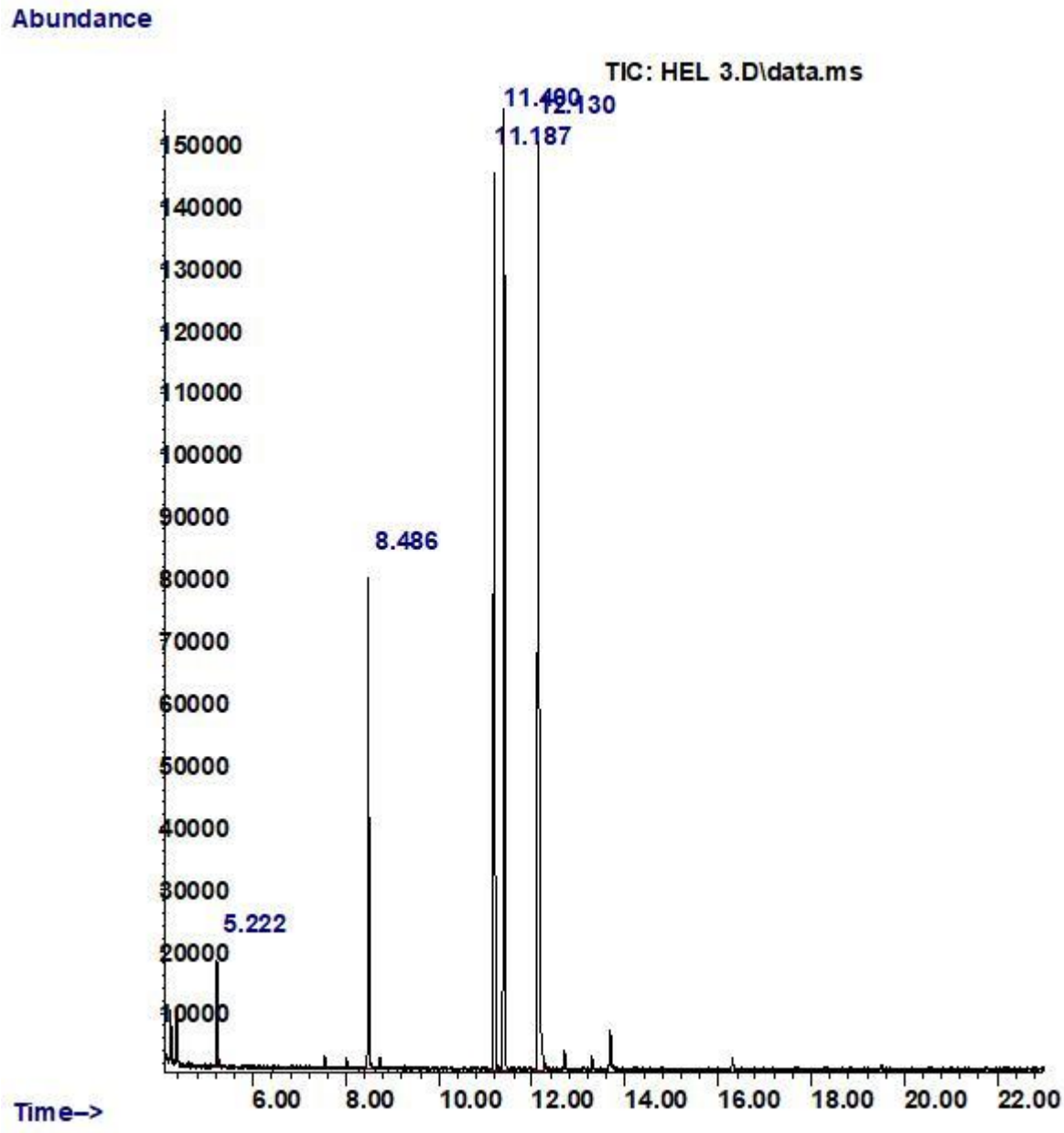
BEER SAMPLE

HEL 2 chromatogram.



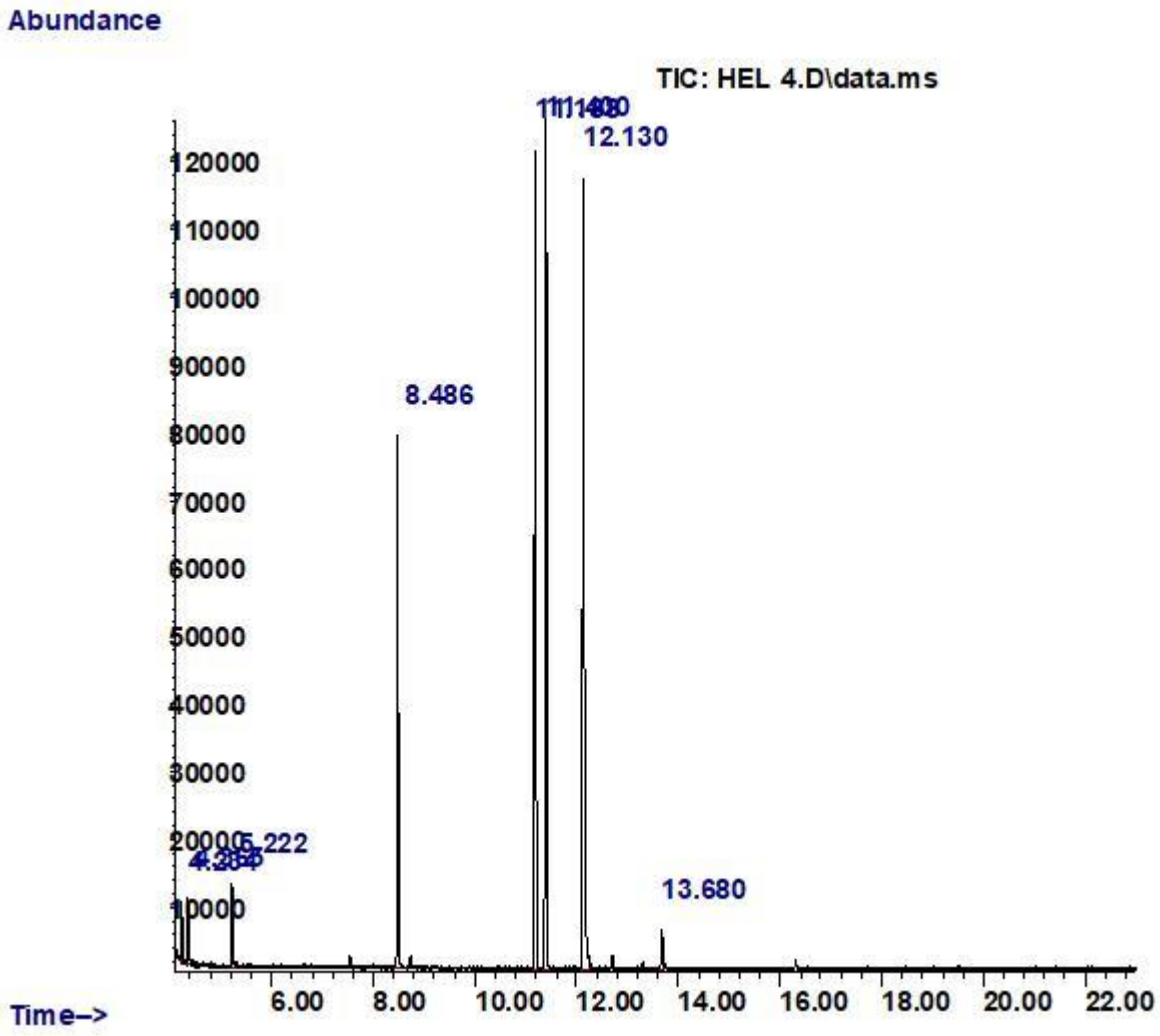
BEER SAMPLE

HEL 3 chromatogram.



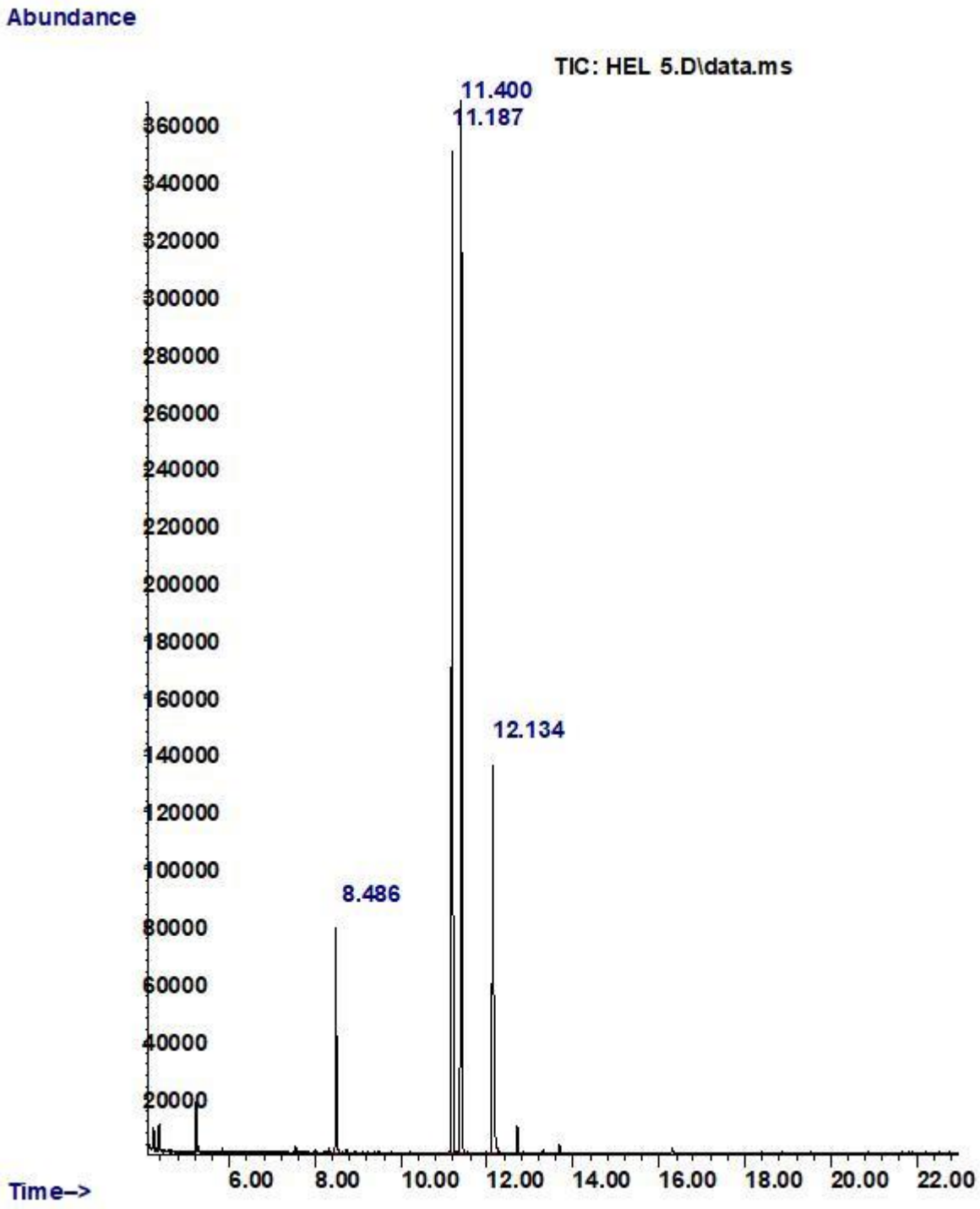
BEER SAMPLE

Hel 4 chromatogram.



BEER SAMPLE

HEL 5 chromatogram.



BEER SAMPLE

HEL 6 chromatogram.