eman ta zabal zazu
Universidad del País Vasco
Euskal Herriko Unibertsitatea
ZIENTZIA ETA TEKNOLOGIA FAKULTATEA
FACULTAD DE CIENCIA Y TECNOLOGÍA

Bachelor Degree Final Proyect
Biotechnology

Beer composition analysis

Abundance evolution and characterization of beer flavor active compounds by GC-MS and determination of its ethanol concentration using MIR spectroscopy.

Student:
Leire Martínez de Butrón Revuelta

Directors:
Dr. Gorka Elordi Foruria
Dr. Pedro Castaño Sánchez

Leioa, 20th of June of 2018

INDEX

1. INTRODUCTION

Beer has become one of the most widespread and commonly consumed alcoholic beverages in the world. Its brewing process is a very antique practice that undoubtedly represents mankind's oldest biotechnology. Despite the massive technological advance that separates ancient brewing from today's high-tech breweries, the basis of its production process remains entirely unchanged (Bamforth, 2017). As seen in **Figure 1,** beer brewing consists of 6 consecutive steps: Malting, Milling, Mashing, Boiling, Fermentation and Packaging.

During malting, barley grains are induced to germinate over a limited period of time so as to start synthesizing glucoamylases and α and β amylases, which are the enzymes that will transform barley starch into fermentable sugars. Meanwhile, cellulose degrading enzymes are also produced, which will soften the grain wall making it more millable. The germination phase is ended through a kilning process that dries out the grains. The end product of malting is germinated barley, which is known as malt. After several weeks of storage, the malt is milled to produce more extractable particles and it is prepared for mashing. The most important process occurring during mashing is the breakdown of malt starch into fermentable sugars.

To initiate this process, the milled malt is soaked into hot water at a pre-specified temperature to produce a slurry mixture known as mash. At this point, the starch granules become gelatinize and susceptible to amylase digestion, which breaks them down into sugars. As a result, a watery liquid rich in fermentable sugars is produced. Such liquid is known as wort, which is the ultimate precursor of beer. Before undergoing fermentation, the wort is boiled with different hops to extract its bitterness and aroma. Finally, yeast is added to the wort to carry out fermentation. During this stage, the yeast transforms the sugars present in the wort into different compounds such as ethanol, CO_2, glycerol and higher alcohols. Once the process is over, yeast is removed and beer is subjected to different stabilization and clarification treatments before being packed (Bamforth, 2017). To generate a high quality final product, it is crucial to control and monitor the appearance and evolution of several influencing parameters throughout the production line. One of the parameters that affects beer quality the most is its flavor. Flavor is described as the set of sensory impressions

experienced when ingesting a substance. It is mainly determined by the sense of taste and smell. In this regard, aroma, which is the most deterministic parameter that affects the scent of a product, has an indirect impact on flavor. During the brewing process, many flavor active compounds are generated. All of them can be classified into 3 groups; malt derived compounds, hops derivatives and yeast fermentation by-products, which are each produced at different stages within the brewing process. In terms of flavor compound production, the brewing process can be divided into 3 different stages (**Figure 1**).

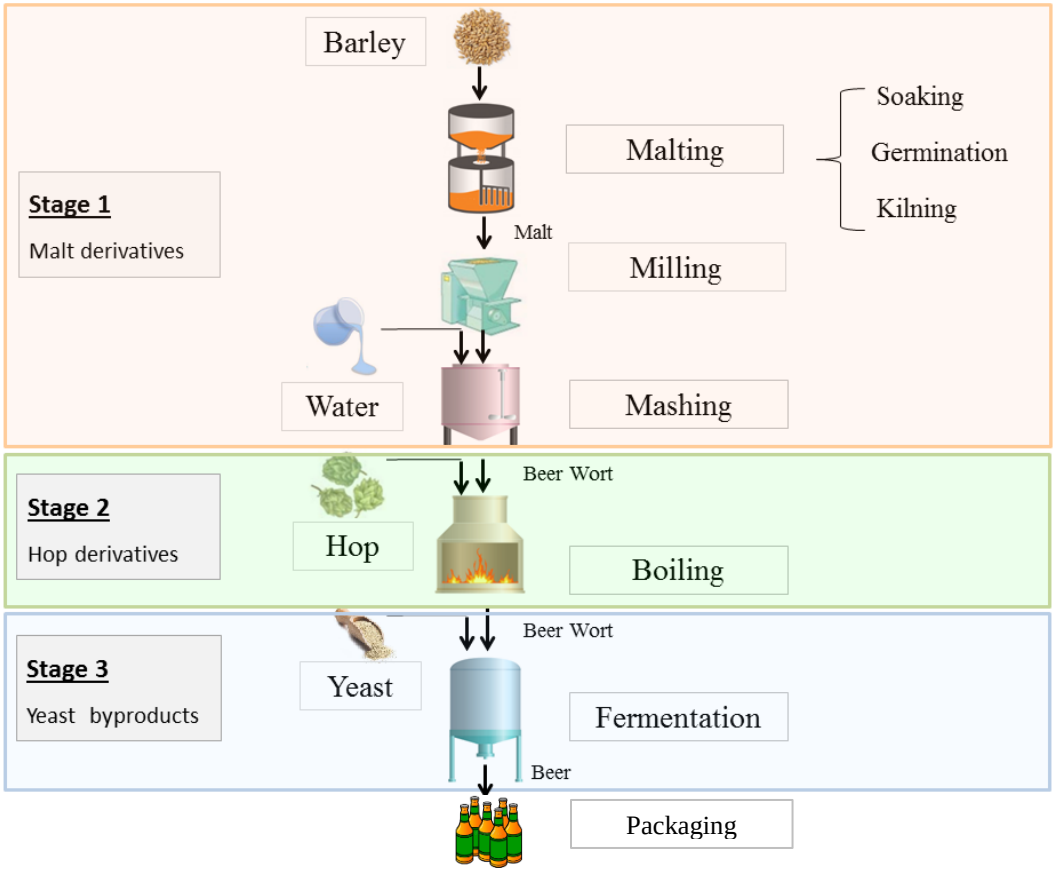

Figure 1: Classification of the brewing process into 3 stages regarding the flavor active compounds that are produced at each step of the production line. The 6 consecutive steps that make up the brewing process are labeled on the right hand of the figure. These are grouped into 3 different stages depending on what of the 3 types of flavor active compounds (malt derivatives, hop derivatives or fermentation by-products) are produced when they occur. The first stage, represented in an orange box, includes malting, milling and mashing and corresponds to the phase when flavor active compounds are produced. The second, which is represented in a green box, corresponds to the boiling process in which hop derived flavor active compounds are extracted. The third, which is represented in a blue box, coincides with fermentation, when fermentation organoleptic by-products are produced. The steps at which each of the 4 main raw materials of beer (barley, water, hop and yeast) is added, is also pointed out at the diagram.

During the first stage, the predominant flavor active compounds that are produced are malt-derived aromatic volatile compounds. These are predominantly formed at the final stage of malting, during the kilning process, when the Maillard reactions occur. The chemistry underlying the Maillard reaction is very complex as it encircles a whole network of various reactions (Pires, 2014). As a result, highly flavor active compounds such as furanol, maltol and isomaltol are produced, which give beer a caramel-like flavor. Many heterocycles are also generated as byproducts of the Maillard reaction, from which the most flavor active ones are thiazoles, thiazolines, pyridines, pyrrolizines and pyrazines, which respectively have a malty, oxidized, sweet, meaty and burnt flavor (Ademola, 2017). However, a significant amount of these heterocycles are lost during boiling.

The second stage includes the boiling process, when hop derived flavor active compounds are extracted from the proper hop into the wort. Some hops are known as "flavor hops" while others as "bitter hops." Hop iso-α-acids such as humulone, cohumulone and adhumulone are responsible for the so characterizing bitter flavor found in beers, whereas hop aroma is tied to essential oils found in hop cones.

At the last stage, the main organoleptic compounds produced are higher alcohols, aldehydes, esters and vicinal diketones (VDKs) , which are all volatile compounds (VOCs) produced during fermentation. From them, higher alcohols are the ones produced in highest quantities during fermentation. The most abundant higher alcohol found in beer is ioamyl alcohol, followed by isobutanol, 3-methylbutanol and 1-Propanol. If found in optimal concentrations, they give beer a desirable mouth warming sensation (Ademola, 2017).

Most higher alcohols are produced as derivatives of their corresponding aldehydes. Therefore, there is an intimate link between higher alcohols formation and aldehydes reduction. Such aldehydes are formed during wort preparation, from processes such as Maillard reactions and lipid oxidation. Regarding the flavor effect they have in beer, aldehydes are much more flavor-active than their corresponding alcohols and are generally considered as flavor put-offs. Acetaldehyde is the major aldehyde as it is produced as an intermediate in the formation of ethanol and acetate. It produces a crisp green apple flavor in beer. Other secondary aldehydes such as 2-butenal, nonenal and

pentenal also affect beer flavor. It is thought that nonenal is responsible for the stale flavor that appears during beer aging.

Esters, on the other hand, add a pleasant fruity-flowery flavor to beer. Nevertheless, if overproduced, they can give beer a bitter, over-fruity taste, which is considered undesirable by most consumers (Ademola, 2017). Esters are primarily produced during the phase of primary fermentation as a result of the condensation of organic acids and alcohols. Volatile esters in beer can be divided into two major groups: the acetate esters and the ethyl esters. Although dozens of different esters can be found in any beer (Engan, 1974), six of them are of major importance to its flavor: ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), isobutyl acetate (fruity aroma), phenyl ethyl acetate (roses and honey aroma), ethyl hexanoate (sweet apple aroma) and ethyl octanoate (sour apple aroma) (Pires, 2014).

VDKs, are considered as flavor spoilers as they give an unappetizing 'butterscotch' flavor. The most noteworthy flavor active VDKs, are 2,3-butanedione and 2,3-pentanedione, which are produced as a result of valine and isoleucine anabolism respectively (Bokulich, 2013). The concentrations of both VDKs are of critical significance to beer flavor. At the end of the main fermentation, most diacetyl content is re-assimilated by yeast and is reduced to acetoin and 2,3-butanediol, which have relatively high flavor thresholds. (Krogerus, 2013; Ademola, 2017).

The ratio of flavor active compounds found in beers is strictly dependent on the barley, hop and yeast strain used, therefore, its selection process is vital to produce a determine flavor profile (Pires, 2014). Some flavor active compounds, such as higher alcohols, have a desirable effect of beer flavor; however others such as VDKs can affect it very negatively. Therefore, it is vital that all flavor-active components are kept within certain limits; otherwise, the flavor balance could be destroyed, resulting in a distasteful product (Ademola, 2017). It is absolutely necessary to provide a high number of analyses that ensures the quality of the final product. This, together with the need for a fast response to detect changes in the production process of beer, has encouraged the commercialization of specific instruments for beer analysis (Cascant , 2017; Andrés-Iglesias, 2015).

One of the most popular and accurate analytical techniques for beer is gas chromatography mass spectrometry (GC-MS). Given the volatile nature and low concentrations of most of the flavor components of interest in beer, gas chromatography (GC) has become the optimal technique for beer flavor analysis (Rossi, 2014). To identify the different organoleptic molecules found in the samples, GC is often combined with detectors such as mass spectrometers (MS), which enables a classification of such molecules regarding their mass to charge ratio (m/z). When analyzing beer using GC-MS, head space (HS) sampling is often employed, which allows only volatile compounds to be injected into the column. To optimize the efficiency of the HS injection technique, the samples are often submitted to a previous extraction process. Solid phase microextraction (SMPE) has come forward as the most successful extraction method owing to its high sensitivity, reproducibility and low cost. Hence, in last decade, the combination of these 4 techniques, HS-SPME-GC-MS, has been implemented as the state-of-the-art method for the analysis of beer flavor (Andrés-Iglesias, 2015).

Besides, other techniques such as mid-infrared spectroscopy (MIR) are gaining importance in beer composition analysis. MIR spectroscopy is a nondestructive vibrational spectroscopy technique that permits the identification of the molecular makeup of a sample by analyzing its corresponding absorbance spectrum in the mid-infrared region. This analysis is based on the principle that every molecule produces a specific absorbance spectrum when irradiated with mid-infrared light (400-4000 cm^{-1}). This absorption is strictly determined by the chemical functional groups present in the molecules structure. Following such principle, MIR spectrometers are able to determine the molecular composition of a sample by comparing the resulting MIR spectrum of the sample, which is a mixture of molecules, with the specific MIR absorbance spectrum produced by isolated molecules (Lucas, 2017).

One of the main challenges that MIR faces, is that it requires a rather complex sample preparation. To solve this problem, MIR is combined with the sampling technique of attenuated total reflectance (ATR) which enables samples to be examined directly in solid or liquid state without further preparation. An attenuated total reflection accessory operates by measuring the changes that occur in a totally internally reflected infrared beam when it contacts a sample located on the ATR-crystal. A mid-infrared

beam is directed at a certain angle onto the optically dense crystal which has a high refractive index. This internal reflectance creates an evanescent wave that extends beyond the surface of the crystal into the sample placed on the crystal. However, this evanescent wave only penetrates a few microns (0.5 µ - 5 µ) into the sample, hence; there must be an excellent contact between the sample and the surface of the crystal. In those regions of the infrared spectrum where the sample absorbs energy, the evanescent wave will be attenuated. The attenuated energy from each evanescent wave goes back into the MIR beam, which then exits the opposite end of the crystal and is passed to the detector of the spectrometer. The system then generates an infrared absorbance spectrum. The biggest challenge faced when working with MIR spectroscopy is its calibration, as it requires a high level of expertise, particularly in chemometrics or multivariate data analysis in order to make the MIR application successful.

Other analytical methods such as nuclear magnetic resonance (NMR) are also becoming more relevant in beer analysis. This technique enables a more rapid and non-invasive characterization of foods, potentially providing information about a very wide range of different compounds. In brewing, NMR has been mostly applied to study specific problems, such as the identification and quantitation of malt and hop constituents, like polyphenols, humulones or isohumulones (Mannina, 2016). It has been recently proven that NMR spectroscopy enables a rapid and direct overview of the chemical composition of beer without the need for pre-treatment aside from degassing (Lachenmeier, 2015).

Currently, two analytical technologies are emerging. These are electronic noses (e-nose) and electronic tongues (e-tongue) (Andrés-Iglesias, 2015). The former aims to simulate the taste detection modality of the human tongue by means of electrochemical sensors that are sensitive to several components in the measured sample (Nery, 2016). E-noses, on the other hand, are focused on identifying the aroma-producing compounds found in a sample.

HS-MS-e-noses are the most promising as they can carry out analyses in very short times with minimum sample preparation. Using this technology, it has been possible to unravel the overall volatile composition of a sample in a few minutes (Vera, 2016). Most studies using electronic tongues and noses are focused towards the differentiation and characterization of beers, but their use as analytical tools in brewery applications is very promising.

In this project the flavor and ethanol composition of a craft Pilsen beer provided by a micro-brewery called Boga will be analyzed. The first aim of this study is to identify the flavor active compounds found in such beer and study their respective abundance evolution throughout fermentation using the GC-MS technique. The second aim is to determine its ethanol concentration through MIR spectrometry.

2. MATERIALS AND METHODS

2.1 BEER SAMPLES USED

To carry out the study, 8 different beer samples were collected at different stages of the production line. Most flavor changes in beer occur during primary fermentation, which corresponds to the first 6 days of fermentation. Thus, it is crucial to collect as many samples as possible during this period of time. The samples analyzed and the production line stage at which each was collected is shown of **Table 1.**

Table 1 : Beer samples used to carry out the analysis of a Pilsen beer produced at Boga brewery.

Sample nº	Sample Composition	Production line stage
1	Beer Wort	Before fermentation
2	Day 1 of fermentation	Primary fermentation
3	Day 2 of fermentation	Primary fermentation
4	Day 3 of fermentation	Primary fermentation
5	Day 5 of fermentation	Primary fermentation
6	Day 8 of fermentation	Secondary fermentation
7	Day 14 of fermentation	Secondary fermentation
8	On store	End of fermentation

2.2. GC-MS ANALYSIS CONDITIONS

A Shimadzu GC-MS system equipped with a split/splitless inlet and a thermal conductivity detector (TCD) was used to detect the different flavor active compounds found in the 8 different beer samples. This technique allows a qualitative and semi quantitative analysis of the compounds found in each sample. The chromatographic conditions used are shown in **Table 2**.

Table 2: Chromatographic conditions used for the GC-MS analysis

Apparatus:	GC-MS-QP2010SE equipped with AOC-20i
Carrier:	Helium (He ≥ 99.99%). 1.68 mL/min constant flow
Column:	BPX5 Thickness: 0.25 µm Diameter: 0.22 mm Length: 50 m
Injection Mode:	Direct Sample Injection (DI)
Inlet:	Split/Splitless. 250 °C.
Inlet liner:	Shimadzu Inert Focus liner (p/n 092062)
MS:	TCD (detector) Ion source temperature: 200 °C Interface Temperature: 300 °C Solvent cut time : 3 only for analysis done at 24 h of fermentation
Oven:	45 °C for 1 min and then increased to 290 °C at a rate of 10 °C/min
Pressure:	300.5 kPa
Sampler:	AOC-20i autosampler with 7 position trays nº of rinses with solvent(Post run) : 6 nº of rinses with sample : 3 Plunger speed (Suction): Medium

2.3. MIR SPECTROSCOPY ANALYSIS CONDITIONS

A Portable IRSphinx spectrometer was used to determine the characteristic ATR spectrum in the mid-infrared region of the bottled beer and of different standard samples. The beer sample used to carry out this study was sample nº 8, as it is the one that corresponds to the bottled beer. The standard samples employed were deionized water, commercial alcohol free beer, ethanol 5%, ethanol 10 % ethanol 7.1%, and maltose 1% (v/v). All samples were degassed before being placed on the ATR crystal to avoid air bubbles from staying in the optical path and interfere in the sample-crystal

intimate contact. The ATR crystal used for the analysis was made up of ZnS which is a relatively cheap material with a high refraction index (n=2.4). However, this type of crystals are highly sensitive to strong acids and are easy to scratch, therefore, they must be handled with extreme caution. The ZnS crystal is cleaned with deionized water using a soft, lint-free cloth and must be dried in a current of warm air so that there is no possibility of condensation forming on the window. To minimize the noise in the sample reading, the scan count (N) was adjusted to 400 which is the maximum possible and will provide the most sensible analysis. The conditions used to carry out this analysis are shown in **Table 3.**

Table 3: Analysis conditions for MIR spectroscopy

Apparatus	Portable IRSphinx spectrometer
Sample interface	ATR
ATR crystal material	ZnS (zinc selenide)
ATR Surface area	17 x 27 mm
Detector	128-pixel uncooled pyroelectric array
Spectral Range	5500-11000 nm (1800-900 cm^{-1})
Cleaning liquid	Deionized water
Scan count (N)	400
Frecuency	8 Hz
Measurement time	≈ 60 s. (For N=400)
Background	Deionized water

3. RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF THE FLAVOR-ACTIVE COMPOUNDS

The GC-MS analysis provided a profile for each of the studied samples. As a representative example of the results, the spectrum that corresponds to day 3 of fermentation is shown in **Figure 2.**

This chromatogram, was selected for being the one that presented the greatest number of flavor active compounds. To avoid the high concentration of ethanol from saturating the injector, a solvent cut at minute 3 was used in the analysis of the samples collected from day 3 of fermentation onwards.

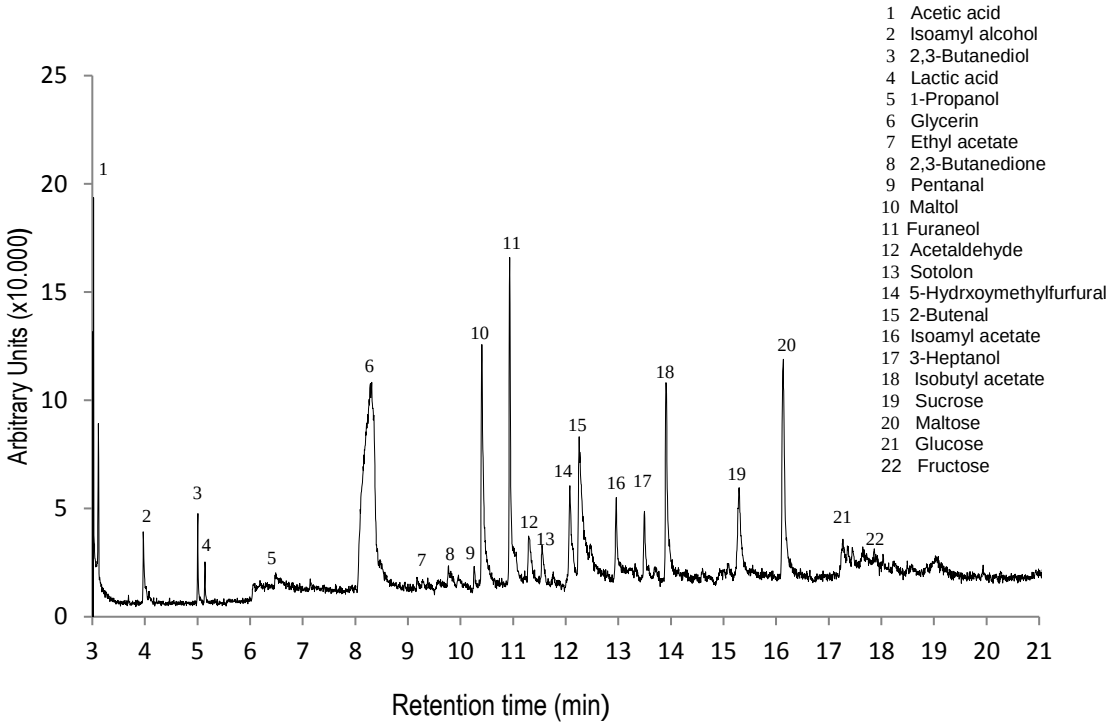

Figure 2: GC-MS chromatograph corresponding to a 1mL Pilsen beer sample took on day 3 of fermentation. The identification of the 22 flavor active compounds detected in this spectrum is shown on the right hand of the figure. Each compound is labeled at their corresponding retention time within the chromatogram. A solvent cut at minute 3 was used to avoid ethanol form saturating the detector.

From the analysis of the 8 chromatograms obtained, a vast array of flavor active compounds was identified. Depending on their chemical nature, they were classified into 7 chemical groups: Higher alcohols, esters, VDKs, Maillard reaction products, aldehydes, sugars and others. The classification of all the detected compounds into the 7 chemical groups together with the flavor effect that each of them produce in beer and their respective organoleptic threshold value is shown in **Table 4.**

Table 4: Characterization of the effect that each of the different flavor active compounds detected by GC-MS analysis have on beer flavor and representation of their respective organoleptic limit threshold. Source: (Ademola, 2017; Charles 2009). *The organoleptic threshold shown for ethanol, corresponds exclusively to Pilsen beers.

Compounds	Flavor effect in Beer	Organoleptic Treshhold (ppm)
Higher alcohols		
3-Heptanol	Herbaceous	2.5
Isoamyl alcohol	Alcohol	70
2,3-Butanediol	Butterscotch	17
1-Propanol	Alcoholic, Solvent	800
Esthers		
Isoamyl acetate	Banana, apple	1.2
Isobutyl acetate	Pinapple	0.7
Ethyl acetate	Fruity	33
Maillar reaction		
Maltol	Caramel	9
Furaneol	Roasted	0.6
Sotolon	Caramel	0.9
VDKs		
2,3-Butanedione	Butterstoch	0.15
Aldehydes		
4-Pentenal	Grass, apple, cheese	1
2-Nonenal	Cardboard, stale	0.0001
Acetaldehyde	Green leaves, fruity	25
2-Butenal	Apple, almond	8
5-Hydrxoymethylfurfural	Spicy	17
Sugars		
Sucrose	Cidery" or "vinous	2.600
Maltose	Sweetness	undertermined
Fructose	Sweetness	undertermined
Glucose	Sweetness	undertermined
Others		
Lactic acid	Sour	400
Acetic acid	Sour, vinegar taste	280
Glycerol	Flavor enhacor	1.000
Ethanol	Alcoholic, warm	60.000 *

3.2 ABUNDANCE EVOLUTION OF FLAVOR-ACTIVE COMPOUNDS THROUGHOUT FERMENTATION

The spectrum area percentage that each of the compounds represent is provided by the GC-MS apparatus. As shown in **Table 4,** each compound is classified into one of the following chemical groups: Higher alcohols, esters, VDKs, Maillard reaction products,

aldehydes, sugars and others. The total area percentage that each of these chemical groups show, can be obtained by summing up the area percentage of each of the compounds that make it up. The data obtained for the 8 chromatograms is collected in **Figure 3.** The spectrum area percentage for ethanol was not included in this analysis because the solvent cut used from day 3 of fermentation onwards, signify a lack of sufficient chromatographic data for this compound.

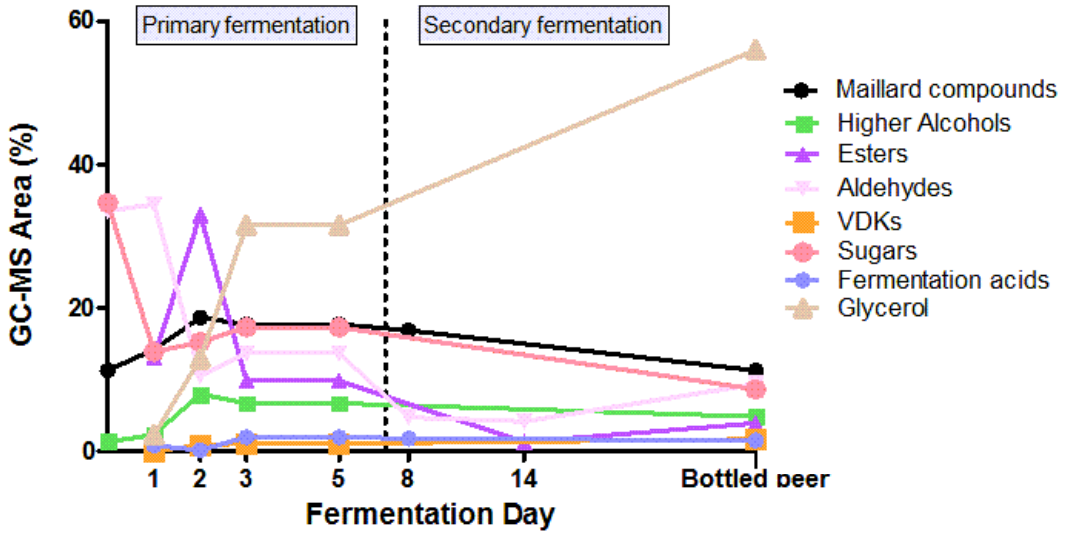

Figure 3: Evolution of the GC-MS Chromatographic Area% that each of the chemical groups represent along the fermentation process of Pilsen beer. Each chemical group follows the point-symbol and color scheme pattern shown in the legend. The primary fermentation duration phase has been proportionally enlarged, to facilitate the visual analysis of the variations that occur during this initial stage of fermentation.

The data provided by the GC-MS analysis permits a semi-quantitative study of the abundance evolution of the different flavor active compounds of beer. The spectral peak that corresponds to each of the compounds, represents a specific area percentage from the total area of the spectrum. It is possible to do an approximate prediction on whether the amount of each compound is increasing or decreasing by comparing the evolution of the area percentage that each of the peaks suffer throughout the process. **Figure 3** shows how the area percentage of each sample evolves along the fermentation process. By studying this data, one can determine the abundance evolution of all flavor-active compounds throughout fermentation.

Regarding the concentration of sugars, it is noticed that as soon as yeast is added onto the wort at day 1 of fermentation, its amount is steeply decreased. This occurs as a result of the quick sugar consumption carried out by metabolically active yeast. The greatest reduction in the amount of sugars occurs during the first day of fermentation, when the yeast is massively growing and thereby, when sugar uptake is maximal. After this initial decrease, the sugar concentration decrease becomes more progressive and extends throughout the whole fermentation process (Ademola, 2017).

The abundance of glycerol on the other hand, increases as fermentation advances. By day 3 of fermentation its concentration has already outstripped the rest of the studied compounds by a large amount (**Figure 3)**. This is due to the fact that glycerol is one of the main fermentation by-products produced by yeast. In fact, it is the most abundant constituent in beer after carbon dioxide and ethanol. Most extensive studies have shown that, depending upon the yeast strain, medium and process conditions, 4 to 10% of the carbon source is converted into glycerol. As a major byproduct, it serves critical roles in yeast osmo-regulation and redox balancing, and acts as the carbon competitor against ethanol in alcoholic fermentation. Glycerol does not contribute to flavor due to its non-volatile nature but it does contribute to the smoothness and body of beer. It has also been reported to have a positive influence in flavor intensity (Zhao X, 2015). Hence, increasing glycerol yield benefits both the flavor and ethanol reduction for the fermented beverages.

In the same way, the amount of higher alcohols and fermentation acids, which are also fermentation byproducts, increases as fermentation goes by. However, they are generated in relatively low quantities in comparison to glycerol. Therefore, their respective concentration in the final beer product is much lower than the one corresponding to glycerol.

The concentration of aldehydes in wort is relatively high as they are primarily formed during wort preparation. However, during the first stage of primary fermentation, most aldehydes are reduced by yeast into their corresponding higher alcohols, resulting in a significant decrease in its concentration. It is observed in **Figure 3** that the point at which aldehydes concentration begins to decrease, coincides with the moment when the level of higher alcohols starts rising. At the last phase of primary fermentation, the

concentration of aldehydes stops decreasing to suffer slight increase. This increment is owed to a minimal production of aldehydes through the oxidation of higher alcohols and isohumulones or/and to the Strecker degradation of amino acids. Among these new aldehydes, nonenals stand out for their influence in beer flavoring, as they are believed to be responsible for the characteristic stale flavor of old beers (Jeroen , 2015).

In relation to esters abundance, it is shown in **Figure 3** that as the population of yeast increases throughout the first days of fermentation, esters production rate rises. This occurs because esters are produced as a result of yeast metabolism; hence, the amount of esters found in beer is directly proportional to the abundance of metabolic-active yeast. This results in a progressive increase in ester concentration at the growing stage of fermentation, reaching its maximum at day 2. However, in the last stage of primary fermentation its concentration suffers a steep decrease and from this time on, it continues to decrease less abruptly throughout secondary fermentation. This reduction is owed to esters degradation carried out mainly by esterases that are being secreted into the mediun by dying yeast cells (Loviso, 2018).

Among all the compounds produced by yeast, VDKs are the ones found in lowest amounts. Their concentration suffers very small variations during fermentation that do not significantly affect beer flavor. It is crucial to keep these compounds below their flavor threshold value to avoid a potent ‘butterscotch’ flavor that could damage the flavor balance.

It is evidenced in **Figure 3** that the concentration of Maillard reaction by-products suffers unnoticeable variations throughout fermentation. These products have very complex structures that cannot be easily broken down by yeast. The degradation of these compounds is mainly triggered by the exposure to high temperatures. Therefore, the process that influences their concentration the most is wort boiling rather than fermentation. As boiling occurs before fermentation, the concentration found in wort is approximately the same as that found in the final beer product

3.3. ETHANOL CONCENTRATION

Figure 4 shows the ATR spectra provided by a Portable IRSphinx spectrometer, used under the experimental conditions collected in **Table 2.** Every absorbance attenuation indicates the presence of a certain compound that absorbs light at that specific wavenumber.

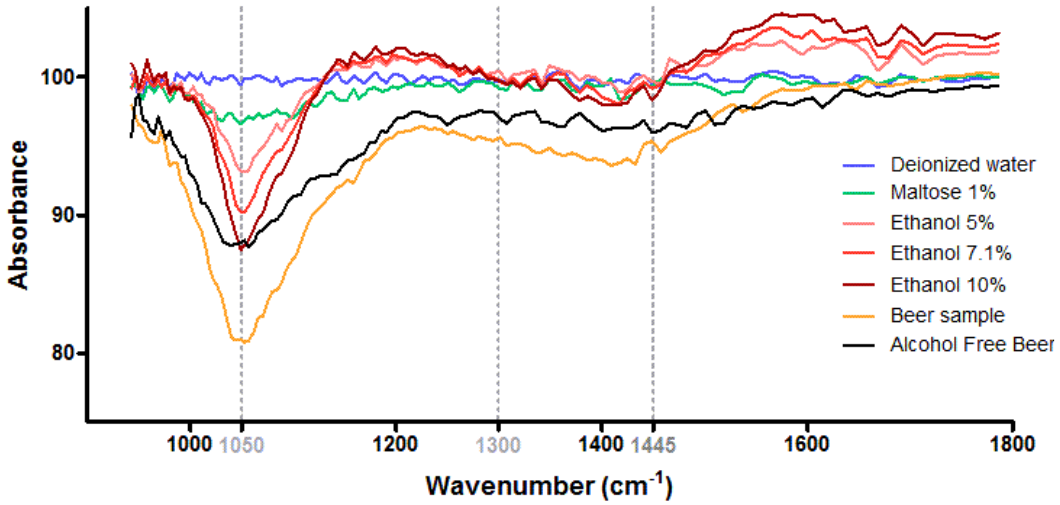

Figure 4: ATR-Spectrum in the mid-infrared region, produced by Pilsen beer and standard samples. The spectra follow the color scheme shown in the legend. The wavenumbers of interest for the analysis of ethanol concentration in beer are pointed out on the figure with dotted lines.

The water content of beers is approximately 90–92% (m/m), so the characteristic vibrational spectra of beers are strongly dominated by water and affected by the contribution of two other main constituents: ethanol and carbohydrates. By studying the ATR spectra (**Figure 4)**, it can be appreciated that both, ethanol and maltose, present absorption bands in the mid-infrared region. It is possible to determine the concentration of ethanol in a beer sample by comparing its ATR spectrum with the one produced by standards samples whose alcohol concentration is known.

However, as beer is a mixture of many constituents, its characteristic spectrum will be the result of the sum of the absorption bands corresponding to each of its constituents. Hence, to carry out this analysis it is crucial to determine the ATR spectrum generated by an alcohol-free beer that has an analogous molecular composition to regular beers

except for the presence of ethanol. Thereby, the respective ATR spectrums of both beer types will be very similar except for that the alcohol-free beer, will not present the bands corresponding to ethanol absorption. This difference is crucial for determining which peaks are possible valid candidates to be used for an ethanol concentration reading.

Maltose presents an absorption band around 1050 cm^{-1} while ethanol presents absorption bands at, 1053 cm^{-1} (asymmetric C—C—O stretching), 1300 cm^{-1} (CH_2 wagging) and 1455 cm^{-1} (C—OH bending) (**Figure 4)**. It is evidenced that both compounds absorb mid-infrared light around the same wavenumber (1050 cm^{-1}). The beer sample spectrum shows absorption activity at all of these wavenumbers, however, the alcohol free beer only shows absorption bands around 1050 cm^{-1}. As the only difference between both beers is the ethanol content, the fact that alcohol free beer does not show absorption at 1300 cm^{-1} and 1455 cm^{-1} but regular beer does, indicates that the absorption experienced at these wavenumbers by the alcohol containing beer, is exclusively owed to the presence ethanol. Therefore, these bands are possible candidates to determine the ethanol concentration in beer. To do so, the absorbance attenuation peaks at both regions are analyzed.

To calculate the absorbance attenuation value that occur at the attenuation absorbance peaks found at 1300 cm^{-1} and 1455 cm^{-1}, it is necessary to determine the absorption value at the maximum and minimum point of the absorption peak. The former correspond to the absorbance base-value, which correlates with the spectrum point where there is no absorption decrease. The later, correspond to the absorbance value detected at 1300 cm^{-1} and 1455 cm^{-1}. The respective absorption values produced by each of the ethanol standards and beer samples at these wavenumbers are collected in **Table 5.** The data supplied by the MIR-spectrometer did not provide the absorption values at 1300 cm^{-1} or 1455 cm^{-1}. Instead the data corresponding to 1301 cm^{-1} and 1458 cm^{-1} was used.

Table 5: Absorbance values produced by each of the ethanol standards and beer samples at specific wavenumbers. The wavenumbers at which ethanol presents absorption bands (1458 and 1301 cm-1) correspond to the minimal base point of the absorbance peaks. The wavenumbers that will be used as base-values (1540 and 1220 cm-1) correspond to the maximum point of the absorbance peaks.

	Absorbance				
Wavennumber(cm^{-1})	Ethanol 5%	Ethanol 7.1%	Ethanol 10%	Beer sample	Alcohol Free Beer
1540	102,46	102.83	103.56	97.85	97.73
1458	99.49	99.48	99.15	94.58	96.16
1301	100.42	99.73	99.73	95.64	97.04
1220	100.99	101.14	101.55	96.32	97.49

The absorbance attenuation at 1300 cm^{-1} and 1455 cm^{-1} increases as the concentration of ethanol rises. In other words, the more ethanol present in the sample, the steeper the absorbance attenuation peaks will be.The alcohol concentration of the beer sample can be stablished by comparing the depth of the absorbance peak of beer with the one produced by the standard samples. These values are calculated by substracting the value of the absorbance at 1300 cm^{-1} or 1455 cm^{-1}, which correspond to the minimum point of their corresponding peaks, to the absorbance base-value of each peak, which correspond to the highest point of the peaks. Once the absorbance attenuation at each wavenumber is calculated, the ethanol concentration in beer can be easily stablished by comparing the absorbance attenuation produced by the different ethanol standards and the one generated by beer. Using this analytical method it has been determined that the beer sample has an ethanol concentration between 5.15-5.75 %. These calculations are shown in **Table 6.**

Table 6: Determination of ethanol % in beer by analyzing the absorbance attenuation produced by ethanol standards and beer sample at 1301 cm^{-1} or 1458 cm^{-1}. The error found by using this analysis is ≈ 5.5%.

	Absorbance Attenuation				
Wavenumber(cm-1)	Ethanol 5%	Ethanol 7.1%	Ethanol 10%	Beer sample	Ethanol % Beer
1458	2.98	3.35	4.41	3.28	≈ 5.70
1301	0.57	1.41	1.82	0.69	≈ 5.15

4. CONCLUSIONS

The GC-MS technique was found to be a convenient and appropriate technology to undergo a reliable qualitative and semi-quantitative analysis of beer flavor. This method enabled the detection and identification of a vast array of beer flavor active compounds comprising aldehydes, VDKs, higher alcohols, esters, Maillard reaction products, sugars, glycerol and fermentation acids. GC-MS also provides sufficient data to carry out a semi-quantitative abundance analysis for each of the compounds. From it, it was determined that glycerol was quantitatively in greatest abundance, followed at a far distance by aldehydes, higher alcohols and Maillard reaction products.

It was evidenced that the concentration of yeast fermentation by-products such as glycerol, higher alcohols, fermentation acids and VDKs, increases as fermentation proceeds. Sugars, on the other hand, are consumed throughout the process. Regarding esters abundance, its concentration suffers a steep increase at the initial phase of fermentation followed by a drastic fall, owed to a rapid esterase-mediated degradation. Aldehydes concentration undergoes a steep initial decrease as they are being reduced to produce their corresponding higher alcohol. Nonetheless, its concentration value is kept within significant levels in beer. On the other hand, Maillard reaction compounds suffer no significant abundance changes throughout fermentation as they are difficult to degrade by fermenting microorganism.

The analysis system described appears to be highly appropriate for analyzing volatile flavor active compounds; however, it is not suitable for tracing ethanol in beer because the solvent cut used at minute 3 to avoid the ethanol concentration from saturating the GC-MS detector, signifies a total loss of its chromatographic data.

For this purpose, MIR spectrometry focused on the absorption bands found at 1300 cm^{-1} and 1455 cm^{-1}, has come forward as an efficient and reliable analytical method. The absorbance attenuation shown at these wavenumbers is exclusively owed to the absorption carried out by ethanol. Hence it has been proven that it is possible to determine the ethanol concentration of a beer sample, by comparing the absorption attenuation produced by beer and at these wavenumbers with the attenuation shown by standards samples with known ethanol concentration (v/v).

These absorbance bands, 1300 cm^{-1} and 1455 cm^{-1}, could be used to carry out the chemometric calibration of the MIR spectrometer to enable faster analysis of ethanol concentration in alcoholic beverages. In this sense, advances could be expected in the coming years in the automation of MIR spectrometry by an extensive calibration of the apparatus, which could provide continuous sensitive fast measurements of specific components in beer samples. Thus, it can be concluded that we are at the beginning of a long journey in which the molecular composition of beers obtained by MIR spectrometry will be of a great value for beer analysis.

5. ACKLOWLEDGEMENTS

This work was supported by the University of The Basque Country, in special by the department of chemical engineering. I particularly thank Dr. Beatriz Valle Pascual for assistance with GC-MS analysis.

6. BIBLIOGRAPHY

Ademola O., Lettisha H., Mduduzi P., Mokoenaand B. P. 2017. Flavour-active volatile compounds in beer: production, regulation and control. J. Inst. Brew. Dig. 123(1): 13–23.

Andrés-Iglesias C., Montero O., Sancho D., Blanco C. A. 2015. New trends in beer flavour compound analysis. J. Sci. Food. Agric. 95(8):1571–1576.

Bamforth C. W. 2017. Progress in Brewing Science and Beer Production. Annu. Rev. Chem. Biomol. Eng. 8:161–176.

Bokulich A. N., Bamforth C. W. 2013. The Microbiology of Malting and Brewing. Microbiol. Mol. Biol. Rev. 77(2): 157–172.

Cascant M. M., Breil C., Fabiano-Tixier A. S., Chemat F., Garrigues S., de la Guardia M. 2018. Determination of fatty acids and lipid classes in salmon oil by near infrared spectroscopy. Food Chem. 239:865–871.

Castritius S., Kron A., Schäfer T., Rädle M., Harms D. 2010. Determination of alcohol and extract concentration in beer samples using a combined method of near-infrared (NIR) spectroscopy and refractometry. J. Agric. Food. Chem. 58(24):12634–12641.

Charles W. B. 2009. Beer: A Quality Perspective, 1st ed. Elveiser. In: Paul H. Beer Flavor, 61–83.

Engan S. 1974. Organoleptic threshold values of some alcohols and esters in beer. J. I. Brew. 78:33–36.

Escobar JD., Alcaniz M., Masot R., Fuentes A., Bataller R., Soto J., Barat JM. 2013. Quantification of organic acids using voltammetric tongues. Food. Chem. 138(2–3):814–820.

Jeroen J., Baert, Jessika D. C., Paul S. H., Luc C., Guido A. 2015. On the Origin of Free and Bound Staling Aldehydes in Beer. J. Agric. Food. Chem. 60(46):11449–11472.

Krogerus K., Gibson BR. 2013. Diacetyl and its control during brewery fermentation. J. Inst. Brew. 119:86–97.

Lachenmeier D.W., Frank, W., Humpfer, E. et al.2015. Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis. Eur. Food. Res. Technol. 220:215–221.

Loviso CL., Libkind D. 2018. Synthesis and regulation of flavor compounds derived from brewing yeast: Esters. Rev. Argent. Microbiol. 7541(17):30186–30184.

Lucas V., Johnathon B. L., John D. S. 2017. The application of near-infrared spectroscopy in beer fermentation for online monitoring of critical process parameters and their integration into a novel feedforward control strategy. J Inst Brew 119:86–97.

Mannina L, Marini F, Antiochia R, Cesa S, Magrì A, Capitani D, Sobolev A. P. 2016. Tracing the origin of beer samples by NMR and chemometrics: Trappist beers as a case study. Electrophoresis. 37(20):2710–2719.

Nery E. W., Kubota L. T. 2016. Integrated, paper-based potentiometric electronic tongue for the analysis of beer and wine. Anal. Chim. Acta. 918:60–68.

Pires, E.J., Teixeira, J.A., Brányik, T. et al. 2014. Yeast: the soul of beer's aroma a review of flavour-active esters and higher alcohols produced by the brewing yeast. Appl. Microbiol. Biotechnol. 98(5):1937–1949.

Rossi S., Sileoni V., Perretti G., Marconi O. 2014. Characterization of the volatile profiles of beer using headspace solid-phase microextraction and gas chromatography-mass spectrometry. J. Sci. Food. Agric. 94(5):919–928.

Sterckx F. L., Saison D., Delvaux F. R. 2010. Determination of volatile monophenols in beer using acetylation and headspace solid-phase microextraction in combination with gas chromatography and mass spectrometry. Anal. Chim. Acta. 676:53–59.

Vera L., Aceña L., Guasch J., Boqué R., Mestres M., Busto O. 2016. Characterization and classification of the aroma of beer samples by means of an MS e-nose and chemometric tools. Anal. Bioanal Chem. 399(6):2073–2081.

Zhao X., Procopio1 S., Becker T. 2015. Flavor impacts of glycerol in the processing of yeast fermented beverages: a review. J. Food. Sci. Technol. 52(12):7588–7598.