Study of hop enzymatic activity during dry-hopping and its impact on yeast physiology and on the beer aroma profile: A sugar story

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Abstract:

The *Humulus lupulus* L. inflorescence, also called hop, is almost exclusively used in the brewery field as one of the main ingredients to perfume and conserve beer. The current trend in craft breweries of heavy dry-hopping as attested by the increasing hopping rate in recent years sometimes leads to uncontrolled and aberrant aroma profile production. The aim of this work is to determine whether part of the enzymatic content of hop, namely α-amylase and β-amylase, could influence the aroma profile of dry-hopped beer consecutively to yeast fermentation of the fermentable carbohydrates produced by their activity.

To do so, spectrophotometric methods of enzyme activity quantification were designed to assess the content within hop. Moreover, liquid chromatographic method (HPLC-ELSD) was developed to determine the impact on the sugar profile of the beer by production of glucose and maltose and degradation of the higher degree of polymerization sugars by these enzymes.

Furthermore, gas chromatographic techniques (GC-ECD/FID) were used to assess yeast metabolism using vicinal diketones (butane/pentanedione) as a marker of the fermentation. Finally, a principal components analysis evaluating global change by monitoring ester (ethyl and acetate), higher alcohols and aldehydes demonstrating the impact on the aroma profile of this yeast and hop interaction.

Résumé:

L'inflorescence du houblon (*Humulus lupulus* L.) appelée aussi cônes est presqu'exclusivement utilisée dans le domaine de la brasserie comme l'un des principaux ingrédients pour parfumer et conserver la bière. La tendance actuelle des brasseries artisanales d'houblonnage à cru conséquent conduit parfois à une production de profils aromatique incontrôlée et aberrante. Le but de ce travail est de déterminer si une partie de la teneur enzymatique du houblon, à savoir l'α-amylase et la β-amylase, pourrait influencer le profil aromatique de la bière houblonnée à crû consécutivement à la fermentation par la levure des hydrates de carbone produits par ces enzymes.

Pour ce faire, des méthodes spectrophotométriques de quantification de l'activité enzymatique ont été élaborées pour évaluer le contenu au sein du houblon. De plus, une méthode de chromatographie liquide (HPLC-ELSD) a été utilisée pour déterminer l'impact sur le profil des sucres de la bière par la production par ces enzymes de glucose et de maltose à partir de sucres de plus haut degré de polymérisation.

En outre, des techniques de chromatographie en phase gazeuse (GC-ECD/FID) ont été utilisées pour évaluer la métabolisation éventuelle par la levure en utilisant des cétones vicinales (butane/pentane dione) comme marqueurs de la fermentation.

Enfin, une analyse en composantes principales évaluant le changement global en surveillant la concentration en esters (éthyle et acétate), alcools supérieurs et aldéhydes démontre l'impact sur le profil aromatique de cette interaction levure-houblon.

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List of abbreviations

Aatase = Alcohol acetyl transferase
ABV = Alcohols By Volume
AHA(S) = Acetohydroxyacid (Synthetase)
AS = Alsace Strisselspalt
ATP = Adenosine Tri-Phosphate
BCAA = Branched Chain Amino Acid
DHA = Dihydroxyacid
ECD = Electron Capture Detector
ELSD = Evaporative Light Scattering Detector
FID = Flame ionization detector
GC = Gas Chromatography
HHE = Hallertau Hersbrucker
HPLC = High Performance Liquid Chromatography
MS = Mass Spectrum
OHAI = Overall Hop Aroma Intensity
PCA = Principal Components Analysis
RE = Real Extract
VOCs = Volatile Organic Compounds

1) General purpose of the study

Though the brewing process has been extensively studied, some experimental facts still fail to be explained by theory. Indeed, the idea behind this study came from the empirical observation in different breweries that a differential attenuation (percentage measuring the conversion of sugar to alcohol) in beer exists between different batches where the only changing parameter is the form of hop used to dry-hop the beer.

This observation could have remained anecdotal because it did not lead to huge changes in the taste or quality of the beer, but it highlights a more fundamental process that takes place during the ripening of the beer. Indeed, it was shown as early as 1893 that hop possesses a small “diastatic power” (ability to degrade the starch). This enzymatic activity has long been ignored due to the fact that hop was mainly added during a boiling stage in which those enzymes were inactivated.

Nevertheless, the ongoing trend in the brewery field which consists of producing heavy dry-hopped beer could turn this small effect into something much more significant for the final product.

Therefore, the aim of this work is to demonstrate the role of the enzyme brought by hop during the ripening of a dry-hopped beer. This illustrates that the role of hop during dry-hopping involves far more than the simple dissolution of volatile compounds. Indeed, fermentable sugar liberated by this process could stimulate yeast activity and change the aroma profile of resulting beer.

To conclude, the general purpose of this study is to investigate the interaction between yeast and hop during dry-hopping and demonstrate whether or not these enzymes could play a role in the process.

2) Introduction

2.1) *Humulus lupulus* L.

Historical evidence shows that hops have been used in a variety of ways since ancient times. Indeed, the first recorded use of the plant dates back as early as the ancient Greeks when Pliny mentions it in his Natural History as "*lupulus salictaffies*", a plant eaten as an appetizer or a green salad (Edwardson, 1952).

Another culinary purpose was the cultivation of wild yeast to make bread. To do so, a decoction in water was prepared and mixed with the dough to flavour and prevent spoilage (Robbins *et al.*, 1917). A similar method is still used nowadays in East Africa (Neve, 1976; Robbins et Ramaley, 1933).

Furthermore, apart from this dietary purpose, hops were prescribed by physicians to cure many different kind of illness: "They were supposed to free the blood of all impurities, tumors and flatulence, to cure itchitching and other skin diseases, and to relieve the liver and spleen" (Edwardson, 1952).

Though the art of brewing began as long as 5.500 years ago in the Mesopotamian world with different kind of cereals, it is much later that hops joined the history of beer. Indeed, there is no evidence that hop was used in beer until the 8th century, when beer was perfumed with a mix of aromatic plants named "gruit". This mix could contain hop and other plants such as rosemary, sage or myrtle. The first written evidence of its cultivation came from a Bavarian monastery in the 9th century. The hopped beer produced by monasteries rapidly gained in notoriety until it became the norm in the 14th century. (Wilson, 1975)

Nowadays beer-making is virtually the only market for hop cultivation with a total worldwide annual production varying between 80.000 and 100.000 tons of hop (European statistics). In the last few decades, craft beer has benefited from a boom in sales undoubtedly linked to the recent consumer craze for heavily hopped beer, resulting in a supply deficit.

2.2) Botanical characteristics

The *Humulus* genus belongs to the C*annabaceae* family (within the Rosales order) which is composed of two genera. In this genus three distinct species can be observed: *Humulus lupulus* Linneus, *Humulus japonicus* Siebold & Zucc. and *Humulus yunnanensis* Hu (Neve, 1991)

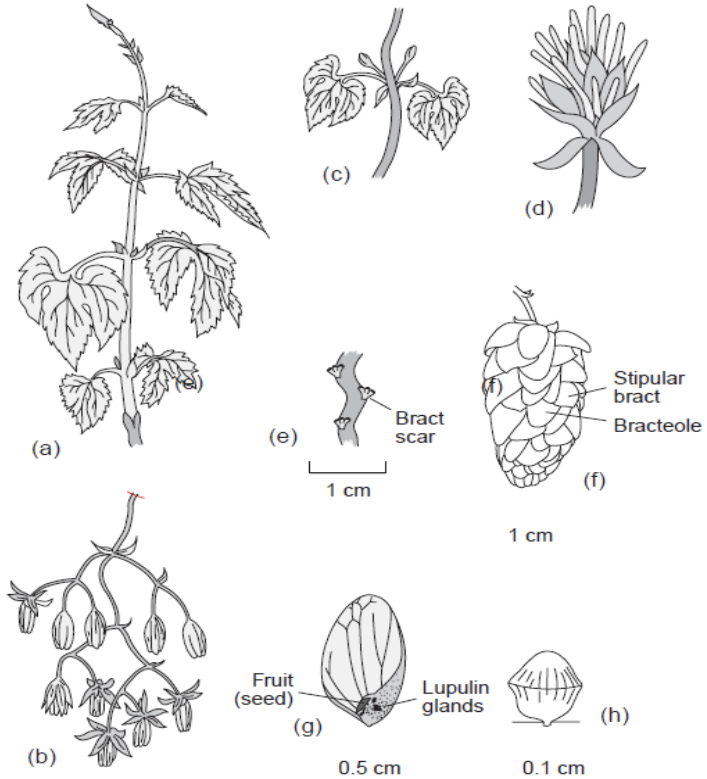
Within the *Humulus lupulus* species five different taxonomic varieties have been distinguished based on morphological (numerical analysis of leaf shape/pubescence) as well as geographical characteristics:
Origins in Europe: *H. lupulus var. lupulus*

Origins in Asia: *H. lupulus var. cordifolius*

Origins in North America: *H. lupulus var. pubescens, H. lupulus var. neomexicanus H. lupulus var. lupuloide* (Small, 1980).

Humulus lupulus L. is an annual climbing vine which grows up to 6 - 9 m in height from perennial underground rootstock. The rhizomes require a dormancy period during which the above ground part dies off. The leaves are opposite, with a heart shape, a dark green colour, a long petiole and a rough surface. The natural geographic area of the plant is distributed in the northern hemisphere, though it can be cultivated from 35° to 55° north or south of the equator. The main producers nowadays being Germany and USA (Burgess, 1964).

The plant is dioecious with 2n chromosomes (20), meaning that the male and female flowers grow on separate individuals although monoecious plants exist in north American wild populations. They can be distinguished only by their respective inflorescence, the male inflorescences being racemes of 7,5 - 12,5 cm whereas the female inflorescences are catkins (called strobiles) of 2 to 5 cm composed of up to sixty individual overlapping flowers. The strig or central axis is surrounded by stipular petals. These structures are called ‘bracts’ and ‘bracteoles’ at the base of which a small akene is found next to yellow trichomes (called lupulin gland) producing the lupulin (the resin used by the brewing sector). All those organs are represented in figure 1. The males are cultivated only for hybridization purposes to create new varieties (Haunold, 1991;Haunold *et al.*, 1993).



(a) young shoot;
(b) male flowers;
(c)`pin', young flowering shoot developing in the leaf axils;
(d)`burr', young female inflorescence with papillated stigmas;
(e) part of axis (`strig') of cone;
(f) single mature hop cone;
(g) bracteole with seed and lupulin gland;
(h) lupulin gland

Figure 1: Hop (*Humulus lupulus L.*) (Briggs *et al*., 2004)

2.3) Physico-chemical composition of hop

As listed on table 1 below, hop is composed of many different fractions namely resins, essential oils, proteins, carbohydrates, polyphenols, waxes, etc. The leafy nature of the petal provides ubiquitous amounts of carbohydrates, polyphenols and proteins. Furthermore, there is a wide chemical diversity within all these fractions. The fractions mainly responsible for the value of hop cones in the brewing process are the resins secreted by the lupulin glands which give bitterness to the beer as well as the essential oil giving the product its aroma (Almaguer *et al.*, 2014a).

Table 1: Average chemical composition of dried hop cones (Almaguer *et al.,* 2014)

Constituent	Amount (%)
Total resins	15–30
Essential oil	0.5–3
Proteins	15
Monosaccharides	2
Polyphenols (tannins)	4
Pectins	2
Amino acids	0.1
Waxes and steroids	traces–25
Ash	8
Moisture	10
Cellulose, etc.	43

2.3.1) Resins

The total resin content can be subdivided into two fractions: soft and hard resins. The former contains primordial compounds of hop such as α-acids and β-acids, also called humulones and lupulones. Primordial because they yield the bittering agents iso-α-acids or isohumulones after reaction during the brewing process (boiling). These acids are synthesized by the lupulin glands present in the cones of the female plant (De Keukeleire *et al.*, 2003).

Another interesting fraction inside the hard resin is the xantohumol (prenylflavonoids) which is being studied extensively at the moment for its medical properties (potent cancer chemopreventive properties) (Gerhauser *et al.*, 2002).

2.3.2) Polyphenols

In beer the polyphenol content is due to both malt and hop and plays crucial role regarding: beer stability (colloidal instability due to the interaction between protein and polyphenols), taste (catechin and epicatechin are responsible for astrengincy and flavonols play a part in perceived bitterness), colour (with the formation of chromophores under enzymatic oxidation), health properties (cardioprotectives and antioxidant effects) (Collin *et al.*, 2013; Mikyška *et al.*, 2002).

To conclude, it is interesting to make the link between the chemicals and their locations inside the hop cone. Indeed, though the lupuline synthesizes the two main active compounds resin and essential oil, the polyphenols are mainly produced by the bracts and bracteoles (Biendl *et al.*, 2014). Therefore, the manufacture of hop products and their use in the brewing process will impact the final physico-chemical composition of the beer.

2.3.3) Essential Oil / Volatile organic compounds of hop

The major fraction at harvest are hydrocarbons consisting mainly of myrcene, humulene, caryophyllene and farnesene (Aberl et Coelhan, 2012). However, as shown in figure 2, the diversity of compounds present in the essential oil of hop is wide. It contains hydrocarbons, oxygenated and sulfur compounds, which makes their identification a very laborious undertaking, usually achieved by gas chromatographic process. Many different techniques are applied (headspace, Solid Phase Fiber Micro extraction, comprehensive multidimensional chromatography,…), depending on whether it is directly hops or beer being examined and the method selection is of primary importance. The research conducted nowadays uses these chromatographic techniques to separate the volatile organic compounds contained in hop in order to allow their identification. Especially, in the varieties added after the boiling process in beer undergoing the maturation phase. Indeed, the chemistry associated with this is not yet fully understood and some researchers suggest that more than a thousand different compounds are present in the essential oil part of the hop (Roberts *et al.,* 2004).

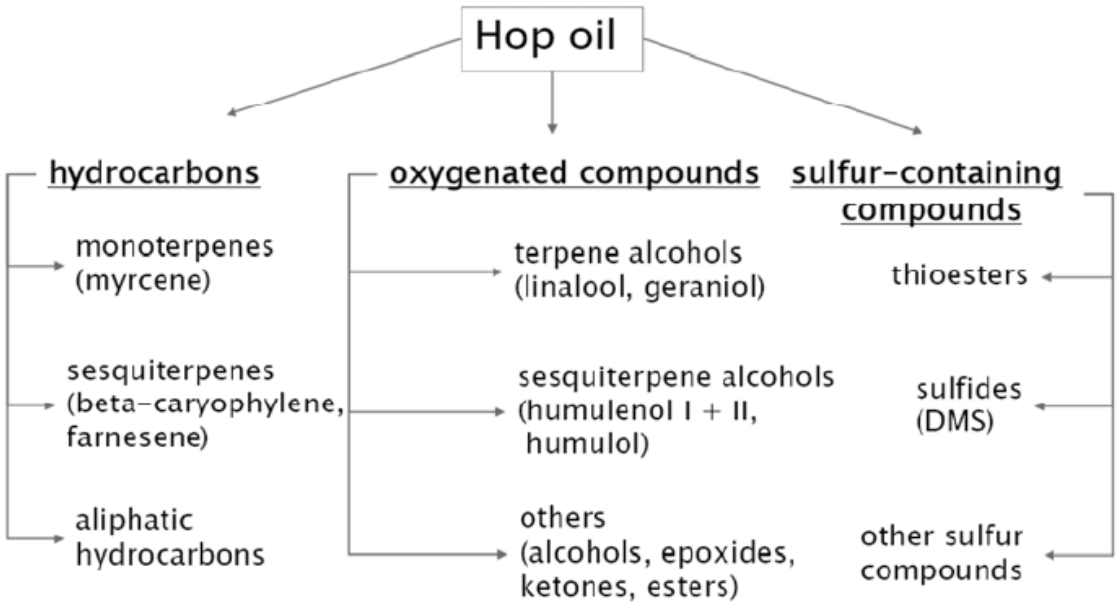


Figure 2: Hop oil classificaton (Schönberger et Kostelecky, 2011)

Even so, researchers still fail to explain which mix of aroma compounds are responsible for the so-called noble hop flavour given to beer after the dry-hopping process.

2.3.4) Enzymatic fraction of hop

Besides the direct contribution of the essential oil and resins to the beer aroma profiles, there is also a significant amount of proteins (15%) within which some enzymes have been identified. While these enzymes are denatured by the boiling stage, the dry-hopping techniques allow their extraction in the active forms within the beer, which seems to have a number of effects on the secondary fermentation going on during this period. Indeed, the first reference to the influence of hops on secondary fermentation during the dry-hopping process goes back to 1893: "We can briefly indicate that we have found that the acceleration of secondary fermentation depends on the presence in the strobile (female flower) of a small but appreciable amount of diastase sufficient to slowly hydrolyse the (non-crystallisable) starch transformation products left in the beer and reduce them to a state in which they can be seized and fermented by the yeast " (Brown et Morris, 1893).

This work was later extended in 1941 in an experiment during which the researchers showed two really important facts. Firstly, according to (Janicki *et al.*, 1941), "Examination of thirty-three samples of different seeded hops showed that they did not vary widely in saccharifying activity towards soluble starch at pH 4.8, irrespective of their variety, country of origin, age (up to 3 years) and conditions of storage (cold-store or warehouse)." Secondly, that seedless hops showed less saccharifying activity than seeded hops, meaning that about half the activity would appear to be contained in the seeds. Nevertheless, when added to a dextrinous beer in cask and not to a starch solution, the degree of saccharifying activity exerted by equal weights of different hops does not seem proportional to the laboratory results. This fact indicates that other factors play a role, such as activators of diastase or activators of yeast “zymase”. (Janicki *et al.*, 1941)

For around 70 years, this topic was not investigated to our knowledge. However, with the growing trend towards dry-hopped beer in order to produce Indian Pale Ales, American researchers brought this topic back into the spot light. Though they analyzed only one hop variety (namely Cascade), their conclusions are more than interesting. Indeed, they accurately measure the enzyme activity as listed on table 2, and though this is small, it can degrade residual dextrins to fermentable sugars, glucose and maltose, in beer causing significant changes in beer real extract (RE, °P), alcohols by volume (ABV, %), and CO_2 (v/v). They also highlighted the fact that dry-hopping parameters (temperature and time) influence the enzyme activity of Cascade hops in finished beer (Kirkpatrick *et al.*, 2017).

Table 2: Enzyme activity in hop pellets and malt (Unit/gram) (Kirkpatrick *et al.,* 2017)

Enzyme	Hop (Cascade)	Malt (130 dp)
Amyloglucosidase	0,02	NA
Alpha-Amylase	0,35	198
Beta-amylase	0,41	13
Limit dextrinase	< 0,01	NA

2.4) Definition of the amylases enzymatic activity

These two particular classes of enzymes are ubiquitously distributed in plants, animals and the microbial kingdom. Furthermore, they are of paramount importance in today's biotechnology, applied broadly in the food, fermentation or textile and paper industries (Van Der Maarel *et al.*, 2002).

These enzymes are proteins, biologically catalyzing a particular reaction in the cell. They increase the rates of chemical reactions occurring in the cell without themselves being altered. Most of the time these proteins need a non-protein component called a co-factor in order to be active (Palmer, 1991).

Each enzyme can be defined by its specific activity, and a nomenclature has been systematically established by the Committee of the International Union of Biochemistry and Molecular Biology (NC - IUBMB).

The α- and β-amylases are classified as 3.2.1. The first digit refers to the type of reaction being catalyzed (3 = hydrolysis reaction), the second to the bond being hydrolyzed (2 = glycosidic, unit linking carbohydrates), the third further describing the bond hydrolyzed (1 = enzymes hydrolysing O- and S-glycosyl compounds). They both act on starch, glycogen and related polysaccharides and oligosaccharides as represented in figure 3 (Palmer, 1991).

More specifically, the α-amylase (EC 3.2.1.1) more accurately called 1,4-α-D-glucan glucanohydrolase, catalyzes the hydrolysis of 1,4 α linked D-glucose units in a random manner, producing low molecular weight dextrins and glucose. The β-amylase (EC 3.2.1.2) 1,4-α-D-glucan maltohydrolase hydrolyzes the α-1,4-glucosidic linkages from non-reducing ends producing maltose as shown in figure 3. The terms 'α' and 'β' do not refer to the configuration of the hydrolyzed link but to the initial anomeric one of the sugars delivered (Palmer, 1991).

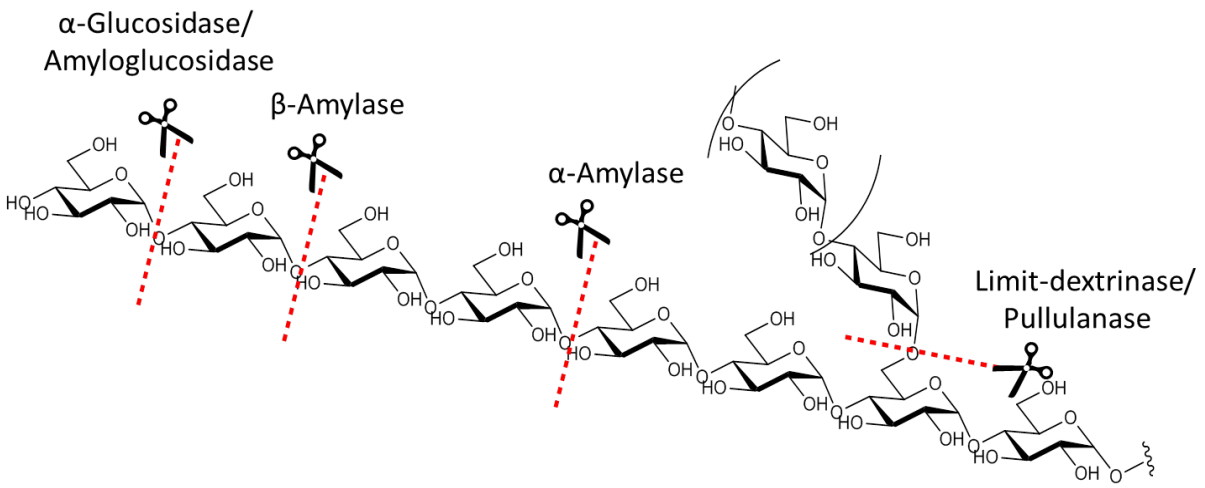


Figure 3: Schematic representation of the hydrolytic action of starch degrading enzymes (Megazyme)

2.5) Roles and impacts of enzymes, hop and yeast during the brewing process

2.5.1) Hop products

Although it represents a quantitatively small amount compared to the other ingredients in the beer-making process (water, malted barley and yeast), hop as a minor component has three crucial impacts.

- Firstly, hop is responsible for the typical bitter taste and aroma as well as the perceived hop character.

- Secondly, the acids contained in hop play a key role in ensuring microbial stability against gram^{+} bacteria.

- Finally, the resins of hop contribute to developing and stabilizing the beer foam (Almaguer *et al.*, 2014a).

As one can see, hop, even though present in small amounts, plays many crucial roles in the brewing process. By understanding which natural compounds within hop are responsible for these different properties, we gain a better fundamental understanding of the reactions occurring in the beer, thereby leading to improved management of the process.

The form in which as well as the stage of addition at which the hop is added to beer (during boiling or after/downstream products) will have a huge impact on the product. Indeed hop products may be distinguished by their specific application and dosage (aromatization, foam improvement, stability, bitterness,…). Hop products, presented in figure 4, especially type 45 pellets or extracts combine many advantages when compared to classical raw hop.

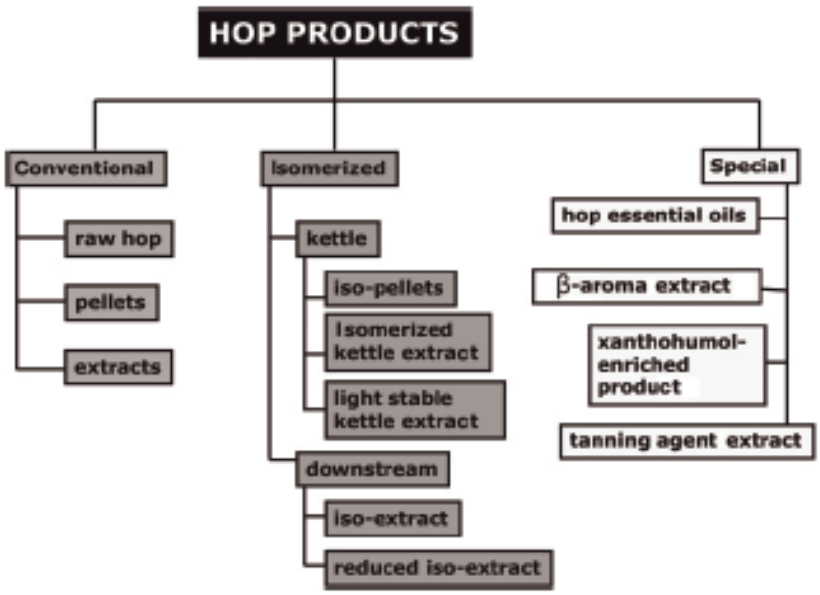


Figure 4: Classification of hop product (Eßlinger, 2006)

Indeed, by the reduction of packaging size, pellets facilitate the logistics and good storage to preserve the product from oxidation (allowing longer conservation) as well as an exact dosage due to the homogeneous distribution and extraction of α-acids. Besides that, the amount of pesticides and other undesirable chemical products is reduced during the process. The two types of pellets result from a process in which hop is cooled to -30°C and then crushed in a hammer mill, the resulting powder then being homogenized and compressed as such (type 90), where as for type 45 the powder goes through a sieve concentrating the lupulin gland but reducing the conversion ratio from 90 % to 45 %. During this compression, temperature may rise and its control is fundamental to avoid deterioration of the products (Briggs *et al.*, 2004).

2.5.2) Malting and brewing process in a nutshell

The brewing process can be summarized as the production of an alcoholic beverage using yeast to convert sugar from starch-containing materials (usually barley). The final product results from the succession of these different steps.

Malting

Barley cannot be used directly in brewing and will first need to undergo the malting process. This step begins with the germination of seeds (development of the coleoptile) by increasing their moisture content by steeping (immersing them in water). The germination triggers the conversion of the starchy raw material, usually barley (but also oats, rye, wheat, millet or sorghum are also used), by hydrolytic enzymes (α- and β-amylase) which will partly mobilize the sugar reserves of the seeds. Beside the hydrolysis of protein (proteolysis) that of the cell walls (cytolysis) also occurs, liberating free amino nitrogen. Finally, when the degradation is sufficiently advanced, all these enzymes are temporarily inactivated by drying with hot air, a step also called kilning (Briggs, 1998).

Milling

The aim of this step is to expose the carbohydrates contained in the cotyledon so that it can be extracted during mashing by breaking apart the kernel while preserving the husk that will later be used for separation. The malt is sometimes mixed with other cereals, called adjuncts. The resulting product of this operation is known as grist.

Mashing

The grist (milled grain) is placed in contact with hot water in a vessel called a “mash tun”. The operation consists in the solubilization of the malt component using physical, chemical and enzymatic processes. This enzymatic breakdown is controlled by several parameters such as temperature, pH and

viscosity. The amylases manage to convert the starch to dextrins and then into fermentable sugars such as maltotriose, maltose and glucose, whereas the proteolysis activity allows the liberation of both low and high molecular weight proteins required for fermentation by yeast and stability of haze. Many different parameters are to be considered as they have an influence on the type of beer obtained *i.e.* depending on the amylase favoured during the mashing process. The result will be a fuller bodied beer or a beer with a higher alcohol content. Finally, a "mash out" completes the process by inactivating the enzymes with a heating step up to 78°C. In addition, that organic phosphate is cleaved, creating a buffer effect; the polyphenols are oxidized and lipids undergo auto-oxidation. Numerous aspects must therefore be considered (Eßlinger, 2006).

Lautering or wort filtration

In this step the solubilized materials such as carbohydrates, proteins, polyphenols and lipids are separated from the insolubilized ones by using the husk as a filtration bed and by washing them with hot water to extract the residual sugar.

Boiling

Wort boiling is critical to allow many chemical reactions to occur. Hop is added during this stage to allow the extraction and isomerization of its α-acids as presented on figure 5. Besides that, it sterilizes the wort by killing unwanted bacteria and it coagulates the proteins, hence preventing the beer from becoming turbid. The vapour produced concentrates the wort as well as volatilizing unwanted off flavour such as dimethyl sulfide precursors and other volatile elements derived from hop and malt. Furthermore, many different complexes are formed (proteins- polyphenols, flavours and colours) that also reduce redox potential protecting the wort during the next steps. (Denk *et al.*, 2000)

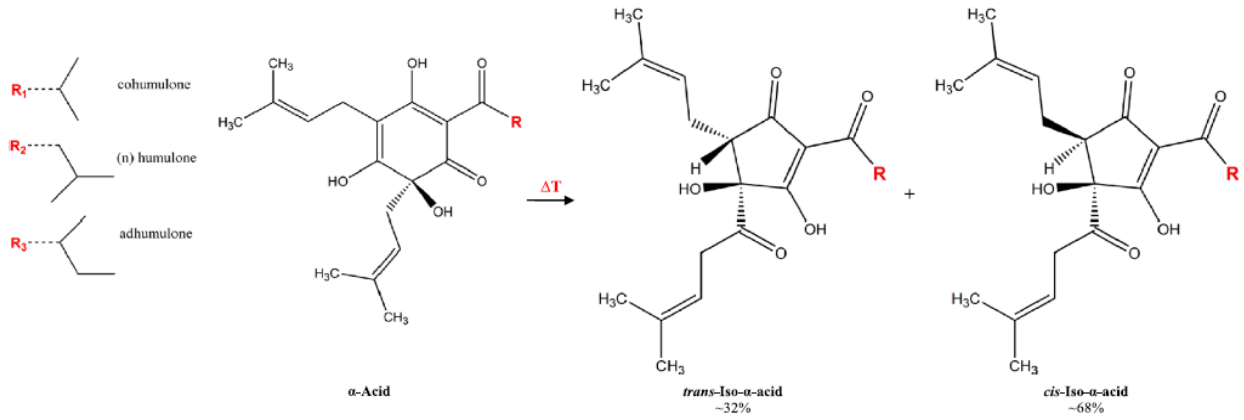


Figure 5: « The isomerization of hop α-acids thermally induced during wort boiling to produce the diastereomeric trans- and cis-iso-α-acids, being the bittering agents of beer» (Almaguer *et al.*, 2014b)

Cooling

After being boiled, the wort must be cooled in order to reach its fermentation temperature (7 - 8°C for bottom fermentation and 15°C for top fermentation). This is usually achieved by using a plate heat exchanger with water as cooling fluid. This step must be quick enough to avoid spoilage micro-organisms developing before the yeast is added.

Fermentation

Fermentation is the process through which the yeast turns the wort into beer. It is usually classified into three types: warm, cold and spontaneous, according to the conditions and micro-organisms used. These single-cell microorganisms are biologically classified as fungi belonging to the genus *Saccharomyces cerevisiae* or *uvarum,* respectively responsible for top and bottom fermentation, thereby producing ale or lager. Spontaneous fermentation occurs without pitching the wort with other microorganisms. The process starts due to microorganisms found in the close environment such as *Brettanomyces* or other bacteria genera. The fermentation consists in the production of adenosine tri-phosphate (ATP) through substrate-level phosphorylation. During the process, glucose is partially oxidized, contrary to what occurs during respiration in which it is totally oxidized, resulting in the production of ethanol and carbon dioxide as main by-product. Besides, these major by-products many other types of compound are synthesized during the fermentation process such as higher alcohols and esters. They are respectively formed by amino acid anabolism (Ehrlich pathway) and organic acids with alcohols enzymatic condensation (Lodolo *et al.*, 2008). For a better understanding of the aroma compounds production during this process the yeast metabolism is further described in the 2.6 part (metabolism of yeast during fermentation).

Maturation/Aging

After the primary fermentation the beer is called “green”. It contains small amounts of CO_2 and has less flavour than the matured beer ready for commercialization. Traditionally, this maturation process is carried out by adding small amounts of yeast causing a secondary fermentation limited in rate by the smaller amount of fermentable carbohydrates available and lower temperature.

Like any food product, beers possess a shelf life which varies from months to years depending on the type of beer. Indeed, during storage many processes causing instability are ongoing (microbial, colloidal, colour, foam and especially flavour). Flavour deterioration is both due to reactions of formation and degradation of molecules above or below their flavour threshold. Besides, the existing interactions between them will increase or decrease this effect (Meilgaard, 1975).

From a chemical point of view, many different reactions such as oxidation of higher alcohols, Strecker degradation of amino acids, aldol condensation, degradation of hop bitter acids, oxidation of unsaturated fatty acids, auto-oxidation of fatty acids, enzymatic breakdown of fatty acids, Maillard reaction, synthesis and hydrolysis of volatile esters, formation of dimethyltrisulfide, degradation of polyphenols occur both simultaneously and concurrently (Vanderhaegen *et al.*, 2006).

2.5.3) Dry-hopping techniques

The hop volatile compounds present in the essential oil part are also subject to dissolution though their apolar nature and are for their part responsible for the distinctive hop aroma. These terpenic compounds from hop aromatic varieties are very sensitive to heat degradation, justifying their introduction at the end of the process to extract and preserve them in the wort (late-hopping). Despite these precautions, processes of beer production such as pasteurization, strong primary fermentation (departure with CO_2) or long storage can cause their disappearance from the beer (Hough *et al.*, 1982).

Therefore, other hopping techniques such as dry-hopping have been developed and introduced at different stages of production, with a view to obtain strong hop flavour. For this technique the hop products are infused into cold beer to transfer the aroma compounds with minimum loss by evaporation and reduced chemical transformation. The technique being still novel and used mainly in small breweries, relatively little information is available on the technology and techniques of dry-hopping. Indeed, the fate of the aromatic product resulting in the final beer (linalool, β-citronellol and geraniol particularly) depends on many factors (Forster et Gahr, 2013).

As stated earlier, dry-hopping consists of the infusion of hop materials or extract to wort or beer during a time ranging from days to weeks. The most common techniques consist in the maceration of 1 - 12 g/L of whole cones or pellets (either 45 or 90) in a maturation tank (containing green beer) at a temperature from 1 to 20°C which results in a so-called cold extraction. The dry-hopping can be static when hop materials macerate without stirring and dynamic when pump or CO_2 is used for stirring it. Beside hop parameters (variety selection, harvest date, rate of addition, oil content of the selected hop harvest) this extraction is influenced by the alcoholic content of the beer due to the solvating power of ethanol leading to extraction of unwanted vegetative materials (Wolfe, 2012; Sharp *et al.*, 2014).

Nevertheless, from the late 1980', some brewers used liquid CO_2 hop extract to shorten this period. The common thought being that only the hop-derived volatiles contained in it characterized a dry-hopped beer and that the sole addition of these essential oil would be enough to impart the aroma profile (Laws *et al.*, 1983).

To sum up the dry-hopping factors recognized to determine dry-hop aroma of beer are:
- Time
- Temperature
- Hop (variety, harvest state, oil content)
- Hopping rate
- Hop dispersion methods (static / dynamic)
- Beer type

However, recent studies show that besides this aromatic extraction the content of some non-volatile hop acids such as humulinones, iso-α-acids, α-acids increases after long dry-hopping (over 2 weeks), which imparts the perceived bitterness (Parkin et Shellhammer, 2017).

Furthermore, a study demonstrate that the hop oil content, a parameter used by brewers to dose hop in beer, is not linked to the overall hop aroma intensity (OHAI). They hypothezised that "It is in addition to the number of factors and interactions affecting hop material there are an equal, if not greater, amount of contributing downstream factors and interactions that influence the aroma potential of hop material in the brewing operation." (Vollmer et Shellhammer, 2016).

Finally, besides these considerations, the presence of yeast greatly complicates the process by metabolizing the dissolved oxygen (DO) that could lead to beer oxidation and change the aroma profile by its fermentation process (Oladokun, 2017).

2.6) Metabolism of yeast during fermentation: production of flavour compounds

In figure 6, below we see the basic process taking place within yeast, leading to the production of aroma compounds. For example, Pyruvate fermentation (red and green boxes) leading to ethanol and carbon based compounds (such as acetaldehyde), anabolism of amino acids leading to vicinal diketones formation (pink box), metabolism of amino acid leading to higher alcohol and ester production (purple box).

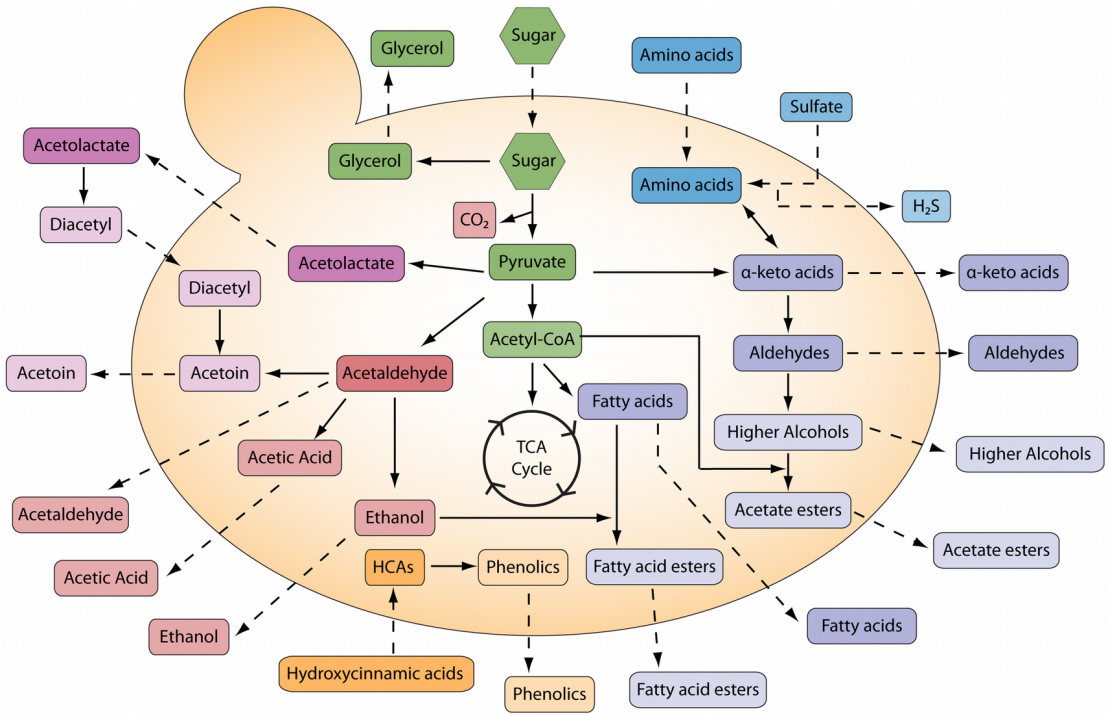


Figure 6: Overview of flavour compounds production (Dzialo *et al.*, 2017)

2.6.1) Formation of vicinal diketones

The 2,3-butanedione (commonly named diacetyl) as well as the 2,3-pentanedione originate from the endogenous production by the yeast of amino acid needed for its own metabolism. Their presence in beer is commonly seen, except for some rare cases, as a defect due to their unpleasant flavour (butter-like and toffee-like) and their really low flavour threshold (0.1 - 0.2 ppm and 0.9 - 1.0 ppm depending on the taster) (Krogerus et Gibson, 2013).

The fermentation performance is greatly impacted by the assimilation of nitrogen compounds of wort. Indeed, during growth the yeast cells need nitrogen in order to be able to assemble themselves, this nitrogen being principally in the form of amino acids, ammonium and small peptides. Nevertheless, yeast does not use these previous compounds as such to synthesize new biomolecules. Indeed, they first need to be catabolized, and it is these intermediate catabolites that are used (see Ehlrich pathway for further explanation) (Pierce, 1987).

More specifically, as presented in figure 7, the commonly accepted pathway shows that they respectively originate from non-enzymatic spontaneous oxidative decarboxylation of α-acetolactate and α-acetohydroxybutyrate that are intermediate products in the biosynthesis of valine and isoleucine. Indeed, they are extracted in the wort by the yeast due to the limiting reaction rate between α-acetolactate and 2,3-dihydro-isovalerate to prevent carbonyl stress. Therefore, the diacetyl excretion rises with biosynthesis of valine, depending on cell requirement and its availability within the yeast environment. Rapid yeast growth as well as insufficient free amino nitrogen leads to high diacetyl content (Ryan et Kohlhaw, 1974).

Previous studies calculate that the minimum free amino acid content required to sustain a healthy yeast growth and good attenuation at the end of fermentation is around 100 ppm. Inadequate concentration can lead to slow and incomplete fermentation as well as high diacetyl content (Krogerus et Gibson, 2013).

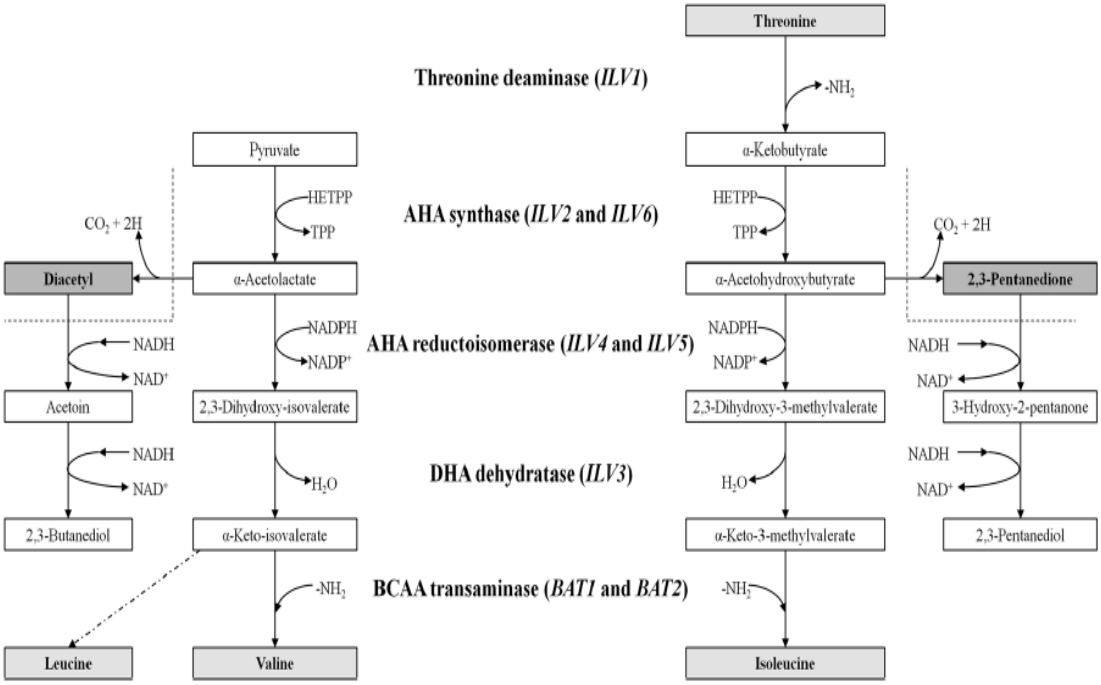


Figure 7: « The pathways for diacetyl and 2,3-pentandione formation and reduction, as well as valine and isoleucine synthesis, in *Saccharomyces* spp. yeast. AHA, acetohydroxy acid; DHA, dihydroxyacid; BCAA, branched chain amino acid » (Krogerus et Gibson, 2013)

Finally, another aspect being researched is the increase of these vicinal diketones following dry-hopping. Indeed, this metabolite plays a huge role in the metabolism of yeast and therefore in the production of fermented beverages. In beer production, the vicinal diketones and especially the diacetyl are considered to be a spoilage product which gives an undesirable buttery, butterscotch-like flavour, and bottle refermentation traditionally aims to reduce its content in beer. In the case of fully attenuated beer an increase in VDKs is observed after dry-hopping suggesting that: "Yeast is utilizing added sugar in a nitrogen deprived environment and is autonomously producing amino acid". (Baillo, 2017)

2.6.2) Formation of higher alcohols and esters

Besides the vicinal diketones two other classes of compounds originating from yeast contribute to the flavour profile, namely esters and higher alcohols.

In order to incorporate the amino group into its own structure, the brewing yeast absorbs wort amino acid, and the final by-products of this reaction chain (represented in figure 8 below) are these higher alcohols. Their production is therefore influenced by the wort amino acid content as well as the genetic regulation of yeast by nitrogen catabolite repression (NCR), which is the control system associated with it (Pires *et al.*, 2014b).

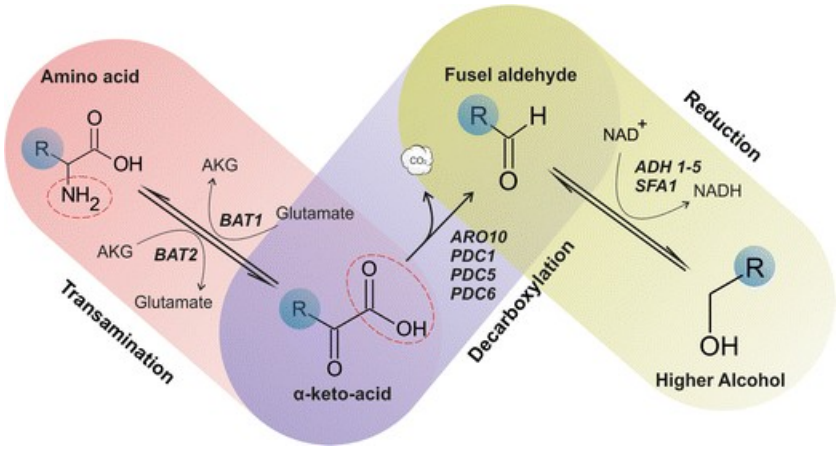


Figure 8: Ehrlich pathways from(Pires *et al.*, 2014b)

The three main amino acids used by brewer's yeast are leucine, valine and isoleucine, which yield different fusel alcohols at the end of the Ehrlich pathway, respectively isoamyl alcohol, isobutanol and methylbutanol. The threonine leads to propanol as fusel alcohols (Hazelwood *et al.*, 2008; Eden *et al.*, 2001).

Esters are formed in the cytoplasm of brewing yeast by the enzymatic condensation of alcohols and organic acids which, due to their lipophilic nature, easily cross the membrane to dissolve in the fermentation media. Despite their very low trace concentration, they have a huge impact on the flavour by their very low threshold bringing fruitiness to beer. Two different kinds of ester can be distinguished: acetate ester when higher alcohols are associated with acetyl-coA by alcohol acetyl transferase (AATase), and ethyl ester when ethanol is associated with acyl-coA (derived from middle-chain fatty acid) (Pires *et al.*, 2014b).

In spite of their low concentrations due to synergy between them, small variations in the ester content can greatly impact the beer aroma profile (Verstrepen *et al.*, 2003).

As represented on figure 9, though dozens of esters can be formed by different combinations, only six of them contribute to the vast majority of the aromatic constituent. Two main factors control ester production, namely the concentration of substrates and the activity of enzymes involved in the reactions. Therefore, all parameters affecting these factors will impact ester production, leading to extreme difficulties in predicting and controlling ester formation within alcoholic beverages resulting in insufficient or aberrant ester production for many producers (Verstrepen e*t al.*, 2003).

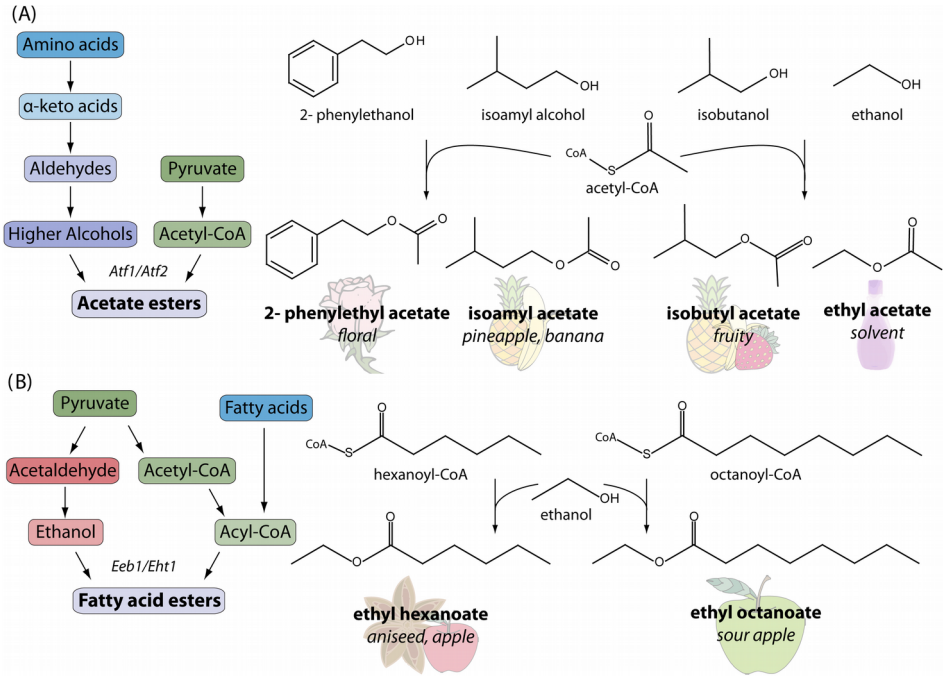


Figure 9: General schema for both types esters synthesis (Dzialo *et al.*, 2017)

The table 3 below resume the typical concentration rate found in beer as well as the aroma impression and flavour threshold associated to them.

Table 3: Flavour threshold of the main esters and fusel alcohols (Pires *et al.*, 2014a)

Compound	Threshold (mg L^{-1})	Concentration range (mg L^{-1})	Aroma impression
Acetate esters			
Ethyl acetate	25–30	8–32	Fruity, solvent
Isoamyl acetate	1.2–2	0.3–3.8	Banana
Phenylethyl acetate	0.2–3.8	0.1–0.73	Roses, honey
MCFA ethyl esters			
Ethyl hexanoate	0.2–0.23	0.05–0.21	Apple, fruity
Ethyl octanoate	0.9–1.0	0.04–0.53	Apple, aniseed
Higher alcohols			
n-Propanol	600	4–17	Alcohol, sweet
Isobutanol	100	4–57	Solvent
Isoamyl alcohol	50–65	25–123	Alcoholic, banana
Amyl alcohol	50–70	7–34	Alcoholic, solvent
2-Phenylethanol	40	5–102	Roses

2.7) Transformation of hop volatile compounds by yeast *Saccharomyces cerevisiae*

Among the three main groups present in the hop derived aroma compounds (mono and sesquiterpene, sulfur compounds and oxygenated compounds), different bio-transformation reactions occur upon fermentation by *Saccharomyces cerevisiae*.

For the hydrocarbon terpene compounds which are the main components of the hop essential oil, namely β-myrcene, α-humulene and β-caryophyllene, no transformation products have been detected. In beer their concentration drops during the first few days due to the adsorption on hydrophobic membranes of yeast. On the contrary, the epoxides derived from these compounds have much higher chance of remaining in beer (Praet *et al.*, 2012).

Regarding the sulfur compounds, as opposed to terpenes, their concentration is much lower (0,015 − 1,296 mg/kg) (versus 4.000 − 8.500 mg/kg for terpenoids), but their odour perception threshold also makes them an important contributor to the hop aroma of beer. Furthermore, these polyfunctional thiol concentrations in final beer are higher than one would expect from the hops free thiol content. This rise is due to biotransformations by yeast β-lyase from heavy precursors (S-cysteine conjugates) to those odorant thiols (Gros *et al.,* 2012; Kankolongo Cibaka *et al.,* 2016).

Finally among the oxygenated compounds (carbonyl, ethers, esters,…) the prominent reaction is the bio-transformation of monoterpene alcohol, geraniol and linalool, by the yeast enzymes to citronellol and terpineol as represented on the figure 10 (King et Dickinson, 2003).

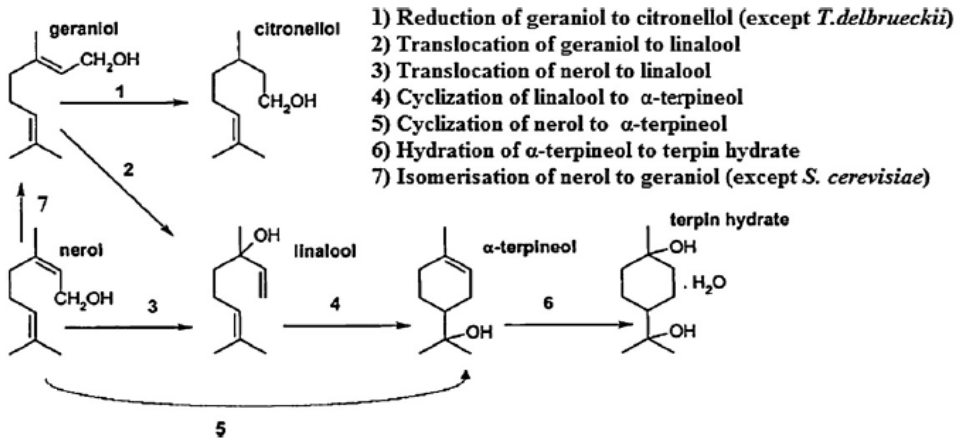


Figure 10: « Scheme showing the monoterpenoid biotransformation reactions catalyzed by *Saccharomyces cerevisiae, Torulspora delbrueckii* and *Kluyveromyces lactis* » (King et Dickinson, 2000))

To conclude a last group of aroma compounds can be liberated by the yeast enzymatic activity (β-glucosidase) this group is the glycosidically bound aroma or glycosides. Indeed, depending on the variety, significant amounts of aroma compounds such as terpene alcohols and norcarotenoids compounds can be produced by the hydrolysis of these glycosides (Praet *et al.*, 2012; Haslbeck *et al.*, 2017).

3) Aims of the study

The aim of the present work is to highlight the potential impact in beer of two enzymes contained in hops, namely α and β amylase. This potential impact will be studied in the context of dry-hopping techniques allowing their extraction without denaturation, which is not the case with classic hopping (hop kettle).

The first part will aim to demonstrate the absolute variability in the specific activity of these enzymes extracted from hop products (pellet 45, whole hop, pellet 90) from different years and varieties.

Later, the impact on the sugar profile will be investigated, especially regarding the production by these enzymes of mono and disaccharides from a higher level of polymerization sugars originating from the brewing and fermentation processes.

Furthermore, the impact of the production of these fermentable sugars on yeast physiology will be explored by monitoring different volatile organic compounds, especially vicinal diketones as explained in the previous part.

Finally, other tendencies in specific compounds representative of the beer aroma profiles will be investigated.

To conclude, four more specific goals are targeted:

1) To evaluate the specific enzymatic activity in different varieties and forms of aromatic hop.

2) To dry-hop a characterized beer and follow the fermentable sugar production within the beer resulting from this enzymatic activity.

3) To examine the yeast metabolism after hop addition by following the production of vicinal diketones (2,3-butanedione and 2,3-pentanedione).

4) To evaluate the aroma profile modification generated by yeast activity (aroma compounds production and bio-transformation).

4) Materials and methods

This chapter will be divided following the aims of the study previously presented :

- Firstly, enzyme activity assessment by two specific spectrophotometric methods.

- Secondly, the dry-hopping designs and conditions providing samples for the three further goals will be presented.

- Thirdly, sugar profile evolution during dry-hopping using a liquid chromatography technique.

- Fourthly, vicinal diketone variation will be monitored using GC-ECD as well as other aroma modifications of dry-hopped beer with GC-FID and GC-MS.

- Fithly, free amino nitrogen content will be evaluated for its ability to influence fermentation.

- Finally, the statistical analysis procedure applied to the results will be described.

4.1) Specific activity assay of α-amylase and β-amylase

4.1.1) Starch based method

In order to quantify the specific enzymatic activity of α- and β-amylase present within the samples described below, a first method was developed, inspired by (Lebon *et al.*, 2016).

Samples and reagents

The hop materials were provided by Orval brewery in bags under inert nitrogen to saveguard materials from oxidation. Two different varieties from 2016 were provided, namely Alsace Strisselspalt and Hallertaü Hersbrücker, under whole hop (4 kg bags) and pellet type 45 forms (5 kg bags). These initial samples were repackaged under vacuum in approximately 200 g bags for the following analysis. Furthermore, samples from 2017 were provided in 100 g bags for the Strisselspalt variety in whole hop, type 90 and type 45. These seven samples are listed in table 4 below.

Table 4: Hop samples

Year	2016		2017
Variety	Strisselspalt	Hersbrücker	Strisselspalt
Whole hops	X	X	X
Pellet 90			X
Pellet 45	X	X	X

- Extraction buffer: 0,1 M Tris-HCl (pH 6,5) containing: magnesium chloride ($MgCl_2$, 8 mM), disodium ethylenediaminetetraacetic acid (Na_2EDTA, 2 mM), 1,4-dithiothréitol (DTT, 1 mM), Phenylmethylsulfonyl fluoride (PMSF, 0,1 mM).
- α-amylase substrate: acetate buffer 50 mM (pH 4,8) containing: starch 0,2 %,

calcium chloride ($CaCl_2$, 20 mM), sodium chloride (NaCl, 50 mM).

- α-amylase stopping reagent (acidified iodine solution): potassium iodine (KI, 38,3 mM)

iodine (I_2, 2,8 mM), hydrogen chloride (HCl, 0,25 mM).

- β-amylase substrate: citrate buffer 50 mM (pH 3,6) containing

starch 1%, disodium ethylenediaminetetraacetic acid (Na_2EDTA, 0,78 mM)

-β-amylase stopping reagent: 3,5-dinitrosalicyclic acid (DNS, 43,8 mM), sodium hydroxyde (NaOH, 0,4 M) sodium/potassium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$, 1,06 M).

Sample preparation

Hop samples were crushed with liquid nitrogen in a high speed mill (IKA© 2000) to obtain a homogeneous powder. 5 g of powder were mixed with 50 mL of extraction buffer on ice for one hour. The homogenate was then centrifuged at 10.000 g for 10 min and filtered to obtain the enzyme extract. In Lebon *et al.*, 2016, this extract was used as such to assess the activity of the enzymes. However, the quantity of enzymes within hops being much smaller, a precipitation step was added to concentrate it and bring it within the quantification limit of the method. In order to do so, a specific ammonium sulfate range of 40 - 60 % was used to precipitate protein, especially amylases. The resulting pellets after a second centrifugation at 20.000 g for 30 min were re-suspended in 20-times smaller amounts of water and aliquots were made to assess specific activity. The whole process of sample preparation is represented on figure 11 below.

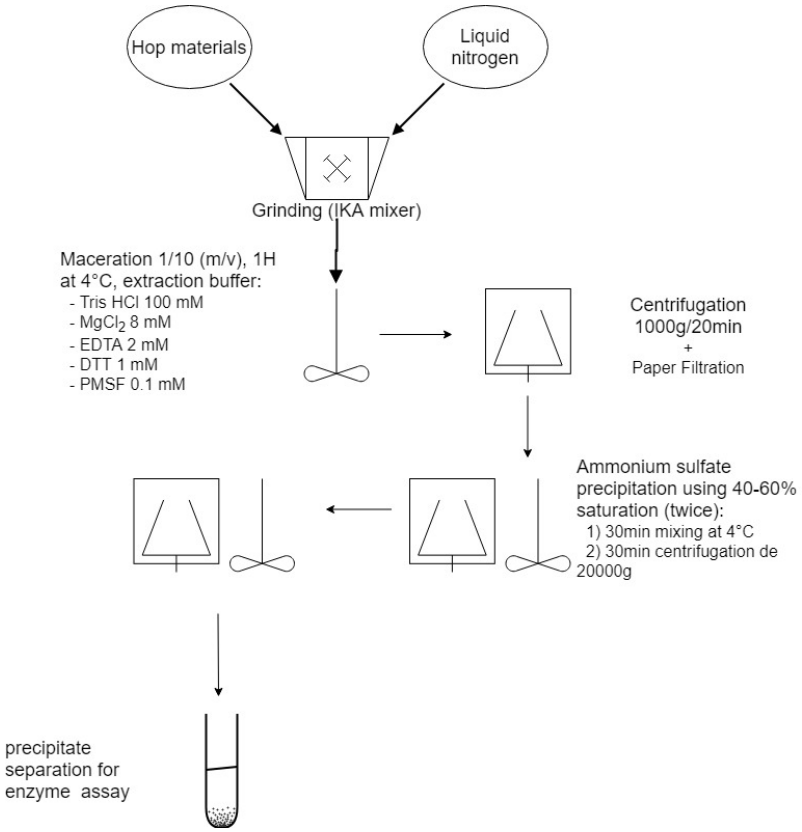


Figure 11 : Hop materials sample preparation for enzymes assay

Calibration

Pure α and β-amylase were purchased at Sigma-Aldrich©, respectively α-amylase from *Aspergillus oryzae* (83 U/mg), β-amylase from barley *Hordeum vulgare* (19,3 U/mg) and calibration curves were performed in triplicates at concentrations of 0; 20; 30; 40; 50; 60; 80 U/L for β-amylase and 0; 6,64; 13,28; 19,92; 26,56; 33,2 U/L for α-amylase.

Equipment and conditions

Absorbance of the enzyme assays were measured by a Ultrospec 7000 spectrophotometer thermostated at 25°C with a PCB 1500 Water Peltier System (DBS©).

Enzyme assay

For α-amylase, an aliquot of 100 µL was combined with 200 µL of α-substrate and the whole was incubated for an hour at 37°C. Afterwards the reaction was stopped by adding 800 µL of iodine solution and 3,2 mL of water. The residual starch content was assessed spectrophotometrically at 620 nm.

For β-amylase, an aliquot of 200 µL was mixed with 200 µL of β-substrate and incubated at 20°C, also for an hour. After 400 µL of DNS solution were added, the samples were then heated in a 95°C water bath for 5 min and then cooled on ice before being assessed spectrophotometrically at 540 nm.

The differential in absorbance with a blank reaction is converted using the calibration curve previously established into an international unit of activity which represents the quantity of enzymes which will liberate 1.0 mg of maltose from starch in 3 min at pH 4,8 at 20°C.

4.1.2) Megazyme amylases assay kit

The second quantification method was performed by adapting two amylase assay kits: Betamyl-3® and Ceralpha® from Megazyme© as suggested by the Megazyme technical support team and professor Tom Shellhammer (Oregon State University).

Sample and reagents

The same hop samples as described in the previous assay method and presented in table 4 were also assayed using this kit containing:

- Ceralpha substrate consisting of p-nitrophenyl-α-D-maltoheptaoside (BPNPG7), thermostable α-glucosidase and stabilisers.

- Betamyl substrate consisting of p-nitrophenyl-β-D-maltotrioside (PNPβ G3), thermostable β-glucosidase and stabilisers.

- Ceralpha buffer A: Sodium malate/sodium chloride (1 M), Calcium chloride ($CaCl_2$, 40 mM), sodium azide (NaN_3, 0,02 % w/v).

- Betamyl buffer A: Tris/HCl buffer pH 8 (1 M), disodium ethylenediaminetetraacetic acid (Na_2EDTA, 20 mM) and sodium azide (NaN_3, 0,02 % w/v).

- Betamyl buffer B: 2-(*N*-morpholino)ethanesulfonic acid buffer pH 6,2 (MES, 1 M) disodium ethylenediaminetetraacetic acid (Na_2EDTA, 20 mM), Bovine serum albumin (BSA, 10 mg/ml) and sodium azide (0,10 % w/v).

- Stopping reagent: Tris buffer solution pH 8,5 (1 % w/v).

Sample preparation

First 0,5 ± 0,01 g of homogenated hop powder is mixed with 5 mL of betamyl buffer A for one hour on ice (at 4°C) to allow enzymes extraction with short vortexing (10 s) each 10 min. The samples are then centrifuged (5.000 g, 10 min) and filtrated on a 0,45 µm nylon syringe filter.

Equipment and conditions

Absorbance of enzyme assays was measured by Ultrospec 7000 spectrophotometer thermostated at 25°C with a PCB 1500 Water Peltier System.

Enzyme assay

In order to assess the β-amylase activity, 0,2 mL of filtrate is diluted in 4 mL of betamyl buffer B, and 100 µL of this solution is incubated with 100 µL of substrate at 40°C for 1.000 min. When the maltose is liberated, the resulting product is cleaved by the glucosidase to liberate the nitrophenyl group and the absorbance of the solution is read at 400 nm (as represented in figure 12).

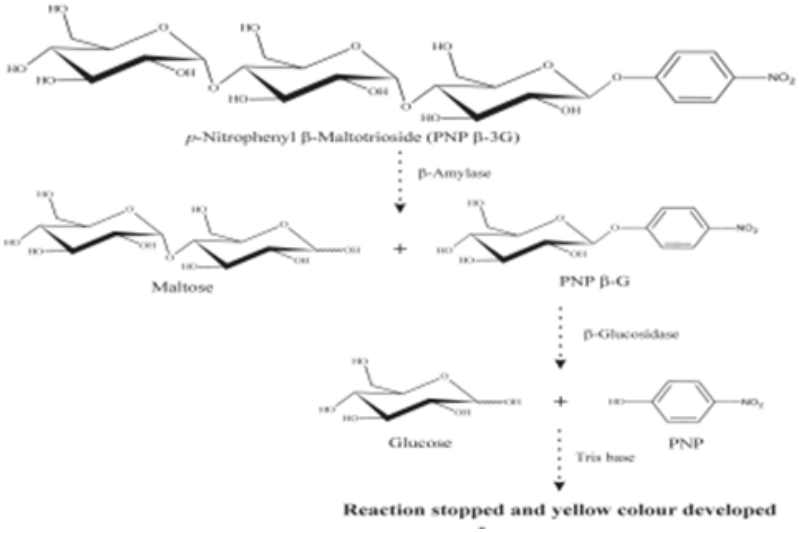


Figure 12: Representation of the β-amylase assay (Megazyme)

For the α-amylase, 0,2 mL of diluted extract is mixed with 3 mL of buffer A. An aliquot of 100 µL is incubated with the same volume of substrate at 40°C for 1.000 min. The nitrophenyl liberated by glucosidase (as represented in figure 13) is then assessed by reading at 400 nm.

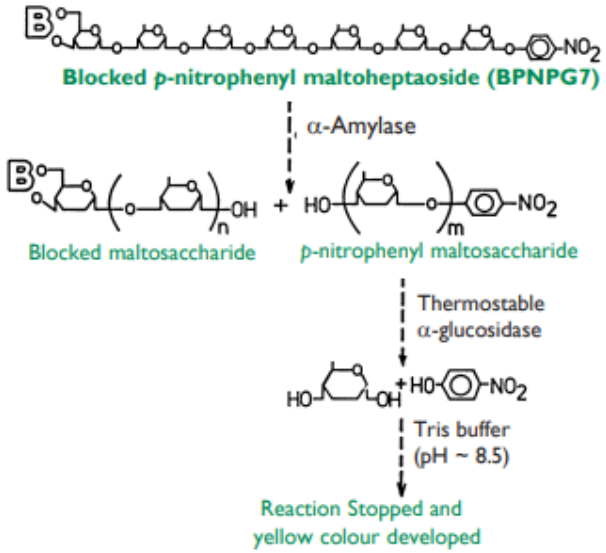


Figure 13: Representation of α-amylase assay (Megazyme)

“Calculation of activity per gram of hop

$$\text{Units of Amylase } (\alpha\text{- or } \beta\text{-}) \text{ / g of flour} = \left| \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{E_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution} \right|$$

ΔE400 = Absorbance (sample) - Absorbance (blank)
Incubation time = 10 min
Total volume in cell = 3,4 mL
Aliquot assayed = 0,2 mL
EmM *p*-nitrophenol = 18,1 (at 400 nm) in 1% Tris buffer solution
Extraction volume = 5 mL per 0,5 g of malt
Sample weight = 0,5 grams
Dilution = 0,2 mL to volume of 4,2 mL (i.e. 21-fold) for
β-amylase; then a further 0,2 mL to 3,2 mL
(16-fold) for α-amylase (i.e. total 336).”

“One unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of p-nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed a Ceralpha® Unit.”

“One unit of activity is defined as the amount of enzyme, in the presence of excess thermostable β-glucosidase, required to release one micromole of p-nitrophenol from PNPβ-G3 in one minute under the defined assay conditions, and is termed a Betamyl-3® Unit.” (from malt amylase assay procedure megazyme).

4.2) Experimental design to assess hop enzymes impact during dry-hopping

After the assessment of the absolute activity of these specific enzymes within the hop cones, two experimental plans were designed to assess the possible impact within the beer matrix during dry-hopping.

4.2.1) Laboratory design

First, a dry-hopping test was performed in the laboratory with 36 samples in triplicates as represented on the table 5 below. Indeed to do so, a sample of beer was either combined with yeast (20 x 10^6 million cells/ml) or with sodium azide (NaN_3 20 mM) to prevent microbial development. Six different modalities were evaluated over a 14-day period at 17°C, which represent the classic dry-hopping period in the brewery field. These six modalities were chosen in order to be able to isolate the impact of the hop alone (modalities 2 and 3) with concentrations either close to brewing practice (5 g/L) and a stronger one (25 g/L) to see how far the reaction could go. Modalities (5 and 6) aimed at evaluating the interaction that could exist with the yeast. The last two modalities (beer and beer + yeasts) were blank modalities performed to ensure that the change observed in the other could not take place without hop, yeast or the interaction of the two. To conclude, in this experiment three factors are taken into account to explain the variability observed, namely hop (0, 5 or 25 g/L), yeast (0 or 20 x 10^6 million cells/mL) and time (1, 2, 3, 4, 7, 14 days) as represented on the table 5.

Table 5: Dry-hopping laboratory samples (modalities)

Modalities / Days	1	2	3	4	7	14
1) Beer	B1	B2	B3	B4	B7	B14
2) Beer + Hop 5 g/L	B1H5	B2H5	B3H5	B4H5	B7H5	B14H5
3) Beer + Hop 25g/L	B1H25	B2H25	B3H25	B4H25	B7H25	B14H25
4) Beer + Yeast	B1L	B2L	B3L	B4L	B7L	B14L
5) Beer + Yeast + Hop 5g/L	B1H5L	B2H5L	B3H5L	B4H5L	B7H5L	B14H5L
6) Beer + Yeast + Hop 25g/L	B1H25L	B2H25L	B3H25L	B4H25L	B7H25L	B14H25L

Sample and reagent

The Strisselspalt 2016 whole hop sample was used to dry-hop the three beer repetitions at the previously specified rate. The beer was collected at the so-called green beer state at the end of the fermentation step and filtrated to get rid of the yeast. The analysis by Anton-Paar procedure at the brewery gives its characteristic before the dry-hopping on table 6 below.

Table 6: Beer sample characteristics for laboratories dry-hopping

Samples	Alcohol (ABV%v/v)	Real extract (°P)	Apparent extract (°P)	Original extract (°P)	Colour (EBC)	pH
Repetition 1	6,67	3,75	1,37	13,81	20,8	4,1
Repetition 2	6,56	3,91	1,57	13,81	20,4	3,98
Repetition 3	6,61	3,73	1,37	13,78	19,4	3,98

Sample preparation

In order to avoid oxidation and other degradation reactions as much as possible, 40 mL of beer (either with yeast or sodium azide) were dry-hopped in 50 mL falcon hermetically sealed after the addition of 0; 0,2 or 1 g of hop powder (obtained after nitrogen grinding). These samples were briefly vortexed for 10 sec before being kept in the dark at 17°C in a cold chamber for the duration of the dry-hopping (1, 2, 3, 4, 7, 14 days)

4.2.2) Industrial design

Sample collection

A second experiment was designed by following the process in industrial tanks to assess whether the changes occurring at the laboratory scale were also taking place. Dry-hopping takes place with whole hop. For this experiment, dry-hopping is also tested with pellet 45. The factors taken into account were here limited to hop form and times, the yeast supposedly being present at the same concentration for each maturation tank. 50 mL of beer were collected from facilities located at the base of the four different tanks after the same period of time (1, 2, 3, 4, 7 and 14 days) than the laboratory design as represented in table 7. Furthermore, they were kept in a freezer before application of the two analytic protocols described in parts 4.3 and 4.4

Table 7: Industrial analysis design

Industrial sample	1	2	3	4	7	14
Maturation tank 4 (whole hop)	T4C1	T4C2	T4C3	T4C4	T4C7	T4C7
Maturation tank 5 (pellet 45)	T5P1	T5P2	T5P3	T5P4	T5P7	T5P7
Maturation tank 18 (whole hop)	T18C1	T18C2	T18C3	T18C4	T18C7	T18C7
Maturation tank 19 (pellet 45)	T19P1	T19P2	T19P3	T19P4	T19P7	T19P7

4.3) Determination of carbohydrates by High Performance Liquid Chromatography (HPLC) with Evaporative Light Scattering Detector (ELSD) within dry-hopped beer

Though the official EBC method recommends the use of refractive index detector (HPLC-RI) for this analysis, as demonstrated by (Floridi *et al.*, 2001), the evaporative light scattering detector allows the use of gradient of elution and therefore the separation of sugars with a higher degree of polymerization, whereas the RI allows only the separation of monomers. This, in our case, is of particular interest because it will make it possible to assess the enzyme activity by the degradation of substrate (non fermentable sugar) as well as the production of fermentable sugar.

Sample and reagents

The dry-hopped beer samples result from the application of the experimental designs previously presented (4.2). The acetonitrile HPLC ultra grade was purchased from Sigma-Aldrich.

Sample preparation

The beer or wort samples were homogenized and degassed by vortexing them and then filtrated on a 0,22 µm nylon syringe filter before analysis.

Evaluation of the total carbohydrate contents by reduction to glucose of all the oligo and polysaccharides is of primary importance in understanding the total substrate content for the enzyme and in explaining a part of the variability between different batches. To do so, a hydrolysis protocol was applied to beer sample before dry-hopping. In order to perform an acid hydrolysis of the higher degree of polymerization sugar to glucose, different chloridric acid concentrations were used from 1 M up to 6 M for 30 min at 80°C. NaOH 4 M were added to bring pH > 2 and avoid degrading the column. The same filtration on a 0,22 µm nylon syringe filter was applied before analysis.

Furthermore, in order to identify the quantity of sugar originating from hop (glucose and fructose) without the enzyme activity, dry-hopping were performed in presence of amylase inhibitor silver nitrate ($AgNO_3$, 80 mM) allowing the quantification for a "zero" day.

Calibration

Calibration curves were established at concentrations from 0,2 - 1 g/L and 1 - 10 g/L with the available laboratory compounds, fructose, glucose, sucrose and maltose. Despite any reference compounds were available, the relative retention time and response factors allowed identification of the polymers such as maltotriose, maltotetraose up to heptaose as presented in annexes.

Equipment and conditions

The apparatus was an Agilent 1200 series equipped with an ELSD detector and drift tube temperature of 40°C. The column used for this analysis was a NH_2 spherisorb from Waters with dimensions of 250 mm x 4,6 mm x 5 µm.

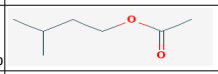
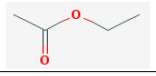
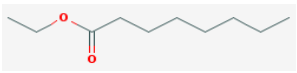
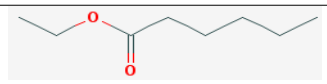
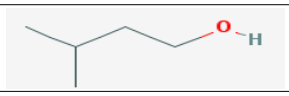
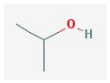
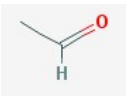
For each analysis the run lasted for 35 min with an eluent flow rate of 1 mL/min. During the first 10 min, the eluent was composed of 75 % acetonitrile in water, before decreasing to 50 % over a 15 min period. A plateau of 5 min at this concentration finished the run, the increase in water content increasing the polarity, which allowed the separation of these polysaccharides. Finally, a cleaning procedure before each batch of analysis was applied by increasing water content to 90 %.

4.4) Determination of volatiles by gas chromatography (GC) within dry-hopped beer

4.4.1) Flame ionization detector (FID)

This detector allows the quantification of volatile organic compounds produced by yeast during the fermentation process in different groups of VOCs, the most characteristics of beer being chosen and presented on table 8.

Table 8: List of standard used with the flame ionisation detector

Compound familly	Compound name	IUPAC	CAS number	Provider	Purity	Molecular structure
Acetate	Isoamyl-acetate	(3-Methylbutyl) ethanoate	CAS 123-92-2	Sigma-Aldrich	99,00 %	
Acetate	Ethyl acetate	Ethyl ethanoate	CAS 141-78-6	Sigma-Aldrich	99,00 %	
Ethyl esther	Ethyl caprylate	Octanoic acid, ethyl ester	CAS 106-32-1	Sigma-Aldrich	99,00 %	
Ethyl esther	Ethyl caproate	Hexanoic acid, ethyl ester	CAS 123-66-0	Sigma-Aldrich	99,00 %	
Alcohols	Isoamyl Alcohols	3-Methyl-1-butanol 2-Methyl-1-butanol	CAS 123-51-3	Sigma-Aldrich	99,00 %	
Alcohols	Iso butanol	2-Methyl-1-propanol	CAS 78-83-1	Sigma-Aldrich	99,00 %	
Alcohols	Propanol	N-propanol	CAS 67-63-0	Sigma-Aldrich	99,00 %	
Aldehydes	Acetaldehydes	Ethanol	CAS 75-07-0	Sigma-Aldrich	99,00 %	

The protocol described here came from an internal procedure of the Orval brewery (BRA-LABO-22).

Sample and reagents

The dry-hopped beer samples result from the application of the experimental design previously presented.

Sample preparation

2 mL of the dry-hopped beer was encapsulated in a headspace vial and then injected into the column under specific conditions.

Calibration

Standard preparation:

- Solution 0 is prepared by diluting pure ethanol to a 5 % solution with distilled water.

- Solution 1 (higher alcohol) is prepared by weighting n-propanol (± 2 g), isobutanol (± 2 g) and isoamyl alcohol (± 12 g); (60 % 3-methyl-1-butanol (9 mL) and 40 % 2-méthyl-1-butanol (4mL)) in 100 mL flask and filling it to 100 mL with 40 % ethanol solution.

- Solution 2 is prepared by weighting acetaldehyde (± 400 mg) in a 100 mL flask and filling it with solution 0.

- Solution 3 (vicinal diketones stock solution) is prepared by weighting diacetyl (± 250 mg) and pentanedione (± 50 mg) in a 100 mL flask and filling it with 40 % ethanol.

- Solution 4 is prepared by diluting 100-times solution 3 with ethanol 5 % (solution 0).

- Solution 5 (esters) is prepared by weighting ethyl acetate (± 3 g), isoamyl-acetate (± 250 mg), ethyl caproate (± 0.03 g) and ethyl caprylate (± 0.03 g) and filling it with pure ethanol.

- Solution 6 (calibration stock solution) is prepared by adding 1 mL of solutions 1, 2, 5 and 2 mL of solution 4 in a 100 mL erlenmeyer before being filled with solution 0.

Calibration solution.

Four calibration solutions, cal 1, cal 2, cal 3, cal 4 were prepared from solution 6 by adding 5, 10, 15 and 20 mL in 100 mL flasks filled with solution 0. A volume of 2 mL of these solutions were placed in a headspace vial for each analysis run of 36 samples.

Equipment and conditions

The apparatus was a Perkin Elmer AutoSystem Gas Chromatograph equipped with Perkin Elmer Headspace Sampler HS40. The column was a CP WAX 52CB 50 m x 0,32 mm x 1,2 µm. The samples were thermostated for 20 min at 70°C before being injected on the column. The temperature programme started at 50°C, held for 2 min then increased to 80°C at 3°C/min, with a final increase to 140°C at 15°C/min. The detector temperature was fixed at 150°C.

4.4.2) Electron capture detector (ECD)

The vicinal diketones 2,3-butanedione and 2,3-pentanedione are highly volatile and their quantification in trace concentrations found in beer (1 - 150 ppb) requires specific derivatization or the use of an electron capture detector. Indeed, though this type of detector is mainly used for halogen and nitro substitute compounds (which are the first group of electrophores defined by inventor I. E. Lovelock), a second group of specific conjugate electrophores exists. These electrophores are typically found for groups which alone do not absorb but do so if connected by bridges.

The same sample reagents and protocols as in the previous analysis were applied, the two detectors being installed on the same chromatographic apparatus.

4.4.3) Mass spectrum detector (MS)

The previously evaluated volatile compounds all arise from the yeast, though hop by itself liberates many aromatic molecules during dry-hopping. The majority of its essential oils being composed by terpenic molecules (mono, sesqui and alcohol terpenes), a mass spectrum detector was used to assess the variation in beer terpene profile.

Sample and reagents

The dry-hopped beer samples result from the application of the experimental design previously presented.

Sample preparation

A specific method using dynamic headspace inspired by (Durenne *et al.*, 2018) was used to concentrate these volatiles, the beer matrix being too complex to be injected as such. A 20 mL vial was filled with 2 mL of dry-hopped beer and 0,8 g of NaCl.

Equipment and conditions

The gas chromatographic apparatus was composed of an Agilent 7895A equipped with a Gerstel© automatic sampler.

DHS procedure

A 500 rpm agitation was held for 30 min at 40°C to concentrate the volatile within the headspace. A volume of 600 mL from the headspace was fixed on the tenax adsorbant tube with a purge flow of 20 mL/min and then dried with a vent (50 mL/min at 2 min) to avoid ice formation during cryofocusing (further describe).

TDU/CIS procedure

Samples injections were made employing a multipurpose sample (MPS). First a thermal desorption of the volatiles from TENAX was performed using a thermal desorption unit (gerstel, TDU2) in splitless mode from 40°C up to 260°C at rate of 100°C/min and hold during 5 min. The TDU was connected with a cooled injection system (CIS) allowing a cryo-focusing of volatiles achived at -60°C previous injection in column by heating the CIS/PTVinlet to 250°C at a rate of 12°C/s hold for 2 min. The molecules were then injected in an HP5 column (30 m x 0,25 mm x 0,25µm) with a temperature programme from 35 to 280°C at 5°C/min. The carrier gas was helium at constant flow of 1,6 mL/min.

Mass spectrometer parameter

To allow semi-quantification of these specific terpenic compounds, an internal standard was used: n-butyl benzene, considered to have the same chromatographic behaviour at a concentration of (50 mg/100 mL) with a direct injection of 1 µL on the adsorbant tube. Furthermore, detection was made using a quadrupole-type massspectrometer (MS 5975C; Agilent Technologies). An electron impact (70 eV) was performed to acquire the mass spectra in SCAN mode with a range of 35 to 350 amu for m/z ratios.
Both selected ion monitoring (SIM) (with only ion 93 recorded) and full-scan modes were used in the same run of 52 min. The quadrupole temperature was of 150°C and MS source of 230°C. Data analysis of mass spectra were performed on Masshenter (Agilent Technologies). The identification of terpenes was obtain with a Wiley275 massspectral database and confirmed by major ions and supplemented by calculation of the linear retention index.

4.5) Free amino nitrogen content of beer

The ninhydrin international method can be used to determine the free amino nitrogen content, which is the amount of amino nitrogen available for yeast before fermentation in wort or after to quantify the amount remaining in beer. In order to generate this chromophore, a purple dye now called Ruhemann's purple (RP), which is the 2-(1,3-dioxoindan-2-yl)iminoindane-1,3-dione, the amino group must be condensed with the ninhydrin to form a Schiff base. Therefore, only amino acid, amonia and to some extent the end group of peptides and small proteins react.

Sample and reagent

The three beer samples before dry-hopping were analyzed with this procedure.

To prepare the colour reagent 10 g of $Na_2HPO_4.12H_2O$; 6 g of KH_2PO_4; 0,5 g of ninhydrin and 0,3 g of fructose were weighted in a 100 mL flask filled with distilled water.

The dilution solution was prepared by weighting 2 g of KIO_3 in distilled water (600 mL) made up to 1 L by 96 % ethanol.

Calibration

A glycine standard solution of 2 mg amino nitrogen/L was prepared by 100-times dilution of a stock solution prepared by weighting 107,2 mg in a 100 mL flask filled with distilled water.

Equipment and conditions

Absorbance of the enzyme assay were measured by Ultrospec 7000 spectrophotometer thermostated at 25°C with a PCB 1500 Water Peltier System.

Sample preparation

Each sample was analyzed in triplicate.

For wort analysis, a dilution by 100 is necessary before adding 2 mL of sample to the test tubes.

For beer analysis, a dilution by 50 is enough before adding the 2 mL of sample to the test tubes.

For blank reaction, 2 mL of distilled water is used instead of the sample.

For the calibration standard, 2 mL of the glycine standard solution are used.

To each test tube, 1 mL of colour reagent is added before being heated in a boiling water bath for 16 min. The tube is then cooled for 20 min in a 20°C water bath. Furthermore, 5 mL of dilution reagent are added to all tubes. Finally, the test tubes are mixed meticulously before spectrophotometric absorbance measurement against distilled water at 570 nm.

4.6) Statistical analysis of the results

All the data were processed by the Minitab© software on which two types of statistical procedures were performed, namely the analysis of variance (ANOVA) and the principal components analysis (PCA).

Analysis of variance (ANOVA):

The conditions required to apply this procedure are a normal distribution of the studied parameters as well as the equality of the variance. The number of repetitions being fixed at three ($n = 3$) the normal distribution is therefore supposed. Indeed, the test is only applicable when ($n > 10$). Therefore, the Levene test was used to demonstrate the equality of the variance because it did not assumed a normal distribution. The results associated to these tests are presented in annexes.

All the samples and variables are independent to each other, allowing the use of two- and three-ways ANOVA. The significant level is fixed for a P-value lower or equal to 0,05. The null hypothesis implies that the means for all factors are equal and that there is no interaction between them.

Principal components analysis (PCA):

This mathematical procedure is designed to transform a large number of possibly correlated variables into a small number of uncorrelated variables. It allows the compression of data by creating principal components which are a combination of the factors explaining the maximum of variance. In our case, it will allow us to assess the total change in the volatile compounds observed during the dry-hopping by reducing the ten dimensions of analysis for each sample to just two or more components.

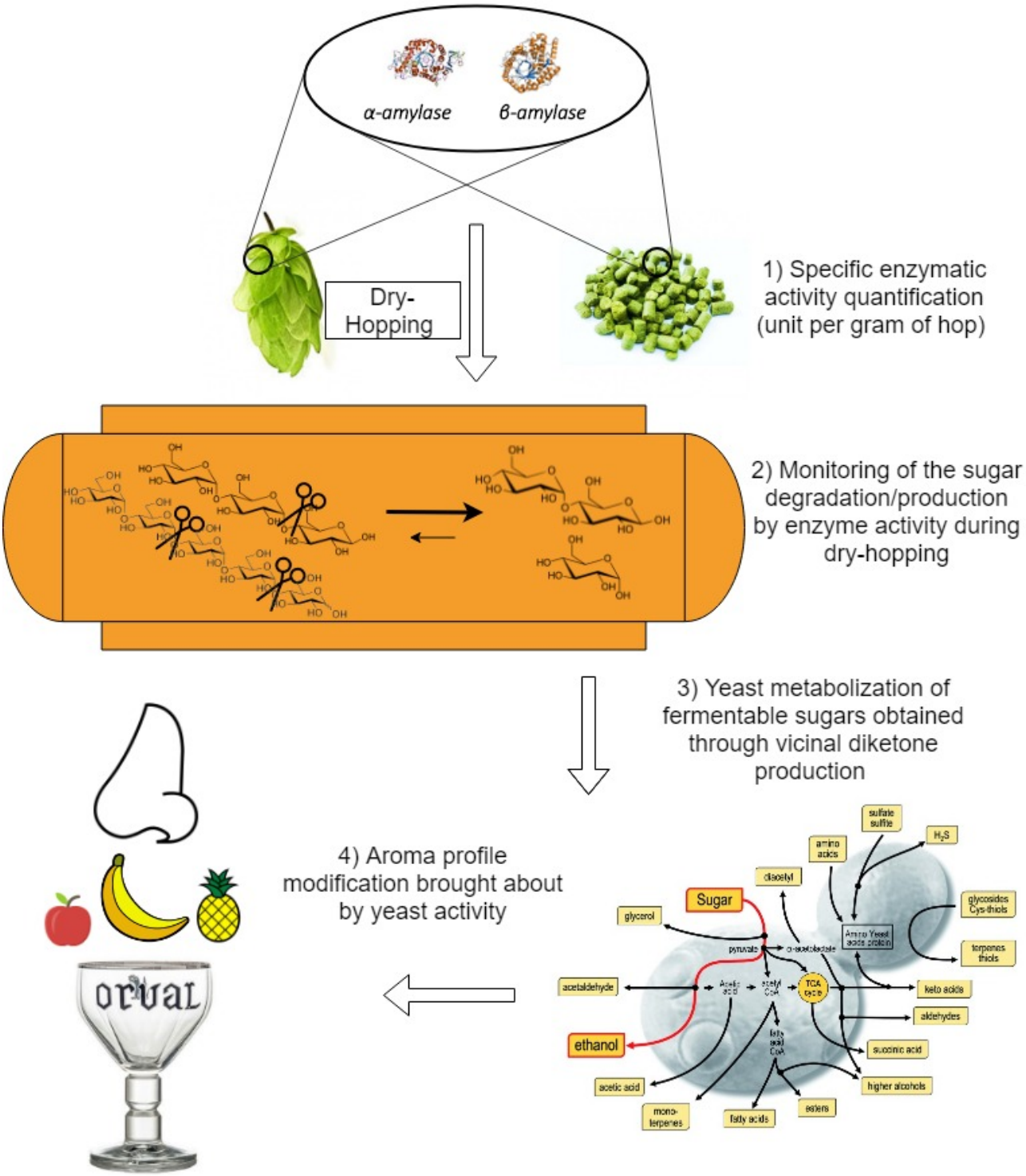


Figure 14: Schematic representation of objectives and results

5) Results and discussion

This chapter will be divided into four main parts as presented in figure 14 on the previous page:

- The first part will concern the enzymatic activity measurement obtained by the two methods developed in the materials and methods (part 4.1).
- The second part will discuss the impact on the beer sugar profile at the laboratory and industrial scale as set out in the two experimental designs.
- The third part will assess the impact on the yeast physiology using volatiles production as a marker of the metabolism.
- The fourth part will aim to evaluate the impact on the beer aroma profile as well as the interaction existing between hop aroma and yeast by looking for developments in the aroma profile of beer.

5.1) Results of the α-amylase and β-amylase activity quantification

5.1.1) Results of starch based method for enzyme assay

The initial step for this experiment was to assess the sensitivity of the method using pure enzymes. As can be seen on the calibration curves obtained after one hour of reaction at different enzyme concentrations (figure 15 and 16), this method should be sensitive enough to detect activity as low as 5 mU/mL which, if we refer to table 2 (in the introductory part), should be enough to assess our enzymatic reactions.

After obtaining negative results trying to evaluate the enzymatic activity of the extract, it was decided to complete this first method with a precipitation step to allow concentration of the enzymes as well as separation from the possible inhibitor of amylase also present within hop namely sugar, and other polyphenols which stay in solution after addition of ammonium sulfate which pelletizes the proteins.

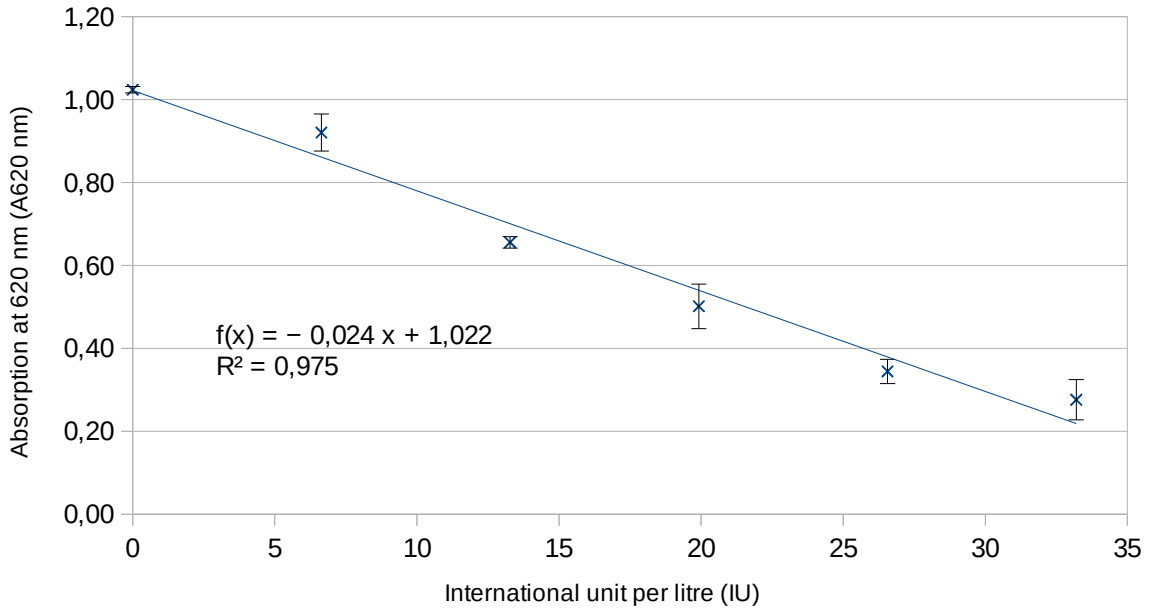


Figure 15: Absorbance evolution at 620 nm of enzyme assay at different α-amylase concentrations

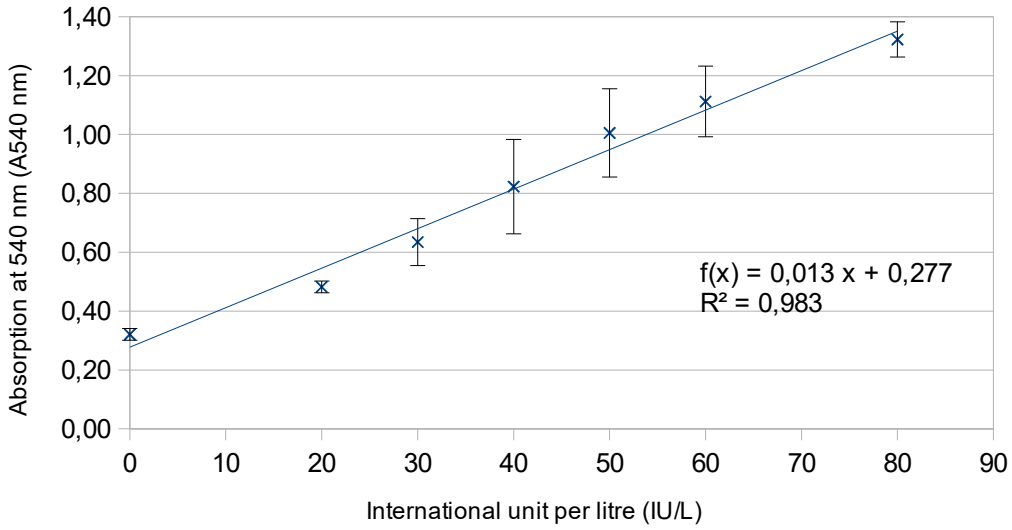


Figure 16: Absorbance increase at 540 nm of enzyme assay at different β-amylase concentrations

As we can see on table 9 below, in spite of the concentration step, it seems obvious that this method was not specific or sensitive enough to assess the enzyme present in our biological materials. Either the process in itself or its conditions (temperature, concentration and time) led to no extraction or denaturation of the enzymes extract, or the method used to quantify was not specific enough to study the reaction, the latter being unlikely because of the reaction with pure enzymes.

Table 9: Enzymatic activity for the 2017 samples with starch substrates

Samples 2017	α-amylase (U/g)	β-amylase (U/g)
Strisselspalt whole hop	0,02	0,12
Strisselspalt pellet 90	0	0,03
Strisselspalt pellet 45	0	0,01

Therefore seeing that this experiment was unsuccessful, it was decided to use a different approach to try to assess this particularly small activity using more specific substrates than boiled wheat starch for the enzymes. Other solutions include extracting, concentrating and purifying the enzymes to quantify their activity by specific methods such as ultra-filtration, affinity chromatography or other. However, this lies beyond the scope of this work, which aims to assess this activity during the dry-hopping of beer and not to study in detail the enzyme in itself.

Before discussing the results acquired for this second method of evaluating enzymatic activity, it is important to acknowledge the fact that these activities are not expressed in international units like the previous one but in specific ceralpha and betamyl-3 units of activity. Eventhough conversion factors exist between the two, as mentioned in the materials and methods sections they are specific for each enzyme source and cannot be applied as such. Nevertheless, the aim of comparing our hop samples with each other can still be achieved.

5.1.2) Results of Megazyme amylase assay kit

A clear distinction appears between the activities measured for α-amylase in figure 17. Three factors were taken into account in the model to explain the variability between the samples, namely the variety (Alsace Strisselspalt (AS), Hallertaü Hersbrücker (HHE)), the year of production (2016 and 2017) and the type (whole hop, pellet 90, pellet 45). The variance should therefore have been analyzed by three-way ANOVA, though it was not, due to the fact that 5 samples were missing. As a result, the statistical analysis was broken down as shown in table 10 into two AV 2 (green and yellow) and one AV 1 (red).

Table 10: Simplified analysis of variance due to missing samples

Strisselspalt	2016	2017	Hersbrücker	2016	2017
Whole hop	X	X	Whole hop	X	
Pellet 90		X	Pellet 90		
Pellet 45	X	X	Pellet 45	X	

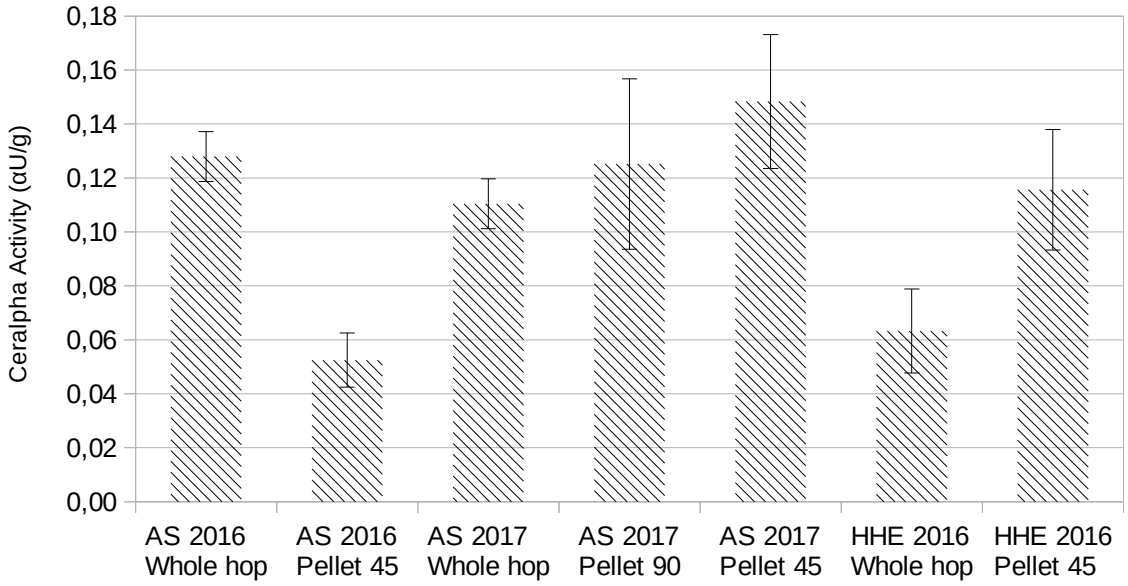


Figure 17: α-amylase activity (Ceralpha Unit per gram) within different hop varieties, types and years

No significant difference between the types of sample from 2017 can be observed as show by figure 18 and table 11. Though the mean increases with refinement (Pellet 45 > pellet 90 > whole hop), due to the size of the standard deviation, no conclusion can be drawn regarding this tendency. The size of the standard deviation can be explained by the very low trace activity measured.

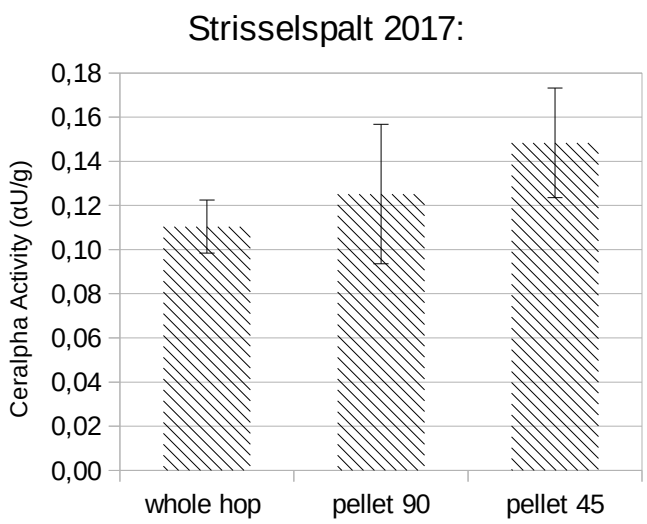


Figure 18: α-amylase activity per gram of hop within the different Strisselspalt 2017 samples

Table 11: Analysis of variance for α-amylase content within 2017 samples

AV1, Factor = type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	2	0,002022	0,001011	1,72	0,257
Error	6	0,003533	0,000589		

The 2016 samples are represented in figure 19 and the variance analysis associated to them figure on table 12. As it can be seen, an interaction between the factors imposes the breakdown of the AV2 according to the most important factor, which is the type. Indeed, the variety being two close taxons, not much of the variance should arise from it. The resulting AV1 for each variety is shown on the next page.

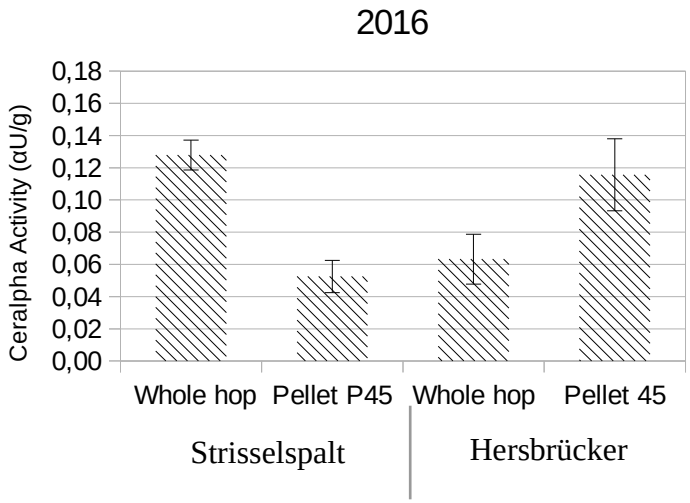


Figure 19: α-amylase activity per gram of hop within the different 2016 samples

Table 12: Analysis of variance for α-amylase content within 2016 samples

AV2, Factors = variety and type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	1	0,0006750	0,0006750	3,12	0,116
Variety	1	0,0000083	0,0000083	0,04	0,849
Type*Variety	1	0,0126750	0,0126750	58,50	0,000
Error	8	0,0017333	0,0002167		

These two analyses of variance (tables 13 and 14) show us that significant differences exist between pellet 45 and whole hop for each variety, but unfortunately not in the same way. Indeed, pellet 45 α-amylase content compared to whole hop is higher in Hersbrücker variety and lower in the Strisselspalt case. Many lines of explanation could be presented to explain these differences. The main ones being that longer time elapses before the pelletizing process takes place in each producer and that the process itself is more or less gentle on the product.

Table 13: Analysis of variance for α-amylase content within 2016 samples (Strisselspalt)

Variety = Strisselspalt

AV1, Factor = type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	1	0,009600	0,009600	96,00	0,001
Error	4	0,000400	0,000100		
Total	5	0,010000			

Table 14: Analysis of variance for α-amylase content within 2016 samples (Hersbrucker)

Variety = Hersbrücker

AV1, Factor = type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	1	0,003750	0,003750	11,25	0,028
Error	4	0,001333	0,000333		
Total	5	0,005083			

For analysis of year and type within Strisselsplat in figure 20, the same interaction between the factors occur as show on table 15, also resulting in the breakdown in AV1 according to the type of hop (still the most important factor).

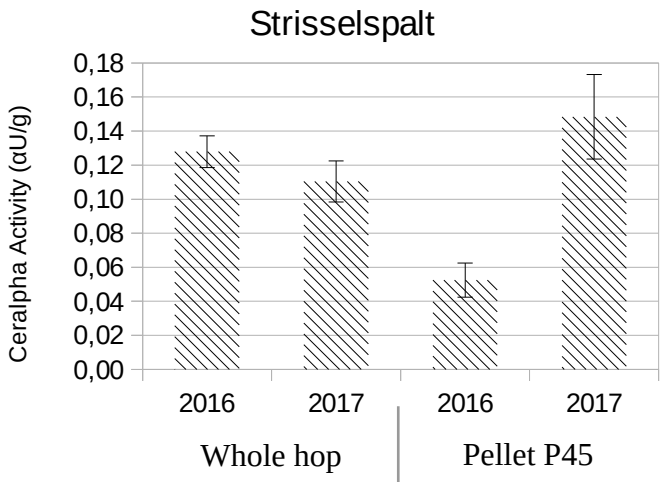


Figure 20: α-amylase activity per gram of hop within the Strisselspalt samples

Table 15: Analysis of variance for α-amylase content within Strisselspalt samples

AV2, factors = year and type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	1	0,0014083	0,0014083	6,04	0,040
Year	1	0,0044083	0,0044083	18,89	0,002
F1*F2	1	0,0102083	0,0102083	43,75	0,000
Error	8	0,0018667	0,0002333		
Total	11	0,0178917			

The analysis shown on table 16 is the same as for the previous AV2 breakdown leading to the same significant differences between pellet 45 and whole hop for the year 2016 (table 13), whereas no significant differences exist between those two types for the year 2017 on table 17.

Table 16: Analysis of variance for α-amylase content within Strisselspalt 2016 samples

Year = 2016

AV1, Factor = type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	1	0,009600	0,009600	96,00	0,001
Error	4	0,000400	0,000100		
Total	5	0,010000			

Table 17: Analysis of variance for α-amylase content within Strisselspalt 2017 samples

Year = 2017

AV1, Factor = type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	1	0,002017	0,002017	5,50	0,079
Error	4	0,001467	0,000367		
Total	5	0,003483			

The same protocol of analysis was applied to the β-amylase results presented here in figure 21. As opposed to α-amylase content, that of β-amylase does not show great variation, implying ubiquitous distribution of the enzyme within the cones and stability throughout the years and process.

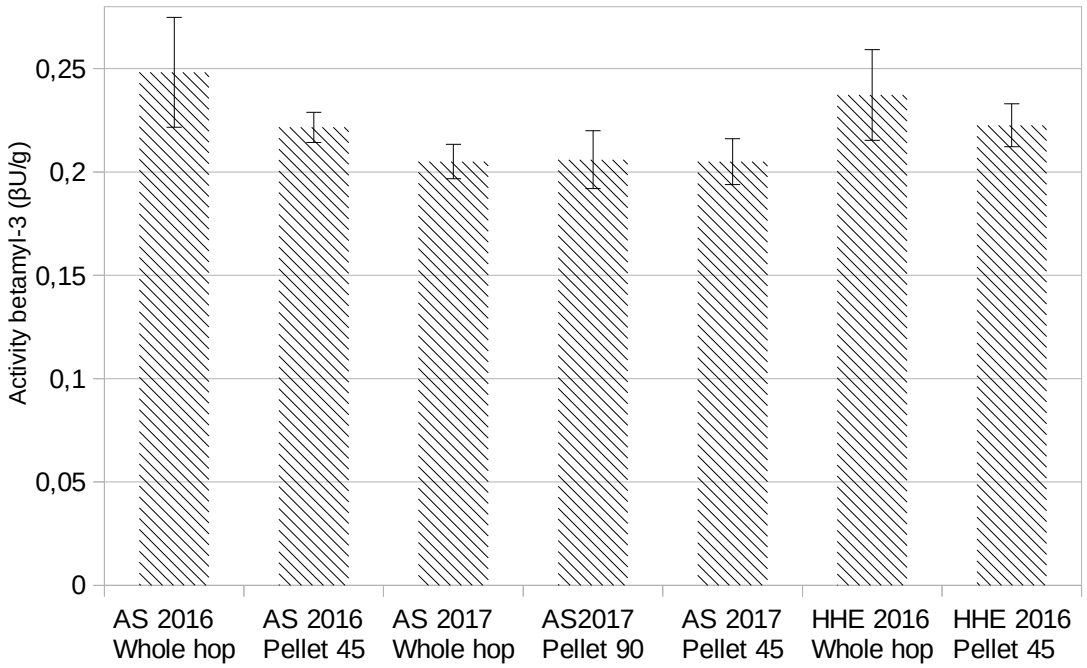


Figure 21: β-amylase activity (betamyl-3 unit per gram) within the different hop varieties, types and years

First, regarding the 2017 samples in figure 22, the p-value for this analysis on table 18 indicates that almost no difference exists for the β-amylase content within the different samples.

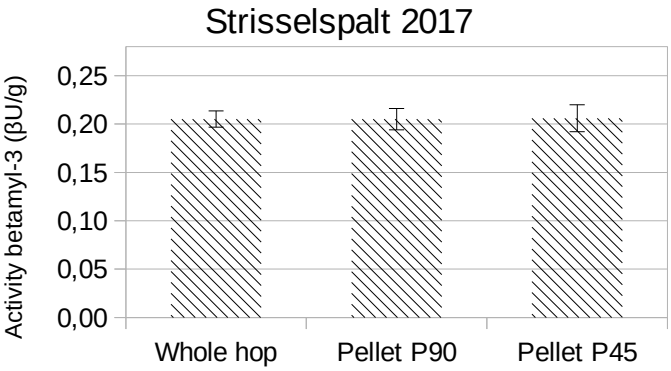


Figure 22: β-amylase activity per gram of hop within the different Strisselspalt 2017 samples

Table 18: Analysis of variance for β-amylase content within 2017 samples

AV1, Factor = type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Type	2	0,000022	0,000011	0,07	0,936
Error	6	0,001000	0,000167		
Total	8	0,001022			

Second, concerning the 2016 samples in figure 23, even if no interaction takes place in this two-way analysis of variance of the β-amylase activity as shown on table 19, no significant differences can be observed between the samples. This implies that varieties and processing do not influence its content in the final hop product.

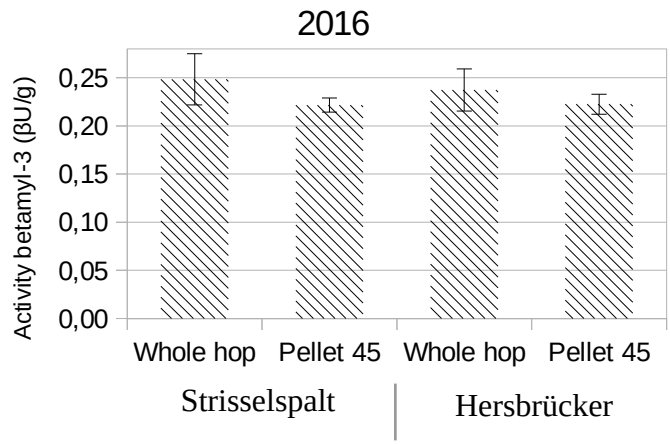


Figure 23: β-amylase activity per gram of hop within the different 2016 samples

Table 19: Analysis of variance for β-amylase content within 2016 samples

AV2, Factors = variety, type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Variety	1	0,0002083	0,0002083	0,60	0,463
Type	1	0,0010083	0,0010083	2,8	0,128
F1*F2	1	0,0000750	0,0000750	0,21	0,656
Error	8	0,0028000	0,0003500		
Total	11	0,0040917			

Third, for hop from Strisselspalt variety in figure 24, even if no interaction takes place in the two-way analysis of variance on table 20, no significant conclusion can be drawn concerning differences in the β-amylase content within hop samples, except between those from years 2016 and 2017. The latter presents a significantly higher content, which seems to imply that this enzyme is ubiquitously distributed within the hop flower, stable in time, and that growing conditions as well as the stage of maturity of the plant could explain this shift from one year to another.

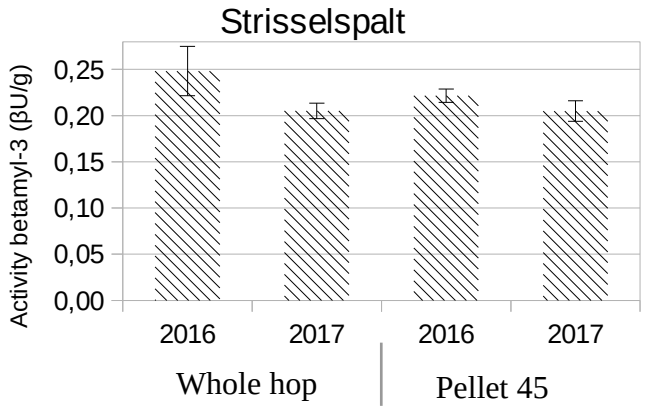


Figure 24: β-amylase activity per gram of hop within the Strisselspalt samples

Table 20: Analysis of variance for β-amylase content within Strisselspalt samples

AV2, Factors = years, type

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Year	1	0,0027000	0,0027000	9,53	0,015
Type	1	0,0003000	0,0003000	1,06	0,334
F1*F2	1	0,0005333	0,0005333	1,88	0,207
Error	8	0,0022667	0,0002833		
Total	11	0,0058000			

5.2) Sugar profile of the beer

Figure 25 represents the chromatograms obtained with the HPLC-ELSD procedures described earlier after one day of dry-hopping for the modalities beer and beer + hop (either 5 or 25 g/L).

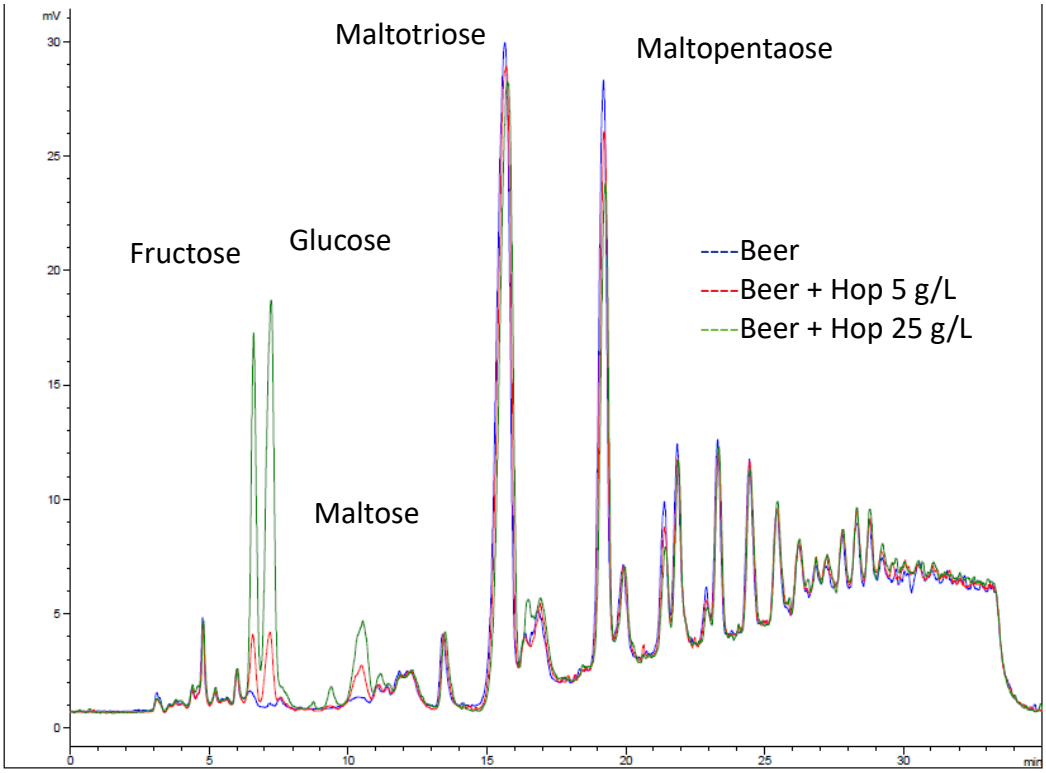


Figure 25: Chromatograms of HPLC-ELSD for beers after one day dry-hopping

Though an increase in base line appears due to the gradient applied in the elution solvent, the resolution of the different peaks is more than sufficient to quantify our sugar by manual integration of the area. The doubling of some peaks as well as little change in the retention time are due to the mutarotation phenomenon characteristic for sugar in light scattering detection, and therefore a standard mix was injected for each run of samples to ensure the right identification was obtained.

Concerning the analysis of these laboratory results, it is useful to know that for the dry-hopping in itself the hop chosen to carry out the process was the Strisselspalt whole hop 2016 because it presents a higher enzyme content. Furthermore, to ensure hop dissolution in beer the hop was crushed in liquid nitrogen which is not the case in breweries, this being justified by the wish to observe the maximum action. However, this powder once dissolved in beer shows no difference with dissolved pellets.

As one can see in figure 25, we clearly observed changes in the area of the 5 sugars even after only one day of dry-hopping. First, the fructose was released by the hop as well as a part of the glucose. Second, the remainder of glucose supposedly produced by α-amylase activity. Third, maltose is produced by the β-amylase activity, fourth and fifth being the main substrates for the enzyme activity, namely the maltotriose and maltopentaose.

First, when we look at the variation in the fructose concentration after dry-hopping in figure 26, we see that the modalities with hop bring about a certain concentration, which makes sense due to the monosaccharide content of hop. This concentration does not move over time except for the modalities with yeast in which it is consumed.

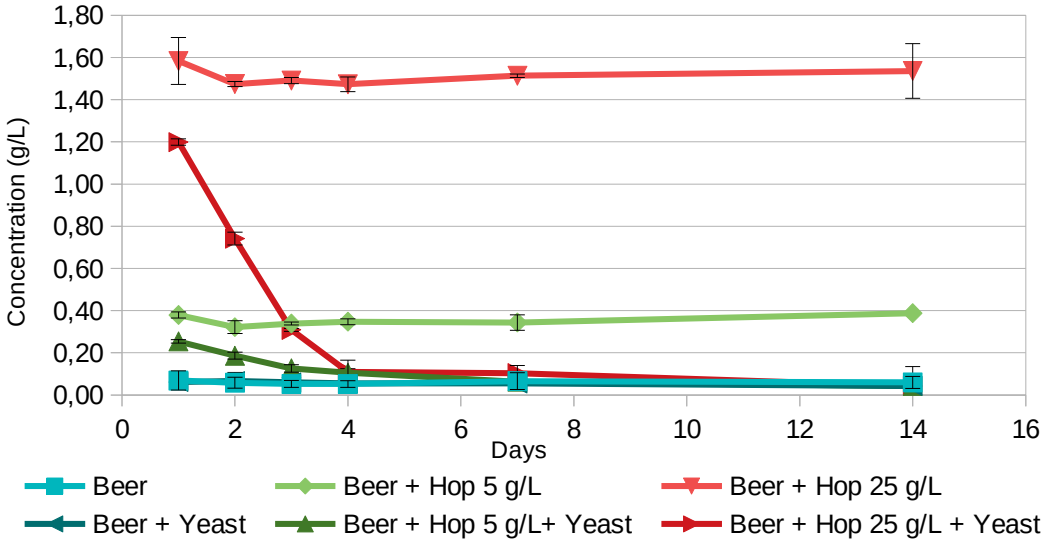


Figure 26: Variation in the fructose concentration after dry-hopping

Second, when we examine the glucose concentration in figure 27 over 14 days for the six modalities, we clearly distinguish 3 different patterns. A) Two of the modalities (beer and beer + yeast) remain along the base line which confirms that higher degree of polymerization sugars degradation does not occur by itself. B) Moreover, for the beer + hop modalities, either 5 or 25 g/L, we clearly see production of glucose over time with a sharp increase the first day, then constant linear increase.

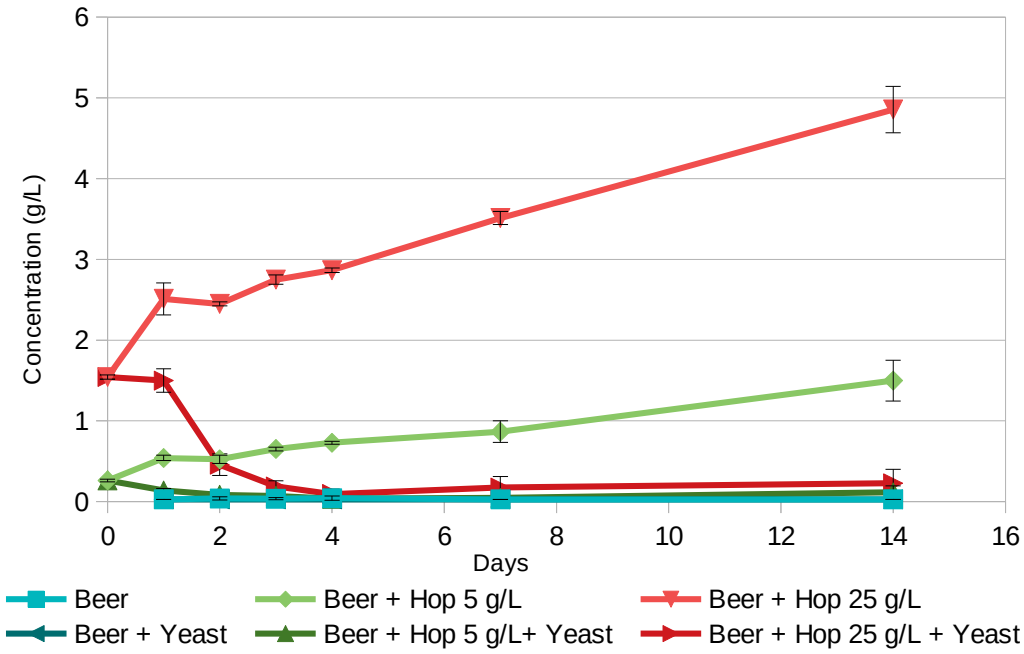


Figure 27: Variation in the glucose concentration after dry-hopping

The “0” day being evaluated by inhibition of hop enzymes with the $AgNO_3$ procedure. It is important to highlight the fact that, from the beginning the value obtained for higher hop concentration is not five times greater than the lower one, which means that the substrate is limiting the reaction. C) Besides, the modalities of beer, hop and yeast do show a small increase after one day, which then falls to join the base line. This means that all the sugar produced is almost directly metabolized by the yeast, indicating a secondary fermentation.

Third, regarding the maltose concentration, the exact same three patterns can be deduced regarding the β-amylase activity when looking at figure 28. Though the standard deviation occurring between the repetitions is higher, the results gathered are still very significant for the sugar profile with production of maltose up to 4,5 g/L.

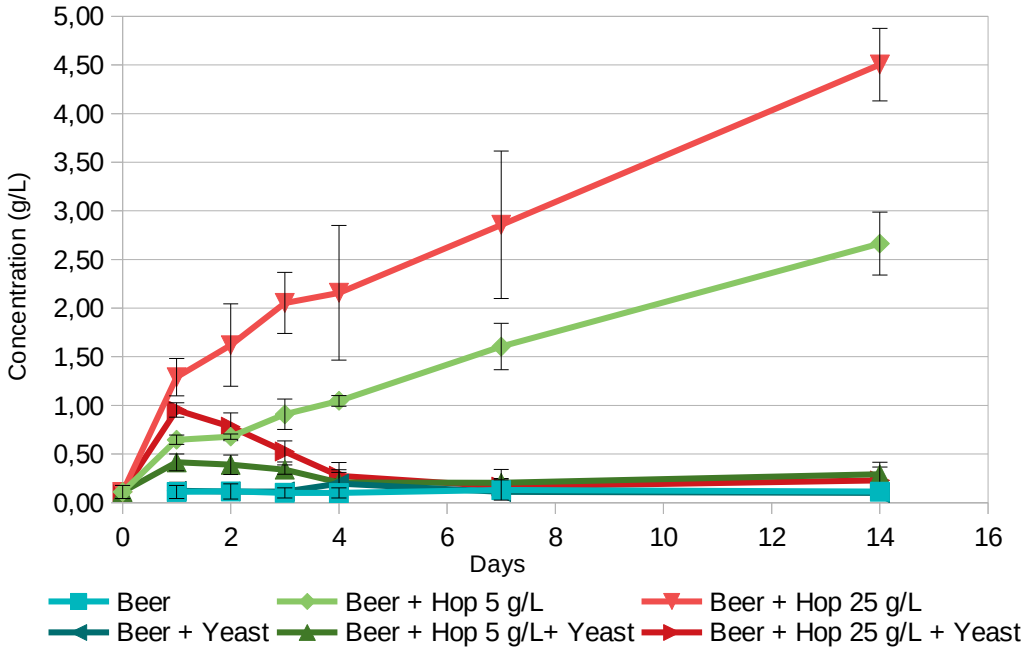


Figure 28: Variation in the maltose concentration after dry-hopping

To conclude, we have taken a close look at what happens in one aspect of the studied enzymatic reaction (product side). Although, as explained above, these reaction products are involved in other reactions by yeast fermentation. Therefore, it is essential to look at the substrate consumption of the reaction to assess this activity for the modalities of beer supplemented with both hop and yeast. This is the main reason why, despite the small increase in the baseline, this chromatographic technique has been preferred to others.

By inspecting figure 29, which represents modalities with yeast after 14 days, we clearly notice, as opposed to figure 25, that a strong attenuation occurs for maltotriose and maltopentaose, and that even sugars of higher degree of polymerization are degraded. This informs us on the selectivity of the enzymes present within hop and its stability over time.

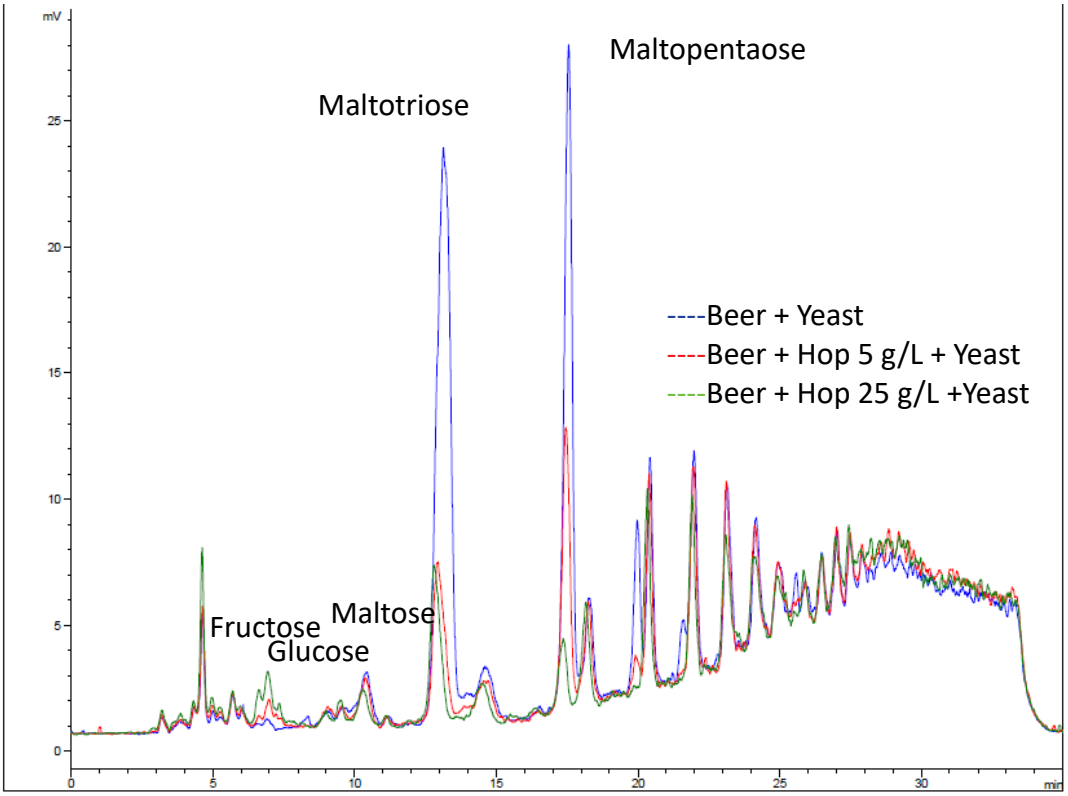


Figure 29: Chromatograms of HPLC-ELSD for dry-hopped beers after 14 days in the presence of yeast

Therefore, when we look at the variation in the area of these specific sugars for the 5 and 25 g/L modalities with yeast, we see a clear decrease, more especially in the maltotriose, for which the initial decrease is faster for the 25 g/L modalities though resulting in the same final area.

In this case, however, it is not possible to separate the activity of each enzyme, as is the case for the product. Indeed, only a global coefficient of activity can be deduced from this observation, but this will allow a comparison with the activity found within industrial tanks. Besides, it is important to remember that some varieties of yeast bear membrane transporters for maltotriose and therefore have the ability to metabolize it.

Figures 30 and 31 represent the variations in maltotriose area for modalities with or without yeast after dry-hopping (either 0,5 or 25 g/L). Firstly, for three modalities without yeast, we witness significant decrease for modalities with hop up to 50 % of the maltotriose content for the higher one.

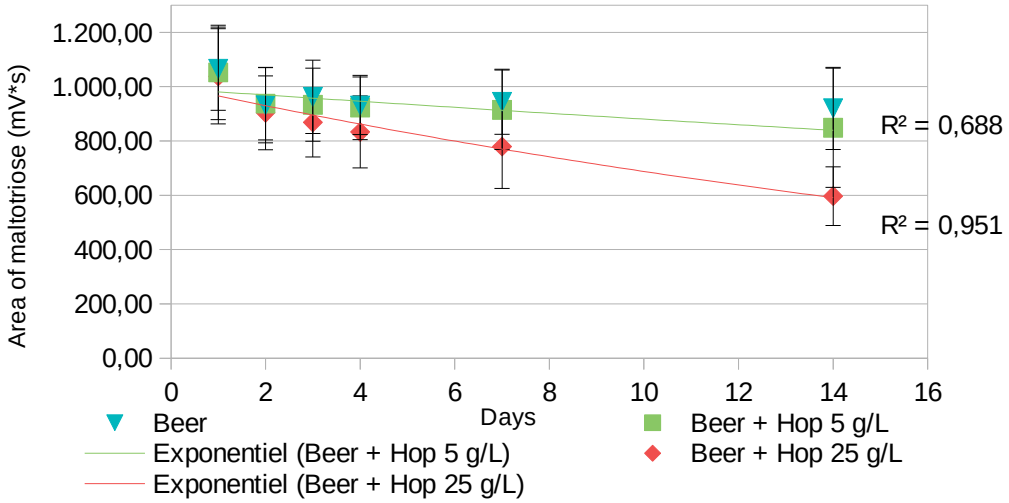


Figure 30: Variation in the maltotriose area after dry-hopping in the absence of yeast

Secondly, for modalities with yeast, we notice for the blank modalities a 30 % decrease in the area due to the starving yeast trying to metabolize any available substrates. Nevertheless, this is far less than the identical decrease of 90 % observed for the modalities 5 and 25 g/L of hop. The exponential decrease is characteristic of a Michaelis-Menten enzymatic reaction with higher initial speed due to higher enzyme content for the 25 g/L but resulting in the same final concentration due to substrate deficiency. The exact concentration not being calculated plus the fact that this is a two enzymes numerous substrates reaction system make it impossible to calculate the kinetics constants associate with it. Besides, the observed decrease is far superior than the one observed without yeast meaning that the continuous consumption of the enzymatic products allow the enzymes to perform at full initial rate without retro-inhibition by the product as seen for enzyme alone.

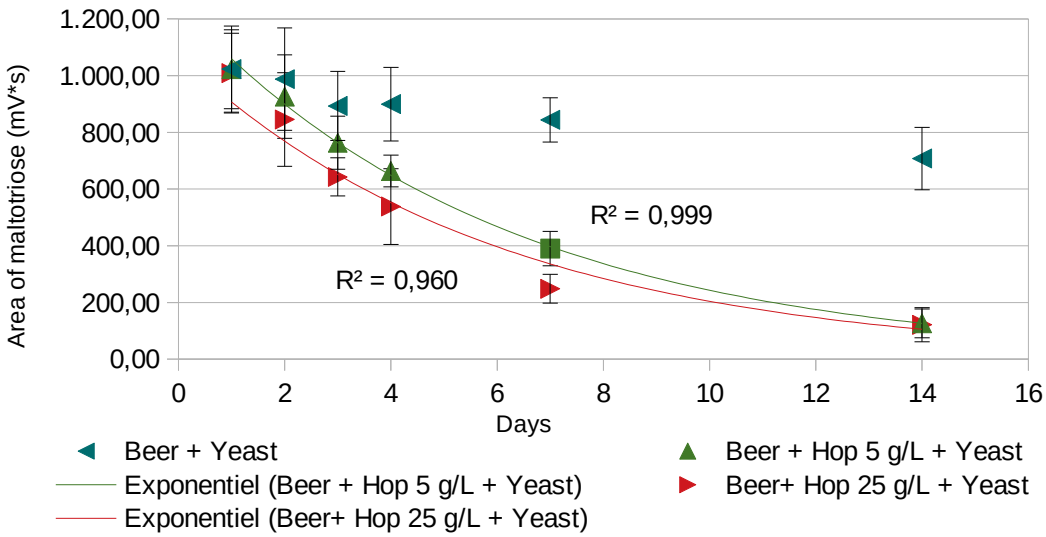


Figure 31: Variation in the maltotriose area after dry-hopping in the presence of yeast

Furthermore, if we look at the maltopentaose area variation in figures 32 and 33, we first see that hop is sufficient to trigger a decrease of 25 and 60 % of the area.

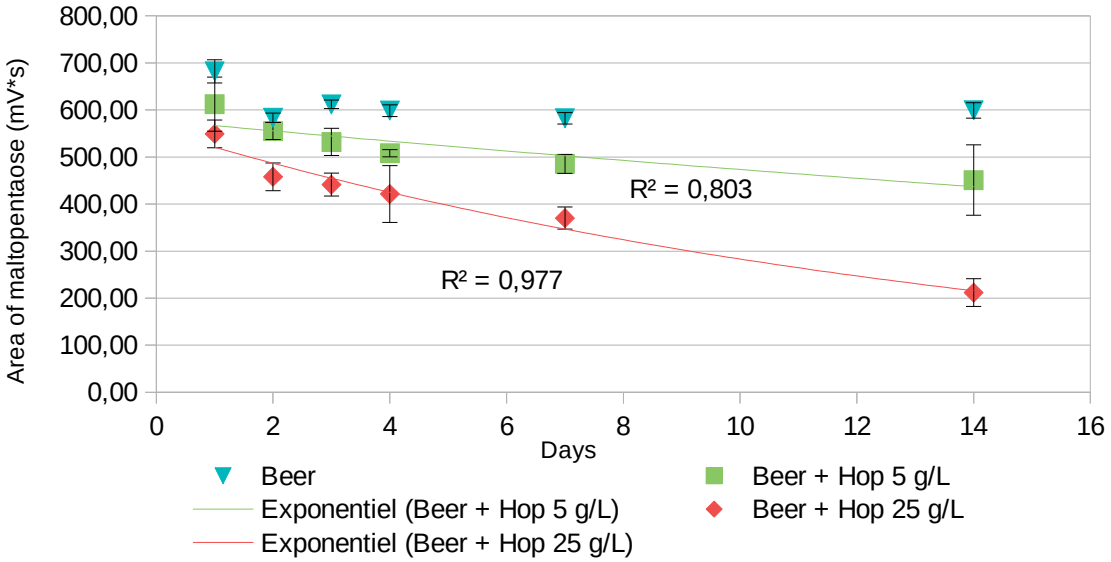


Figure 32: Variation in the maltopentaose area after dry-hopping in the absence of yeast

Secondly, as opposed to maltotriose, no change occurs with the yeast alone meaning that yeast cannot metabolize it, demonstrating again the absence of a diastatic activity for yeast alone. On the contrary, the area strongly decreases (50 % and 80 %) for the modalities with hop resulting from this enzymatic activity combined with continuous consumption of the product.

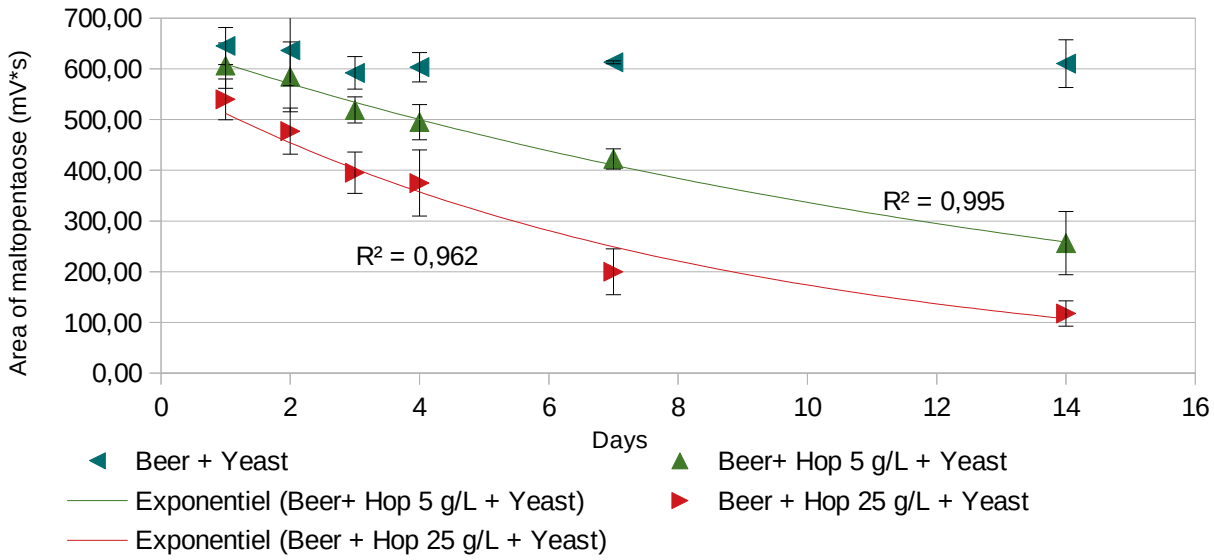


Figure 33: Variation in the maltopentaose area after dry-hopping in the presence of yeast

Finally, it is really interesting to highlight the fact that the 5 g/L modalities result in the same degradation of substrate, as shown by tables 21 and 22 of total chromatogram area, which leads to the remarkable conclusion that despite of its smallness, enzymatic activity is not the limiting factor in this reactions sequence. Even the amount of enzymes contained in 5 g/L, which is in the range used in breweries (1-12 g/L), can be sufficient to induce yeast activity and lead to strong attenuation of the beer content.

Table 21: Total chromatogram area (mV) of final sample

Sample	Total area (mV)
B14H5L	1.139,94
B14H5L	1.181,21
B14H5L	1.364,55
B14H25L	905,79
B14H25L	1.014,76
B14H25L	1.149,01

Table 22: Analysis of variance for total chromatogram area

AV1, Factor = hop

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Sample	1	63.273	63.273	4,34	0,106
Error	4	58.275	14.569		
Total	5	121.548			

If we now take a close look at the industrial results presented in figures 34 and 35, we see that the same phenomenon takes place with either whole hop or pellets, which is surprising because the dry-hopping being static, nothing ensures the dilution of the enzymes as in the laboratory (where grinding and mixing steps occur).

Among the four sampled tanks, pellets were added to two of them, whole hop to the others but no clear distinct patterns arise from these two modalities. Indeed, repetition 2 seems to have higher attenuation for each modality, whole hop 2 presenting the strongest attenuation. Regarding the hopping rate, it is important to note that it was 5 times higher in the whole hop case compared to the pellets, implying that their better dissolution (liberating proportionally more enzymes) compensates for their smaller quantities. The impossibility of obtaining representative samples due to the in-homogeneity of the beer within the tanks probably accounts for the absence of a marked distinction between the samples.

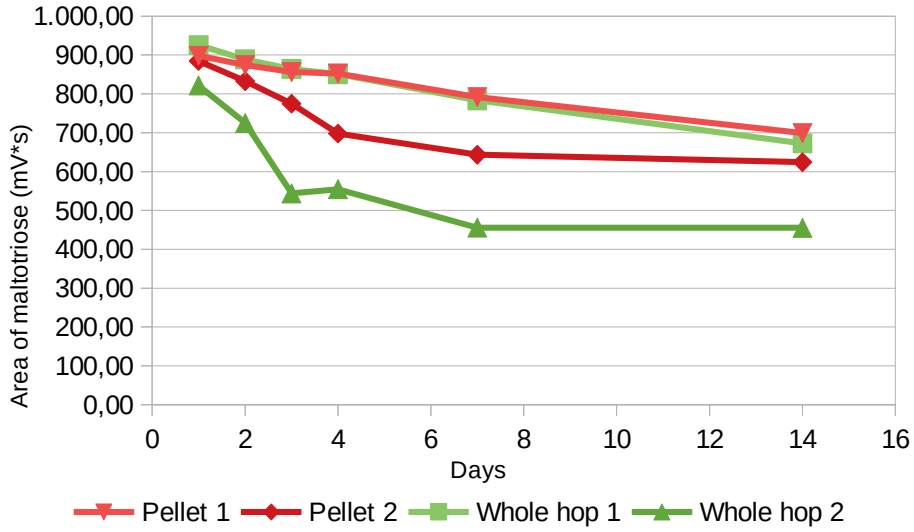


Figure 34: Variation in the maltotriose after dry-hopping in industrial tanks

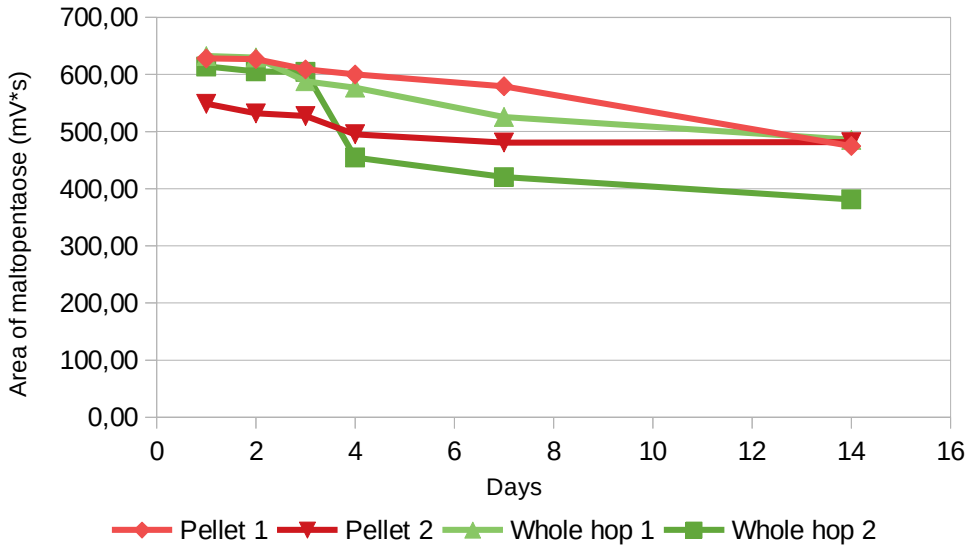


Figure 35: Variation in the maltopentaose after dry-hopping in industrial tanks

To conclude, both maltotriose and maltopentaose area have presented significant decrease during dry-hopping in brewery condition (50 % and 30%). Therefore, it implies that α- and β- amylase contained in hop operate in industrial condition leading to considerable attenuation of the sugar profile as well as yeast activity. The further challenge being to demonstrate this activity of the yeast and the impact it could have on the beer aroma profile.

5.3) Gas chromatographic analysis of volatile organic compounds (VOCs)

5.3.1) Vicinal diketones (ECD)

As explained previously, the two vicinal diketones, butanedione and pentanedione, were selected to monitor the activity of the yeast due to the fact that their production is directly related to the mitochondrial metabolism of yeast. Indeed, as shown in figure 7 of the introduction, the diacetyl is related to the pyruvate metabolites, the latter being directly related to the absorption of fermentable sugar by the yeast, justifying the variations in this volatile concentration as an indicator of fermentation. In figure 36, we can follow these variations in concentration for the 6 modalities.

Firstly, regarding the butanedione (diacetyl), we observe that modalities containing both hop and yeast present a clear increase after one to three days following dry-hopping. Besides these peaks, we observe a small rise for the modalities with hop alone especially for the 25 g/L. These facts can be easily explained by the Maillard reaction between the reducing sugar liberated by hop (glucose, maltose, fructose) and the remaining amino acids in beer the diacetyl being one of the product of this non-enzymatic reaction (Hollnagel et Kroh, 1998). Furthermore, this phenomenon has already be observed during beer aging (Vanderhaegen *et al.*, 2003).

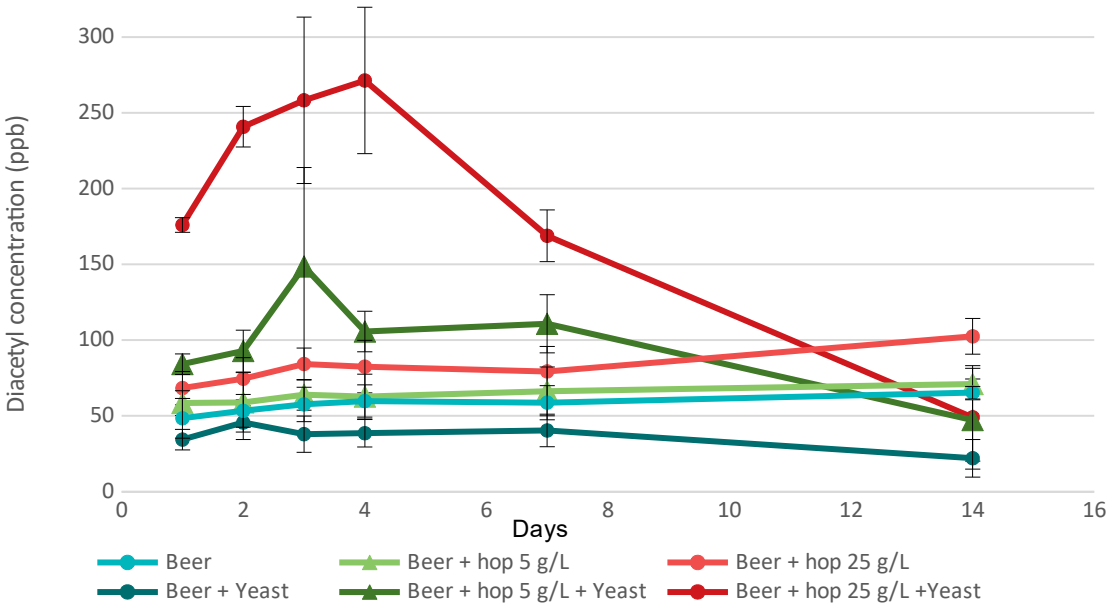


Figure 36: Variation in the butanedione (diacetyl) content in beer after dry-hopping

Although we clearly see that the modalities with yeast and hop produce butanedione as expected, a significant variation appears in between the repetitions leading to the important standard deviation observed.

Indeed, even if standardized conditions have been applied content varies greatly as represented in figure 37 and 38. This variation implying that other factors must be considered besides the fermentable sugar available for yeast to explain these vicinal diketone productions. Indeed, other environmental parameters such as pH, oxygen, and medium composition control its production by yeast (Dzialo *et al.*, 2017).

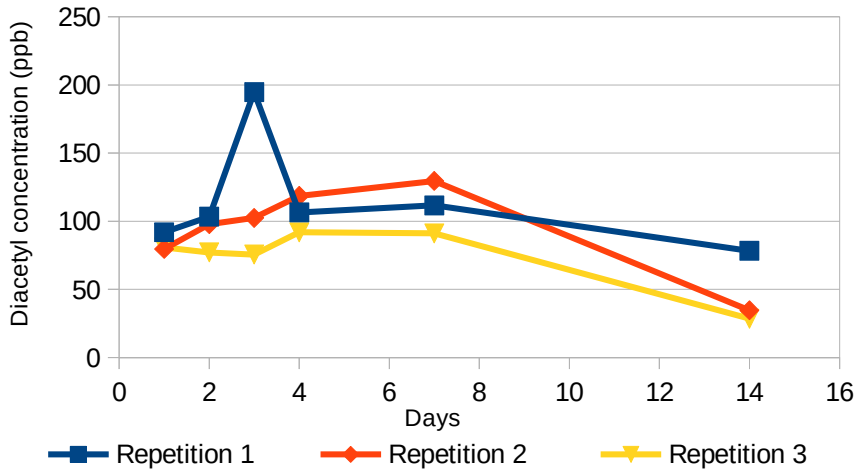


Figure 37: Variation in the diacetyl content within the different hop 5 g/L and yeast repetitions

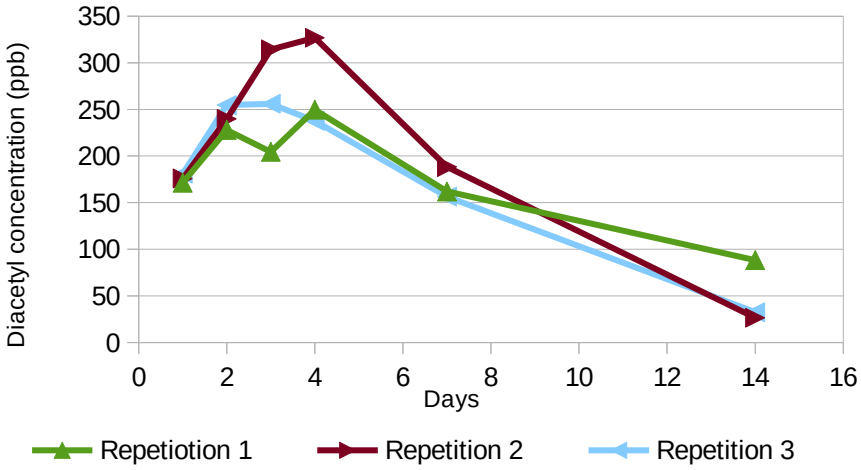


Figure 38: Variation in the diacetyl content within the different hop 25 g/L and yeast repetitions

In conclusion, during the dry-hopping process, the sugar liberated by both the hop and its enzymes lead to secondary fermentation of yeast in an environment exhausted of nitrogen resulting in the production of vicinal diketones that may potentially alter the product. Therefore, besides transferring hop aroma, dry-hopping in the presence of yeast is likely to alter the aroma profile of beer by producing all the aroma compounds associated to yeast. This aspect will be examined in greater details below.

Secondly, as stated in figure 7 of the introduction, the pentanedione emerges from the threonine (amino acid pathway), which, at first, seems non-directly related to the sugar adsorption as for diacetyl. Nevertheless, contrary to animals, yeast possesses a branched chain amino acid (BCAA) biosynthesis pathway with the acetohydroxyacid synthetase enzyme (AHAS EC 2.2.1.6) which produces 2-aceto-2-hydroxybutyrate from pyruvate and 2-ketobutyrate.

When we look at the variation in the concentration obtained after dry-hopping for the six modalities in figure 39, we witness the same significant increase for hop and yeast compared to the other modalities. Besides, on the contrary of diacetyl, the pentanedione is not produced by Maillard reaction but by oxidation of acetoin and 2,3 butanediol which content stays identical after dry-hopping (not as the reducing sugar ones).This justifying that the content of those other modalities remains constant over time. Though the same standard deviation occurs, arising from identical differences in-between batches as represented in figures 40 and 41.

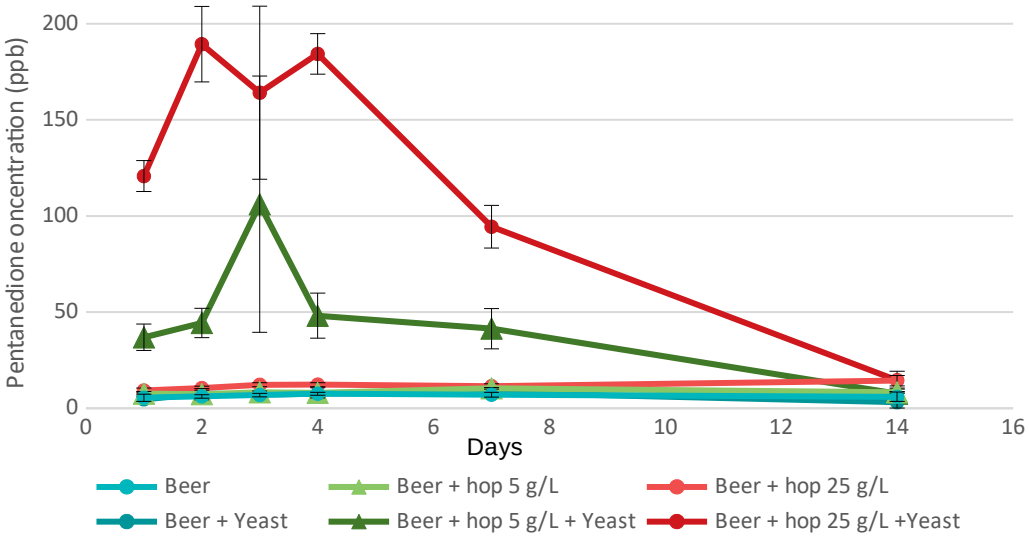


Figure 39: Variation in the pentanedione content after dry-hopping

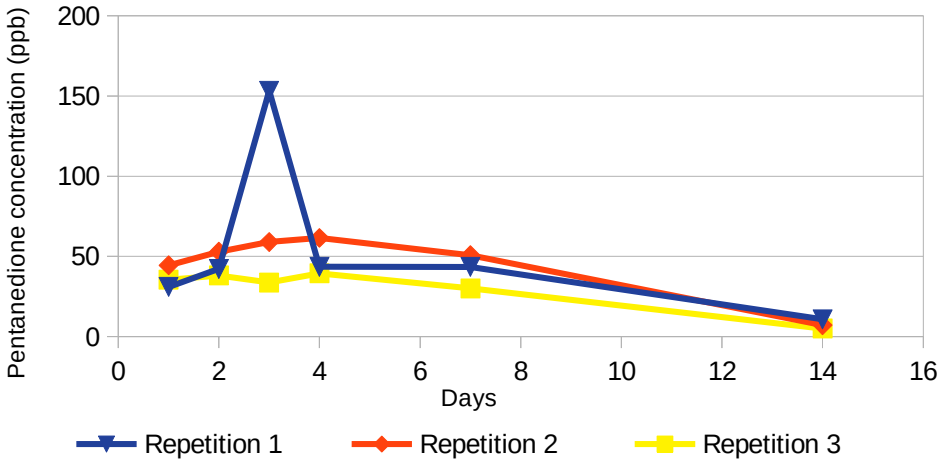


Figure 40: Variation in the pentanedione content within the different hop 5 g/L and yeast repetitions

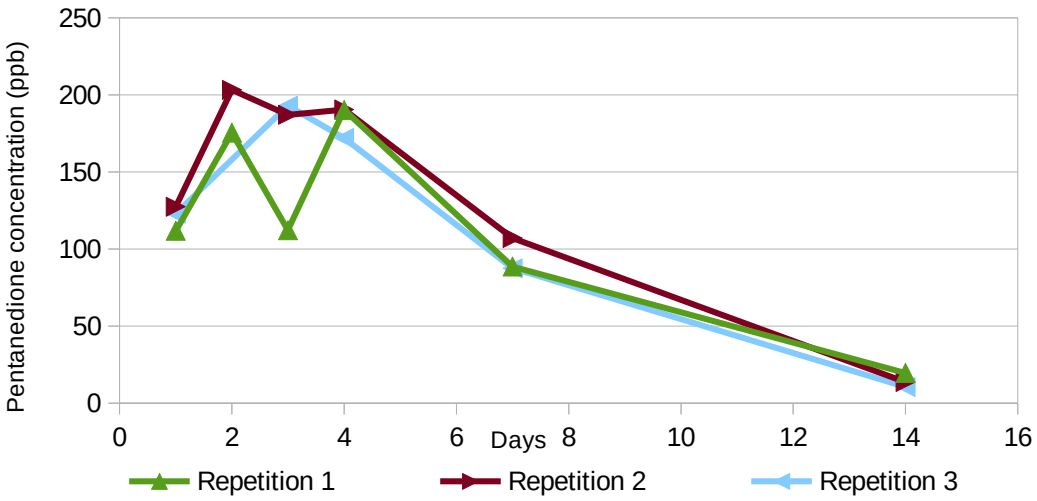


Figure 41: Variation in the pentanedione content within the different hop 25 g/L and yeast repetitions

By way of general discussion for those two ketones, we can state that the yeast activity was considerably increased thanks to the activity of amylases contained in hop. Indeed, the higher the hop concentration was, the greater was the increase in ketone production by yeast due to the higher fermentable sugar content available. Furthermore, as shown by the control samples (beer + yeast), this variety of yeast did not possess a diastatic power, asserting the previous conclusion of the necessity of the hop enzymes action to deliver this impact on yeast. Finally, we may wonder which factor not included in the model could explain such fermentation performance differences between the batches. Indeed, the main nutritional requirement for yeast is sugar as a carbon source plus a nitrogen requirement for structural protein and enzymes.

Regarding Free Amino Nitrogen (FAN) content as shown by table 23 and the associated analysis of variance on table 24, we see no significant differences between the batches. However, this analysis shows only a total content available for yeast but does not give an idea of specific amino acid content such as isoleucine, valine and threonine. As demonstrated by figure 7 in the introduction, this plays a major role in butanedione and pentanedione production. Even so, the content is far below the 100 mg/L limit for good fermentation, implying a nitrogen deprived environment leading to the relatively high vicinal diketones production observed previously.

Table 24: FAN content of beer before dry-hopping

FAN (mg/L)	Repetition 1	Repetition 2	Repetition 3
	42,22	43,11	46,46
	45,12	44,45	44,90
	46,02	46,69	47,58
Mean	44,45	44,75	46,31
Standard deviation	1,99	1,81	1,35

Table 23: Analysis of variance for the FAN content of beer before dry-hopping

AV1, Factor = repetition

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Repetition	2	5,992	2,996	1,00	0,423
Error	6	18,053	3,009		
Total	8	24,045			

Regarding the total carbohydrate content displayed in table 25, we observe clear differences in the total sugar content of beer after hydrolysis, as confirmed by the analysis of variance (table 26), which could partly explain the better performance observed for the first repetition as well as difference between repetitions in the fermentable sugar production by enzyme, as observed in the previous results (5.2 Sugar profile of beer). However, repetition 2 shows greater diketone production with 25 g/L of hop demonstrating the necessity to include more environmental parameters to understand yeast behaviour as well as the physiological state of the yeast at time of addition.

Table 25: Total carbohydrates content of beer before dry-hopping

Total carbohydrates content (g/L)	Repetition 1	Repetition 2	Repetition 3
A	157,88	144,89	147,40
B	150,33	136,96	144,40
C	152,44	133,99	138,21
Mean	153,55	138,61	143,34
Standard deviation	3,89	5,64	4,69

Table 26: Analysis of variance associated to the total carbohydrate content of beer before dry-hopping

AV1, Factor = repetition

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Repetition	2	349,7	174,86	7,61	0,023
Error	6	137,8	22,96		
Total	8	487,5			

5.3.2) Other volatile compounds: higher alcohols, esters, aldehydes (FID)

Variation in higher alcohol content increases to a greater or lesser degree depending on the modality assessed. Indeed, we observe a sharp increase in the n-propanol content in figure 42 for the 5 g/L and 25 g/L modalities with yeast, the content of which is almost double when compared to beer alone. This indicates a strong activity of yeast concerning threonine absorption by the Ehrlich pathway. Unfortunately, unlike the others, the 25 g/L modalities alone also show an inexplicable rise. However, the flavour threshold of the n-propanol being 600 mg/L, the change is certainly not detected.

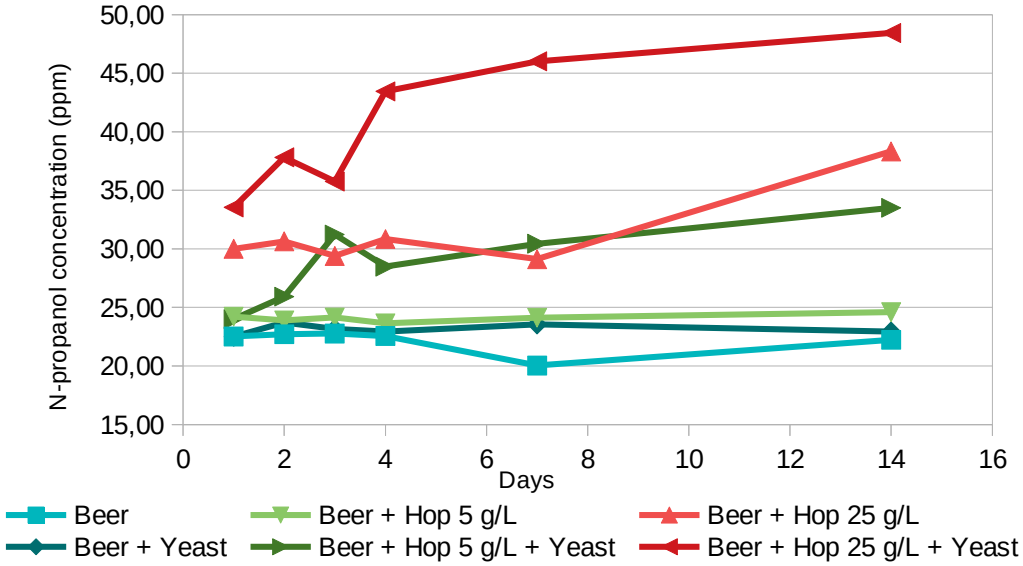


Figure 42: Variation in the n-propanol concentration after dry-hopping

The isobutanol content represented in figure 43 also increases for the modalities with hop and yeast, separating from the other modalities but less spectacularly. Interestingly, two main production phases can be distinguished, one after 3 days, as for the ketones, and the other at the end of dry-hopping. The flavour threshold being around 100 mg/L, the concentrations observed are far lower.

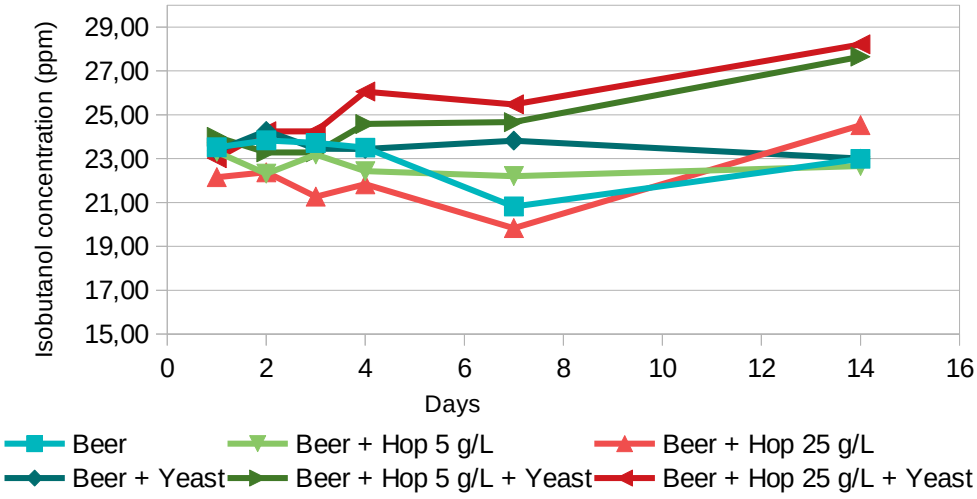


Figure 43: Variation in the isobutanol concentration after dry-hopping

Finally, for the isoamyl alcohol content shown in figure 44, the modalities beer and beer + 25 g/L present great unexplained variations during dry-hopping, though modalities with hop and yeast still present a higher content at the end. Regarding the concentration, they are above the flavour threshold, is located between 50 - 65 mg/L, implying that even small changes in concentration would impact the perceived aroma.

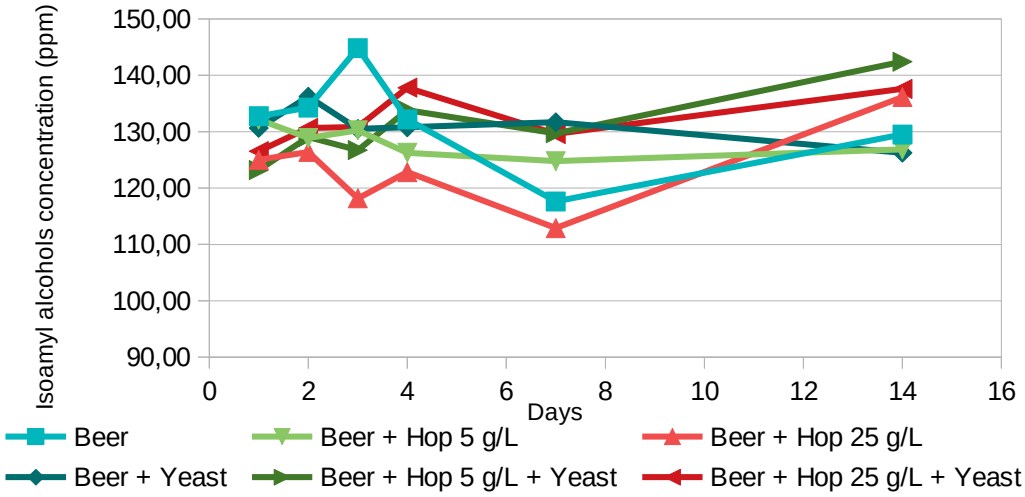


Figure 44: Variation in the isoamyl alcohols concentration after dry-hopping

To conclude, we observe an increase in higher alcohol production for samples with both hop and yeast, though only n-propanol seems to show differences between 5 g/L and 25 g/L of hop modalities, implying that, for isoamyl alcohols and isobutanol, other factors are limiting their production, among which amino acid content (leucine and valine) must play a great role.

Concerning the esters, ethyl esters, ethyl caproate and caprylate all show a decrease over time of storage, a well-known phenomenon due to trans-esterification and hydrolysis (Vanderhaegen *et al.*, 2006b). However, no clear tendency can be distinguished between the modalities for ethyl caprylate presented in figure 45. The flavour threshold, being between 0,9 and 1 mg/L, concentration falls way under for each modality. However the decrease seems to be correlated to the quantity of hop added, the more hop added, the more the ester content decrease even in the absence of yeast.

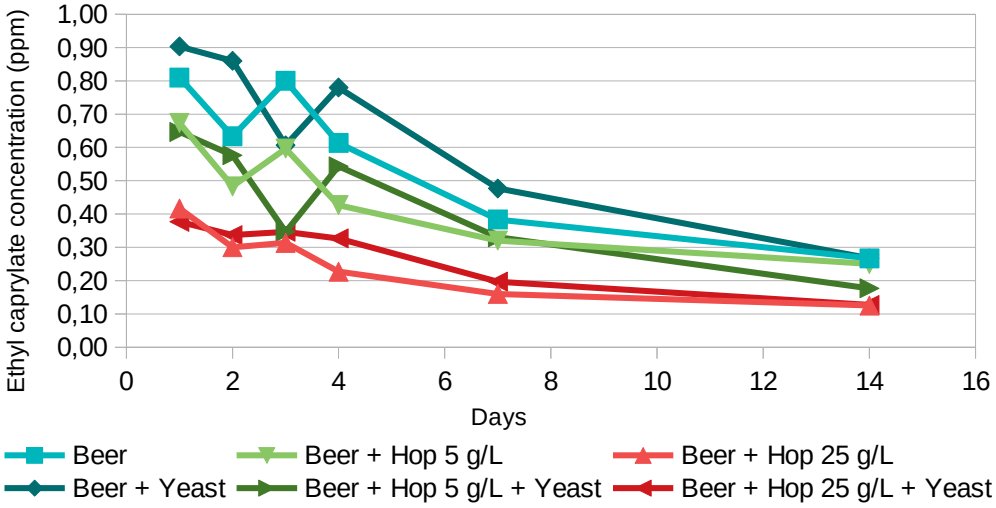


Figure 45: Variation in the ethyl caprylate concentration after dry-hopping

Regarding the ethyl caproate in figure 46, the modalities with yeast tend to have higher content, though no clear distinction appears among them. It is interesting to note that the content tends to approach the odour threshold located around 0,2 mg/L.

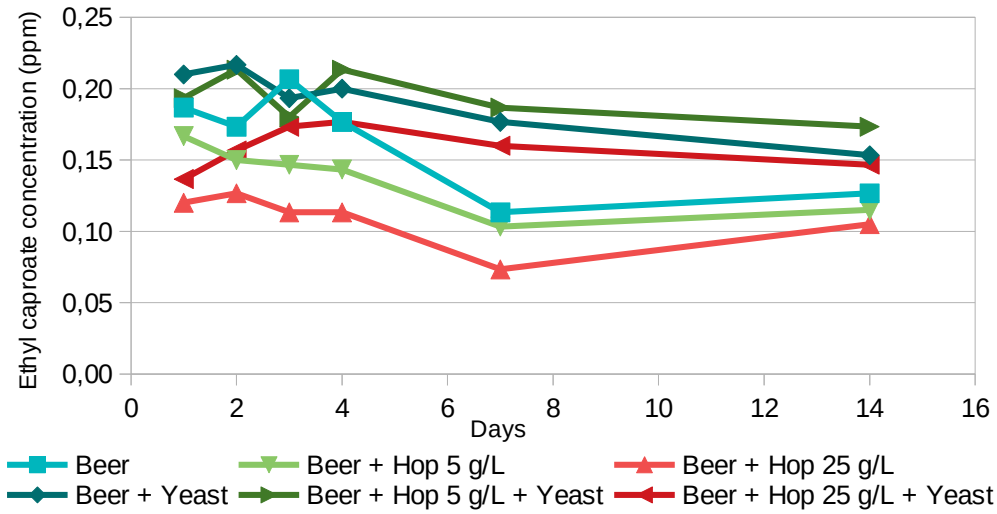


Figure 46: Variation in the ethyl caproate concentration after dry-hopping

To conclude concerning ethyl esters, though the alcohol content increased (as previously demonstrated) which is one of the two substrates for their formation together with acyl-coA and acetyl coA (produced by sugars and lipid metabolism), it seems that only limited esters are synthesized. Besides the regulatory gene of ester synthetase ATF1 being influenced by oxygen, unsaturated fatty acids, fermentable sugars and nitrogen, it seems that modification of only the fermentation sugars at the experimental rate alone is not in itself sufficient to induce differential formation by yeast.

Futhermore, the two measured acetate esters, namely ethyl acetate and isoamyl acetate, show different tendencies. For the former, an increase in concentration for hop and yeast modalities is observed in figure 47, whereas for the latter the opposite is observed in figure 48.

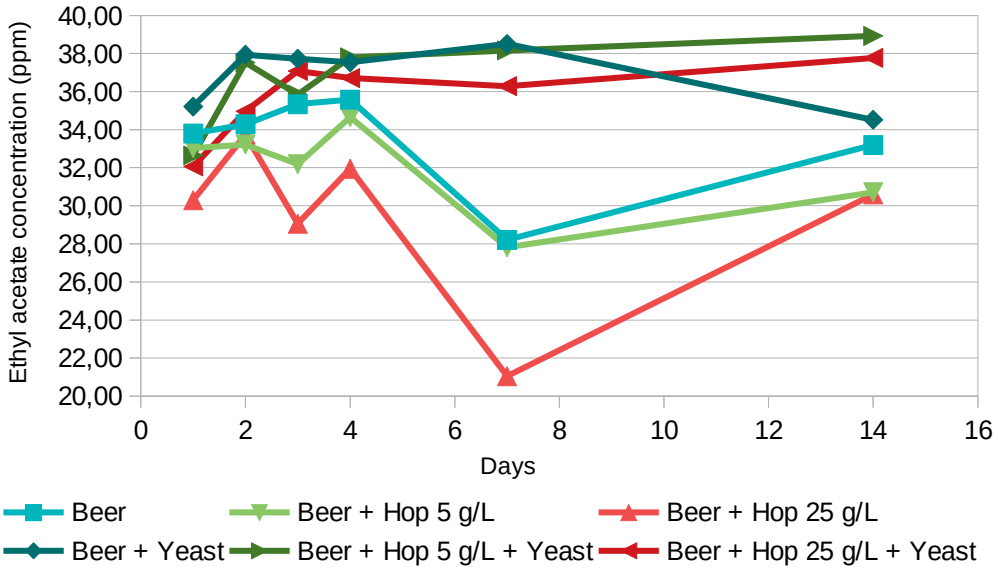


Figure 47: Variation in the ethyl acetate concentration after dry-hopping

The isoamyl acetate originates from the isoamyl alcohols and acetyl-coA condensation, and though the concentration in the former tends to increase, as seen in figure 48, the content in acetate decreases for each modality, especially the one with hop. It has been shown that isoamyl acetate was degraded by both chemical and enzymatic hydrolysis (yeast possessing specific esterase) (Neven et Delvaux, 1997). However, the sharp decrease even after one day for 25 g/L modalities compared to the other one imply that hop could possess other enzyme such as esterase able to hydrolyze this compounds. This hypothesis explaining the identical variation observed regarding ethyl caprylate.

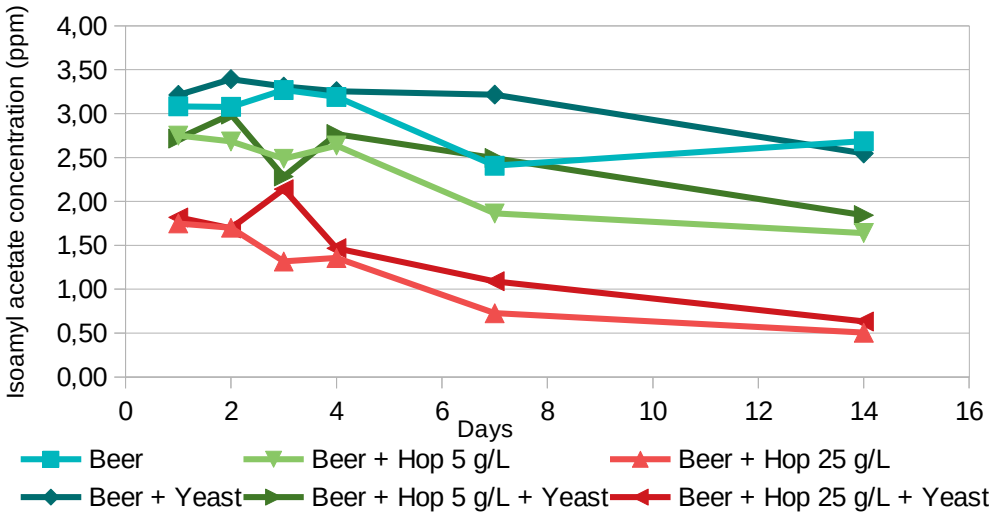


Figure 48: Variation in the isoamyl acetate concentration after dry-hopping

As a conclusion regarding the ester content, both ethyl and acetate, the ones with a longer chain, isoamyl acetate and ethyl caprylate, show a decrease in their respective content depending on the hop addition (the more hops the sharper the decrease). These facts leading to the formulation of the hypothesis that hop could possess an esterase hydrolyzing more specifically these types of compounds. The presence of esterase in flower trichomes has been demonstrated in the past and is widely dispersed in the plant kingdom and within the cannabaceae family (Truţa *et al.*, 2002; Schilmiller *et al.*, 2008). Whereas shorter chain esters ethyl acetate and ethyl caproate present increase for modalities with hop and yeast implying that, in this specific conditions, yeast preferably produce these compounds. The modalities with hop alone also present decrease in concentration especially for the 25 g/L reinforcing the hypothesis previously formulated.

Lastly, the acetaldehydes content (figure 49) rises drastically for modalities containing yeast probably due to a high dissolve oxygen content. Indeed, it is formed from ethyl alcohol and can be used as a marker of oxidation (Vanderhaegen *et al.*, 2003). However, it is interesting to notice that afterwards content decreases for modalities with hop whereas it increases for yeast alone.

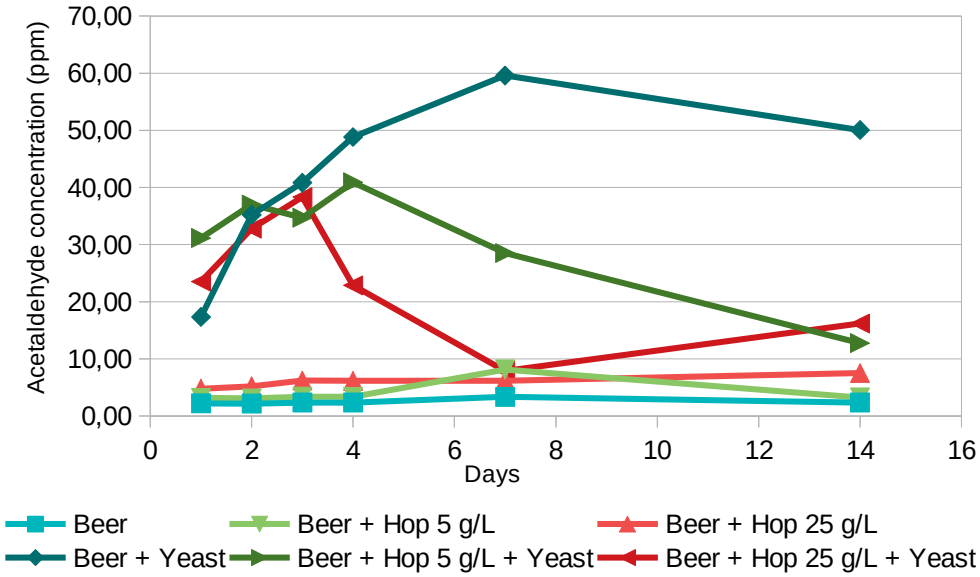


Figure 49: Variation in the acetaldehyde concentration after dry-hopping

To conclude, when analyzed alone, these other volatiles do not all show such a clear tendency as the vicinal diketones. Therefore, in order to go further and assess global change in total aroma profile between the modalities principal components multivariate analysis was used. This analysis aims to create principal components among possibly related variables and gives as a result a correlation matrix in which the Eigenvalue shows the parts of variance observed for each of the components it creates.

In our case, we see in table 27 that 73,4 % of the variability can be explained by the first two principal components. Furthermore, by exhibiting the link between the principal components and each of the original variables, the correlation matrix indicates how each one is built by their combination. Indeed, if we examine the magnitude of the coefficient associated with each of our variables, the largest one in absolute value being the main used for the calculation of the principal component.

Table 27: Eigenanalysis of the Correlation Matrix (PCA) for laboratory design

Eigenvalue	3,8427	3,4977	1,3730	0,7730	0,2230	0,1707	0,0538	0,0406	0,0152	0,0103
Proportion	0,384	0,350	0,137	0,077	0,022	0,017	0,005	0,004	0,002	0,001
Cumulative	0,384	0,734	0,871	0,949	0,971	0,988	0,993	0,997	0,999	1,000
Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Diacetyl	-0,043	0,435	-0,408	-0,339	-0,158	-0,031	-0,184	-0,098	0,547	-0,399
Pentanedione	0,030	0,429	-0,455	-0,260	-0,173	-0,068	0,283	0,125	-0,427	0,478
Acetaldehyde	0,269	0,103	-0,421	0,723	0,165	-0,420	-0,038	0,067	0,048	-0,069
Ethyl acetate	0,449	0,169	0,084	0,206	-0,285	0,550	-0,350	0,342	0,172	0,259
Propanol	-0,087	0,499	0,149	0,037	0,484	0,235	-0,170	0,195	-0,439	-0,413
Isobutanol	0,281	0,351	0,411	0,073	0,019	-0,028	0,728	0,026	0,295	-0,074
Isoamyl acetate	0,374	-0,320	-0,195	-0,094	-0,392	0,100	0,224	0,140	-0,358	-0,591
Isoamyl alcohol	0,372	0,170	0,414	-0,250	-0,184	-0,623	-0,388	-0,001	-0,161	0,014
Ethyl caproate	0,491	0,006	-0,108	-0,090	0,275	0,235	-0,043	-0,768	-0,103	0,079
Ethyl caprylate	0,341	-0,291	-0,173	-0,407	0,582	-0,075	0,040	0,454	0,205	0,095

Therefore, when looking at the correlation matrix on table 27, also represented in figure 50 below, we see that the principal component is built with ethyl acetate and ethyl caproate for the first, propanol, diacetyl and pentanedione for the second. A value of 0,4 was arbitrary selected but we can note that iso-amyl acetate and ethyl caprylate also participate greatly at the first component as well as isobutanol for the second. Implying that esters contribute to x axis and ketones and higher alcohols (except for isoamyl alcohols) to y axis.

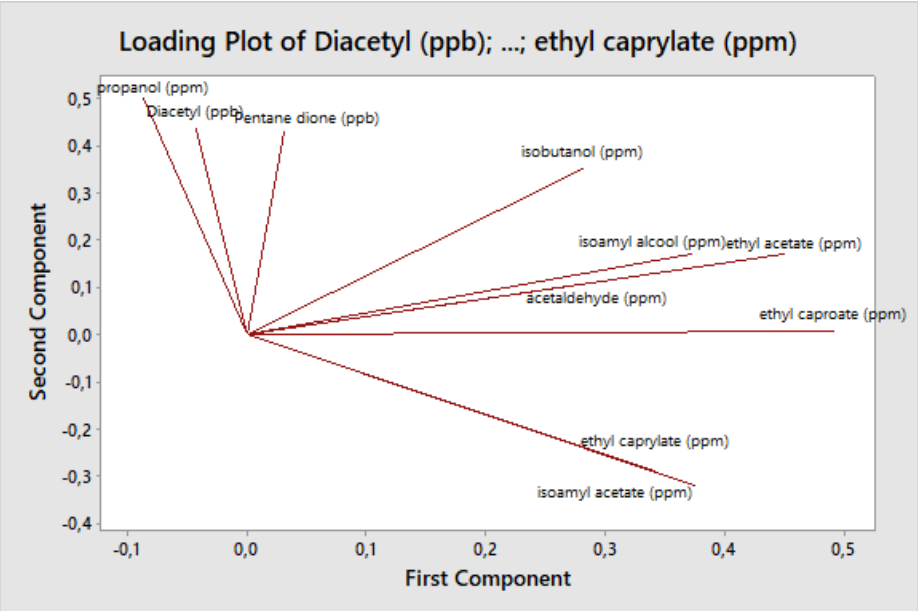


Figure 50: Representation of the variable combination forming the principal components (PCA)

In the score plot in figure 51 below the dots represent each of the 36 samples means comprising the 10 volatiles measured. If we examine this, we clearly witness clusters of sample separating from each other depending on the hop and yeast content implying by their clustering the same modification of the volatile profiles.

Firstly, modalities hop 25 g/L and yeast, on the top of the graph, tend to have higher propanol, diacetyl and pentanedione content (y axis) as well as little higher ethyl acetate and caproate (x axis).

Secondly, modalities of beer alone and beer + hop 5 g/L are located the closest from the center meaning that they present the least modification in the volatile compounds measured.

Thirdly, the modalities with 25 g/L is located on the left side of the graph implying a significant decrease in ethyl acetate and caproate, as well as the two other esters, possibly explained by the presence within hop of esterase hydrolyzing them.

Fourthly, beer + yeast have tend to keep higher esters content no yeast or enzyme activity hydrolyzing them. Besides their content in ketones and alcohols do not move implying the absence of significant activity from yeast.

Fifthly, the beer hop 5g/L and yeast tend to be located in between the previously described modalities.

To conclude, regarding these yeast-derived volatiles it seems that this amount of hop (enzymes) is sufficient to bring yeast activity allowing the conservation of beer esters without producing too much off flavours.

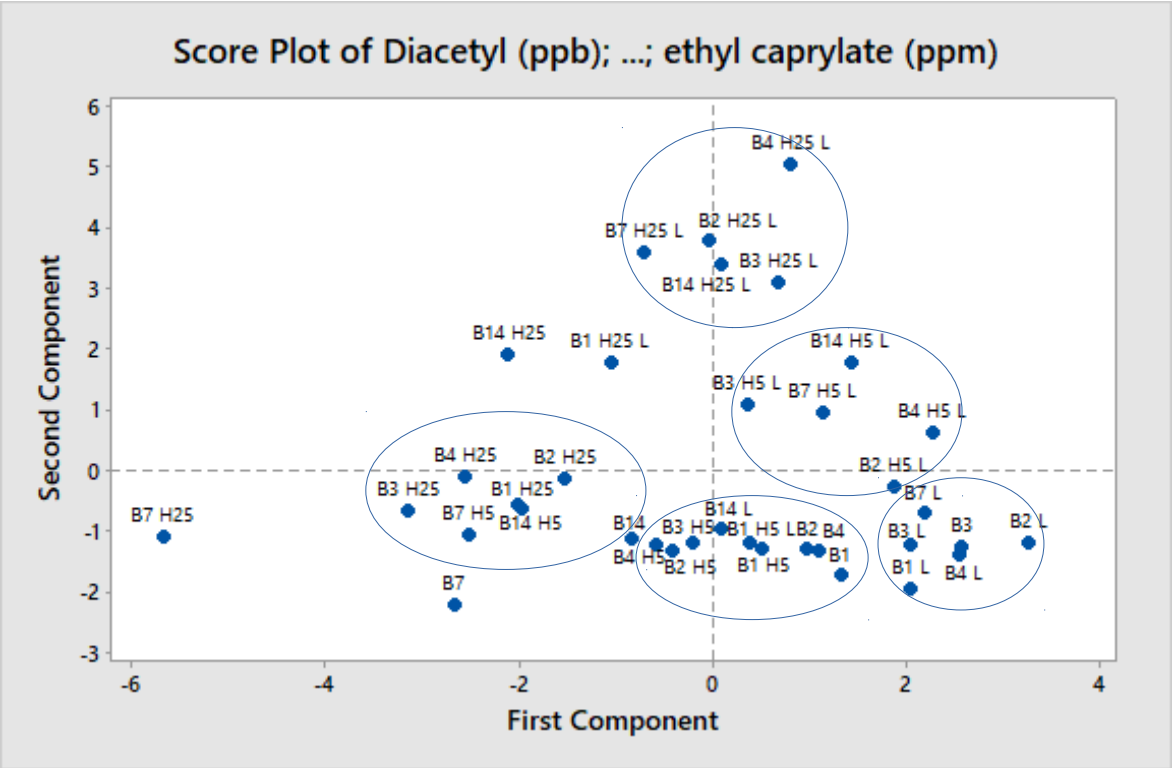


Figure 51: Score plot of the principal components analysis laboratory samples volatiles

VOCs content variation in industrial tanks

Concerning the variations in vicinal diketones concentration, as one can see when studying table 28, the same increase appears one day after dry-hopping but almost only for the whole hop, especially repetition 2, which shows the greatest sugar decrease. These facts consolidate the stated hypothesis that hop enzymes could lead to yeast metabolism activation through fermentable sugar production.

Table 28: Variation in diacetyl and pentanedione concentration in industrial tanks (ppb)

	Whole hop 1		Whole hop 2		Pellet 1		Pellet 2	
Days	Diacetyl	Pentanedione	Diacetyl	Pentanedione	Diacetyl	Pentanedione	Diacetyl	Pentanedione
0	29,31	2,26	31,25	2,45	29,31	2,26	31,25	2,45
1	29,37	5,02	40,8	7,4	22,37	1,07	27,86	2,7
2	21,28	2,32	24,54	3,38	19,23	0,6	23,02	1,39
3	16,98	0,82	18,87	1,09	17,55	0,55	22,16	1,45
4	14,09	1,02	21,15	2,06	18,76	1,08	21,62	1,49
7	14,6	0,48	16,78	0,83	18,47	0,38	17,61	0,5

Finally, regarding the other volatiles, the same multivariate analysis (PCA) was applied to determine the evolution in the total profiles and, as we observe in table 29, the first two components are composed respectively from the highers alcohols for the first (n-propanol, isobutanol and isoamyl alcohol) and vicinal diketones (diacetyl, pentanedione) for the second, which in the light of the metabolic pathway presented in the introduction makes perfect sense.

Table 29: Eigenanalysis of the Correlation Matrix (PCA industrial tanks)

Eigenvalue	4,5498	2,5165	1,6210	0,6445	0,3290	0,2005	0,0688	0,0339	0,0322	0,0039
Proportion	0,455	0,252	0,162	0,064	0,033	0,020	0,007	0,003	0,003	0,000
Cumulative	0,455	0,707	0,869	0,933	0,966	0,986	0,993	0,996	1,000	1,000

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Diacetyl	0,092	0,562	0,065	0,337	-0,170	-0,557	0,446	-0,087	0,109	-0,027
Pentanedione	0,189	0,514	-0,103	0,364	0,337	0,110	-0,643	0,038	-0,121	0,023
Acetaldehyde	0,069	0,435	0,272	-0,724	-0,340	-0,065	-0,289	-0,031	0,059	-0,023
Ethyl acetate	0,379	-0,310	0,117	-0,060	0,189	-0,516	-0,212	0,515	0,351	0,090
Propanol	0,437	-0,095	-0,205	-0,111	0,163	0,017	-0,003	-0,710	0,420	0,196
Isobutanol	0,405	0,077	-0,345	-0,218	0,005	0,010	0,250	0,194	-0,545	0,517
Isoamyl acetate	0,361	0,129	0,388	0,200	-0,173	0,611	0,235	0,271	0,325	0,158
Isoamyl alcohol	0,421	0,043	-0,324	-0,135	0,039	0,121	0,164	0,126	-0,056	-0,797
Ethyl caproate	0,185	-0,070	0,676	-0,073	0,487	-0,070	0,151	-0,227	-0,405	-0,132
Ethyl caprylate	0,333	-0,309	0,153	0,322	-0,641	-0,118	-0,303	-0,206	-0,318	-0,080

The score plot concerning these industrial maturation tank samples reveals that, as for the vicinal diketones, the whole hop triggered a stronger modification of the aroma profile. Indeed, as opposed to the pellet dots in the centre of figure 52, the dots representing them are located on the extremities of the graph. This implies a stronger content in volatile composed by the principal components, which are the higher alcohols and ketones. However, the variation within each modality over time does not show a clear tendency. On the contrary of figure 51, the esters do not take part of as major flavour of the principal components due to the fact the modalities here represent only combination of both hop and yeast.

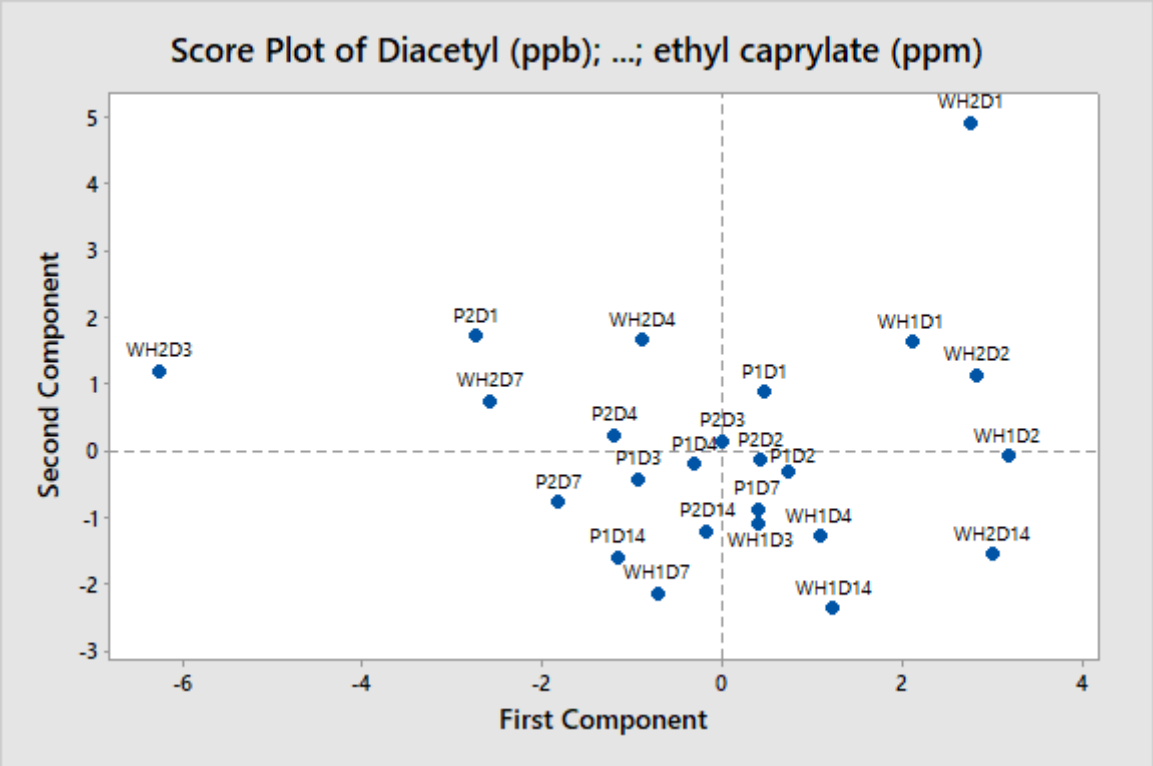


Figure 52: Score plot of the principal components analysis for the industrial samples volatiles

5.3.3) Terpenic compounds (Mass spectrum detector)

In figure 53, we observe the total ion chromatogram obtained by application of the GC-MS-DHS procedure, which presents too broad and irregular peaks due to the higher content in solvent (hexane, ethanol and methylbutanol) compared to other beer volatiles.

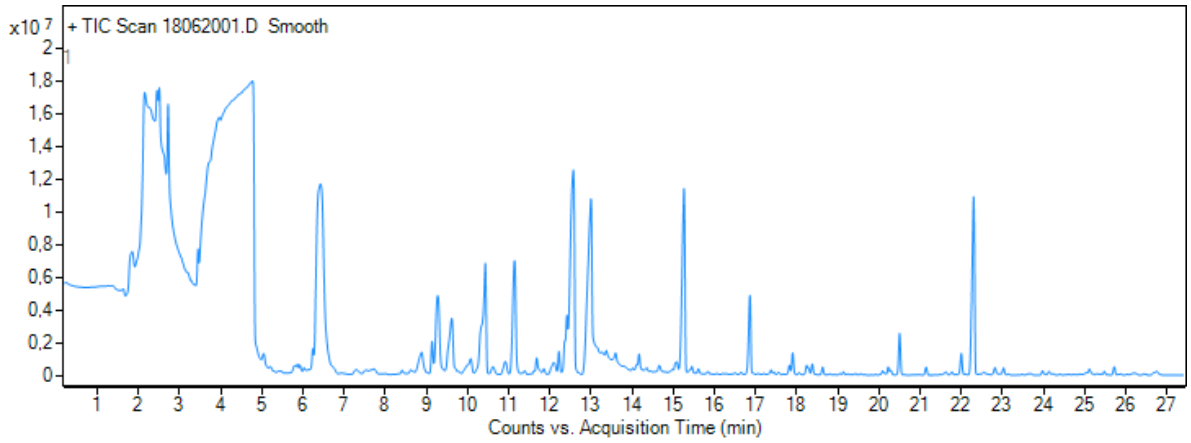


Figure 53: Total ion chromatogram acquired with the GC-MS-DHS procedure for dry-hopped beer

On the contrary, the single ion monitoring of m/z = 93, represented in figure 54, shows that this procedure allows us to get rid of all these parasite signals and quantify terpenes also presenting this ion, such as myrcene, linalool and humulene, whose molecular structures are presented above their respective peaks.

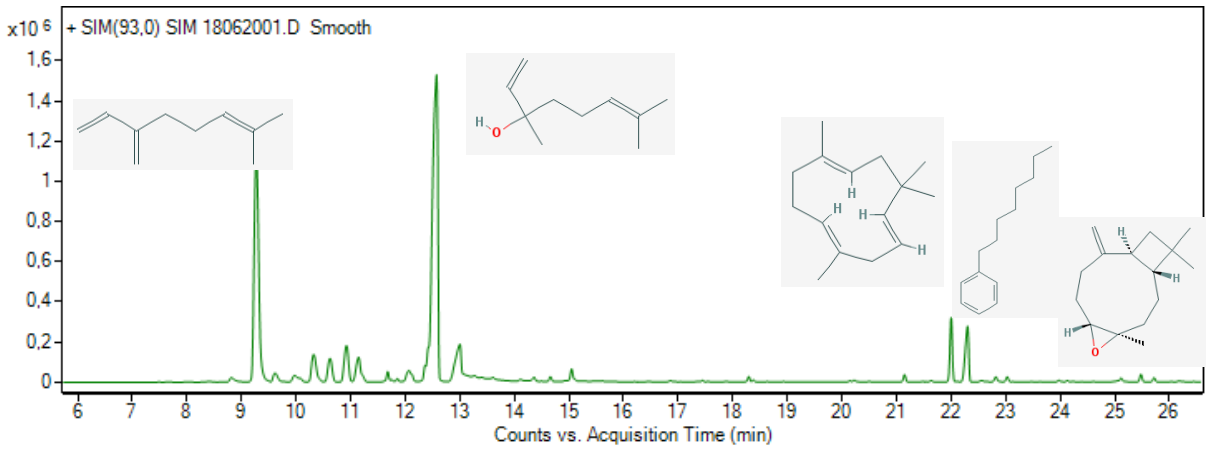


Figure 54: Single ion monitoring (m/z = 93) for dry-hopped beer

In table 30, we can see the terpenes identification and their confirmation with majors ions and linear retention index as well as the area measured for each.

Table 30: Terpenes identify by the gas chromatographic mass spectrum method

Retention time	Name	CAS number	Formula	LRI (th)	LRI (exp)	Majors ions (relative intensity)	Match rate	Area (TIC)	Area (m/z=93)
9,278	Beta.-Myrcene	123-35-3	C10H16	988	993,70	69 (75) ; 93 (100) ; 108 (11)	91,16	29103897	6696820,38
12,591	Linalool	78-70-6	C10H18O	1095	1.108,40	71 (100) ; 93 (84) ; 121 (30)	98	82934000	10287498,31
22,002	Humulene	6753-98-6	C15H24	1452	1.457,03	93 (100) ; 121 (30) ; 147 (20)	96,09	3986190	380741,07
22,306	Benzene, octyl-	2189-60-8	C14H22	1461	1.469,29	92 (100) ; 105 (11) ; 190 (31)	94,69	25854933	1248172,94
25,11	Caryophyllene oxide	1139-30-6	C15H24O	1582	1.586,86	93 (84) ; 107 (56) ; 121 (42)	91,71	1606428	98326,4

Figures 55 and 56 represent the concentration variation of these terpenes during dry-hopping. On the one hand, the decrease in beta myrcene content can be attributed to both oxidation to linalool and absorption in the yeast membrane. On the other hand, the linalool first decreases presumably oxidized to trans-linalool oxide and then the content sharply increases during the three to four days which correspond to the yeast activity peak detected previously. This implies that this yeast strain possessess enzymes able to liberate it from glycoside. Indeed, the beta myrcene decrease alone could not explain the total increase observed.

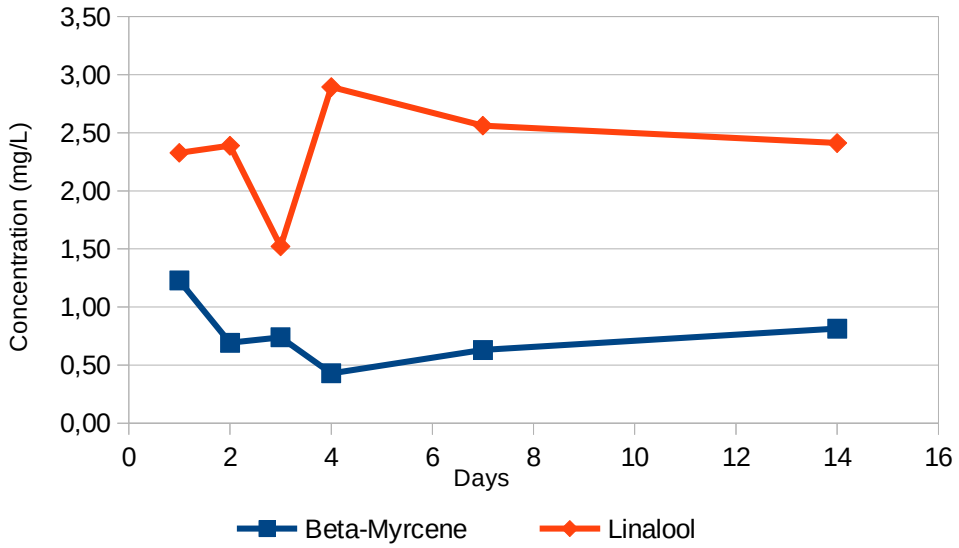


Figure 55: β-myrcene and linalool content in dry-hopped beer (25 g/L + yeast)

Regarding caryophyllene oxide and humulene, no clear tendency appears for the former, whereas the latter shows greater variation. However, these variations cannot be explained by any clear pattern as for the previous terpenes.

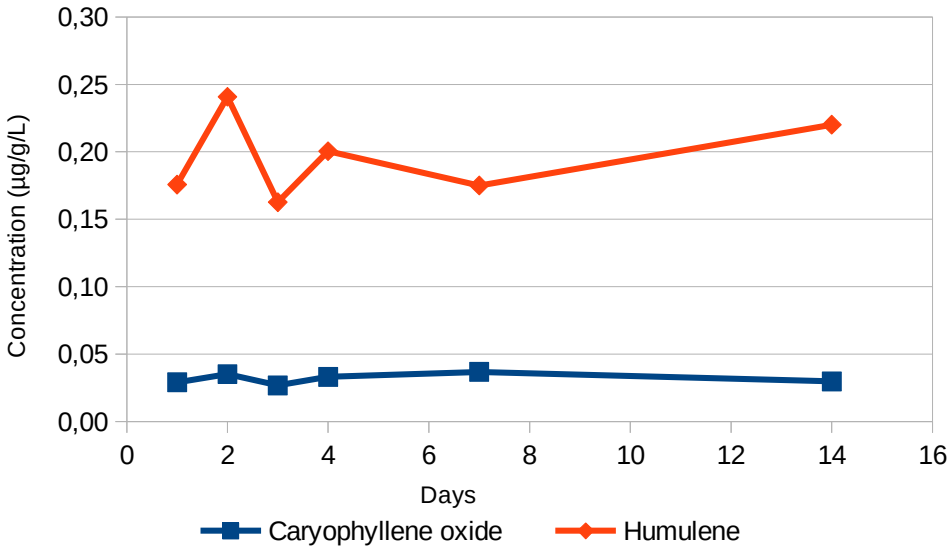


Figure 56: Caryophyllene oxide and humulene content in dry-hopped beer (25 g/L + yeast)

6) General Discussion

The dry-hopping of beer is an increasing trend in both craft and industrial breweries throughout the world. Though it corresponds roughly to a cold extraction of specific hop components in green beer, especially volatile compounds, due to the high complexity of hop, many other phenomenona take place simultaneously, making it highly complicated to predict the resulting product.

Besides, factors controlling the composition of hop and those of the process affecting the dissolution of compounds, research has shown that interaction with the yeast remaining from the fermentation could also have a high impact on the resulting product. Indeed, bio-transformation of hop-derived components as well as the liberation of hop glycoside by yeast activity has been demonstrated (Daenen *et al.*, 2008), (King et Dickinson, 2000). Changes in the ABV % (ethanol content) and density of beer have been observed during dry-hopping, and it has been further suggested that "Consequently, the presence of suspended yeast in beer during dry-hopping may have further significant impacts on overall beer flavour beyond those already observed in relation to volatile hop compounds". (Oladokun, 2017) Therefore, the aim of this work was to investigate whether or not enzyme specific activity of hop amylases during dry-hopping could have an impact within the beer matrix through yeast metabolization of the fermentable sugar produced.

Firstly, in spite of their trace amount, specific methods were adapted to assess the absolute α and β amylase activity of hop extract which unveil more variable content for the former (α). However not more than two close varieties and years of all types were analyzed. Therefore, further investigation should be initiated in order to identify factors influencing their content such as genetic aspect, growing condition, processes, etc. Besides, many other enzymes are known to be able to degrade starch-derived sugars such as pullulanase, limit dextrinase or amyloglucosidase. It could therefore be useful also to assess their activity with similar methods. Furthermore, enzymatic activity is known to be affected by different types of inhibitors such as polyphenols, and the activity observed could therefore result from partial inhibition. Separation and purification of these enzymes could be useful in determining their specific characteristics such as Km and turnover (1/s) value, optimal pH and temperature, molecular weight, quaternary structure, and substrate specificity. Though for the latter, this work tends to show results for substrates (maltotrioside and maltoheptaoside) smaller than starch.

Secondly, variations in the whole residual starch-derived sugars profile of beer was used to assess the activity of these enzymes during dry-hopping, which is to our knowledge an original experiment. Although involving a shift in the retention time, this experiment allows the quantification over time of both substrates and products of these enzymatic reactions in the particular condition of dry-hopping (acid buffered

pH and low temperature). Only one kind of beer and hop were exploited to perform this experiment, it will therefore be really interesting to use other beers as substrates with a distinctive characteristic sugar profile, alcohol content, pH, etc. Indeed, as stated previously, enzyme activity is greatly impacted by temperature and pH. Therefore very different activity could be observed depending on the condition of the dry-hopping process.

Thirdly, the impact on yeast by vicinal diketone production as well as the impact on the aroma profile of beer by volatile compound production (higher alcohols, esters, aldehydes) show great variation depending on hop concentration, as demonstrated by the principal components analysis. This fact demonstrates all the interest of further studies regarding these enzymes in their ability to modify the resulting aroma profile in dry-hopping following yeast metabolization. Nevertheless, only one kind of yeast at a specific concentration of viable cells was used whereas many different kinds are used by brewers during this step. Besides, even if the pitching rate was controlled through this parameter of viable cells, it provides no information on the physiological state of the yeast cells (glycogen and nutrient reserve). This physiological state will have a great impact on the selected metabolism pathway as well as environmental conditions such as amino acid content, dissolved oxygen and lipid content, which should be monitored to deepen understanding of the results. Besides, it would seem obvious to undertake similar experimental designs in which yeast strain as well as its environmental conditions are modified to gain insight into those volatiles produced by yeast. Finally, other yeast such as *Brettanomyces* are sometimes used in the maturation process and variation in-between the results from laboratory and industrial tanks can be explained by their presence.

Fourthly, regarding the modification in the aroma profile monitored by principal components analysis though discussion of the results takes into account the aroma threshold it probably does not reflect the actual change in perception. Indeed it has been demonstrated that the overall perception was impacted by synergism and antagonism interactions between those compounds (Meilgaard, 1975). Therefore, small concentration change could lead to bigger perception change but these having to be analyzed by trained sensorial pannels. Finally, as shown by our last results, hop-derived compounds found in beer can be modified by yeast, either by absorption or by bio-transformation which could also impart the overall hop aroma intensity.

To conclude, as for all the other processes ongoing within beer, yeast plays a tremendous role if suspended in beer during dry-hopping to modify the beer aroma profile through the cascade of reactions highlighted by this work.

7) Conclusion

If we look back at the targeted aims of this study, we see that the four goals were reached.

Firstly, the specific activity of α-amylase and β-amylase was calculated within two varieties of hop (Hallertau Hersbrücker and Alsace Strisselspalt) from different types (whole hop, pellet 90 and pellet 45) and years (2016, 2017). The results obtained for β-amylase content do not vary greatly between the samples, ranging from 0,20 ± 0,01 betamyl-3 U/g to 0,25 ± 0,03 U/g. Whereas the results gathered for α-amylase show great variability, from 0,05 ± 0,01 Ceralpha U/g to 0,15 ± 0,02 U/g.

Secondly, the next purpose was to determine whether this activity in spite of its trace level could lead to significant changes in the sugar profile of the beer during dry-hopping at 17°C up to fourteen days on laboratory and industrial scale. The production of fermentable sugar, namely maltose and glucose, using higher degrees of polymerization sugars as substrate rises as high as 4,5 and 5 g/L respectively. These observations of specific product creation and substrate degradation for these enzymes strongly confirm the results obtained in the first stage.

Thirdly, by adding yeast during the dry-hopping of these beers, the purpose was to determine whether the sugar production could lead to its metabolization by the yeast. The 90 % decrease in both maltotriose and maltopentaose as well as the vicinal diketone production demonstrate this fermentation. Indeed, the butanedione (diacetyl) as well as the pentanedione sharp rise three days after dry-hopping up to 250 and 200 ppb, confirming the yeast activity in a nitrogen deprived environment.

Fourthly, monitoring of the most important esters, both ethyl and acetate, aldehydes and higher alcohols, as well as the aroma profile global evaluation by principal components analysis confirms that the presence of yeast during dry-hopping results in changes of the aroma profile of beer, compared to hop alone. Furthermore, as demonstrated by the increase in linalool content the yeast bio-transforms hop-derived compounds.

To conclude, we demonstrate in this study the enzymatic potential of hop in the beer matrix as well as the consequent impact it can have on the beer aroma profile via yeast metabolism. This highlights the fact that dry-hopping is far more than the dissolution of the hop-derived volatiles in beer and that a much more complex interaction between hop and yeast takes place.

8) Perspectives

Like any scientific work, this study was limited in time and investment leading to prioritization of certain objectives at the expense of others.

Firstly, the enzyme content was analyzed on the only hop materials available in sufficient quantity at the brewery at that time, which were two close taxons differing from the Cascade content analyzed by (Kirkpatrick *et al.,* 2017). The botanical localization on the hop flowers (close or not to the lupulin gland) as well as the impact of the hop process on its content should therefore be investigated on many more samples.

Secondly, the temperature at which the dry-hopping takes place is of enormous importance, as for any enzymatic reaction, and lower levels should be tested seeing the usual range in breweries varies from 1 to 20°C. Furthermore, the dispersion method, especially the dynamic one, should be investigated in the light of these results to explain less attenuation of beer due to lack of time for this reaction.

Thirdly, as well as producing volatile compounds (only some of which were analyzed) yeast is known to bio-transform compounds and produce volatiles from glycoside precursors. These other effects should be investigated to determine positive and negative outcomes of this reaction sequence in terms of the aroma profile. Furthermore, the hypothesis of additional esterase in the hop trichomes should also be investigated as it could lead to consequent modification of the ester content of dry-hopped beer.

Lastly, behaviour of yeast in a deprived environment should be investigated for a better understanding of its interaction with hop leading to re-fermentation.

To conclude, the inability of hop oil extract to develop the same aroma profile as classical dry-hopping could be demonstrated by this.

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Annexes :

Index of annexes

α-amylase Megazyme results

Strisselspalt 2016 whole hop			Strisselspalt 2016 Pellet P45			ɔrücker 2016 whole hop			Hersbrücker 2016 Pellet 45		
Sample Name	A400nm	Enzymatic Unit	Sample Name	A400nm	Enzymatic Unit	Sample Name	A400nm	Enzymatic Unit	Sample Name	A400nm	Enzymatic Unit
Blank AS 0min	0,021	/	B AS 0min	0,017		B H0min	0,016		B H0min	0,02	
Blank AS 1000min	0,021	/	B AS 1000min	0,029		B H0min	0,028		B H0min	0,02	
AS1 1000min	0,065	0,14	AS1 1000min	0,049	0,06	H1 1000min	0,053	0,08	H1 1000min	0,064	0,14
AS2 1000min	0,059	0,12	AS2 1000min	0,043	0,04	H2 1000min	0,043	0,05	H2 1000min	0,056	0,11
AS3 1000min	0,063	0,13	AS3 1000min	0,046	0,05	H3 1000min	0,049	0,07	H3 1000min	0,052	0,10
	Mean	0,13		Mean	0,05		Mean	0,06		Mean	0,12
	Standard deviation	0,01		Standard deviation	0,01		Standard deviation	0,02		Standard deviation	0,02
	Variation coefficient	7,25		Variation coefficient	19,11		Variation coefficient	24,59		Variation coefficient	19,35

Strisselspalt 2017 Whole hop			Strisselspalt 2017 Pellet P45			Strisselspalt 2017 Pellet P90		
Sample Name	A400nm	Enzymatic Unit	Sample Name	A400nm	Enzymatic Unit	Sample Name	A400nm	Enzymatic Unit
B AS 0min			B H0min			B AS 0min		
B AS 1000min	0,028		B H0min	0,022		B AS 1000min	0,025	
AS1 1000min	0,067	0,12	H1 1000min	0,077	0,17	AS1 1000min	0,054	0,09
AS2 1000min	0,059	0,10	H2 1000min	0,06	0,12	AS2 1000min	0,071	0,14
AS3 1000min	0,065	0,11	H3 1000min	0,072	0,15	AS3 1000min	0,071	0,15
	Mean	0,11		Mean	0,15		Mean	0,13
	Standard deviation	0,01		Standard deviation	0,02		Standard deviation	0,03
	Variation coefficient	10,89		Variation coefficient	16,74		Variation coefficient	25,22

β-amylase Megazyme results

Strisselspalt 2016 whole hop		
Sample name	A400nm	Enzymatic Unit
B AS 0min	0,132	
B AS 1000min	0,132	
AS1 1000min	1,352	0,23
AS2 1000min	1,558	0,28
AS3 1000min	1,35	0,23
	Mean	0,25
	Standard deviation	0,03
	Variation coefficient	10,72

Strisselspalt 2016 Pellet P45		
Sample name	A400nm	Enzymatic Unit
B AS 0min	0,145	
B AS 1000min	0,157	
AS1 1000min	1,27	0,22
AS2 1000min	1,315	0,22
AS3 1000min	1,339	0,23
	Mean	0,22
	Standard deviation	0,01
	Variation coefficient	3,31

Hersbrücker 2016 whole hop		
Sample name	A400nm	Enzymatic Unit
B H0min	0,126	
B H0min	0,144	
H1 1000min	1,24	0,21
H2 1000min	1,401	0,24
H3 1000min	1,452	0,25
	Mean	0,24
	Standard deviation	0,02
	Variation coefficient	9,24

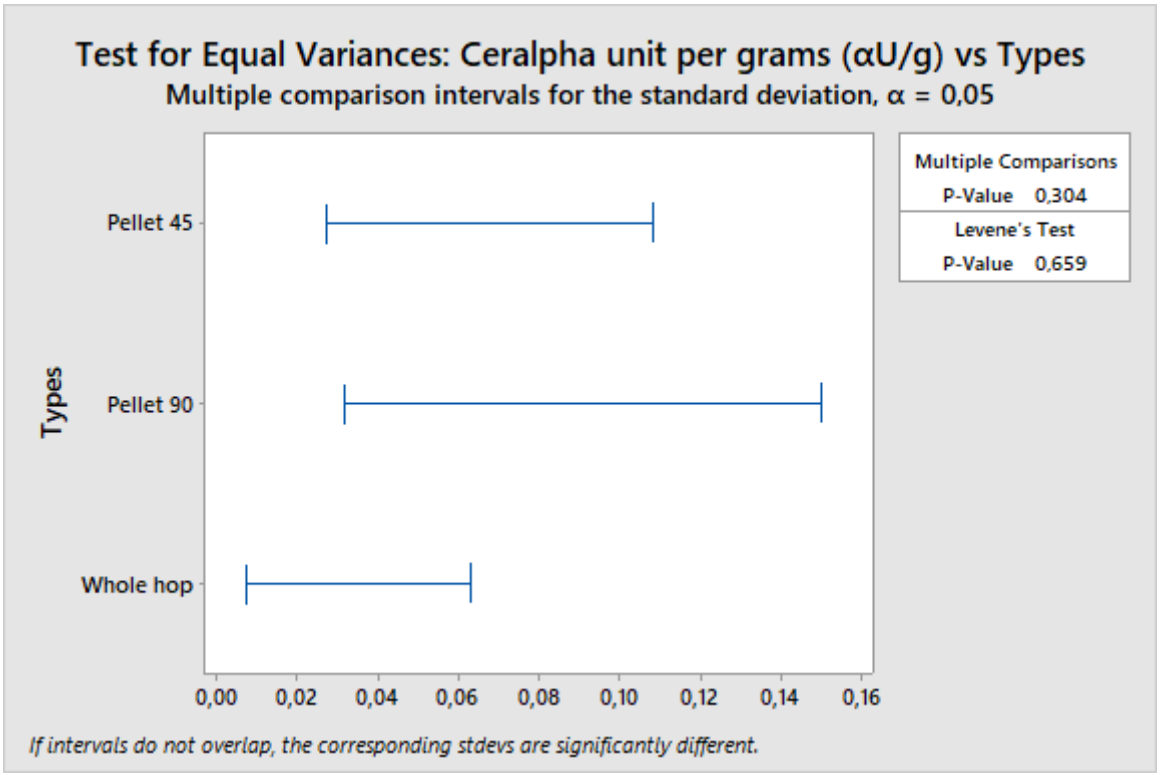
Hersbrücker 2016 Pellet 45		
Sample name	A400nm	Enzymatic Unit
B H0min	0,137	
B H0min	0,159	
H1 1000min	1,233	0,21
H2 1000min	1,331	0,22
H3 1000min	1,321	0,23
	Mean	0,22
	Standard deviation	0,01
	Variation coefficient	4,67

Strisselspalt 2017 Whole hop		
Sample name	A400nm	Enzymatic Unit
B AS 0min	0,147	
B AS 1000min	0,147	
AS1 1000min	1,165	0,20
AS2 1000min	1,174	0,20
AS3 1000min	1,282	0,21
	Mean	0,21
	Standard deviation	0,01

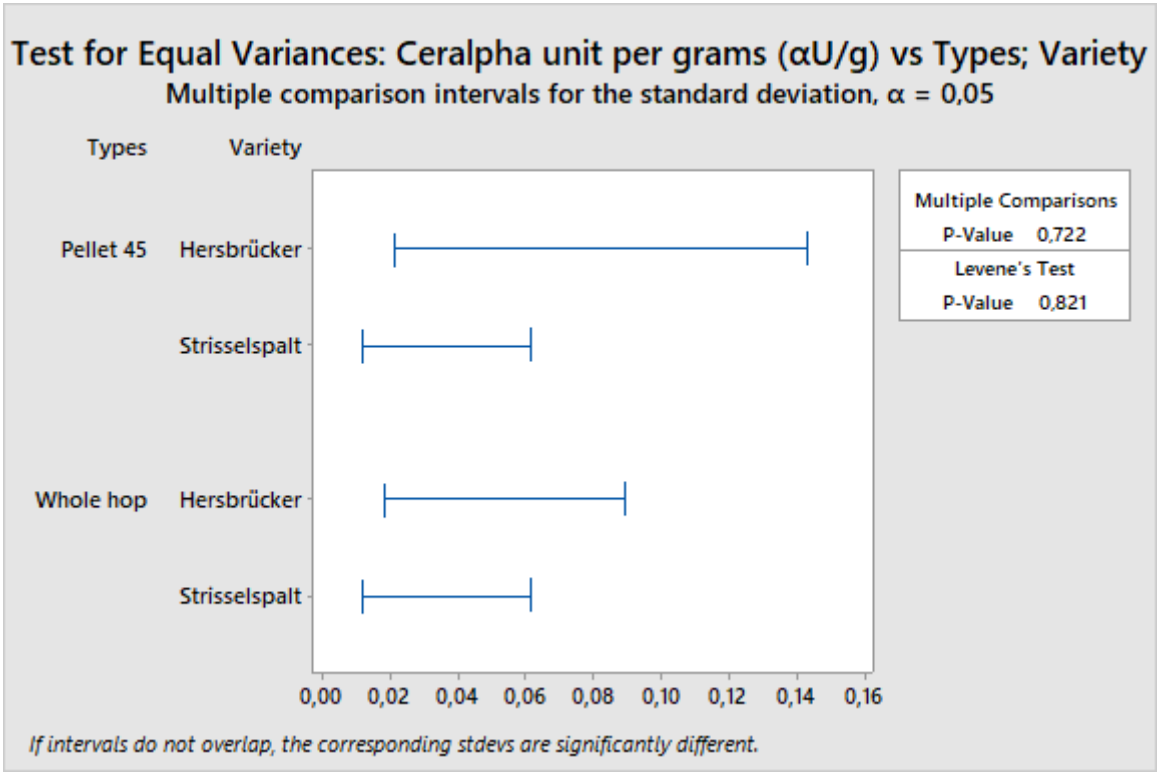
Strisselspalt 2017 Pellet P45		
Sample name	A400nm	Enzymatic Unit
B AS 0min	0,146	
B AS 1000min	0,146	
AS1 1000min	1,155	0,19
AS2 1000min	1,221	0,21
AS3 1000min	1,237	0,22
	Mean	0,20
	Standard deviation	0,01

Alpha amylase equality of the variance levene test results :

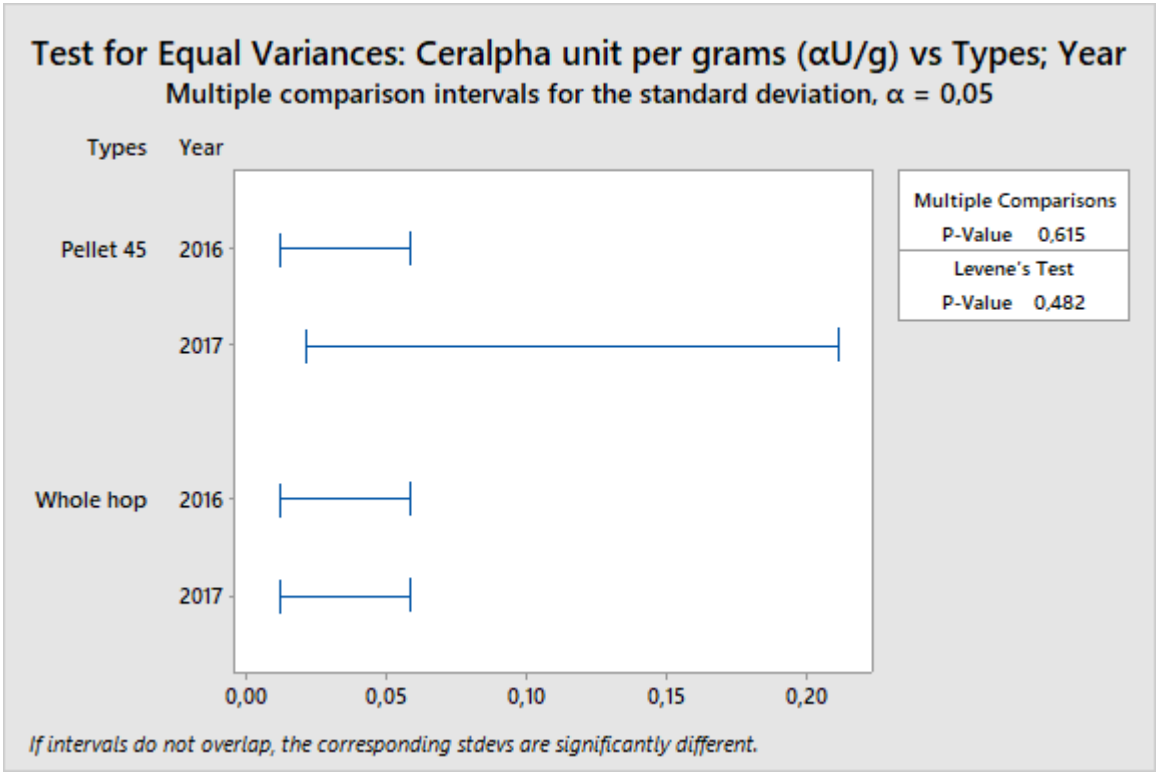
Strisspalt 2017 :



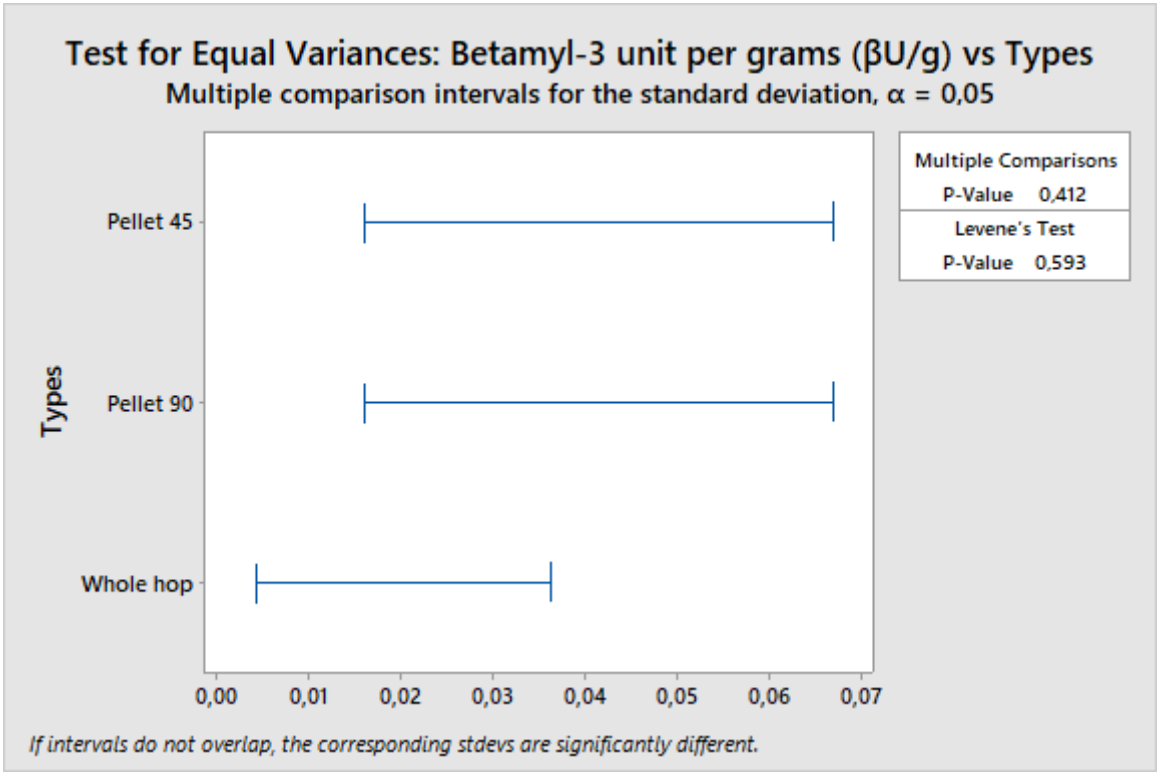
AV 2 variety types :



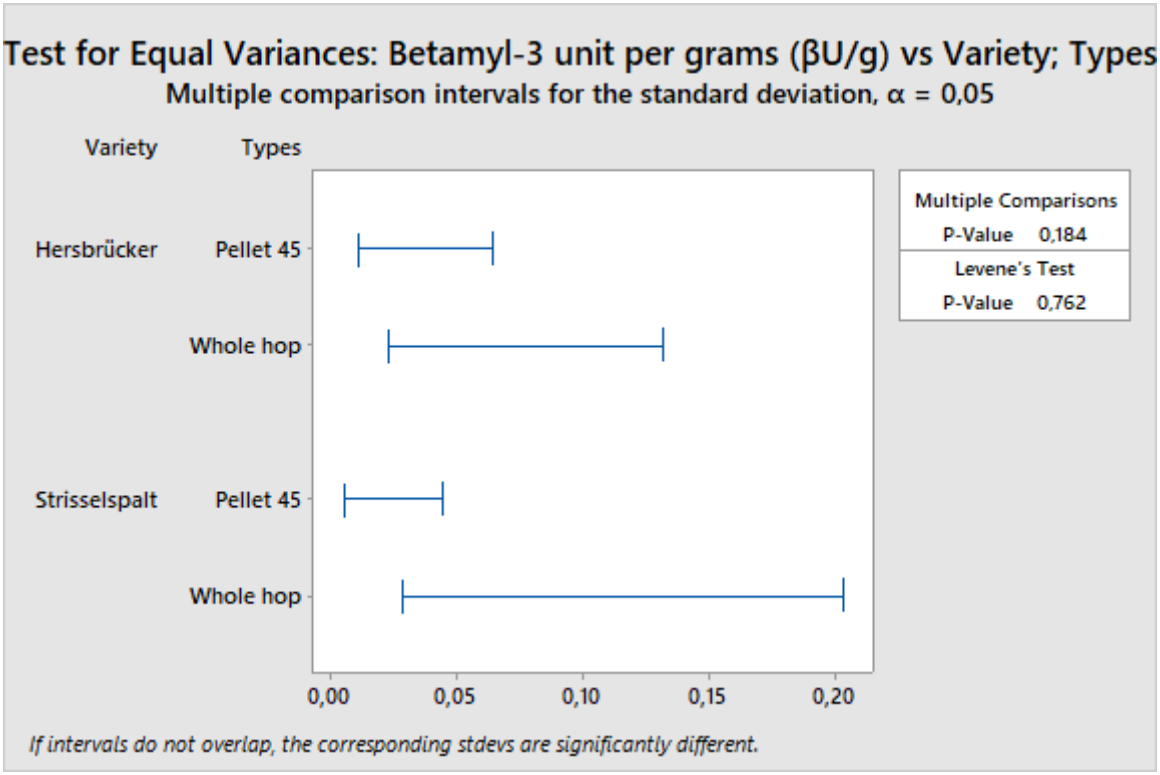
AV2 Year types



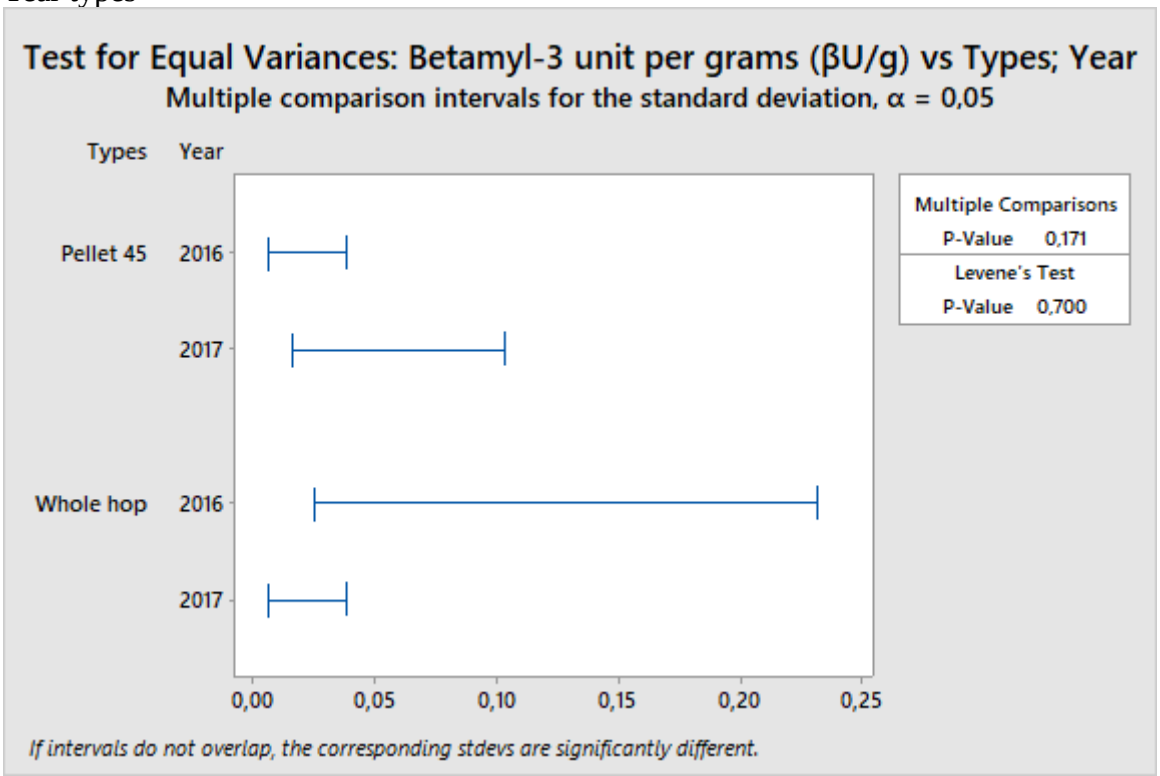
<u>Beta Amylase Results Levenes test :</u>



AV 2 Variety types

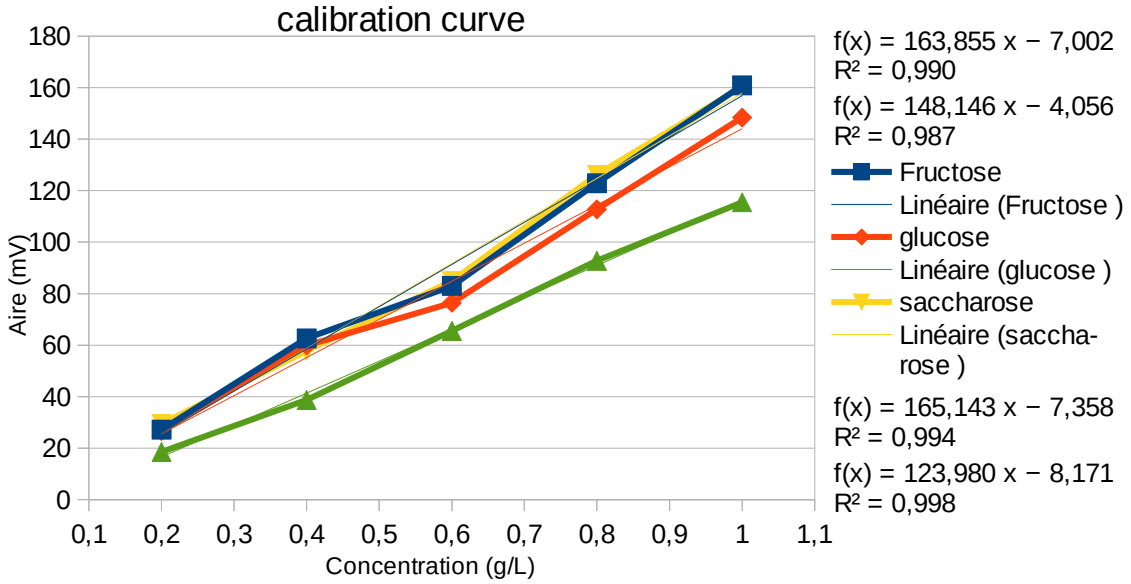


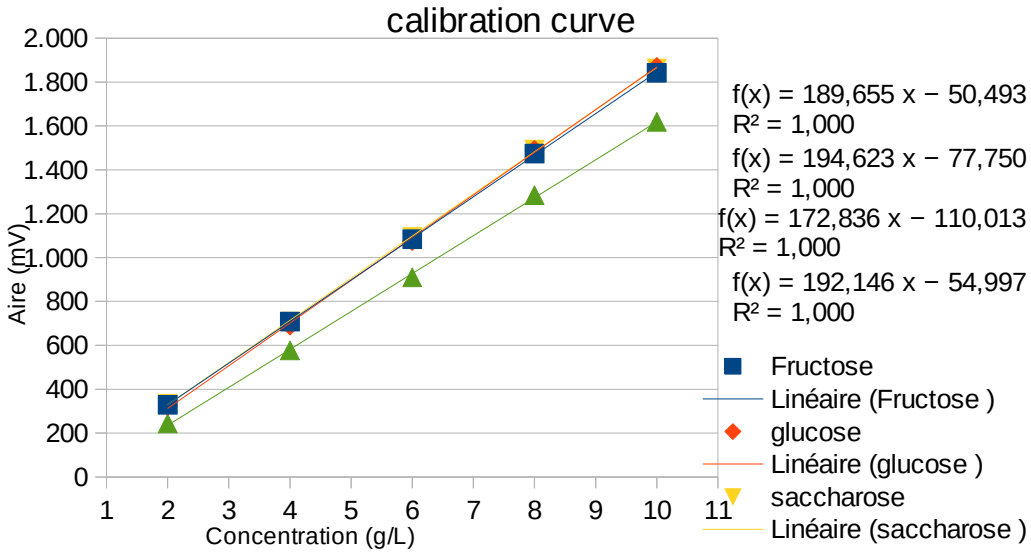
AV2 Year types



Identification of sugar based on the Relative retention time :

N°	carbohydrates	Retention time (RT)	Relative retention time (RRT)	theoritical RRT
1	fructose	7,42	1,00	1
2	glucose	8,84	1,19	1,2
3	sucrose	12,267	1,65	1,7
4	maltose	14,081	1,90	2
5	maltotriose	19,098	2,57	2,6
6	Maltotetraose	20,106	2,71	3
7	Maltopentaose	22,077	2,98	3,2
8	Maltoheaxose	24,107	3,25	3,4
9	Maltoheptaose	24,66	3,32	3,5
10	DP8	25,573	3,45	3,6
11	DP9	26,099	3,52	3,7
12	DP10	27,216	3,67	3,8
13	DP11	28,194	3,80	3,8
14	DP12	29,025	3,91	3,9
15	DP13	29,709	4,00	4
16	DP14	30,02	4,05	4,1
17	DP15	31,224	4,21	4,2





Mean of sugar profile for the three repetitions

Sample Name	Time (days)	Hop (g/L)	Yeast (0/1)	Fructose (mV)	Glucose (mV)	Maltose (mV)	Maltotriose (mV)	Maltotetraose (mV)	Maltopentaose (mV)	maltoheaxose (mV)	Maltoheptaose (mV)	DP8 (mV)	DP9 (mV)	DP10 (mV)	DP11 (mV)	DP12 (mV)	DP13 (mV)
B1	1	0	0	14,95	7,87	6,83	1.066,19	146,69	682,05	272,05	189,48	127,69	93,92	69,93	36,29	40,47	33,76
B1H5	1	5	0	54,06	74,79	71,10	1.052,51	117,43	612,51	239,95	171,20	129,15	106,44	66,04	35,59	40,98	36,10
B1H25	1	25	0	247,36	411,91	150,47	1.037,82	127,69	549,12	227,12	174,24	123,59	96,34	70,09	37,70	46,88	41,03
B1L	1	0	1	3,88	0,00	7,70	1.023,23	126,55	645,18	275,16	182,34	118,48	95,05	65,25	39,81	44,34	41,85
B1H5L	1	5	1	33,35	17,66	42,56	1.022,81	139,27	606,27	244,32	181,74	114,50	97,06	53,92	36,85	39,34	39,94
B1H25L	1	25	1	177,05	214,33	109,60	1.008,55	158,00	539,91	241,88	183,45	120,87	108,56	51,95	38,71	48,91	37,70
B2	2	0	0	3,11	1,80	6,64	931,59	102,08	583,29	245,89	166,94	114,94	86,93	59,72	33,42	37,45	31,93
B2H5	2	5	0	44,69	74,41	70,39	937,55	105,16	555,14	223,91	169,19	130,05	94,58	64,76	34,49	41,17	34,53
B2H25	2	25	0	228,91	397,49	195,91	903,69	133,90	457,86	199,83	155,33	117,95	90,21	65,77	42,43	39,54	33,88
B2L	2	0	1	3,87	0,00	6,27	987,85	118,91	636,36	263,87	186,28	118,48	89,53	58,02	48,73	45,05	41,65
B2H5L	2	5	1	23,77	8,33	37,61	925,90	112,97	584,39	235,24	161,62	115,97	78,30	57,24	43,87	42,61	33,44
B2H25L	2	25	1	94,79	68,83	90,23	845,46	133,69	477,19	210,54	151,55	151,70	111,69	65,30	47,15	52,09	40,35
B3	3	0	0	1,48	1,15	4,93	962,41	105,26	611,95	238,68	177,10	122,44	89,88	58,22	39,98	43,48	39,02
B3H5	3	5	0	48,30	91,55	102,35	933,18	114,48	532,08	215,38	165,01	125,00	86,36	55,07	37,66	37,31	30,12
B3H25	3	25	0	233,38	462,26	242,81	868,35	123,08	441,28	204,34	165,59	123,94	79,95	64,56	40,85	43,07	38,09
B3L	3	0	1	3,20	0,00	5,64	893,13	104,12	592,03	257,84	184,55	122,15	87,44	59,66	38,84	46,02	39,40
B3H5L	3	5	1	13,83	6,16	33,32	763,50	118,52	519,01	208,52	162,81	115,79	78,22	57,31	41,34	45,18	38,72
B3H25L	3	25	1	44,87	25,08	52,77	642,61	114,56	395,24	190,87	149,24	121,32	92,32	54,41	34,92	41,47	39,60
B4	4	0	0	1,58	0,00	3,69	930,01	115,15	598,49	253,99	167,13	115,04	87,57	64,70	33,46	36,99	28,97
B4H5	4	5	0	48,66	102,05	123,81	923,10	94,53	507,85	196,62	144,44	115,13	81,01	58,05	30,69	35,12	29,53
B4H25	4	25	0	230,33	483,47	261,27	833,18	112,95	421,38	191,06	153,63	130,57	90,48	77,97	36,08	37,95	30,27
B4L	4	0	1	0,69	0,00	3,36	899,77	108,91	603,28	242,66	175,21	124,23	80,23	57,55	35,28	37,65	34,25
B4H5L	4	5	1	10,55	1,17	19,41	663,34	95,93	494,97	207,53	137,30	120,42	92,99	63,90	41,08	42,58	29,93
B4H25L	4	25	1	16,88	12,87	29,10	538,35	88,28	374,76	187,58	152,53	140,08	89,31	63,71	44,45	44,49	38,15
B7	7	0	0	4,58	0,00	5,98	944,14	96,83	582,26	255,49	162,87	119,58	84,50	59,14	36,98	38,87	34,39
B7H5	7	5	0	49,32	125,29	196,65	914,64	92,67	485,11	191,31	152,04	122,51	83,50	65,30	44,43	36,35	29,24
B7H25	7	25	0	235,83	608,32	345,31	779,51	110,95	370,26	189,27	142,75	107,32	101,34	76,00	35,19	42,82	36,72
B7L	7	0	1	1,90	0,00	4,18	844,01	93,46	613,05	257,19	176,54	120,92	80,34	64,11	32,11	37,66	33,87
B7H5L	7	5	1	9,41	2,99	24,30	389,94	108,42	422,50	184,85	148,57	125,63	88,84	64,67	38,44	44,54	39,09
B7H25L	7	25	1	28,70	21,63	23,11	248,12	75,06	199,77	129,31	138,59	124,10	95,01	76,00	39,45	32,36	25,91
B14	14	0	0	2,71	0,00	5,28	919,85	155,08	599,07	249,90	174,30	138,78	94,39	59,17	34,67	40,64	36,38
B14H5	14	5	0	56,50	217,93	321,46	848,90	137,60	450,91	187,42	159,52	157,39	101,15	67,03	42,05	39,22	32,21
B14H25	14	25	0	240,79	867,19	549,71	596,97	125,42	211,77	145,40	132,76	145,78	101,76	74,94	39,77	43,96	38,22
B14L	14	0	1	0,00	0,00	0,00	707,51	154,77	610,40	262,38	179,27	149,81	89,90	61,23	34,20	35,58	31,04
B14H5L	14	5	1	0,00	13,13	34,77	126,26	119,71	256,23	147,30	124,24	118,38	84,62	50,92	35,36	36,95	28,24
B14H25L	14	25	1	0,00	29,53	25,47	121,00	118,35	117,64	120,77	112,14	96,14	86,20	70,04	35,75	28,78	27,65

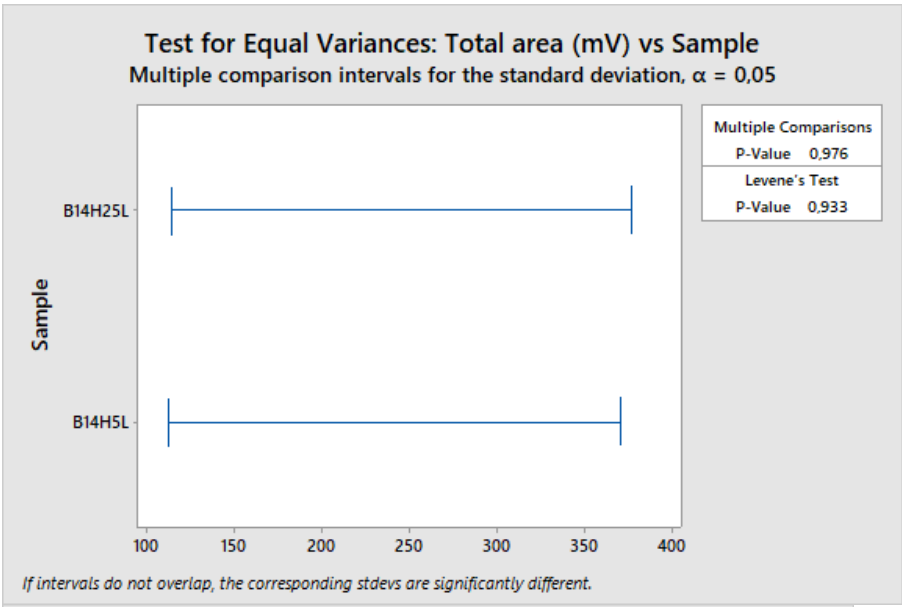
Standard deviation of sugar profile for the three repetitions

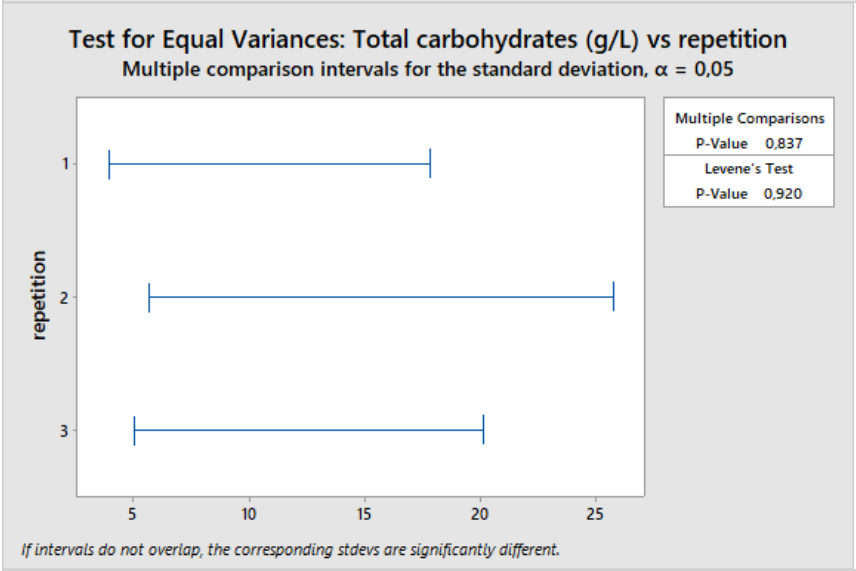
Sample Name	Time (days)	Hop (g/L)	Yeast (0/1)	Fructose (mV)	Glucose (mV)	Maltose (mV)	Maltotriose (mV	Maltotetraose (m	Maltopentaose (m	Maltoheaxose (m	Maltoheptaose (m	DP8 (mV)	DP9 (mV)	DP10 (mV)	DP11 (mV)	DP12 (mV)	DP13 (mV)
B1	1,00	0	0	25,90	13,64	11,84	153,04	65,14	24,84	8,26	15,16	4,85	4,89	10,69	5,28	1,29	5,14
B1H5	1,00	5	0	2,41	6,92	6,46	174,10	48,09	57,51	14,51	25,45	20,53	13,36	14,90	1,24	6,97	4,25
B1H25	1,00	25	0	24,59	37,00	24,92	175,17	70,74	29,39	18,60	26,63	8,19	9,74	12,37	10,52	8,05	2,05
B1L	1,00	0	1	6,72	0,00	13,34	151,74	19,71	36,62	12,41	16,30	18,30	16,64	11,65	8,77	1,33	3,94
B1H5L	1,00	5	1	3,12	3,03	10,86	139,20	44,25	44,85	19,05	24,39	2,66	20,96	19,33	7,39	4,46	6,72
B1H25L	1,00	25	1	13,11	27,96	9,06	140,52	43,71	40,39	22,98	41,85	10,15	6,15	10,70	8,35	3,09	9,86
B2	2,00	0	0	5,39	3,12	11,50	138,92	15,92	9,78	20,36	18,39	2,25	1,39	4,79	6,85	0,84	6,38
B2H5	2,00	5	0	4,65	7,65	10,58	133,85	5,64	18,10	15,60	20,21	18,58	5,51	1,59	0,63	4,80	7,13
B2H25	2,00	25	0	2,42	6,04	47,74	136,10	54,00	29,68	26,80	19,73	9,41	4,53	3,40	5,76	5,84	1,35
B2L	2,00	0	1	6,70	0,00	10,85	180,93	41,27	69,50	28,56	17,82	17,98	13,47	21,39	15,55	9,43	11,37
B2H5L	2,00	5	1	2,68	8,56	15,50	147,36	34,76	68,83	41,69	48,98	7,12	33,66	18,94	14,39	7,65	9,11
B2H25L	2,00	25	1	20,56	28,26	15,47	165,33	32,95	45,44	28,40	15,56	35,62	29,22	19,16	8,51	3,99	3,07
B3	3,00	0	0	2,56	1,99	8,54	134,78	48,94	8,88	7,16	24,44	12,35	5,05	20,80	4,98	2,87	9,03
B3H5	3,00	5	0	0,93	3,71	21,31	134,66	48,98	28,84	31,47	27,74	9,80	5,31	11,57	5,49	0,43	3,02
B3H25	3,00	25	0	4,14	3,09	43,95	126,70	45,99	24,14	29,56	8,26	15,11	3,20	19,38	11,50	2,97	5,80
B3L	3,00	0	1	5,54	0,00	9,77	121,57	29,42	31,96	26,31	30,75	3,18	6,27	14,41	11,60	8,16	3,91
B3H5L	3,00	5	1	2,88	6,09	5,82	93,35	20,91	25,98	11,99	22,77	11,41	9,93	11,70	4,24	8,21	3,06
B3H25L	3,00	25	1	7,27	9,90	7,15	67,33	27,47	40,49	28,42	22,20	12,48	10,46	3,36	2,82	7,04	12,50
B4	4,00	0	0	2,73	0,00	6,38	106,09	31,03	12,28	12,28	12,76	8,29	8,53	12,53	7,06	7,93	4,40
B4H5	4,00	5	0	2,71	5,37	10,83	118,77	24,71	7,66	15,15	12,10	8,79	5,38	7,45	3,07	0,83	3,73
B4H25	4,00	25	0	9,02	6,44	82,59	132,42	64,78	60,12	18,08	7,14	9,89	3,37	14,27	2,93	1,38	2,21
B4L	4,00	0	1	1,19	0,00	5,82	129,82	21,62	28,95	12,81	27,00	15,45	6,50	12,13	5,20	1,27	2,05
B4H5L	4,00	5	1	9,95	2,02	20,28	56,03	17,03	34,54	9,27	36,19	13,29	2,98	9,87	9,42	5,01	1,51
B4H25L	4,00	25	1	5,54	2,24	9,47	133,52	25,27	65,20	40,18	43,54	24,10	9,03	22,78	19,33	15,43	9,63
B7	7,00	0	0	7,93	0,00	10,36	120,04	43,97	12,14	7,09	15,32	16,15	6,58	8,98	3,02	4,21	4,85
B7H5	7,00	5	0	6,00	21,16	27,17	146,15	28,42	20,11	21,58	8,40	16,94	6,88	16,64	9,18	2,45	3,65
B7H25	7,00	25	0	2,61	15,55	93,96	154,45	45,32	23,68	8,02	9,72	8,72	11,72	4,37	3,11	4,78	5,70
B7L	7,00	0	1	3,30	0,00	7,24	77,85	28,31	2,88	26,69	22,72	21,89	8,52	11,31	3,68	5,54	2,04
B7H5L	7,00	5	1	9,19	5,17	14,26	60,82	31,11	19,95	2,01	14,08	14,14	8,98	9,45	5,65	8,13	2,82
B7H25L	7,00	25	1	1,70	20,14	11,71	51,00	13,64	45,00	34,68	21,70	9,95	16,77	7,50	4,53	4,77	11,29
B14	14,00	0	0	4,69	0,00	9,15	151,26	44,17	16,45	11,01	15,33	10,84	9,82	8,49	5,02	1,01	4,80
B14H5	14,00	5	0	15,02	37,47	39,95	219,42	11,51	74,98	22,51	15,85	3,51	29,13	9,16	10,92	9,51	0,94
B14H25	14,00	25	0	24,59	56,13	46,22	108,14	19,00	29,44	38,43	54,28	36,73	18,73	11,20	14,42	19,31	18,56
B14L	14,00	0	1	0,00	0,00	0,00	110,17	28,52	47,08	12,52	10,90	25,79	0,37	11,87	4,66	7,05	2,21
B14H5L	14,00	5	1	0,00	11,39	5,58	50,61	32,55	62,40	18,22	19,76	13,37	6,63	6,03	2,98	6,37	2,42
B14H25L	14,00	25	1	0,00	25,91	6,99	60,09	34,83	24,90	16,73	23,76	21,18	34,15	29,77	20,95	10,73	22,19

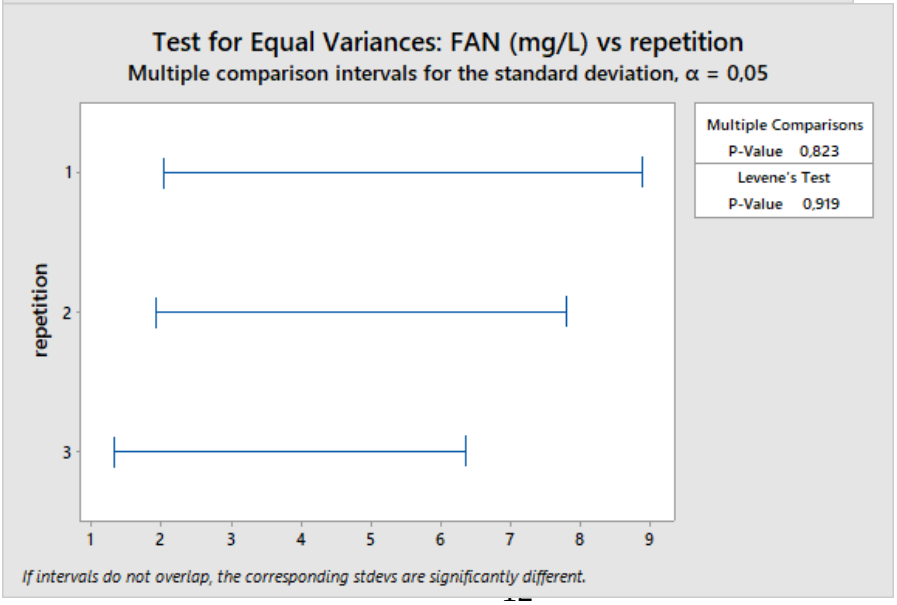
Industrial tank results

		Fructose (mV)	Glucose (mV)	Maltose (mV)	Maltotriose (mV)	Maltotetraose (mV)	Maltopentaose (mV)	maltoheaxose (mV)	Maltoheptaose (mV)	DP8 (mV)	DP9 (mV)	DP10 (mV)	DP11 (mV)	DP12 (mV)	DP13 (mV)
whole hop 1	TC1J1	18,25	0,00	0,00	925,76	131,18	632,25	252,95	174,94	124,09	91,84	52,23	32,72	38,77	32,63
	TC1J2	20,23	0,00	0,00	888,91	138,27	629,65	255,72	186,83	134,09	96,84	59,80	32,62	38,10	29,04
	TC1J3	14,32	0,00	0,00	864,36	131,75	587,97	261,04	195,32	127,61	89,24	53,07	42,36	49,87	35,80
	TC1J4	19,28	0,00	0,00	850,86	130,48	576,88	248,48	201,64	143,92	102,32	71,51	39,45	42,46	37,85
	TC1J7	10,28	0,00	0,00	783,51	132,53	525,60	231,30	168,33	109,39	79,62	49,59	38,03	37,19	33,00
	TC1J14	30,60	0,00	0,00	672,89	145,36	485,66	218,09	155,31	125,55	69,41	53,63	37,37	30,89	26,52
Pellet 1	TP1J1	14,30	0,00	0,00	898,29	130,69	628,13	263,63	197,67	118,12	88,65	59,09	48,76	54,70	42,14
	TP1J2	18,89	0,00	0,00	874,06	124,68	626,94	258,68	182,08	105,51	97,12	50,65	38,36	44,90	33,40
	TP1J3	21,03	0,00	0,00	856,26	117,51	608,88	246,66	183,50	125,08	101,69	68,14	42,08	39,41	35,07
	TP1J4	18,18	0,00	0,00	852,54	145,47	600,06	252,31	182,30	117,32	97,94	53,28	40,55	42,15	36,06
	TP1J7	17,98	0,00	0,00	791,90	110,05	579,27	245,22	185,35	133,97	104,96	73,08	34,04	36,19	36,35
	TP1J14	30,45	0,00	0,00	699,16	169,32	474,72	224,16	156,12	151,79	84,03	53,28	29,65	29,70	29,00
whole hop 2	TCJ1	14,53	0,00	0,00	821,66	184,56	613,90	245,18	142,18	137,16	92,65	68,61	20,14	31,92	22,87
	TCJ2	14,75	0,00	0,00	725,07	174,58	605,50	241,47	147,52	124,66	91,85	55,11	23,24	33,81	20,13
	TCJ3	49,40	0,00	0,00	543,79	195,71	604,40	248,55	151,92	120,28	98,29	70,65	41,51	40,05	37,66
	TCJ4	29,86	0,00	0,00	554,45	147,33	454,60	180,60	111,93	82,78	65,82	46,05	19,87	19,67	16,31
	TCJ7	20,36	0,00	0,00	455,66	122,26	420,38	149,21	110,54	88,30	55,19	33,87	18,74	22,14	17,94
	TCJ14	17,08	0,00	0,00	455,18	114,34	381,41	137,58	104,48	94,91	53,21	38,39	24,12	22,13	15,00
Pellet 2	TP2J1	21,20	0,00	0,00	884,91	168,30	548,58	242,01	149,73	116,02	75,97	51,50	20,28	23,06	18,96
	TP2J2	36,39	0,00	0,00	832,97	165,08	531,98	237,72	159,84	107,40	72,61	57,61	27,81	28,89	23,35
	TP2J3	21,66	0,00	0,00	774,74	154,47	527,13	246,89	123,24	104,15	61,69	51,75	20,07	24,81	23,75
	TP2J4	14,16	0,00	0,00	697,83	142,30	495,17	242,83	130,19	112,55	58,65	57,39	29,62	28,89	25,68
	TP2J7	23,51	0,00	0,00	643,47	140,31	480,88	223,21	135,55	100,17	59,13	48,99	19,66	30,64	24,20
	TP2J14	21,76	0,00	0,00	624,22	131,36	481,54	227,21	153,43	130,38	82,61	64,71	26,63	30,04	32,46

Levene test results for total chromatogram area, FAN and total sugar content







Mean of volatile organic compounds produced by yeast

Echantillon vial	Diacetyl (ppb)	Pentane dione (ppb)	acetaldehyde (ppm)	ethyl acetate (ppm)	propanol (ppm)	isobutanol (ppm)	isoamyl acetate (ppm)	isoamyl alcool (ppm)	ethyl caproate (ppm)	ethyl caprylate (ppm)
B1	48,41	5,46	2,21	33,80	22,52	23,52	3,08	132,73	0,19	0,81
B2	53,33	6,19	2,16	34,28	22,71	23,83	3,08	134,26	0,17	0,63
B3	57,62	6,88	2,35	35,34	22,77	23,71	3,27	144,80	0,21	0,80
B4	59,80	7,57	2,35	35,58	22,55	23,50	3,19	132,17	0,18	0,61
B7	58,77	7,03	3,36	28,21	20,04	20,82	2,41	117,63	0,11	0,38
B14	45,02	5,99	2,37	33,20	22,23	22,99	2,69	129,49	0,13	0,27
B1 L	34,36	4,61	17,33	35,21	22,50	23,36	3,21	130,65	0,21	0,90
B2 L	45,59	7,80	35,22	37,94	23,70	24,27	3,39	136,19	0,22	0,86
B3 L	37,94	7,02	40,82	37,73	23,15	23,45	3,31	130,48	0,19	0,61
B4 L	38,56	7,59	48,82	37,54	22,95	23,44	3,26	130,76	0,20	0,78
B7 L	40,37	8,19	59,60	38,50	23,55	23,82	3,22	131,66	0,18	0,48
B14 L	22,05	3,12	50,06	34,52	22,94	23,00	2,55	126,24	0,15	0,27
B1 H5	58,48	7,57	3,18	33,04	24,21	23,31	2,75	132,14	0,17	0,67
B2 H5	58,92	7,38	3,08	33,22	23,89	22,31	2,68	128,87	0,15	0,48
B3 H5	63,96	8,27	3,37	32,19	24,14	23,18	2,49	130,22	0,15	0,60
B4 H5	62,73	8,04	3,35	34,66	23,64	22,43	2,64	126,26	0,14	0,43
B7 H5	66,23	10,34	8,14	27,82	24,12	22,21	1,86	124,77	0,10	0,32
B14 H5	71,04	8,26	3,25	30,71	24,59	22,67	1,64	126,84	0,12	0,25
B1 H25	68,36	9,26	4,78	30,29	30,00	22,15	1,75	125,00	0,12	0,42
B2 H25	74,55	10,50	5,19	33,73	30,64	22,38	1,70	126,40	0,13	0,30
B3 H25	84,24	12,22	6,21	29,06	29,40	21,27	1,32	118,18	0,11	0,31
B4 H25	82,53	12,30	6,15	31,95	30,84	21,83	1,36	122,81	0,11	0,23
B7 H25	79,36	11,49	6,18	21,05	29,12	19,84	0,73	112,91	0,07	0,16
B14 H25	102,51	14,35	7,52	30,60	38,33	24,53	0,51	136,13	0,11	0,13
B1 H5 L	84,17	36,91	31,13	32,65	24,00	22,02	2,72	123,17	0,19	0,65
B2 H5 L	92,83	44,37	37,02	37,52	25,92	23,29	2,99	129,03	0,21	0,58
B3 H5 L	124,28	81,96	34,70	35,85	31,22	23,29	2,28	126,73	0,18	0,35
B4 H5 L	105,67	48,15	40,89	37,80	28,49	24,58	2,77	133,82	0,21	0,54
B7 H5 L	110,81	41,39	28,52	38,16	30,43	24,67	2,49	129,71	0,19	0,33
B14 H5 L	47,20	7,60	12,75	38,93	33,50	27,65	1,84	142,40	0,17	0,18
B1 H25 L	176,05	120,72	23,51	32,06	33,54	23,02	1,82	126,54	0,14	0,38
B2 H25 L	240,86	189,32	32,78	34,97	37,81	24,25	1,69	130,65	0,16	0,34
B3 H25 L	227,65	142,89	38,31	37,08	35,75	24,25	2,14	130,83	0,17	0,35
B4 H25 L	271,42	184,26	22,86	36,71	43,45	26,06	1,47	137,79	0,18	0,33
B7 H25 L	168,90	94,39	7,89	36,28	46,01	25,47	1,09	129,59	0,16	0,20
B14 H25 L	49,07	14,58	16,18	37,77	48,43	28,21	0,63	137,58	0,15	0,13

Standard deviation of volatile organic compounds produced by yeast

Echantillon vial	Diacetyl (ppb)	Pentane dione (ppb)	acetaldehyde (ppm)	ethyl acetate (ppm)	propanol (ppm)	isobutanol (ppm)	isoamyl acetate (ppm)	isoamyl alcool (ppm)	ethyl caproate (ppm)	ethyl caprylate (ppm)
B1	13,06	0,88	0,43	6,75	1,33	2,53	0,66	12,99	0,04	0,37
B2	10,79	1,75	0,43	8,95	1,99	4,50	0,89	22,66	0,05	0,53
B3	11,33	1,39	0,33	6,79	2,35	4,07	0,53	77,80	0,01	0,31
B4	10,64	1,17	0,27	6,10	1,31	2,73	0,49	14,69	0,02	0,30
B7	11,27	1,87	1,73	13,23	3,12	4,78	1,25	25,83	0,06	0,37
B14	35,31	2,94	66,85	1,32	0,67	2,30	0,10	9,14	0,02	0,14
B1 L	6,76	1,90	7,09	8,92	1,82	3,60	0,85	18,75	0,04	0,22
B2 L	11,17	0,89	7,46	7,24	2,66	4,03	0,59	23,49	0,04	0,35
B3 L	11,99	0,93	3,16	7,43	2,08	3,22	0,63	18,52	0,04	0,39
B4 L	9,12	0,79	9,60	6,07	1,83	4,00	0,56	20,87	0,03	0,23
B7 L	10,72	1,19	12,03	5,88	1,13	2,37	0,54	13,63	0,04	0,29
B14 L	12,45	2,46	2,45	1,92	1,53	1,29	0,08	6,17	0,03	0,12
B1 H5	8,27	1,18	1,05	12,87	3,69	4,08	1,07	24,11	0,06	0,34
B2 H5	19,65	2,91	1,23	9,14	3,43	5,24	0,76	27,23	0,05	0,43
B3 H5	10,15	0,77	0,69	7,36	2,26	3,50	0,57	19,79	0,04	0,31
B4 H5	14,77	2,64	1,17	10,57	4,83	5,40	0,78	33,39	0,05	0,37
B7 H5	16,09	0,42	7,92	10,43	1,74	3,67	0,80	17,70	0,06	0,28
B14 H5	18,61	31,18	19,29	2,77	4,15	2,15	0,03	17,62	0,03	0,13
B1 H25	11,02	1,29	2,78	10,38	2,96	2,88	0,60	17,00	0,04	0,17
B2 H25	13,97	1,03	3,28	7,86	1,84	2,26	0,33	14,34	0,05	0,24
B3 H25	10,49	1,01	2,87	6,72	2,42	3,63	0,25	19,52	0,04	0,14
B4 H25	17,31	1,05	3,56	6,54	2,55	3,63	0,22	21,84	0,04	0,18
B7 H25	16,46	1,18	2,56	15,55	2,44	3,71	0,48	21,31	0,06	0,12
B14 H25	11,75	94,28	10,19	2,49	6,06	2,13	0,01	13,77	0,03	0,06
B1 H5 L	6,77	6,85	5,66	10,69	2,72	3,85	0,92	21,03	0,06	0,20
B2 H5 L	13,81	7,62	2,18	8,22	2,69	3,90	0,62	22,98	0,05	0,21
B3 H5 L	62,57	63,00	0,66	6,94	9,71	2,80	0,37	13,02	0,03	0,07
B4 H5 L	13,39	11,73	7,58	5,69	1,66	2,85	0,37	17,05	0,04	0,14
B7 H5 L	19,24	10,51	14,60	4,12	2,36	1,01	0,31	3,12	0,04	0,16
B14 H5 L	27,14	2,90	5,85	5,25	3,24	1,18	0,12	11,59	0,05	0,06
B1 H25 L	4,78	8,04	4,61	9,08	2,75	3,24	0,44	16,81	0,04	0,10
B2 H25 L	13,38	19,63	14,33	9,50	3,95	4,49	0,31	24,05	0,06	0,12

Industrial sample VOCs

Echantillon vial	Diacetyl (ppb)	Pentane dione (ppb)	acetaldehyde (ppm)	ethyl acetate (ppm)	propanol (ppm)	isobutanol (ppm)	isoamyl acetate (ppm)	isoamyl alcool (ppm)	ethyl caproate (ppm)	ethyl caprylate (ppm)
T18 J+1 WH	29,37	5,02	2,14	39,33	23,84	23,7	3,59	133,95	0,37	0,42
T18 J+2 WH	21,28	2,32	2,46	42,12	26,1	25,09	3,63	141,55	0,4	0,46
T18 J+3 WH	16,98	0,82	1,67	38,47	23,19	22,52	3,33	128,09	0,35	0,4
T18 J+4 WH	14,09	1,02	1,85	39,74	23,83	23,5	3,31	134,15	0,39	0,41
T18 J+7 WH	14,6	0,48	1,13	37,55	22,79	21,41	2,92	120,13	0,29	0,42
T18 J+14 WH	4,16	1,27	1,54	41,03	27,02	25,5	2,86	144,64	0,25	0,39
T19 J+1 P45	22,37	1,07	3,08	37,98	23,32	22,65	3,25	125,05	0,38	0,38
T19 J+2 P45	19,23	0,6	2,38	39,94	23,48	23,26	3,29	128,04	0,41	0,38
T19 J+3 P45	17,55	0,55	2,34	37,24	21,22	20,82	3,08	114,24	0,39	0,38
T19 J+4 P45	18,76	1,08	2,49	38,42	21,9	21,61	3,06	118,44	0,37	0,4
T19 J+7 P45	18,47	0,38	2,28	41,3	23,24	23,21	2,9	128,15	0,37	0,4
T19 J+14 P45	15,9	0,57	1,57	39,21	22,31	21,82	2,51	120,38	0,29	0,39
T04 J+1 WH	40,8	7,4	2,75	35,19	25,78	28,7	3,32	154,79	0,26	0,33
T04 J+2 WH	24,54	3,38	2,36	37,65	25,29	27,65	3,55	149,18	0,28	0,43
T04 J+3 WH	18,87	1,09	2,07	22,51	15,43	16,81	2,36	92,26	0,19	0,28
T04 J+4 WH	21,15	2,06	2,89	31,6	21,78	23,62	2,95	129,39	0,22	0,35
T04 J+7 WH	16,78	0,83	2,38	29,45	21,21	22,69	2,54	124,61	0,19	0,3
T04 J+14 WH	16,26	0,79	1,79	42,31	28,09	29,93	2,85	159,65	0,18	0,45
T05 J+1 P45	27,86	2,7	1,97	28,7	20,79	20,29	2,61	114,9	0,22	0,32
T05 J+2 P45	23,02	1,39	2,12	35,6	22,57	22,24	3,31	125,38	0,29	0,46
T05 J+3 P45	22,16	1,45	2,05	35,1	23,27	22,65	3,18	127,97	0,27	0,4
T05 J+4 P45	21,62	1,49	2,13	34,51	21,19	20,7	3,04	117,98	0,26	0,39
T05 J+7 P45	17,61	0,5	1,71	34,26	21,02	20,96	2,82	118,2	0,23	0,38
T05 J+14 P45	18,01	0,51	1,74	39,9	23,02	23,57	2,76	132,82	0,18	0,41