

Lager Beer Stability During Storage at Room Temperature

MASTER DISSERTATION

Iolanda Cristina de Freitas Pestana

MASTER IN APPLIED BIOCHEMISTRY



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Dissertação submetida à Universidade da Madeira

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Trabalho realizado sob a Orientação de:

Professor Doutor José Carlos Antunes Marques

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The background of the page is decorated with several thin, flowing teal lines that sweep across the page from the bottom left towards the top right, creating a sense of movement and elegance.

Dedicated to my family specially to my son Lucas

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RESUMO

O trabalho apresentado nesta dissertação pretendeu avaliar a estabilidade de uma cerveja do tipo lager quando armazenada à temperatura ambiente, através da aplicação de diferentes estratégias.

Este estudo foi desenvolvido em cinco vertentes que englobaram a análise sensorial, o estudo dos parâmetros de qualidade típicos utilizados pela indústria cervejeira, o estudo da cor, a evolução do 5-hidroximetilfurfural (HMF) e a determinação do perfil volátil da cerveja.

Primeiramente, foram separadas as garrafas de cervejas representativas de um lote de produção colocando uma parte do lote a 4 °C e outra parte à temperatura ambiente (condições normais de armazenagem). As amostras foram analisadas durante 1 ano em condições normais de envelhecimento e para alguns parâmetros, tais como cor e HMF, foi também realizado o envelhecimento forçado de modo a avaliar o seu efeito.

Os dados sensoriais mostram que há um momento de viragem, no qual os provadores profissionais conseguem detetar características de envelhecimento ainda que não perceptíveis ao consumidor corrente. Relativamente aos parâmetros do controlo de qualidade da cerveja, os resultados mostram que apenas os parâmetros cor, turvação e o teor de dicetonas vicinais (VDK) sofrem alterações significativas durante este período. Através da aplicação dos métodos EBC e CIELab, para a determinação da cor da cerveja, observou-se uma evolução crescente dos parâmetros de cor à medida que a cerveja envelhece. Segundo a técnica HPLC-DAD demonstrou-se que o HMF apresenta uma tendência linear crescente ao longo do tempo, confirmando-se a sua forte relação com o processo de envelhecimento. Finalmente, os dados obtidos por SPME-CG-MS mostram que a fração volátil sofre alterações devido ao processo de envelhecimento, principalmente a partir do sétimo mês.

De um modo geral a cerveja evidenciou uma evolução química que não foi detetada pelos consumidores ao fim dos 12 meses em que este estudo foi desenvolvido.

Palavras-chave: Cerveja; Envelhecimento; Análise sensorial; Cor; HMF; Voláteis

SUMMARY

The work reported in this thesis evaluates the stability of a lager beer when stored at room temperature, through the application of different strategies.

This study was conducted in five areas that included the sensory analysis, the study of typical quality parameters used by the brewing industry, the study of colour, development of 5-hydroxymethylfurfural (HMF) and the determination of the volatile profile of the beer.

Firstly, bottles of beers representing a production batch were separated, by placing a part of the batch at 4 ° C and another part at room temperature (under normal storage conditions). The samples were analysed for 1 year of normal ageing. For some parameters, such as colour and HMF, forced ageing was also performed in order to evaluate its effect.

Sensory data show that there is a turning point in which the professional tasters are able to detect some ageing characteristics, even if not perceptible by the regular consumers. For the beers quality parameters, the results show that only the colour, turbidity and the content of vicinal diketones (VDK) undergo significant changes during this period. Through the application of CIELab and EBC methods for determining the colour of beer, there was a growing evolution of the colour parameters with the storage time. According to HPLC-DAD technique it was demonstrated that the HMF concentration has a linear tendency to increase over time, confirming its strong relationship with the ageing process. Finally, the data obtained by SPME-GC-MS showed that the volatile fraction undergoes changes due to the ageing process, especially from the seventh month on.

Generally, beer has showed a chemical evolution that was not detected by regulars consumers in the twelve months that this study was carried out.

Keywords: Beer; Ageing; Sensory analysis; Colour; HMF; Volatile.

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LIST OF ABBREVIATIONS

MBT – 3-methyl-2-butene-thiol

DMS – Dimethyl sulphide

°C – Degree Celsius

°P – Degree Plato

EBC – European Brewery Convention

GC-MS – Gas chromatography-mass spectrometry

g/L – Gram per litre

HMF – 5-Hydroxymethylfurfural

HS-SPME – Headspace solid-phase micro-extraction

HLPC-DAD – High performance liquid chromatography – diode array detection

ISO – International Organization for Standardization

KI – Kovats index

L – Litre

mg/L – Milligram per litre

min – Minutes

m/z – Mass-to-charge ratio

µg/L – Microgram per litre

nm – Nanometres

ng/L – Nanogram per litre

NIST – National Institute of Standards and Technology

PCA – Principal Component Analysis

RSD – Relative standard deviation

SBSE – Stir bar sorptive extraction

UV – Ultraviolet

VDK – Vicinal diketones

Vis – Visible



PART 1

INTRODUCTION

PART 1 – INTRODUCTION

1.1. Brief history of beer production

Beer is defined as a fermented beverage prepared from malted grains (generally barley), hops, yeast and water(1).The art of brewing is as old as civilization and the information that we have today, regarding old brewing techniques, is originated from the work developed by archaeologists in Egypt and Mesopotamia (2).

In ancient Egypt, beer was particularly important in regions where the cultivation of the vine could not be made. At that time, all classes drank beer, from the Pharaoh down to his subjects. A quite large number of different beers were already produced at this time. “Dark beer” is one of the most common examples. In the beginning of the 20th century archaeologists generally began to consider that Egypt was not the oldest beer-producing country in the world. In fact, archaeological studies carried out in the Mesopotamian region found documentation containing reference to beer production (beer recipe). Also in Mesopotamia beer was a popular drink and was consumed by all social classes, including women, and drinking was considered a social activity. Beer was also associated with mythology, religion and medicine (2).

The interest in beer expanded along the centuries mainly throughout the Middle East. The Egyptians followed the Babylonian’s footsteps, and using scientific methods they became such famous brewers. It is also thought that the Egyptians taught the brewing art to the Greeks and to the Romans. Although considered less important than wine, beer production evolved during the period of the Roman Empire, being mainly brewed in the Roman Empire’s outer areas by the Saxons, Celtics, and Nordic and Germanic tribes. During the Middle Ages, the Christian monks were the guardians of the art of beer production, including literature and science. They have optimized the process and have institutionalized the use of hops as both a flavouring agent and preservative (2).

It was only in the 12th century that the interest for the brewing industry developed beyond monasteries walls, with the church also conceding the rights of brewing to private breweries. From the 16th century onwards, brewing expanded throughout the globe, becoming a global beverage.

In ancient times the brewing process was characterized by the experience and tradition, but in 18th century with the Industrial Revolution, the brewing process was dominated by several scientific and technological advances, starting with the introduction of the steam engine, passing by the development of the cold production process and culminating with the work of Pasteur on fermentation and pasteurization. It was in 1881 that a scientist named Emil Hansen isolated and classified the brewery yeasts into top-cropping strains (*Saccharomyces cerevisiae*) and bottom-cropping strains (*Saccharomyces uvarum*, also called

Saccharomyces carlsbergensis). After the Industrial Revolution, the traditional methods have been replaced; nowadays beer production is automated and performed in large factories. The brewing industry has become an enormous worldwide business, with brewers' main concerns being concentrated on the beer quality control.

Malt started being imported to Madeira Island at the beginning of the XIX century, for the production of beer and ginger-beer (beer with ginger, sugar, cream of tartar and lemons) at small rudimentary breweries. At the same period beer was also imported. Madeira brewing industry began to be regulated in 1854 and was driven mainly by British, namely by Henry Price Miles (3). His brewery, called the Atlantic brewery, was founded in 1872 with 10 workers. At the end of the XIX century, the consumption of beer grew considerably and therefore the industry itself, with the entrance of new brewers, namely the Araújo family which owned a small brewery. In 1912 Henry Alfred Miles and Charles Vaughan Miles founded the H.P. Miles & Ca Brewery. Ten years later, the Araújo family and Freitas & Passos's company associated themselves, creating a new association called Araújo, Tavares & Passos which began to producing beer only in 1924.

Empresa de Cervejas da Madeira (ECM) was founded in 1934 by the merger of Leacock & Company with H.P.Miles and Araújo, Tavares e Passos Lda.. The purchase of modern and sophisticated equipment was one of the company's strategies. The production continued and in the following years the company purchased 80% of the Azorean brewery João Melo Abreu Lda.. Since then there were many developments: the acquisition of new bottling lines, the association with others companies like Sociedade Central de Cervejas, Lda., and the acquisition of new distribution centres. The expansion of the brewery continued and a new industrial site was required. In 1993 the foundations of the new brewery were placed in the industrial park of Socorridos. The brewery size together with the modern equipment makes ECM the largest industrial unit in Madeira Island. ECM, a regional company of production and distribution of its own brands of beers, soft drinks and water, also represents brands in other categories (spirits, wines, juices and nectars, waters, milks, oils, vinegars and sauces).

Up to 2013, ECM products have been awarded 118 Monde Selection medals. The company's management system is certified according to the international standards ISO 9001 and ISO 14001 – quality and environment.

Currently, ECM belongs to the Pestana Group (a Portuguese hotel group) and has an industrial experience of more than 140 years.

1.2. The brewing process

The brewing process involves several steps. The main ingredients from which most beers are made are malt (usually barley), water, hops and yeast. The characteristics of beer are derived from these raw materials and from two processes, which have been used in beer production for thousands of years – malting and brewing.

1.2.1. Malting

The malting process, as described in Figure 1.1, consists of three global steps.

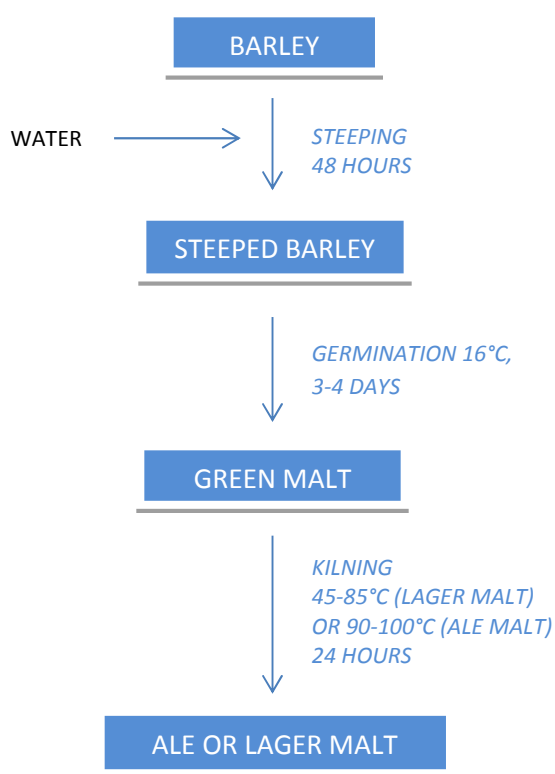


FIGURE 1.1– Simplified diagram of the malting process (adapted from (1)).

The first step consists on the hydration of the grains of barley. This is achieved by steeping the grains of barley in water, raising the water content from 12 to around 45%, through an osmotic process. The increase of water promotes the respiration in the embryo of the grains and the hydration of the starch in the endosperm, beginning the germination step. With the increase of the embryo activity, are produced gibberellins, natural plant hormones. These plant hormones will stimulate the aleurone layer to produce several hydrolytic enzymes, for example amylolytic, proteolytic and cellulytic enzymes. These enzymes move

into the endosperm and initiate the process of breaking down the cell walls, promoting the degradation of proteins, starch and lipids, providing nutrients for the new plant (1). When germination is satisfactorily advanced it is stopped by kilning. At this stage, the barley grains known as green malt (green in the sense of immature, not green in colour) are kilned, this means that the grains are dried and cooked, or cured, with warm to hot air. The higher the temperature and the duration of heat exposure the darker the malt becomes. The step of preparing the malt has a great influence on the fermentation and therefore is crucial for the final quality of beer (1, 4).

1.2.2. Brewing

The brewing process initially converts the malt starch into soluble sugars and then these sugars are fermented by the yeast into alcohol. At the same time proteins are broken into amino acids, which may be used by the yeast as nutrients to produce characteristic flavour compounds. Generally, the brewing process can be described as represented in Figure 1.2.

a) Mashing

In this step the malt is crushed in a mill, exposing the starch of the malt endosperm necessary for the production process. The milling will also reduce the particle size and increase the surface area, which facilitates the absorption of water and consequently increases the starch hydrolysis (1). The resulting crushed malt, known as grist, is mixed with hot water, during 1 hour at about 65 °C. At this temperature malt starch gelatinizes, becoming more vulnerable to enzyme attack. Sometimes, the grist may contain other cereals (adjuncts) in order to confer singular properties to the beer. Cereal starch contains approximately 75% of amylopectin and 25% of amylose (1). During mashing, malt amylolytic enzymes reduce the starch into a sweet liquid syrup called wort, full of fermentable sugars, mainly glucose and maltose, but also with small branched dextrins which are not fermented. Additionally, the wort contains soluble proteins, polypeptides and amino acids (1, 5).

b) Lautering

The step of lautering consists in separating the sugar solution from the spent grains. This is achieved using a vessel with a perforated base plate, which allows the wort be transferred into the next vessel, leaving behind the malt insoluble remains. In most breweries, the spent grains are sold as cattle food (1, 6).

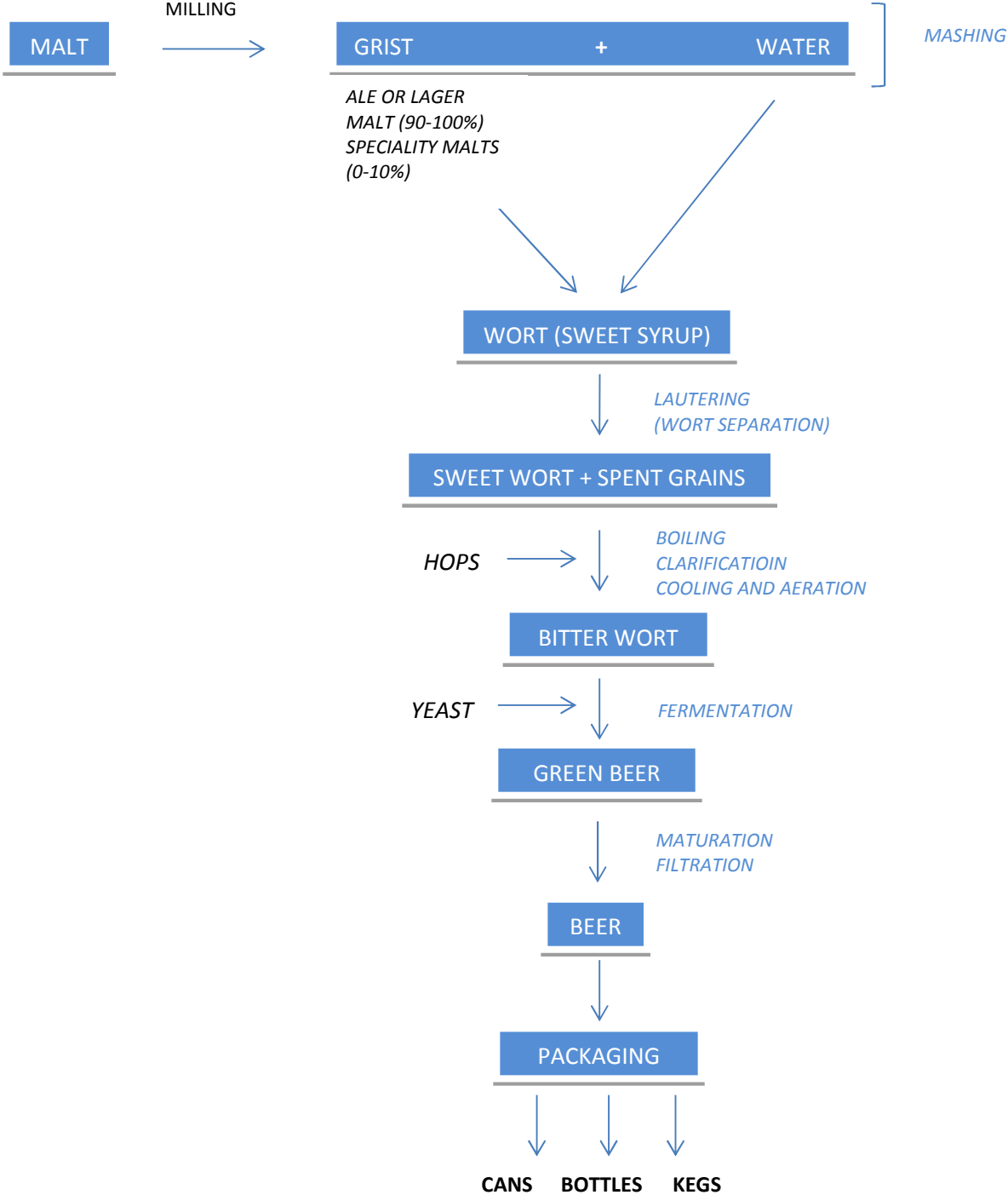


FIGURE 1.2– Simplified diagram of the brewing process (adapted from (1)).

c) Boiling

In the kettle, the wort is subjected to vigorous boiling and hops or hop extracts are added. The boiling step has three functions: sterilization of wort, coagulation of the soluble proteins with formation of insoluble precipitates (called trub) and extraction of α -acids from hops and consequent isomerization into iso- α -acids. Apart from that, the boiling concentrates wort to a certain degree (depending on the rate of evaporation acceptable, ranging from 4 to 12%) (1, 6). During this process some of the volatile components present in essential hop oils may be lost by evaporation.

At this stage, Maillard reactions between reducing sugars and primary amines can occur due to the elevated temperatures employed, contributing to the colour of wort (1).

d) Clarification

Before fermentation, the trub and the hop material are removed from the wort by the whirlpool technique. This practice consists in pumping the wort in a cylindrical vessel where centripetal forces collect the trub, leaving the clarified wort at the surface. Before being sold for cattle food, the removed trub is frequently mixed with the spent grains. (1, 6).

e) Wort cooling and aeration

The resulting wort is then cooled to fermentation temperature using a heat exchanger. Although the fermentation process is anaerobic, wort aeration and oxygen levels control, particularly during the early stages of fermentation, are extremely important as both greatly affect yeast growth and, consequently, beer flavours (5, 6).

f) Fermentation

Fermentation is initiated by the yeast addition to wort (pitching). Yeast is pitched directly into the cooled wort in the fermentation vat, or else released in-line from the heat exchanger to the fermenter (5).

There are two main types of fermentations: ale and lager. Briefly, ale fermentation uses *Saccharomyces cerevisiae*, top-cropping yeast at 14 to 17 °C. This kind of fermentation is fast and exothermic, thus cooling must be applied to maintain a constant temperature. On the other hand, lager fermentation uses *Saccharomyces uvarum*, a bottom-cropping yeast form which ferments at lower temperatures, from 8 to 13 °C, usually for longer time periods (5).

With both kinds of fermentation, yeasts use sugars and proteins to produce alcohol, carbon dioxide, new yeast cells and flavour compounds (5). The produced flavour-active compounds are mainly higher alcohols and esters, however the different profile of each beer depends on the yeast strain. The higher alcohols and esters contribute positively to the flavour of beer, when present in concentrations below their odour thresholds, as for example ethyl acetate (fruity, solvent-like), isoamyl acetate (isopentyl acetate) (pear drops), isobutyl acetate (banana), ethyl caproate (ethyl hexanoate) (apple) and 2-phenylethyl acetate (honey, fruity, flowery). In addition to pleasant flavours, some unpleasant flavours can also be formed during the fermentation process. For example, diacetyl (a rancid butter flavour produced by the yeast from pyruvate) and some sulphur compounds as: SO_2 , H_2S , and dimethyl sulphide. It is convenient that these compounds should be removed or reduced, in the fermentation step or in the following steps of the brewing process. Nevertheless, the CO_2 purging during fermentation may be sufficient to reduce these unpleasant compounds to acceptable levels (5, 7, 8).

Once the yeast has fermented the available sugars, the metabolism slows down and yeast cells start to flocculate together to form clumps. When the fermentation ceases, the vessel is cooled to 0 °C, causing the drop of the clumps to the bottom. The yeast bulk is then separated from the fresh beer by a physical process known as racking (1).

g) Maturation

The young beer, referred to as green beer, still contains undesirable flavour compounds which are removed by cold conditioning or cold storage. In this step a secondary fermentation occurs, with the remaining yeast and remaining sugars to produce carbon dioxide, enabling the carbonating of beer, purging out off-flavour compounds. Moreover, yeast can also chemically remove certain undesired flavour-active components, namely by catalysing the reduction of vicinal diketones (VDK's), such as diacetyl and 2, 3-pentanedione into new compounds with no relevant flavour attributes (1).

h) Filtration

Following conditioning, the beer is then chilled and filtered after being submitted to centrifugation if it is needed remove the remaining yeast (5).

i) Packaging

Before being packaged in bottles, cans or kegs, the beer may suffer some adjustments, specifically in terms of CO₂ levels, colour, bitterness and alcohol strength (5). The packaging has to be extremely efficient, preventing any entrance of oxygen in the system in order to avoid premature ageing.

Generally speaking, malting and brewing means the conversion of the barley starch into alcohol. Consequently, brewers are interested in achieving this with a maximum efficiency, i.e., in terms of highest alcohol yield per unit of starch, but also insist on the consistency of all other attributes of their product, especially foam, clarity, colour, and, of course, flavour (6).

1.3. Beer Quality

Beer visual attributes as well as mouth sensations are equally important to define consumer choice. Certainly that beer drinking can be as visually pleasing as it is thirst satisfying. Some quality attributes of beer can also be perceived by the eye and can undoubtedly influence our perception of flavour (6).

When a consumer has a glass of beer, he immediately looks for various parameters that can demonstrate the quality of the beer. Those parameters are the beer foam, colour and clarity. After the first impact the consumer drinks the beer and then experiences its flavour. Indeed, beer quality is mostly determined firstly by the visual elements and then by its flavour (1).

1.3.1. Foam

Foam may be defined as colloidal dispersions of gas within a continuous liquid phase at high gas volume fractions. Beer foam is widely recognized as a protein stabilised foam system, in which the main foam stabilising material is in the polypeptide form, derived from the solubilisation and proteolytic degradation of cereal proteins during the malting and brewing processes (9).

The presence and persistence of a layer of foam in a glass of beer, together with adhesion of foam to the glass during consumption, are considered highly desirable attributes in the consumer expectation and further evaluation of beer quality (9).

1.3.2. Colour

Colour is a visual quality parameter of beer that is critical for consumers, because enables its immediate classification in lager, ale and stout (dark beer) (Figure 1.3). The used raw materials (water, malt) and the existing brewhouse equipment have a substantial influence on the colour (1, 5).



FIGURE 1.3 - Some colours of beer (adapted from (10)).

In general, the colour of beer is developed during malting and wort production through Maillard reactions, polyphenolic oxidation and metal interactions (iron and copper traces can stimulate oxidation of polyphenols). Also, riboflavin (vitamin B2 present in yeast cells) can also contribute to the colour of the beer (1).

On the European Brewery Convention (EBC) scale, a pale lager scores around 4 units, pale ale 20 and a dark stout around 138 EBC units. The tonality of colour should be brilliant and correspond to the beer type. Changes in colour may result from the use of unsuitable raw materials or water quality, errors during wort production, insufficient tub separation or slow fermentation (10).

1.3.3. Clarity

Beer clarity is readily appraised by the consumer. Though for most beers cloudiness is undesirable, there are a few beers that are intended to be turbid. The measure of beer haze is made through the assessment of the scattered light by particles. The haze is due to the polymerization of polyphenols and their interaction with specific proteins. Also during beer storage, interactions between phenolic polymers and proteins may occur, causing the formation of insoluble complexes and haze (4, 6, 11).

The clarity of fresh beer is dependent of an effective filtration. However, previous processes, like the use of stabilizing agents, may reduce the haze formation during the shelf-life of the beers (1).

1.3.4. Flavour

The flavour of beer results from a wide range of volatile substances, including esters, sulphur containing compounds and other compounds derived from hop essential oils. Furthermore, ethanol also has a crucial role in beer flavour since on the one hand it provides a warming effect; on the other it seems to influence the contribution that other molecules to beer character. Even carbon dioxide has an important role in its character (6).

Beer flavour is in constant evolution. The taste and aroma of a beer suffer changes during the all brewing process, until the moment that the beer is packaged. Moreover, during its shelf-life the beer flavour undergoes several changes (6).

a) Taste

The main tastes that are firstly detected by the tongue are: sweet, salty, sour and bitter. There is also another taste, called umami, defined as a pleasant savoury taste, usually associated with the monosodium glutamate sensation.

The beer sweetness is related to the existence of residual carbohydrates (remaining after fermentation) in the final product.

The salty taste of beer is due to the presence of inorganic anions and cations. These ions come from the water and raw materials, particularly malt.

The sourness of beer is a consequence of the presence of acids in the final beer. The final levels of organic acids in beer, which derived from yeast, depend on the fermentation vigour; more acids are released with faster fermentations.

Bitterness is a sensory attribute well recognised by most consumers. The compounds responsible for this attribute are the iso- α -acids, which derived from the isomerisation of the corresponding α -acids. (1).

b) Aroma

Beer components are often considered to be tasted in the mouth but in fact some are detected through nose perception. This misconception arises from the connectivity of the tongue, throat and the nasal passages. Beer aroma therefore comes not only from sniffing, but also by the volatile evaporation when beer is taken into the mouth. In fact, due to the chemical complexity of beer, aroma is not characterized by a few well-defined components. Instead, many compounds contribute to beer aroma, both individually and in a synergistic or antagonistic sense (1).

The various classes of compounds that contribute to the aroma of beer are briefly described below:

c) Esters

Esters are produced by yeast during the anaerobic metabolism of sugars and they are responsible for the fruity-like aromas. They form the largest group of flavour compounds in beer and are considered positive flavours for the aroma of beer. The most significant esters found in beer and their flavour descriptors are described in Table 1.1. (7, 10).

TABLE 1.1 – Significant flavour-active esters in beer (adapted from (1)).

Ester	Flavour descriptors	Approximate flavour threshold (mg/L) ^a
Ethyl acetate	Solvent, fruity	30
Butyl acetate	Banana, Sweet	7.5
Isoamyl acetate	Banana, apple	1
Ethyl valerate	Papaya	1
Isoamyl propionate	Pineapple, aniseed	1
Ethyl nicotinate	Medicinal	6
Phenylethyl acetate	Roses, honey	4
Methyl caprate	Coconut	1
Octyl caproate	Orange peel	5
Isoamyl caprate	Tropical fruits	3

^a The flavour threshold is the concentration of a substance that must be present in a beer to be detected.

Esters are products of yeast metabolism and for its synthesis are necessary two substrates, alcohol and acyl-coenzyme A and also a catalyser (ester synthase or acyl transferase). The levels of beer esters are influenced by various factors, such as the density of the wort and the amount of oxygen to which yeast is exposed. However, yeast strain by itself is perhaps the major factor affecting the extent of ester production, with some strains readily generating certain esters than others (1, 8).

d) Alcohols

Like esters, also alcohols can affect the beer flavour. The main product of the fermentation promoted by yeast activity is ethyl alcohol (ethanol). Even if not aromatically active, ethanol contributes directly to the beer flavour since it imparts a warming and alcoholic character. Moreover, ethanol can also have influence in the perception of other beer components (1).

However, yeast also produces higher alcohols (i.e. of higher molecular weight than ethanol) in much lower quantities, but yet participates in the flavour of beer (see Table 1.2) (1, 6). Once again, the yeast strains are also important for the production of higher alcohols, with ale strains producing greater amounts than lager strains. In addition, favourable conditions to yeast growth, such as aeration or oxygenation, also promote the formation of higher alcohols (1).

TABLE 1.2– Some of the alcohols commonly found in beers (adapted from (1)).

Alcohol	Flavour descriptors	Flavour threshold (mg/l) ^a
Methanol	Alcoholic, solvent	10000
Ethanol	Alcoholic, strong	14000
1-Propanol	Alcoholic	700
2-Propanol	Alcoholic	1500
2-Methylbutanol	Alcoholic, vinous, banana	65
3-Methylbutanol	Alcoholic, vinous, banana	70
2-Phenylethanol	Roses, bitter, perfumed	125
1-Octen-3-ol	Fresh-cut grass perfume	0.2
2-Decanol	Coconut, aniseed	0.015
Glycerol	Sweetish, viscous	-
Tyrosol	Bitter, chemical	200

^a The flavour threshold is the minimum concentration that must be present in a beer to be detected.

e) Carbonyl compounds

Other abundant class in beers are carbonyl compounds, such as aldehydes and vicinal diketones (VDK's). Their concentrations are also influenced by yeast metabolism during fermentation. As a group, these, generally contribute to beer flavour with negative notes (4).

The most abundant aldehyde in beer is acetaldehyde, which is formed during fermentation, afterwards its concentration falls to lower levels.

In some circumstances, acetaldehyde can be accumulated during fermentation in concentrations above its flavour threshold of 25 mg/L, giving an unpleasant grassy or green apple flavour and aroma to the beer (4).

Regarding vicinal diketones (VDK's), the most abundant is 2, 3-butanedione (diacetyl), but 2,3-pentanedione is also produced in significant quantities during the fermentation of lager beer. These by-products usually impart a negative impact to beer flavour. Both compounds have strong butterscotch or toffee aromas as well as a similar taste. When present in lagers at concentrations higher than their flavour thresholds (around 0.15 mg/L and 0.9 mg/L respectively) they cause an unpleasant flavour defect (4).

f) Sulphur Compounds

Some of the most characteristic flavours of beer are due to sulphur compounds. Several sulphur compounds can be found in beer, each one with a different contribution to the flavour (1).

Hydrogen sulphide (flavour threshold of 5 µg/L) is one of the most common examples and is especially abundant in many ale beers, giving a rotten egg attribute, which is not considered unpleasant, instead, is in fact characteristic of ale beers (1, 6).

Other major sulphur-flavour compound is dimethyl sulphide (DMS), which gives a sweet corn flavour to beer and has a flavour threshold of 7.5 µg/L (1). This compound is commonly found in lager beers, which indeed tend to have a more complex sulphur character.

Another important sulphur compound is 3-methyl-2-butene-thiol (MBT), which is a highly flavour-active molecule, with a flavour threshold of about 10 ng/L, which usually imparts a skunky odour to beer. It is normally formed by the degradation of the bitter iso- α -acids in the presence of sunlight or electric light (1, 4).

Other sulphur compounds are also present in beer, such as thioesters, sulphides, disulphides, polysulphides, thiols and sulphur-containing heterocycles (1).

g) Other Compounds

There are other contributors to the beer flavour, like the unique hoppy aroma imparted by linalool. The linalool flavour threshold in beer is 27-80 µg/L.

Other important compound that has not yet been considered is 4-vinylguaiacol, which is produced by the yeast-induced decarboxylation of ferulic acid. With a flavour threshold of 300 µg/L and an occurrence in beer from 50 to 550 µg/L, this compound can actually change beer flavour due to its potent smoky, clove-like scent. Furthermore, the presence of this compound can also point out that the beer is contaminated with wild yeast strains (1).

1.3.5. Sensorial analysis

The evaluation of the sensory characteristics of beer has been the subject of many scientific investigations. Indeed, several scientific papers have been published on this subject demonstrating this fact (12-14). The efforts developed by the researchers in the last decades ended in the construction of the beer flavour wheel, which is presented in Figure 1.4 (1).

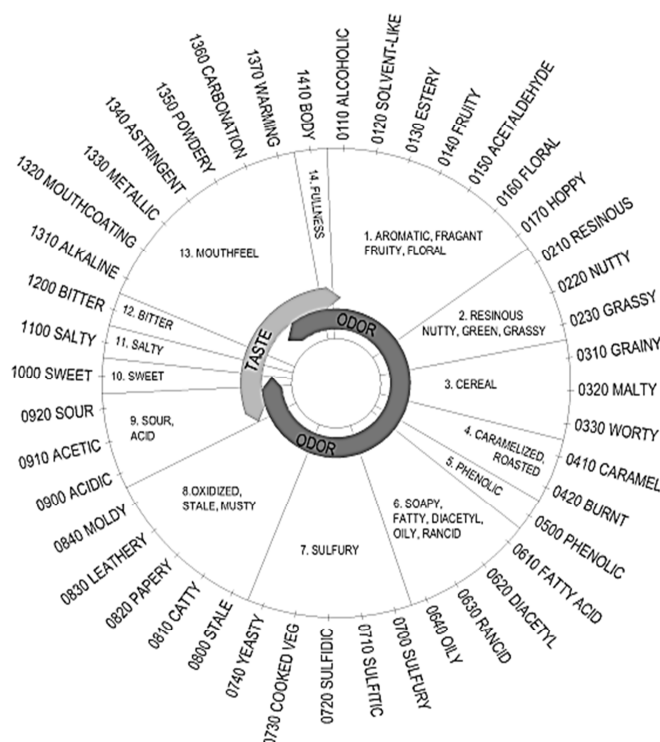


FIGURE 1.4 – Beer flavour wheel showing the class terms and first-tier terms. (Adapted from (15))

The system consists of 14 classes with general names to indicate the area in which a certain flavour should be sought. Only those terms carrying a four digit number are descriptors. Some classes have a broader term (e.g. 0700 sulfury) that serves as a common descriptor for all terms in the class (15).

1.4. Beer ageing: Scientific overview

The organoleptic stability of beer during its shelf-life has been one of the main concerns for brewers, because some quality aspects may vary during the storage time. Beer quality is mostly determined by its foam, clarity, colour and flavour. In the past, the main quality problems of the beer were the presence of haze and the growth of spoilage microorganisms. However, nowadays with the progress in brewing chemistry and technology, these problems are generally under control. Currently, the most important parameter regarding to the beer quality is its flavour, therefore, most of the concern has turned to aspects which affect the changes in aroma and taste. Nevertheless, some consumers like the taste and aroma of an aged beer. Actually, a study developed by Stephenson et al. (16) showed that the most important factor determining the appreciation of a beer were the expectations of the consumers in recognizing the flavour of the particular brand that they generally drink. In this sense, in order to encounter the consumer expectations, the flavour of a specific beer from a particular brand should be always the same. However, the

expected flavour is normally the flavour of a fresh beer, but since bottled beer is constantly changing as a result of beer ageing, such flavour may change and the expected flavour is lost. This should be considered as the most important reason for beer staling being usually seen as a unpleasant phenomenon (16).

The deterioration of beer flavour can be originated from formation or/and degradation reactions. From the formation reactions new molecules are produced, which at concentrations above their flavour threshold generates new detectable effects. With respect to degradation reaction, the existing compounds can be reduced to concentrations below its flavour threshold, causing the loss of the initial flavours typical of fresh beer. In addition, interactions between different aroma volatiles can also occur, leading to an increase or decrease of the flavouring effect of some compounds (17).

Several studies have focused their attention on the chemical aspects of beer during storage and different approaches have been tested. For example, Santos et al. (18) studied the beer ageing determining E-2-nonenal by high-performance liquid chromatography (HPLC) with UV detection, since this aldehyde has raised considerable interest for the brewery industry due to its paper/cardboard unpleasant flavour. Another study developed by Oñate-jaén et al. (19) measured the antioxidant capacity of beers, based on an organic solvent extraction followed by molecular absorption spectrophotometry UV–VIS. Since many antioxidants can be lost as a consequence of food processing as well as during storage, this methodology could be used to follow beer ageing.

As aforementioned, different chromatographic techniques have been used to perform these studies. HPLC but mainly gas chromatography (GC) has shown a wide range of applications in beer analysis namely in beer ageing studies. For instance, Pinho et al. (20) optimized a GC method to analyse the volatile fraction to be used in the study of the volatile profile evolution of a particular beer during ageing.

Furthermore, Vanderhaegen et al. (21) reported a study that also uses GC data, but this time to analyse the ageing characteristics of different beer types during one year of storage, through the determination of the sensory profile and chemical staling markers.

Two years later, Saison et al. (14) carried out a study about the quantification of staling compounds in order to get insight in the flavour changes that occur once again during beer ageing. Headspace solid-phase microextraction (HS-SPME) combined with GC and mass spectrometry detection was used for the quantification of 32 volatiles. In the same year, the same authors investigated the influence of 26 staling compounds in the aged flavour of a Belgian lager beer, through the study of their flavour thresholds. They concluded that encountered flavour thresholds were regularly substantially lower than those previously

reported. Moreover, they also observed the masking effect of isoamyl acetate in addition to various interactions between flavour compounds, which can significantly affect their flavour activity, even when present at concentrations below its threshold. On the other hand, they also matched the cardboard flavour to the aldehyde (E)-2-nonenal. Finally, the same study confirmed methional, 3-methylbutanal, 2-furfuryl ethyl ether, β -damascenone and acetaldehyde as the main contributors to the aged flavour and also, but with a lower contribution, (E,E)-2,4-decadienal, phenylacetaldehyde, 2-methylpropanal, diacetyl and 5-hydroxymethylfurfural.

An exploratory analysis of the volatile profile of beers by HS-SPME-GC was also conducted by Silva et al. (22), using Kohonen Neural Network maps, which proved to be very efficient for the study of the volatile profile of complex matrices that, consequently, generates complex data practically impossible to be analysed by traditional mathematical methods.

Stir bar sorptive extraction (SBSE) coupled with GC-MS was also used by Tsuji & Mizuno (23) to compare the volatile compositions of beers during storage.

Recently, another study proposed a chromatographic methodology based on GC-MS data allied to chemometrics (multivariate analysis) for monitoring the chemical variations occurring in a lager beer during ageing (24).

In general, over the years, many studies were developed based on the chemical aspects of beer ageing, but still nowadays beer ageing remains difficult to control, due to the phenomenon complexity. With the increasing export of beer, caused by market globalisation, shelf-life problems are still an issue of great importance for breweries. In this sense the present study aims to evaluate the changes that occur, due to the natural ageing process, during beer storage, through the determination of standard quality parameters and characterization of the volatile fraction of a lager beer.



PART 2

EXPERIMENTAL

PART 2 - EXPERIMENTAL

2.1. Beer samples

All samples were lager beers from the same brand, produced on the same production line and date. The entire batch was kindly donated by the Empresa de Cervejas da Madeira – Sociedade Unipessoal, Lda. A total of five hundred amber glass bottles of 330 mL were separated. A hundred bottles were stored at 4 °C (fresh beer), while the others were kept in a storage area at room temperature, both during 12 months (aged beer). Beer bottles were analysed every month during this period.

To induce beer forced ageing, accordingly to an internal procedure of the brewery, twenty lager beer samples were stored in an oven at 37 ± 1 °C, in the dark, during 21, 42 and 84 consecutive days, corresponding to 6, 12 and 24 months, respectively.

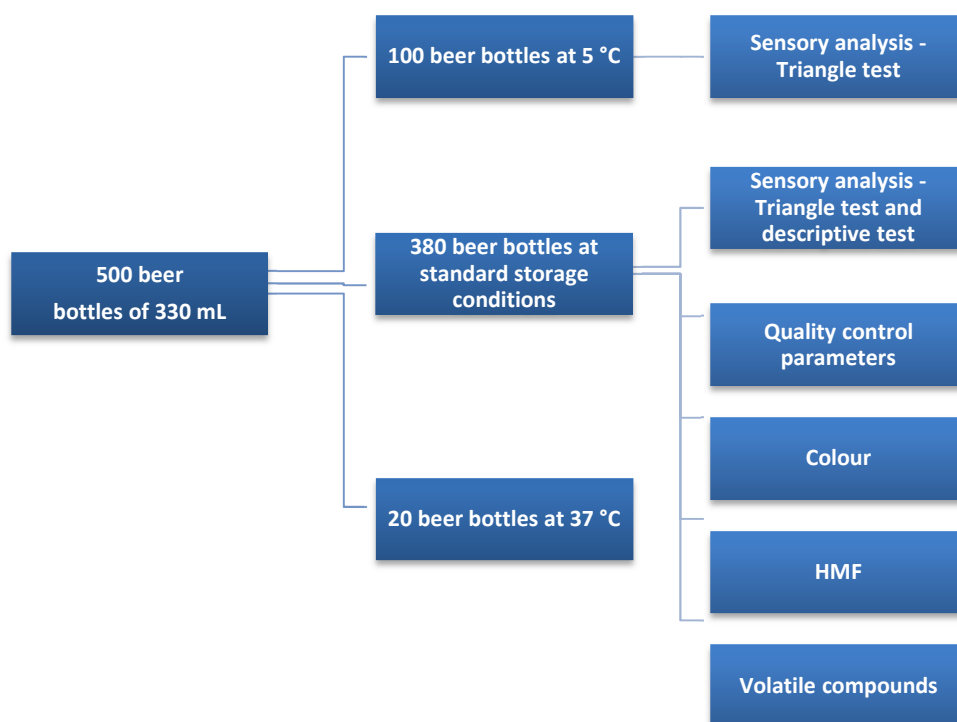


FIGURE 2.1 – Scheme of the beer samples storage used in this study

2.2. Sensory analysis

A systematic examination of the beer's sensory attributes was made monthly by two judge groups during the 12 months. One formed by 10 trained judges, composed by the sensory panel of the Empresa de Cervejas da Madeira, which has good sensory ability and experience, especially on the identification of off-flavours. The other was a group of common consumers (up to 13), which were not trained for this kind of tests.

Before performing any sensory test, all bottles under evaluation were kept at 4 °C for 24 hours. Then the bottles were kept at room temperature for about 15 minutes before the beginning of the tests.

The sensory analysis took place in an adequately isolated room. All beers were evaluated in random order through blind analysis (no subject had previous access to the beer under evaluation).

2.2.1. Triangle test

Twelve beer bottles of each group, aged naturally and fresh, were stored at 5 °C during the 24 hours before the beginning of this test. The aim of this step was to keep all samples at the same temperature, so that judges could not distinguish the beers by their temperature.

A triangle test was used to determine if the samples were significantly different. This method may be applied to any two samples of beer and is recommended when the nature of the difference between samples is unknown. Also, it can be used when the difference may exist in a single or several attributes (25).

Firstly, the group of consumers was familiarized with the procedure of this test and it was considered that they all had the same level of training or experience. All judges were presented to a set of three coded samples, two of which were identical. They were asked to identify the different sample (see Figure 2.2).

Formulário de Prova

Teste Triangular

Provador Data

1 - Tem à sua frente três amostras, duas destas amostras são iguais e uma delas é diferente.

Procedimento:

- Anote os códigos e prove as amostras apresentadas à sua frente da esquerda para a direita.
- Depois compare mentalmente as amostras e tente identificar a amostra diferente.
- Faça um círculo em volta do código da amostra diferente.

Amostras:

<div style="border: 1px solid black; width: 50px; height: 20px; margin: 0 auto;"></div> 1ª Amostra	<div style="border: 1px solid black; width: 50px; height: 20px; margin: 0 auto;"></div> 2ª Amostra	<div style="border: 1px solid black; width: 50px; height: 20px; margin: 0 auto;"></div> 3ª Amostra
--	--	--

Obrigada pela sua colaboração.

FIGURE 2.2 – Score sheet used for the triangle tests.

This is a forced choice method because if the judges cannot identify a difference, they must make a guess. The number of correct responses is added up and compared with statistical tables of significance.

The goal of this test was to identify if there was any noticeable change in the flavour of the beer stored at standard ambient conditions (aged beer), when compared with the beer that was maintained at 5 °C, since the first day of bottling (fresh beer).

2.2.2. Descriptive test

For the descriptive analysis, four aged beer bottles were maintained at 5 °C during 24 hours. The descriptive analysis was performed only with the group of the trained judges, in order to obtain a systematic description of the flavour of the beer samples that have been stored at standard ambient conditions.

The judges were asked to comment on the beer quality based on a test with 23 attributes. The degree of beer ageing was evaluated using a three-point sensory score scale, ranked from 0 (attribute not present) to 3 (very strong attribute), sub-divided by a 1.0 scale, with 1 and 2 corresponding to a weak and moderated attribute, respectively (see Figure 2.3)

 Empresa de Cervejas da Madeira ANÁLISE SENSORIAL

Formulário de Prova
Teste Descritivo

Provedor Data

Amostra n.º Avaliação específica: 0 | 1 | 2 | 3 0 | 1 | 2 | 3 0 | 1 | 2 | 3 0 | 1 | 2 | 3

Aspecto	0	1	2	3
Espuma				
Turvação				
Corpo				
Cheia				
Diluída, aguada				
Carbonatação				
Sabor				
Isoamilacetato				
Etilhexanoato				
Geraniol				
Acetaldeído				
Grão/cereal				
Papel Cartão				
DMS				
Diacetil				
Cerveja ao sol				
Isovalérico				
Caramelo				
Torrado, fumo				
Fenólico				
Ácido				
Acético				
Doce				
Salgado				
Amargo				

Legenda: 0 = atributo ausente 1 = atributo fraco 2 = atributo moderado 3 = atributo forte

Comentários:

Obrigada pela sua colaboração.

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FIGURE 2.3 – Score sheet used for the descriptive tests.

2.3. Analysis of the brewery's standard quality parameters

Some quality parameters were determined monthly for the aged beer, such as pH, carbon dioxide, colour, haze, colloidal stability, foam stability, bitterness, alcohol, original extract, apparent extract, real extract, acetaldehyde, vicinal diketones (VDK), lactic bacteria and wild yeasts. The procedures for these determinations were based on standard methodologies used in the brewery and are briefly described below.

2.3.1. pH

The pH value was measured by potentiometry using the 691 pH Meter apparatus from Metrohm (Switzerland), duly calibrated before the analysis, using the pH 4.0 and 7.0 standards.

2.3.2. Carbon dioxide

The determination of the dissolved carbon dioxide concentration in bottled beer was made by a pressure method. This method is based on Henry's law, which states that at a given temperature, the quantity of a gas dissolved in a liquid is proportional to the partial pressure of the gas above the liquid (26).

The partial pressure of the carbon dioxide gas above the liquid and the temperature of the liquid were measured in equilibrium. Using these two measurements, the concentration of the dissolved carbon dioxide in the beer sample was read in volumes from a CO₂ chart (at 0 °C and 1.013 bar = 1 atm).

2.3.3. Colour

The colour of the beer was determined visually by comparison with standards, using the apparatus Lovibond-2000, and the result being expressed in EBC units. The EBC colour scale was developed by the Institute of Brewing and the European Brewing Convention and is a recognised method for colour grading of beers, malts and caramel solutions (27).

2.3.4. Total oxygen

The oxygen content present in the beverage after the filling is an aspect of major importance for preserving the quality of the beer. Beer is one of the beverages most sensitive to oxygen. The oxygen pick-up in beer promotes the development of ageing processes or a downsized content of antioxidants, so that the beers taste stability may be reduced, and its bitterness may become more pronounced. Continuous cloudiness as well as changes in colour may also occur (10).

The total oxygen in beer was measured only at the beginning of this study, in the fresh bottled beer, with an oxygen analyser (Digox 6, Dr.Thiedig). The principle of this measure is based on an electrochemical reduction of the dissolved oxygen at the measuring electrode.

Immediately after filling the bottles, they were placed on a shaker for 5 minutes at 40 rpm and then, allowed to stand for 5 minutes. To measure the total oxygen, the flow rate was adjusted to 10 L/h. The temperature of the beer was also recorded. The total oxygen was then calculated using the formula: $O_2(\text{mg/L}) = O_{2 \text{ Digox}} \times Z$. The value of Z is obtained from the formula

$$Z = 1 + HS \cdot \frac{3777 \cdot (4.15 \times 10^{-7} \cdot T^2 + 2.0 \times 10^{-4} \cdot T - 0.0701)}{T} \text{ and } HS = \frac{(f-e) - \frac{n-e}{\text{specific gravity}} \times 100}{\frac{(n-e)}{\text{specific gravity}}}, \text{ where } Z \text{ is the correlation}$$

between the oxygen content of a liquid phase and of a gas phase at equilibrium, HS is the headspace ratio, T is the temperature of the sample in degrees kelvin, f is the weight of the bottle brimful of water, e is the weight of the empty bottle and n is the weight of the bottle full of beer (28).

2.3.5. Haze

This parameter was measured using a haze meter calibrated with a formazin standard solution. Approximately 250 mL of beer were placed into the cuvette of the haze meter, avoiding the formation of bubbles. The cuvette was placed in the chamber and the lid was closed. After a few seconds the turbidity of the sample, expressed in EBC units, was read on the display.

2.3.6. Colloidal stability

Colloidal stability is the tendency of a beer to create a haze on storage (6). This parameter was determined using a forcing test. The bottles were stored at 60 °C during 7 days, to accelerate beer ageing, particularly haze formation. Then, the bottled beers were chilled at 0 °C for 24 h and the haze of the beer was measured using a haze meter. High turbidity values correspond to samples with low colloidal stability. Although this test is a long-term forecast, it was implemented to see if the prediction evolved over time.

2.3.7. Foam stability

The beer foam stability was determined using the NIBEM-T meter. The bottled beer was dispensed through a foam flask, in which beer is forced to pass through an orifice under carbon dioxide pressure. This produced a standard glass of beer foam. The resulting glass of beer foam was then placed under the needle electrode system of the NIBEM-T meter, previously calibrated. The Foam Collapse Time (FCT), in seconds, was then displayed on the digital display.

2.3.8. Bitterness

The method used to estimate the beer bitterness, was based on the measurement of bitter substances, mainly iso- α -acids formed from isomerization of α -acids (29).

The bitter substances were extracted from the acidified beer using isooctane as solvent. After centrifugation, the absorbance of the isooctane layer was measured by UV spectroscopy at 275 nm and compared with the reference (pure isooctane).

The results were expressed in units of bitterness (BU) through the expression: Bitterness (BU) = $50 \times A_{275}$, where A_{275} is the absorbance at 275 nm measured against the pure isooctane reference.

2.3.9. Apparent, real and original extracts and alcohol content

These parameters were determined through a density meter (DMA 4500) coupled to an alcoalyzer beer analysing system (Anton Paar - Switzerland).

Before being injected into the equipment, samples were degassed at room temperature by hand shaking with the containing flask closed, to avoid the loss of alcohol per evaporation and ensuring that all carbon dioxide was removed, so that it could not interfere with the analysis. Then the degassed samples were filtered by gravity.

2.3.10. Vicinal diketones (VDK)

The main volatile diketones found in beer are 2,3-butanedione (diacetyl) and 2,3-pentanedione. The content of vicinal diketones (VDK) is the sum of the concentrations of these two compounds.

There are several methods for the determination of VDK. For this measurement it was implemented a method for large-scale brewing industries adopted by the EBC (30). This methodology is based on spectrophotometry and involves a simple distillation, which aims to separate the diketones from the beer. To do so, it was necessary to prepare a solution of phenylenediamine (0.1 g of phenylenediamine in 10 mL of hydrochloric acid 4 N) prior to analysis. Then, 100 mL of beer sample were distilled to give 25 mL of distillate. In turn, 10 mL of the distillate were mixed with 0.5 mL of the solution (phenylenediamine). The obtained mixture was then stirred and kept in the dark for a few minutes. After the addition of 2 mL of hydrochloric acid 4 N the reaction mixture was mixed. Subsequently, the absorbance of the sample (A_{335}) and the absorbance of distilled water containing 0.5 mL of the solution of phenylenediamine (A_{bl}) were measured at 335 nm. The content of vicinal diketones was expressed in mg/L and was calculated using the expression:
$$\text{VDK} \left(\frac{\text{mg}}{\text{L}} \right) = \frac{A_{335} - A_{bl}}{0.250} \times 0.625 .$$

2.3.11. Acetaldehyde

Acetaldehyde concentrations were determined using the SAN++ continuous flow analyser from Skalar. This system incorporates a random access carousel sampler SA1100, a chemistry unit and a data handling.

The chemistry section is based on an integrated concept consisting in a peristaltic pump unit, a segmentation injector with separate air compressor and a chemistry application manifold, the SA 3000. The chemistry application SA 3000 includes all the required components to completely automate the analysis, such as in-line heaters, dialyzers, digesters and distillation units. All parts are integrated into separate sections in the chemistry unit.

The automated procedure for the determination of acetaldehyde is based on an enzyme catalysed reaction. In the presence of aldehyde dehydrogenase (AL-DH), acetaldehyde is oxidised quantitatively by the coenzyme nicotinamide adenine dinucleotide (NAD⁺) to acetic acid and NADH. The amount of NADH formed in the reaction is stoichiometric with the amount of acetaldehyde and is spectrophotometrically measured at 340 nm.

2.3.12. Lactic bacteria and wild yeasts

The microbiological analysis was performed using the universal beer agar CM0651 OXOID, which corresponds to a basal medium for the detection and culture of microbial contaminants in beer.

The medium was prepared according to the instructions of the product. After preparing the medium, a small portion was put into a sterile petri dish, the equivalent of 0.5 cm of thickness. Then, 200 mL of sample were filtered using a sterilized funnel with a 0.45 µm membrane. After filtration, the membrane was removed from the funnel and placed carefully into the petri dish containing the medium, avoiding the formation of air bubbles. The petri dish was then incubated for 5 days at 28 °C, in an anaerobic chamber with the CO₂ generator Anaerogen AN0035 Oxoid.

2.4. Colour study

The colour of beer was determined by three methodologies. The first one was based on EBC colour scale, already described in section 2.3.3. The other two were the reference method of the Analytica EBC (absorbance measurement at a unique wavelength) and the CIELab System. Both colour measurements were performed using 10 mm quartz cells. Before the spectrophotometric analysis, all samples were filtered through a 0.45 µm membrane filter.

In the EBC method the absorbance of the beer was measured at 430 nm, without any dilution. Then, the colour of the beer was calculated using the formula: Colour (EBC units) = $A_{430} \times 25$.

The CIELab parameters (L^* , a^* , b^*) were also determined, measuring the transmittance from 380 to 780 nm at 5 nm intervals and considering the illuminant D65 (daylight source) and the 10° standard observer (human perception). The colour-opponent coordinates, a^* and b^* , correspond to reddish / greenish and yellowish / bluish colours, respectively. The colour lightness, L^* , was evaluated in a black and white scale (ranging from 0 to 100). The psychophysical parameters C^* , H^* and S^* were also estimated (31). The chromaticity (C^*) was calculated as $C^* = \sqrt{(a^*)^2 + (b^*)^2}$, and determines the degree of distinction of each hue when compared with the same lightness grey, while the hue (H^*) was determined as $H^* = \tan^{-1}(\frac{b^*}{a^*})$ and is the attribute that allows the differentiation of a colour with reference to the same lightness grey. S^* represents the saturation and was calculated through the formula $S^* = C^*/L^*$.

Additionally, the measurement of the total colorimetric differences (ΔE^*) between samples over time were also estimated, by $\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$, assuming that ΔE^* higher than 3 units means that the colour of beers is different enough to be easily distinguished by human observers.

All analyses were performed in triplicate and the results were expressed as the mean value \pm standard deviation.

2.5. Determination of HMF

2.5.1. Chemicals

The preparation of the buffered mobile phase used ammonium di-hydrogen phosphate (98.0%) from Panreac Química S.A. (Barcelona, Spain) and phosphoric acid from Acros Organics (Geel, Belgium). Ultra-pure water (18 M Ω) was prepared by means of the Simplicity UV ultrapure water apparatus (type 1), from Millipore (Milford, MA, USA), and it was always used to prepare aqueous mobile phases. Methanol HPLC grade (Sigma–Aldrich, St. Louis, MO, USA) and acetonitrile HPLC grade (Fisher Scientific, Loughborough, UK) were also used. All solvents were previously filtered before being used in the HPLC system, with membrane filters obtained from Pall (0.20 μ m, Ann Arbor, MI, USA).

2.5.2. HPLC-DAD conditions

All beer samples were analysed by direct injection on a Waters Alliance liquid chromatograph (Milford, MA, USA) equipped with an auto-injector (Waters 2695, separations module) and a photodiode array detector (Waters 2996). HMF was separated on an Atlantis T3 (250x4.6 mm id; 5 μ m; Milford, MA, USA) column, kept at 30 °C. The configuration management and the data handling was done by Empower Pro software from Waters Corporation.

The HMF analysis was performed using a method previously calibrated, based on the methodology proposed by (32). Briefly, an elution in gradient mode was followed combining solvent A (10 mM of phosphate solution buffered at pH 2.70), solvent B (acetonitrile) and solvent C (methanol) as follows: within 30 min, 0–10% B and 0–11% C; 30–42 min, only change 11–17% C; 42–55 min, 10–60% B and 17–0% C; 55–58 min, the mobile phase composition was maintained; 58–65 min, washing and column reequilibration time. The flow rate was set to 1.0 mL/min and the injection volume of standard solutions and beer samples was

set to 20 μ L. All standards and samples were filtered through syringe filters 0.45 μ m Acrodisc GHP filters (Pall Gelman Sciences, Ann Arbor, MI, USA) and injected in duplicate. Quantification was achieved using a predetermined external calibration curve.

All determinations were carried out in triplicate and the results were expressed as the mean value \pm standard deviation.

2.6. Volatile compounds

The evolution of a lager beer was studied in terms of volatile compounds, during one year of storage at regular conditions. The volatile profile of three random bottles of beer was analysed monthly through SPME extraction coupled to GC-MS detection. The volatile analysis was performed based on the methodology proposed by Saison et al.(14).

2.6.1. Chemicals

Once again, ultra-pure water (18 M Ω) was used, this time for the sample preparation. 3-octanol was used as internal standard and was purchased to Acros organics. Sodium chloride was from Panreac Química S.A. while pure absolute ethanol was from Sigma-Aldrich. All reagents were of analytical grade with at least 97% of purity.

2.6.2. Sample preparation

To a volume of 50 mL of beer it was added an aliquot of 50 μ L containing 200 mg/L of 3-octanol (diluted in an ethanol solution at 5%) as internal standard. Then, 10 mL of this solution was added to a 20 mL headspace vial, containing 3.5 g of NaCl to promote salting-out. Finally, the vial was immediately capped and vortex prior to the automated HS-SPME extraction. Each bottle was analysed in triplicate.

2.6.3. HS-SPME and CG-MS conditions

The volatile extraction was automated using the TriPlus autosampler (Thermo Scientific, Hudson, NH, USA) in the SPME mode. Firstly, the sample vials were incubated during 10 min at 40 °C. Then, the extraction was performed by exposing the SPME fiber of 50/30 µm Divinylbenzene-Carboxen-Polydimethylsiloxane (DVB-CAR-PDMS, bipolar adsorbent) into the vial for 30 min at 40 °C, under continuous stirring. After that, the compounds were desorbed at 240 °C after inserting the fiber into the GC injector for 5 min. The separation of the sample volatiles was carried out using the Trace GC Ultra from Thermo Scientific, coupled to the iSQ single quadrupole mass spectrometer (electron impact ionization mode). The column was a DB-WAXetr from Agilent J&W (Folsom, CA, USA) of 60 m x 0.250 mm with a film thickness of 0.50 µm. The carrier gas was He at 1 mL/min. The transfer line and ion source were kept at 220 and 230 °C, respectively. After starting at 40 °C, the oven temperature was raised after 1.5 min at 4 °C/min up to 220 °C, and finally kept at 220 °C for 10 min. The total analysis time was 66.5 min. Mass range mode was applied between 30 and 300 m/z and the compounds identification was made by comparison of the mass spectra obtained with those of authentic compounds (standards) and/or with those present in Wiley 6.0 and NIST08 library databases. The results were analysed using Xcalibur software (Thermo, Austin, TX, USA). Moreover, the Kovats indexes were also obtained and compared with the online data base: Pherobase (33). The amount of each volatile compound was expressed in terms of 3-octanol and the relative concentrations were calculated dividing the area of the individual compounds by the internal standard.

2.6.4. Statistics

Microsoft Office Excel 2010 was used to perform the regular statistical analyses, while MatLab (version 7.6, The Mathworks, Inc.) was employed to carry out the Principal Component Analysis (PCA).



PART 3

RESULTS AND DISCUSSION

PART 3 – RESULTS AND DISCUSSION

3.1. Sensory analysis

3.1.1. Triangular test

The triangle test was used to determine if the samples were significantly different. The results obtained for both groups, trained beer judges and regular beer consumers, are described below (Figure 3.1).

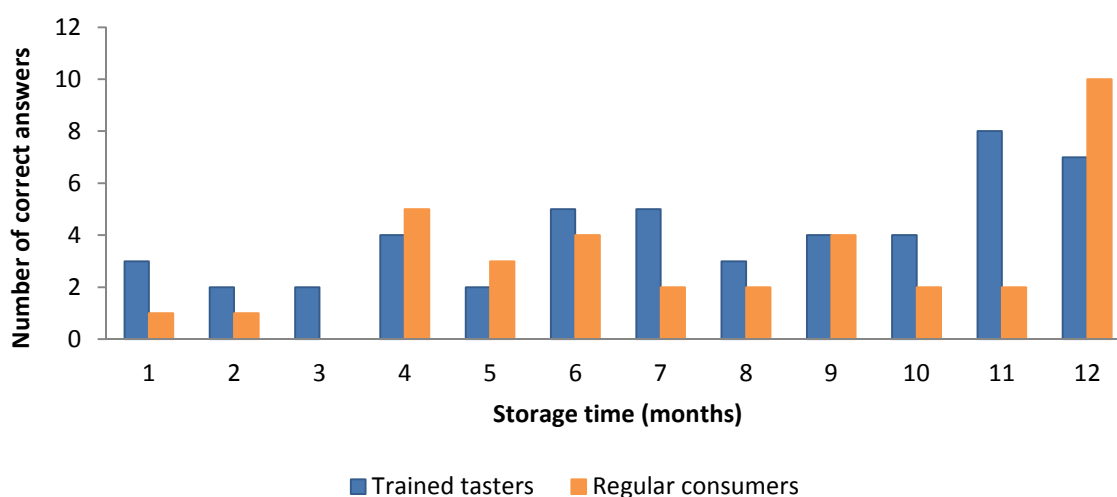


FIGURE 3.1 – Results of triangle test obtained with both groups.

With this test it was intended to find if there was any difference between the samples. Figure 3.1 only presents the number of correct answers, which is not enough to take any direct conclusion. According to this methodology, it must be taken into account the number of judges that performed the test, in each sampling. Thus, if the number of correct responses is greater than or equal to the number given in Table 3.1, it means that a significant difference exists between the samples with a 90% confidence interval, at a specific alpha risk (α). The α risk, also known as risk of “false positive”, corresponds to the risk of concluding that a significant difference exists when, in reality, there is none.

TABLE 3.1 – Minimum number of correct responses required for samples to be considered significantly different at the stated α – level (adapted from EBC Method 13.7 (Sensory Analysis: Triangle Test))

Significance assessments (n)	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Level of $\alpha=0,1$	3	4	4	5	5	6	6	7	7	7	8	8	10	10	10	11	11	12	12	12

Table 3.2 indicates that there is a significant difference between samples from the sixth month, only in the case of the trained judges, with a confidence level of 90% (α risk = 10%). In the case of regular consumers this evidence was not so clear.

TABLE 3.2 – Interpretation of the obtained results for a α risk of 10%.

Storage time (months)	Number of trained judges	Number of correct answers	Are they different?	Number of regular consumers	Number of correct answers	Are they different?
1 st	10	3	No	10	1	No
2 nd	10	2	No	10	1	No
3 rd	10	2	No	10	0	No
4 th	10	4	No	9	5	Yes
5 th	10	2	No	10	3	No
6 th	10	5	Yes	10	4	No
7 th	10	5	Yes	7	2	No
8 th	7	3	Yes	7	2	No
9 th	7	4	Yes	8	4	Yes
10 th	7	4	Yes	7	2	No
11 th	10	8	Yes	6	2	No
12 th	8	7	Yes	13	10	Yes

According to this test, the internal panel of the brewery considered that the beer samples were different, starting from the sixth month up to the twelfth month. However, regular consumers did not detect a turning point, considering that these samples did not present a detectable difference. In this sense, it can be considered that this lager beer can be consumed up to twelve months by most consumers, without being found great differences comparatively to a fresh beer. Only consumers more aware might detect differences.

3.1.2. Descriptive test

The descriptive test was only performed by the trained judges, in order to obtain a systematic description of the flavour evolution of the beer samples over the 12 months. A total of 23 attributes were evaluated and the obtained results are shown in Appendix A.

The degree of beer ageing was evaluated using a three-point sensory score scale ranked from 0 (no present attribute) to 3 (very strong attribute), sub-divided by a 1.0 scale with 1 and 2 corresponding to a weak and moderated attribute, respectively. These attributes were grouped in major classes according to the beer flavour wheel, presented earlier in Part 1 (see Table 3.3). The attributes foam and turbidity were also included in this classification despite both descriptors having particular relevance only in the visual field.

TABLE 3.3 – Distribution of the tested attributes by the various classes that compose the beer flavour wheel.

Classes	Attributes	Comments, definitions*
Aromatic, Fragrant, Fruity, Floral	Acetaldehyde Geraniol Isoamyl acetate Ethyl caproate	Green apples Rose-like Banana Apple-like with note of aniseed
Cereal	Grainy	Raw grain flavour
Caramelized, Roasted	Smoky Caramel	- Burnt sugar, toffee-like
Phenolic	Phenolic	-
Soapy, Fatty, Diacetyl, Oily, Rancid	Diacetyl Isovaleric	Butterscotch, buttermilk Stale cheese, old hops
Sulphury	Dimethyl sulphide (DMS) Lightstruck (3-methyl-2-butene-1-thiol (MBT)	Sweetcorn, cooked vegetable Skunky, sunstruck
Oxidized, Stale, Musty	Papery	Initial stage of staling, cardboard, old beer, oxidized
Sour, Acid	Acid Acetic	Pungent aroma, mineral acid Vinegar
Sweet	Sweet	-
Salty	Salty	-
Bitter	Bitter	-
Mouthfeel	Carbonation	CO ₂ content
Fullness	Body Watery	Fullness of flavour and mouthfeel Thin, seemingly diluted
Visual	Foam	

* Analytica-EBC Section 13 – Sensory analysis, Method 13.12 – Sensory Analysis: Flavour Terminology and Reference Standards.

Figure 3.2 illustrates the tendency of the beer sensory attributes over the storage months, given by the descriptive test.

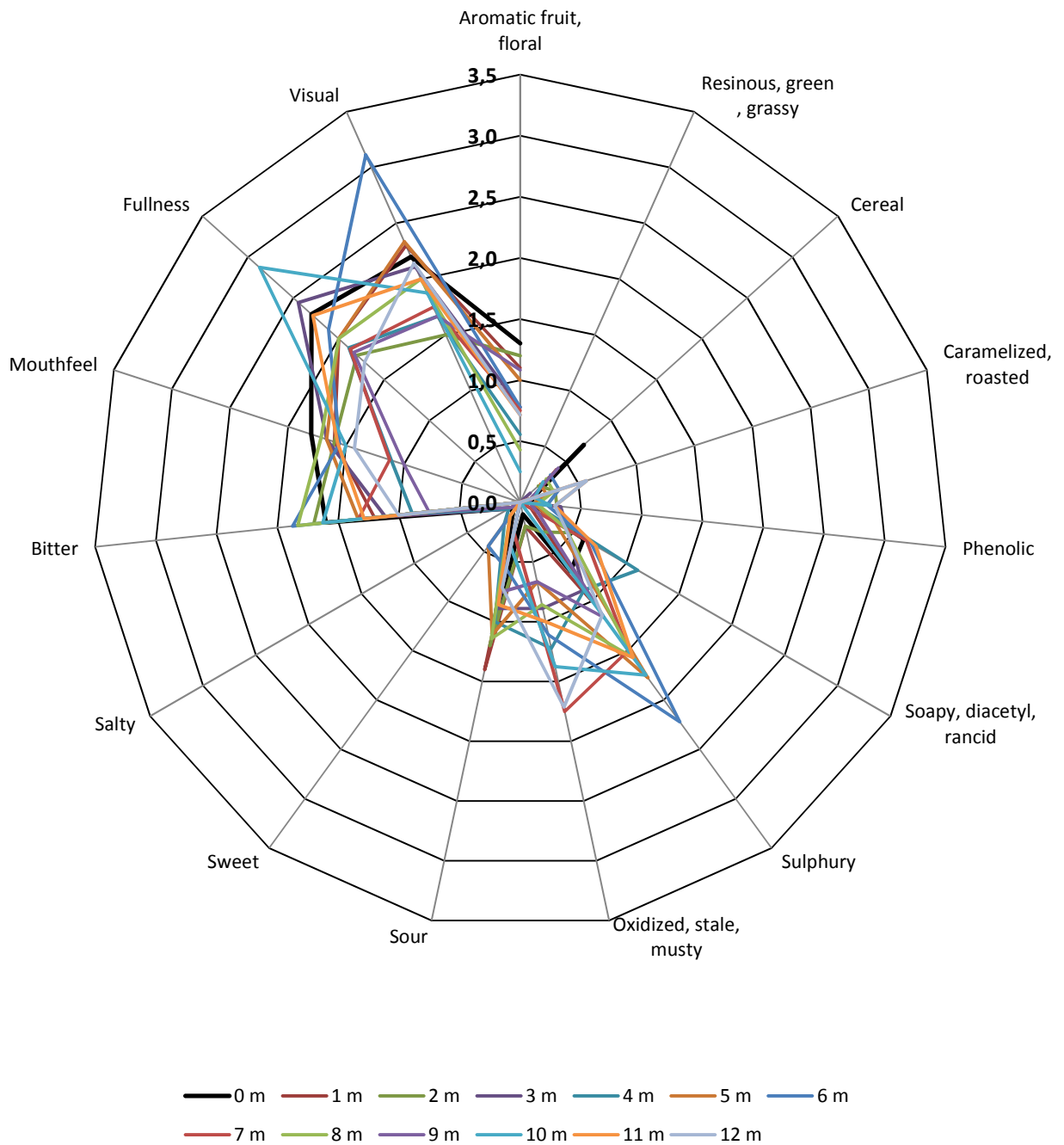


FIGURE 3.2 – Lager beer sensory attributes as a function of the storage months.

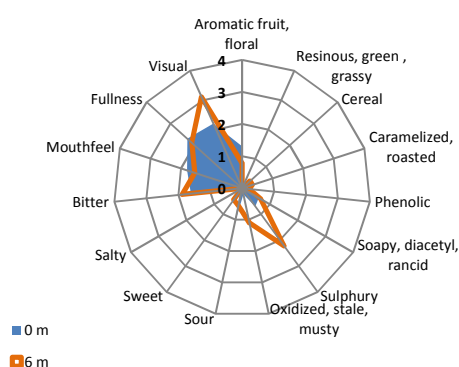
The results showed that the lager beer maintained the aromatic flavour of fruits and flowers, more evident in the fresh beer, even at 12 months, when stored at regular conditions. However, it was observed that the global expression of this kind of attributes presented the tendency to slightly diminish over time. On the other hand, the judges did not decide the same way concerning the cereal attribute, which was weakly detected mostly in the fresh beer (average score of about 1), disappearing from the 11th month onwards.

From Figure 3.2, it can also be verified that caramelized and roasted attributes started to be identified mainly after 11 months of storage. These attributes were indeed detected with a very little expression, not reflecting a great influence in the aroma of this beer. Generally, lager beers should be absent of this kind of attributes.

The two classes of attributes that showed the greatest increase over time were the sulphury notes and the class that includes the oxidized, stale and musty flavours. Both classes are considered off-flavour notes for lager beers. Regarding to the growth of the sulphury notes, this can be related with the increase of the two active components, dimethyl sulphide (DMS) and 3-methyl-2-butene-1-thiol (MBT). Both had a significant impact on the 6-month beer (more than 2.0). DMS can be formed from malt during wort production and/or by contaminant bacteria during fermentation. The typical concentration of DMS in beer can vary between 0.01 to 0.15 mg/L and its flavour threshold is around 0.025 mg/L. MBT is formed in beer due to the exposure to the daylight or artificial light. The typical concentration of MBT can vary from 1 to 5 ng/L for beers kept in the dark and from 0.01 to 1.5 µg/L for beers exposed to light. The flavour threshold of this compound is approximately 4 ng/L (34). In regard to the oxidized, stale, musty class, which also increased, especially due the papery attribute, the main active component responsible for this character is trans-2-nonenal. The development of this character, which is considered as an off-flavour in beer and it is associated with ageing, depends on time and temperature of storage, and also on the oxygen content of the packaged beer. The typical concentration of this compound in fresh beer is less than 50 ng/L and in aged beer is higher than 0.2 µg/L. It has a flavour threshold of 50-100 ng/L (34).

The increase observed by the sulphury and oxidized attributes in the lager beer in study is more evident in the taste of the beers aged for 6 and 12 months, as highlighted in Figure 3.3.

a) 6th month



b) 12th month

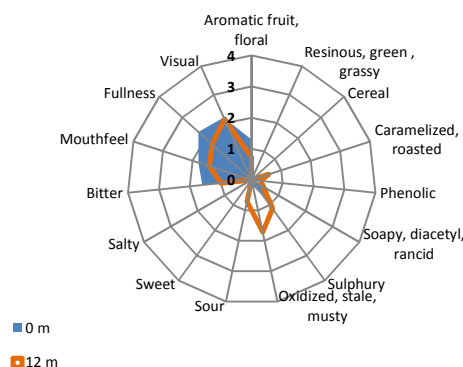


FIGURE 3.3 – Spider web plots only containing the comparison results obtained by the trained panel between the flavour assessment of the initial fresh beer and the 6-month (a) and 12-month (b) beers.

The descriptive sensory analysis was performed by 10 judges, recruited regarding their good sensory ability and experience regarding the identification of off-flavours in aged beer. From this test, it can be concluded that in spite of some variations in the sensory attributes of the lager beer in study it seems that this beer was still organoleptically acceptable for consumption, since most defects did not have great impact, i.e., the average score given by the panel to stale attributes was always less than 2, corresponding to a moderate expression.

3.2. Brewery's standard quality parameters

The quality parameters values determined during the twelve months of storage are showed in Table 3.4. All analyses were performed in duplicate for each beer bottle. Of the 16 analysed parameters, only 3 varied significantly during this period, namely colour, turbidity and VDK.

The colour variation of this lager beer given by the increase of the EBC units, was more evident after 1 month of storage. Then, it remained almost constant up to the 11th month, registering again a new increase at 12 months. The increase of the EBC units, indicates the development of a slight browning and might be related with oxidative processes that might happen during the storage period.

The increase of the VDK content is probably associated with its incomplete elimination during fermentation, namely due to the deficient activity of yeast. More precisely, these compounds are formed by yeast during fermentation, in a reaction where the α -acetolactate precursor leaves the yeast cell to form spontaneously VDK, through an oxidation reaction. Subsequently, yeast itself eliminates the formed VDK but if it is unhealthy the VDK content cannot be removed. The elimination reaction is essential because transforms compounds that have very low flavour thresholds and may give undesirable flavours in beer, such as 2,3-butanedione (diacetyl) and 2,3-pentanedione, into compounds which are not flavour-active, like diols, particularly 2,3-butanediol and 2,3-pentanediol, respectively.

As for turbidity, an increase of haze was observed over the storage period. This was already expected, since the beer is rich in polyphenols and polypeptides that during the beer storage react to form insoluble complexes and haze. Accordingly, the colloidal stability, parameter that is a long term measure, defined as the tendency of a beer to create a haze on storage, also decreased during beer storage.

Regarding to the foam stability, the results indicate a decreasing trend over time. This fact can be associated with the presence of proteolytic enzymes in beer, derived from yeast or from the enzymatic

additions made in the brewing process, which can deteriorate the beer foam during the storage period. In this study, this effect did not have great influence on the foam stability of the lager beer, since the assayed parameter only decreased up to 21% and no major differences were detected in the sensory analysis.

Concerning acetaldehyde, it was denoted some variability in the obtained results. At first, this compound remained practically constant, but then a decrease of 6 and 32% was observed in the 6th and 8th month, respectively. After that, the acetaldehyde content increased about 2.5-fold until the end of the study. This variability may be attributed to some variables that were not taken into account in the apparatus management, which were not identified at the time. Also, this fact can be related with some differences on the oxygen levels in the bottled beers, resulting in oxidative ageing reactions during storage. Contrary to this results, Vanderhaegen et al. (21) observed that the profile of the acetaldehyde slightly increased or remained constant in three different lager beers.

TABLE 3.4 – Results for the quality control parameters.

Months	0	1	2	3	4	5	6	7	8	9	10	11	12
Quality parameters													
pH	4.03	4.08	4.10	4.08	4.10	4.07	4.10	4.09	4.08	4.08	4.10	4.07	4.09
Carbon dioxide (g/L)	5.65	5.20	5.23	5.20	5.20	5.22	5.20	5.21	5.20	5.20	5.20	5.19	5.10
Colour (EBC)	6.30	7.30	7.30	7.30	7.30	7.30	7.30	7.30	7.30	7.80	7.80	7.80	8.70
Total oxygen (mg/L)	0.23	-	-	-	-	-	-	-	-	-	-	-	-
Haze (EBC)	0.54	0.58	0.59	0.61	0.65	0.78	0.85	0.74	0.86	0.90	1.08	1.15	1.35
Colloidal stability (EBC)	2.00	1.95	2.32	1.67	1.98	3.28	3.60	5.02	5.30	5.72	5.72	5.77	5.75
Foam stability (s/cm)	95.00	89.00	85.00	89.00	88.00	85.00	78.00	81.00	79.00	77.00	76.00	76.00	75.00
Bitterness (EBC)	20.75	21.50	21.20	20.00	20.00	20.00	20.60	19.70	19.30	19.05	19.00	19.00	17.10
Alcohol (%p/p)	3.75	3.74	3.79	3.75	3.74	3.70	3.71	3.70	3.70	3.68	3.66	3.70	3.70
Primitive extract (°P)	10.98	10.97	11.12	10.99	10.97	10.88	10.92	10.91	10.91	10.85	10.79	10.86	10.88
Apparent extract (°P)	1.93	1.95	1.98	1.95	1.95	1.95	1.95	1.98	1.98	1.95	1.93	1.93	1.95
Real extract (°P)	3.67	3.69	3.74	3.69	3.69	3.67	3.68	3.70	3.70	3.67	3.64	3.65	3.67
Acetaldehyde (mg/L)	7.16	-	7.20	-	7.33	-	6.75	-	4.84	-	9.21	-	10.38
Vicinal diketones (VDK) (mg/L)	0.09	0.10	0.08	0.11	0.12	0.14	0.16	0.19	0.19	0.16	0.14	0.14	0.14
Lactic bacteria (number/100mL)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Wild yeasts (number/100mL)	n.d.	1.00	1.00	1.00	1.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. – not detected

3.3. Colour characterization

In this study two other methods were applied to measure the colour changes in beer during the 12 months of storage, the EBC method (27) and the CIELab system.

The absorbance readings at 430 nm are reported in Table 3.5. The results showed that the absorbance of beer changes during the time of storage, increasing mostly in the first month (about 20%). Furthermore, an increase of 44% , was also observed at the end of the experiment. The forced ageing assay, obtained for 3 different points of ageing, 6th, 12th and 24th months, also revealed an increase of the beer absorbance, however, not at the same rate.

TABLE 3.5 – Results for absorbance readings at 430 nm.

Samples	A _{430nm}	± SD
0 m	0.25	0.01
1 m	0.30	0.04
2 m	0.29	0.01
3 m	0.30	0.01
4 m	0.30	0.01
5 m	0.31	0.01
6 m	0.31	0.01
7 m	0.32	0.01
8 m	0.33	0.01
9 m	0.33	0.01
10 m	0.34	0.01
11 m	0.35	0.01
12 m	0.36	0.01
6' m	0.30	0.02
12' m	0.31	0.00
24' m	0.33	0.02

6' m, 12' m and 24' m - Corresponding to forced aged beer.

These results can be better explained by the EBC scale. The results expressed in terms of EBC colour units are then presented in Figure 3.4. Figure 3.4a illustrates the colour plot of the natural ageing of the lager beer in study, while Figure 3.4b presents the results of the forced ageing. Analysing both plots, it becomes clear that the beer colour changes during storage, which is shown by the consistent increase of the EBC units. The highest increase is verified right at the 1st month.

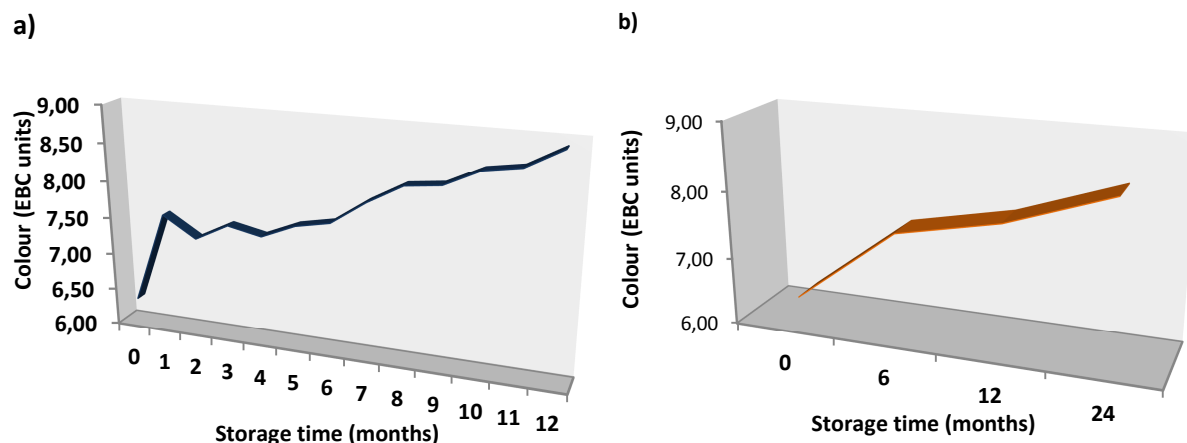


FIGURE 3.4 – Colour evolution of the a) naturally aged and b) forced aged beers during storage, expressed as EBC units: Colour (EBC units)= $A_{430} \times 25$.

CIELab chromatic coordinates a^* , b^* and L^* were also obtained and are represented in Figure 3.5. This methodology also reflected some changes in the lager beer colour along the storage at regular conditions, mostly the increase of the yellow hue, traduced by the increase of the magnitude of b^* positive values, up to 4 units. It could also be observed that the magnitude of a^* negative coordinate slightly decreased up to 1 unit, revealing the green's soft loss. Relatively to L^* values, they decreased despite the irregular trend during storage. This decrease expresses the loss of luminosity. In general, the forced ageing assay revealed similar trends, although the magnitude values were lower, as it was observed before.

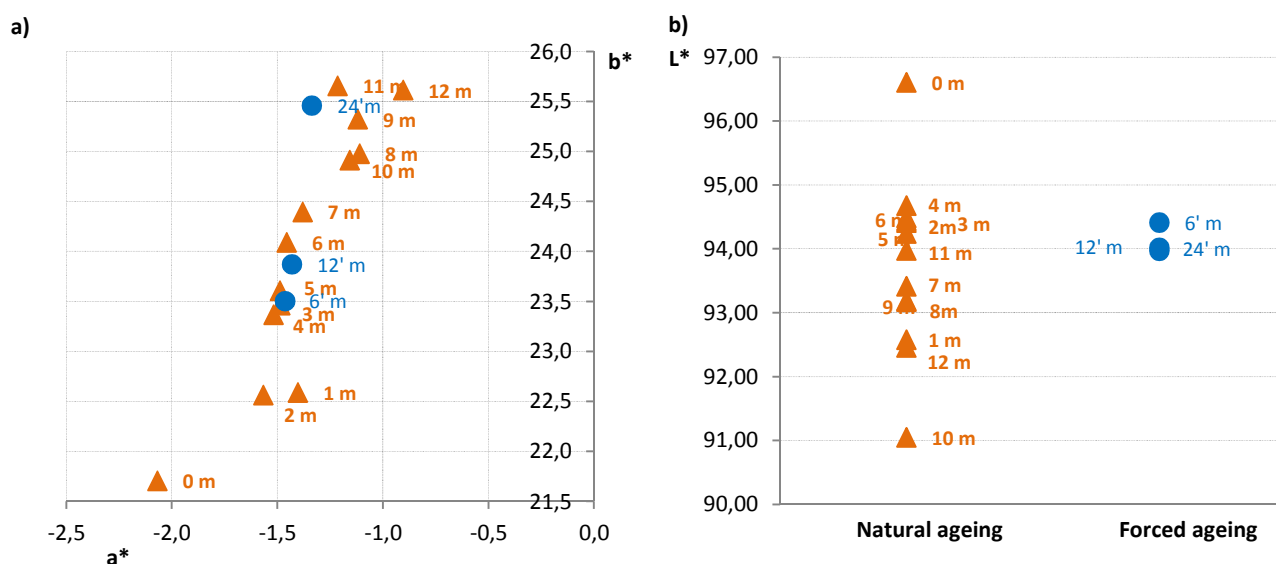


FIGURE 3.5 – Representation of the CIELab chromatic coordinates a^* , b^* and L^* . a) plots a^* vs. b^* and b) plots L^* values during the 12 months of storage. The results marked as 6'm, 12'm and 24'm correspond to beer submitted to forced ageing.

Table 3.6 presents other chromatic parameters that can be calculated from a^* , b^* and L^* . From these results it can be said that significant differences were found mostly after the 7th month, particularly detectable by the human eye, since the colorimetric differences (ΔE^*) were higher than 3.

TABLE 3.6 – CIELab chromatic parameters: chromaticity (C^*), hue (H^*) and saturation (S^*) of the beer during the storage time and of the beer submitted to forced ageing (6' m, 12' m and 24' m)

SAMPLES	C^*	$\pm SD$	H^*	$\pm SD$	S^*	$\pm SD$	ΔE^*
0 m	21.80	0.15	-1.48	0.01	0.23	0.24	-
1 m	22.63	0.45	-1.51	0.01	0.24	0.24	4.11
2 m	22.62	0.26	-1.50	0.00	0.24	0.98	2.34
3 m	23.55	0.33	-1.51	0.00	0.25	0.48	2.78
4 m	23.41	0.07	-1.51	0.01	0.25	0.07	2.51
5 m	23.65	0.24	-1.51	0.00	0.25	1.30	3.00
6 m	24.13	0.27	-1.51	0.00	0.26	1.48	3.15
7 m	24.43	0.32	-1.51	0.01	0.26	0.46	4.14
8 m	25.00	0.28	-1.53	0.00	0.27	0.92	4.69
9 m	25.35	0.38	-1.53	0.01	0.27	0.49	4.92
10 m	24.94	0.22	-1.52	0.00	0.27	0.14	6.38
11 m	25.68	0.27	-1.52	0.00	0.27	0.75	4.69
12 m	25.63	0.51	-1.54	0.00	0.28	0.51	5.64
6' m	23.51	0.16	-1.51	0.01	0.25	0.12	2.78
12' m	23.91	0.28	-1.51	0.00	0.25	2.35	3.34
24' m	25.49	0.38	-1.52	0.01	0.27	0.27	4.53

The results also showed that there was a linear growing trend (see Figure 3.6) in chromaticity (C^*) and saturation (S^*), while in terms of hue (H^*) no defined trend was clearly observed during beer storage.

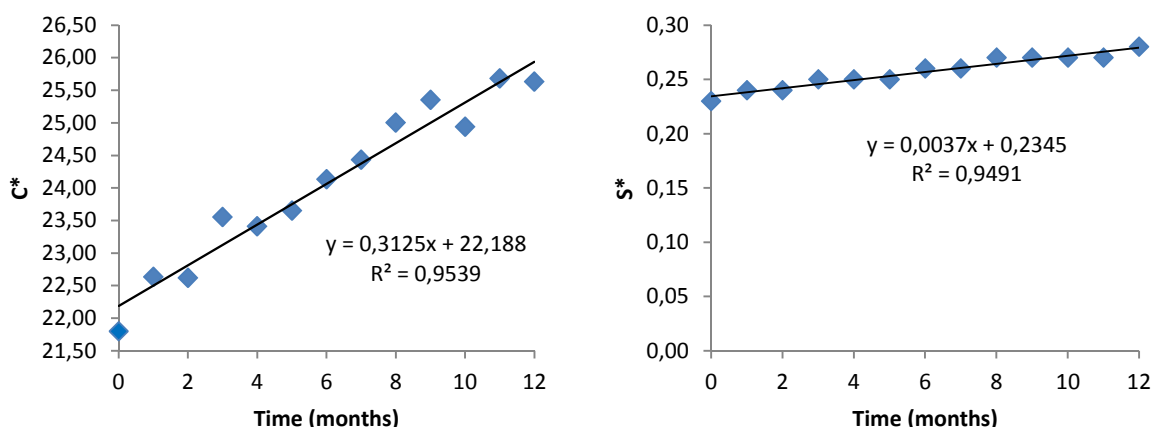


FIGURE 3.6 – Linear tendency of chromaticity (C^*) and saturation (S^*) over the time of storage.

3.4. Determination of HMF

It was observed that HMF increased during storage, as shown in Figure 3.7. In fact, HMF can be considered as an ageing marker of this lager beer, since showed a linear increase ($R^2 = 0.9802$) during the 12 months of storage, under regular conditions (Figure 3.8a). The usefulness of HMF to be considered as a potential ageing marker of this complex food mixture is that opens new insights about the chemistry of beer ageing. Moreover, the results of the beer submitted to forced ageing also showed a similar trend ($R^2 = 0.9897$) (Figure 3.8b). Indeed, this experiment mirrored the beer behaviour during regular storage, in terms of HMF content. Similar results were obtained in others studies revealing that HMF may be an indicator of the beer flavour deterioration (24, 35).

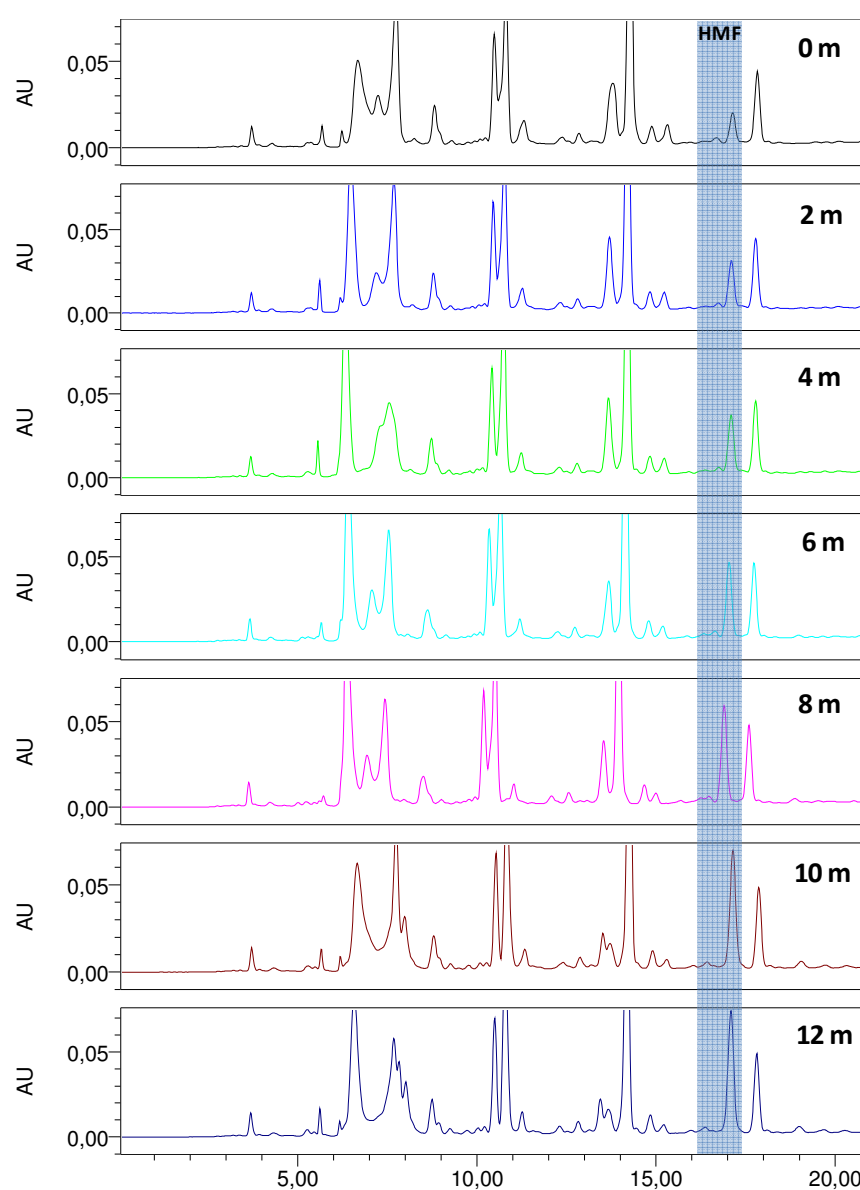


FIGURE 3.7 – Typical chromatograms of HMF during the lager beer storage, at regular conditions.

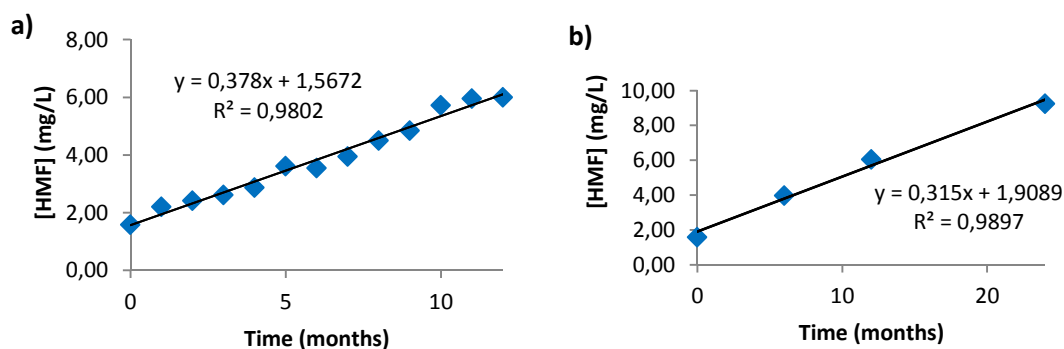


FIGURE 3.8 – HMF concentration during a) the beer storage at normal conditions and b) the same beer submitted to forced ageing.

The variation percentage between the fresh (0 month) and the beer stored for 12 months was of about 278%. Regarding the forced ageing experiment, it was observed an increase of 482%, representing the natural evolution of this lager for 24 months, which is in agreement with the previous results.

3.5. Volatile compounds

As aforementioned the volatile profile of the studied lager beer was monthly monitored during the 12 months of storage at regular conditions. Figure 3.9 shows the typical chromatogram of the volatile compounds found in beer, while in Table 3.8 their identification is presented and some sensorial data of the peaks is given. The GC-MS analyses of the samples allowed the identification of 70 volatile compounds including 25 esters, 15 alcohols, 7 acids, 6 aldehydes and ketones, 7 terpenes, 4 furans and other 6 compounds.

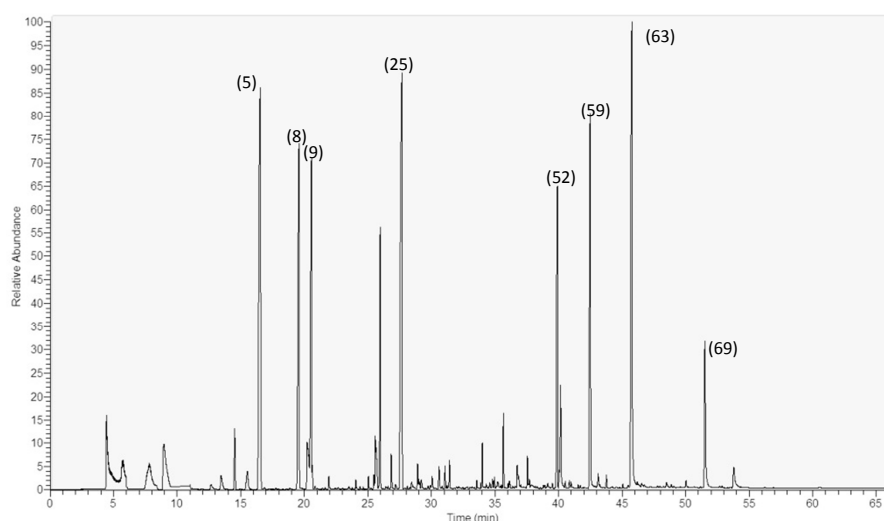


FIGURE 3.9– Typical chromatogram of volatile compounds found in beer. For peak identification please see Table 3.7.

TABLE 3.7 – Peak identification.

#	Compounds	CAS N.º	KI	Common descriptors	Oth(mg/L)
1	ethyl acetate **	141-78-6	872	solvent-like, acid, buttery, pungent ^a	30 ^d
2	isobutyl acetate *	110-19-0	1022	fruit, apple, banana ^b	---
3	ethyl butyrate *	105-54-4	1048	fruity ^a	---
4	isobutanol *	78-83-1	1109	solvent-like, bitter, alcohol, licorice ^a	---
5	isoamyl acetate *	123-92-2	1140	fresh, banana ^a	1 ^d
6	ethyl valerate *	539-82-2	1150	fruity, orange ^a	---
7	myrcene **	123-35-3	1175	metallic, musty, geranium, ^a	---
8	isopentanol **	123-51-3	1226	balsamic, alcohol, burnt, cheese ^a	---
9	ethyl caproate *	123-66-0	1254	fruity, strawberry ^a	0.2 ^d
10	6-methyl-2-heptanone	928-68-7	1262	fresh, sweet ^a	---
11	3-octanone ***	106-68-3	1278	herbal, buttery ^a	---
12	isoamyl butyrate ***	106-27-4	1283	sweet, apricot, banana-like ^a	---
13	hexyl acetate *	142-92-7	1291	fruity, spicy ^a	---
14	p-cymene **	99-87-6	1297	chemical, woody, oxidized citrus lemon ^c	---
15	ethyl 5-methylhexanoate ***	10236-10-9	1303	---	---
16	difurfuryl ether ***	4437-22-3	1312	coffee nutty earthy ^c	---
17	ethyl 3-hexenoate **	2396-83-0	1322	sweet, fruity, pineapple, green ^c	---
18	3-methyl-1-hexanol ***	13231-81-7	1338	---	---
19	ethyl capronate **	106-30-9	1353	fruity ^a	---
20	41+55+69+82+87+110+127+142	unknown	1362	---	---
21	1-hexanol *	111-27-3	1369	flowery, toasty, fruity, herbal ^a	---
22	heptyl acetate ***	112-06-1	1392	green, fatty, citrus, aldehydic ^c	---
23	nonanal **	124-19-6	1421	tallowy, gravy, green, chlorine ^a	---
24	linalool tetrahydride **	78-69-3	1445	floral, citrus, woody, herbal ^a	---
25	ethyl caprylate **	106-32-1	1458	fruity, fatty, green leafy, menthol ^a	0.5 ^d
26	1-heptanol **	111-70-6	1471	fresh, light green, nutty ^a	---
27	acetic acid *	64-19-7	1483	sour, vinegar, pungent ^a	175 ^d
28	octyl acetate***	112-14-1	1495	waxy, floral, apple-like ^a	---
29	furfural *	98-01-1	1495	Papery, husky ^d	200 ^d
30	2-propyl-1-pentanol ***	58175-57-8	1504	---	---
31	decanal **	112-31-2	1527	green, floral, lemon, fatty ^a	---
32	ethyl pelargonate ***	123-29-5	1556	fruity, rose, waxy, rum, wine ^c	---
33	linalool **	78-70-6	1564	muscat, floral, lemon, lavender-like ^a	0.03 ^d
34	furfuryl acetate ***	623-17-6	1567	sweet, fruity banana, horseradis ^c	---
35	1-octanol *	111-87-5	1574	metallic, sulfur ^a	---
36	ethyl 2-nonenolate ***	17463-01-3	1607	---	---
37	myrcenol **	543-39-5	1628	fresh, floral, lavender, citrus ^c	---

TABLE 3.7 – Peak identification. (Continuation).

#	Compounds	CAS N. ^a	KI	Common descriptors	Oth(mg/L)
38	2-decanol ***	1120-06-5	1632	Coconut, aniseed ^d	0.015 ^d
39	carbitol **	111-90-0	1650	slightly ethereal ^c	---
40	ethyl caprate **	110-38-3	1658	grape, fruity ^a	---
41	dimethyloctanol **	106-21-8	1677	floral, rose, waxy, petal ^a	---
42	citronellol acetate **	150-84-5	1684	citrus-like, oily, berry, fragrant, musty, dusty,	---
43	furfuryl alcohol **	98-00-0	1690	fermented, burnt sugar, creamy, caramelize ^a	---
44	2- methylcaproic acid*	4536-23-6	1698	acidic, oily, fatty ^c	---
45	ethyl 9-decenoate ***	67233-91-4	1713	fruity fatty ^c	---
46	methyl geraniate ***	2349-14-6	1726	floral ^a	---
47	43+45+63+68+77+79+105+121+136+151+152	unknown	1752	---	---
48	1-decanol *	112-30-1	1779	fat ^a	---
49	citronellol *	106-22-9	1784	rose ^a	---
50	ethyl phenylacetate **	101-97-3	1815	sweet, fruity, spicy, cinnamon ^a	---
51	propyl laurate ***	3681-78-5	1849	---	---
52	phenyl ethyl acetate *	103-45-7	1864	rose ^a	3 ^d
53	β-damascenone **	23726-93-4	1870	apple, rose, honey ^b	---
54	caproic acid *	142-62-1	1875	sweaty, pungent ^a	8 ^e
55	geranyl acetone **	3796-70-1	1887	magnolia, green ^b	---
56	43+69+71+83+11+155+159+243	unknown	1899	---	---
57	ethyl hydrocinnamate **	2021-28-5	1932	flowery, sweet, pleasant ^a	---
58	agidol ***	128-37-0	1945	phenolic camphor ^c	---
59	phenethyl alcohol *	60-12-8	1962	roses, perfumed, sweet ^a	125 ^d
60	lauryl alcohol **	112-53-8	1984	fat, wax ^a	---
61	phenylethyl butyrate **	103-52-6	2010	dry, sweet, musty, rose-like ^a	---
62	nerolidol **	40716-66-3	2061	floral, citrus, woody, waxy ^c	---
63	caprylic acid *	124-07-2	2090	fatty acid ^a	15 ^e
64	39+43+53+55+57+77+79+91+123+165+180+221	unknown	2121	---	---
65	pelargonic acid **	112-05-0	2198	green, fat, musty, sweaty, sour ^a	---
66	39+41+43+55+67+71+82+93+107+125+161+189	unknown	2211	---	---
67	41+43+55+95+105+119+134+147+162+176+189	unknown	2218	---	---
68	vinylguaicol *	7786-61-0	2251	clove-like, phenolic ^a	---
69	capric acid **	334-48-5	2301	soapy, fatty ^a	10 ^e
70	caproic acid ***	14436-32-9	2365	waxy, fatty, soapy, creamy cheese ^c	---

KI – Kovats index; Oth – odour threshold in beer.

* MS data and Kovats index in agreement with those of authentic compound

** MS data and Kovats index in agreement with those in literature

*** MS data in agreement with those in NIST08 and Wiley 6.0 libraries

Sources: a - (33)
b - (33)
c - (36)
d - (1)
e - (37)

The method showed an average RSD of 9% and the RSDs expressing the sampling variability are shown in the figure 3.10.

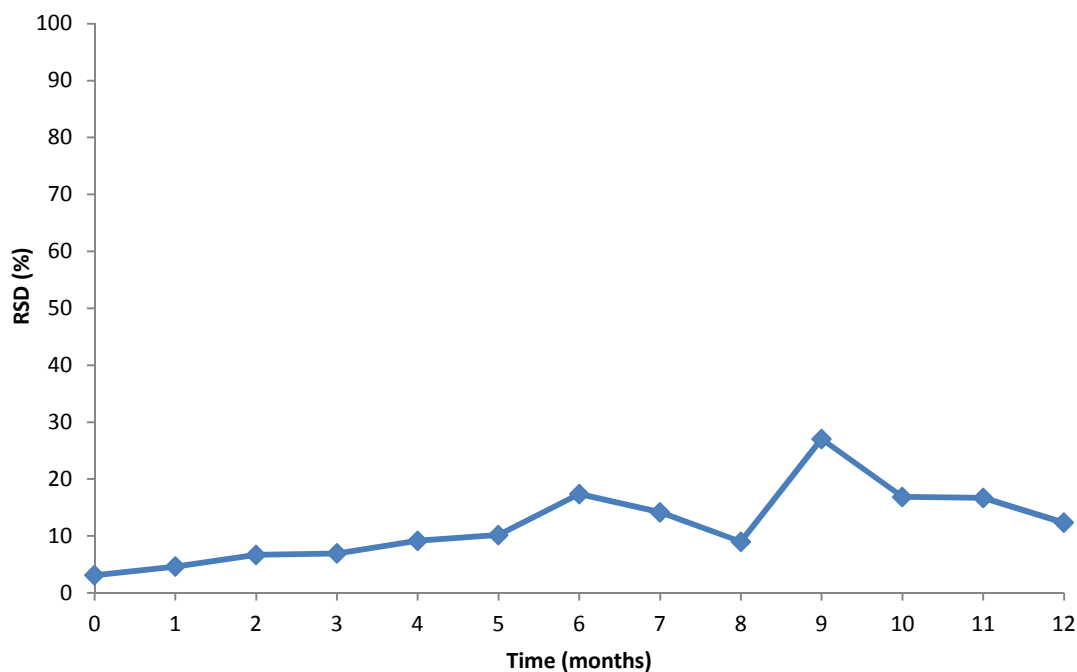


FIGURE 3.10 – Average of the relative standard deviations of the sampling.

The obtained results showed that the volatile fraction of the fresh lager beer is mostly composed by esters (36%), alcohols (21%) and organic acids (10%). Figure 3.11a shows the evolution of these major compounds during the experience period, while Figure 3.11b depicts the natural development of the other chemical classes, which altogether did not represent more than 33%.

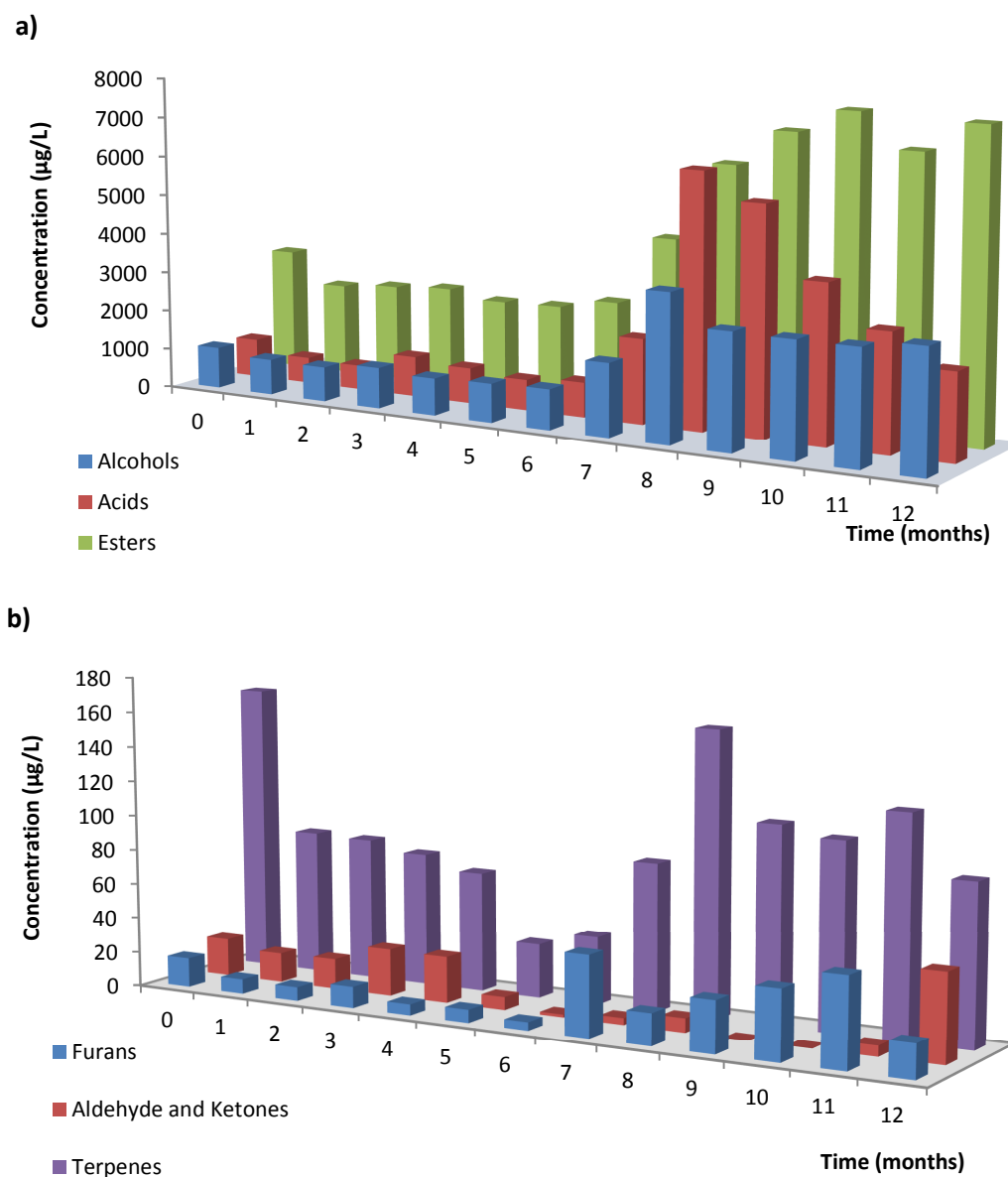


FIGURE 3.11 – Evolution of the chemical families present in beer during the regular storage: a) major compounds and b) minor compounds.

From Figure 3.11a it can be observed that major compounds levels remained almost constant up to the 6th month. From the 7th month onwards, a significant increase was observed in esters and alcohols, up to 152% and 198%, respectively. However, their contents remained almost unchanged after the 10th month. As for the minor compounds, their behaviour was not so consistent: there was an increase of furans, more evident before the 7th month of storage; the class of terpenes increased in the second semester; aldehydes and ketones slightly increased until the 5th month of storage, after the 6th month they almost disappeared, despite increased significantly in last month.

Appendix B presents the evolution of each volatile compound identified in the lager beer during storage for a 12 months period.

3.5.1. Esters

As aforementioned, esters are the most abundant volatiles in the studied lager beer. The esters present in higher concentration were isoamyl acetate, ethyl caprylate, ethyl caproate, phenethyl acetate and ethyl acetate. The levels of these compounds remained almost constant up to the 6th month, after which there was an increase from the 7th month up to the end of the monitoring, up to 3-fold of the initial value (Figure 3.12). In contrast to these results Vanderhaegen et al. (21) observed the decrease of these esters and justified it due to hydrolysis during the storage period. However, the same authors also reported that, when there is a pasteurization process involved - which is the case of the studied beer - the hydrolysis of these esters could not be verified. The identified increase may be justified by the chemical esterification of acids by alcohols, mostly ethanol, during long-term storage. As regards to their contribution to the global aroma of the beer only the isoamyl acetate, the ethyl caproate and the ethyl caprylate were present in concentrations higher than their odour threshold. For isoamyl acetate this fact occurred only after the 6th month of storage; however in the case of ethyl caproate and ethyl caprylate that had been happening since the beginning of the study. Isoamyl acetate may contribute with aromas like: banana, ester or solvent, while ethyl caproate and ethyl caprylate may confer a fruity-like aroma to the beer.

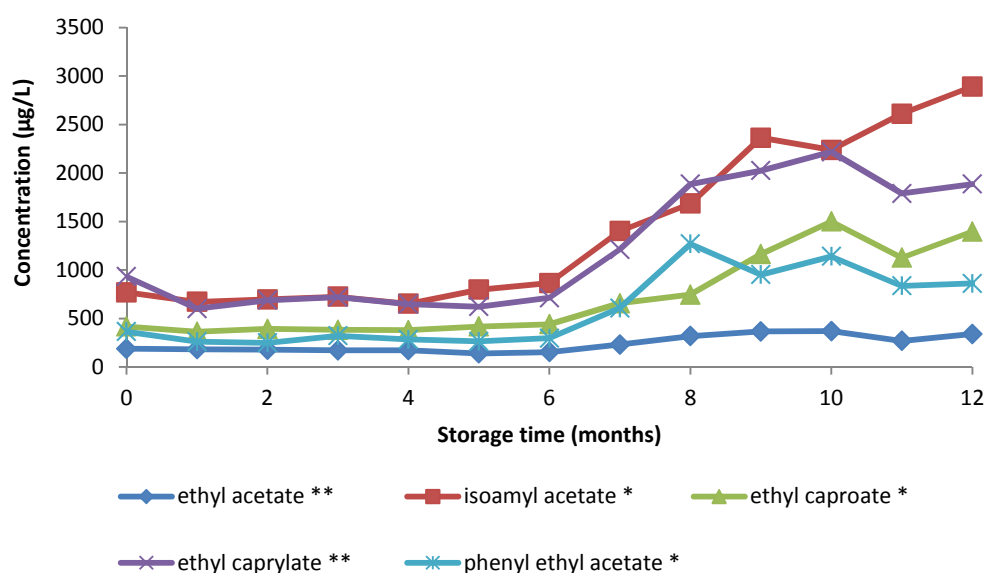


FIGURE 3.12 – Evolution of the major esters during the beer storage at regular conditions.

3.5.2. Alcohols

Besides ethanol, the most predominant alcohols were the higher alcohols isopentanol and phenethyl alcohol. These two alcohols presented a growing tendency especially after 6 months of storage (Figure 3.13). The increase represented about 270% in isopentanol and 173% in phenethyl alcohol. Other alcohols with lower concentrations such as isobutanol, 1-octanol, 2-octanol, dimethyloctanol and decanol also showed a high increase with the storage time (of about 136%, 88%, 22%, 18% and 63%, respectively) (Appendix B). The characteristics that alcohols may confer to the beers aroma are in general a hot, spicy and vinous, as well as a warming sensation that can be detected by the consumer. In this study, however, none of the alcohols have exceeded their odour thresholds.

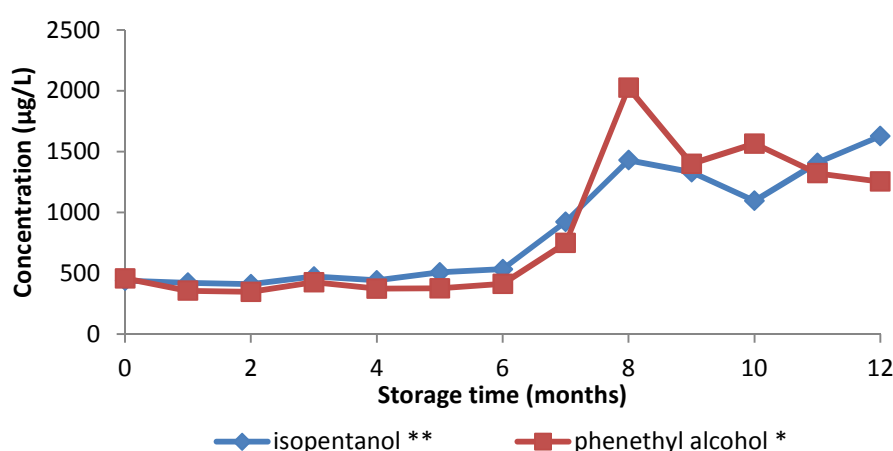


FIGURE 3.13 – Evolution of the major higher alcohols during the beer storage at regular conditions.

3.5.3. Acids

Several compounds belonging to this chemical family were identified in this beer, namely acetic, 2-methylcaproic, caproic, caprylic, pelargonic, capric and caproleic acids. The two most abundant were caprylic acid and capric acid. Both compounds are responsible for conferring to beer the caprylic flavour (12) that resembles the odour of a goat. The most abundant is caprylic acid, but no specific trend was found during the first months of storage (Figure 3.14). Then, from the 7th month onwards, the results showed a significant increase of its concentration, up to 6-fold (on the 8th month) of the level found on the fresh beer. Regarding the capric acid, the second most abundant acid found in beer, its evolution was similar to the caprylic acid, attaining a maximum increase of 755% on the 8th month (Figure 3.13). However, despite being abundant, none of these acids exceeded their flavours thresholds, 15 mg/L to caprylic acid and 10 mg/L for capric acid,

so it may be concluded that they should not have a great contribution to the global aroma of this lager beer, even after 12 months of storage.

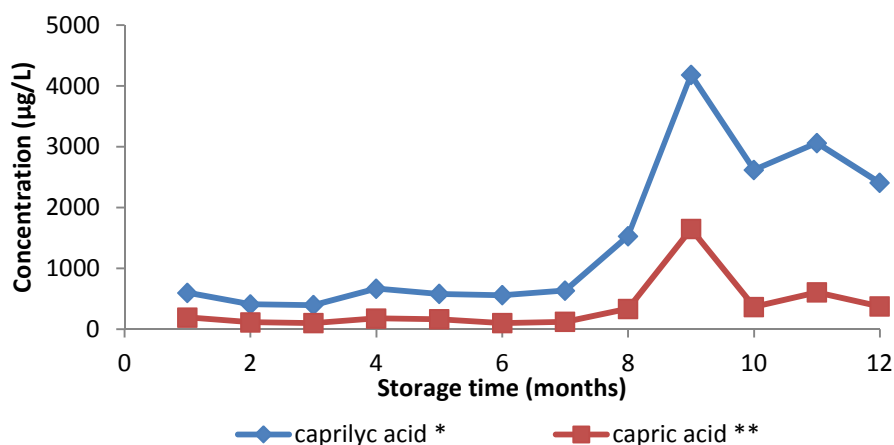


FIGURE 3.14 – Evolution of the most abundant acids in the studied lager beer.

3.5.4. Aldehydes and ketones

The aldehydes identified in this study were linear aldehydes, namely nonanal and decanal. From these two aldehydes only nonanal presented a growing tendency during the first five months of this study (Figure 3.15). After that its trend was not so clear - in the 6th and 7th months it was not detected, thereafter its amount increased again up to 258% (8th month), when comparing to the fresh lager beer. Taking into account its odour thresholds in water (35 µg/L) this compound may have no influence in the aroma of this lager beer, particularly after 8 months of storage. This compound may impart different scents to beer, namely tallowy and fruity odours (38, 39).

A total of 4 ketones were identified in this lager beer namely β -damascenone, geranyl acetone, 6-methyl-2-heptanone and 3-octanone. These ketones are characterized by fresh odours, namely by floral and fruity scents. The obtained results indicate that ketones were only detected in the first months (Figure 3.14). Indeed, this study revealed a decrease of β -damascenone during beer storage, despite in the last month presented an abnormal high value. A contrary trend was found by Chevance et al. (40) who reported that the increased of this compound during storage time could be partially explained by the acid hydrolysis of glycosides. This carotenoid-derived compound, usually associated with red fruits or strawberries flavours,

may affect beer flavour during storage, since even at low concentrations its role in beer aroma could be preponderant due to its very low odour threshold (40).

The fact that ketones disappeared during storage may indicate that the beer aroma might be losing some fresh scents, those usually related with fresh beers.

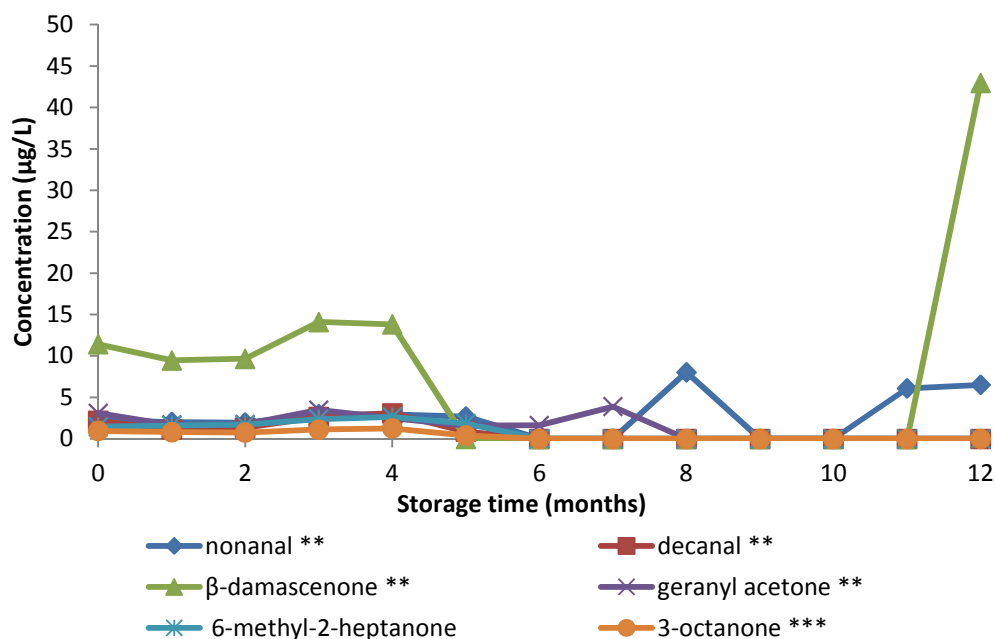


FIGURE 3.15 – Evolution of the most abundant aldehydes and ketones found in the studied lager beer.

3.5.5. Furans

Four furan compounds were identified in this study, namely furfural, furfuryl alcohol, difurfuryl ether and furfuryl acetate. The results showed that furfural appeared after 7 months of storage (Figure 3.16). Thereafter, its behaviour was not clear, however it occurred in older beers, becoming the most abundant furan in this lager beer, with levels ranging between 19 and 40 µg/L. This compound is a sensitive indicator of beer staling, which increases during storage providing a caramel-like and nutty flavour to beer (41, 42). Furthermore, furfural is a dehydration product of pentoses and an important indicator of beer flavor deterioration (41)

Furfuryl alcohol, which is also considered a relevant ageing marker, was found only in the first 3 months and with a decreasing tendency. After that, its behaviour did not follow a regular trend. For these reasons it may not be considered as an ageing marker for this lager beer.

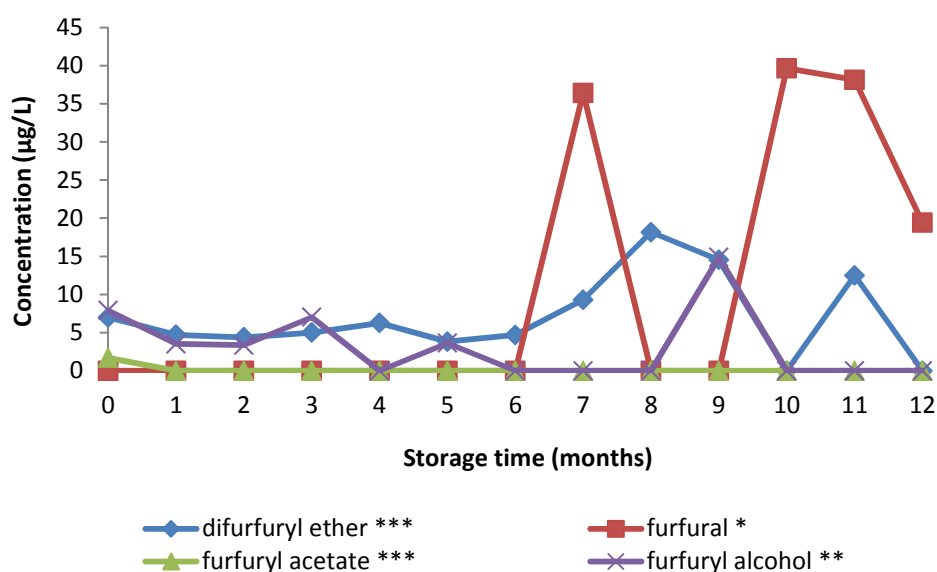


FIGURE 3.16 – Evolution of furanic compounds during lager beer storage.

3.5.6. Terpenes

In the current beer samples, 7 terpenes were found: myrcene, p-cymene, linalool tetrahydride, linalool, myrcenol, citronellol and nerolidol. From these, linalool showed a crescent trend after 6th months storage (Figure 3.17). Linalool levels have indeed reached 2-fold its initial value at the end of the monitoring. Other terpenes also increased, namely nerolidol and citronellol after 6 months of storage. Myrcenol, the most abundant terpene, did not follow a regular trend during storage: first a sharp decrease in the first 5 months, then an erratic behaviour.

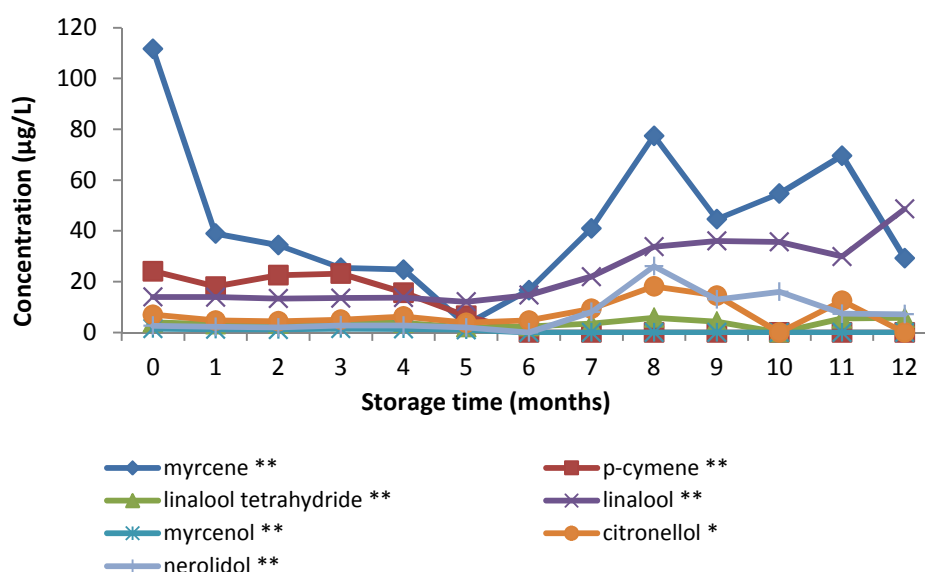


FIGURE 3.17 – Evolution of terpene compounds during beer storage.

3.5.7. Other compounds

In the current study, other compounds were found that may be relevant in the definition of the beer global aroma. One is 4-vinylguaiacol, a volatile phenol usually considered as an off-flavour in lager beers since it imparts phenolic, medicinal, or clove-like scents to beer. It was found that this compound increased during storage, and may be related to the presence of wild yeast strains in the final beer. Its formation is associated with the ferulic enzymatic hydrolysis during fermentation and chemical hydrolysis during long-term storage (40, 43).

3.5.8. Statistics

The analysis performed on the previous sections allows us to understand the evolution of a particular lager beer according to its volatile composition. However, when we are looking for the comparison of all analysed samples, to identify the evolution trends and understand their differences, it makes sense to use all the 70 variables analysed. In this sense, PCA becomes an excellent tool for analysing this multivariate data, since it allows retaining the essential information, aiming to facilitate its visualization and interpretation.

PCA was then applied to all studied beer samples to obtain a more simplified view of their relationship over the storage period. The data matrix was constructed using the 108 samples by 70 variables. Figure 3.18 depicts the first two dimensions of the PCA model which best approximate the original data set, explaining 63.06% of the total variability. The first PC explains the higher percentage of variance (49.88 %) while the second PC explains 13.18%.

From Figure 3.18, two different clusters may be seen showing that the studied samples are well separated according to the storage time. The PC 1 axis is responsible for the complete differentiation between beers with more than 6 months (on the left part of the graph) and those with less than 6 months (on the right part of the graph).

Figure 3.19 shows the weight that each variable has in each principal component. Analysing this figure, it becomes easier to interpret which variables contribute to the differentiation of these clusters. It can be concluded that acids, alcohols, esters and furans are closely related (negatively correlated) with the distinction found across PC 1.

Gathering all the results, especially those from PCA analysis, they lead us to consider that the beer flavour becomes altered after 6 months. The differentiation found by the chemical analysis, however, could not be detected by all beer consumers, as revealed by the sensory analyses.

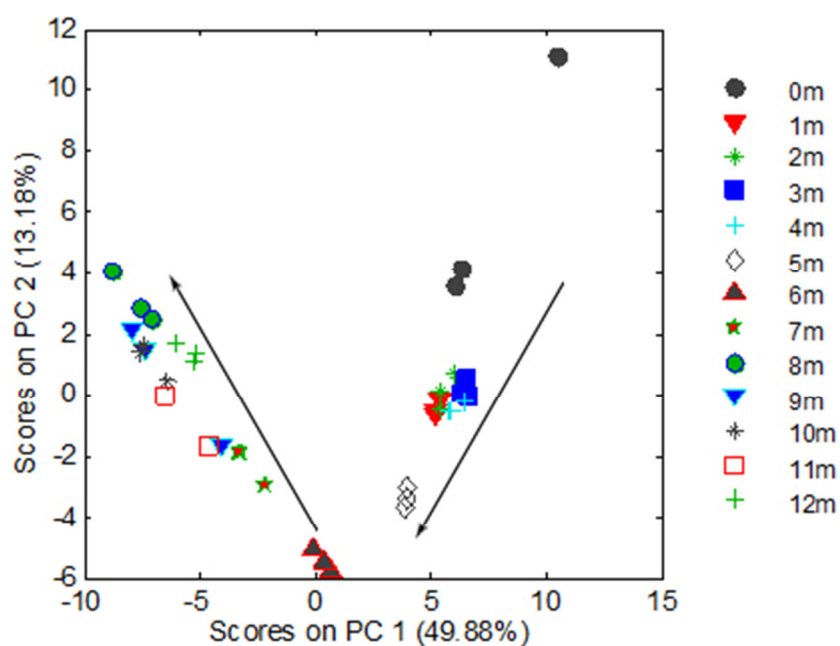


FIGURE 3.18 - PCA analysis of the lager beer in study: beer differentiation

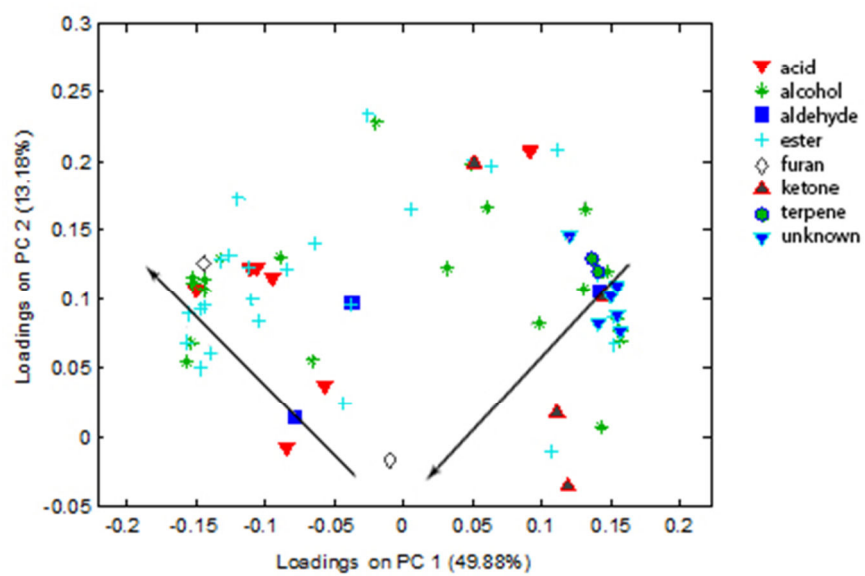


FIGURE 3.19 – PCA analysis of the lager beer in study: variable loadings.



PART 4

FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

PART 4 – FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

The current study showed that the beer suffers changes in its volatile composition during storage at room temperature. However, the changes are not necessary detected by the regular consumers due to the low amounts.

The storage period promoted the increase of the esters, such as isoamyl acetate, ethyl caprylate, ethyl caproate, phenethyl acetate and ethyl acetate, probably because of the chemical esterification of acids by alcohols, mostly ethanol. Only the isoamyl acetate, the ethyl caproate and the ethyl caprylate were present in concentrations higher than their odour threshold. Isoamyl acetate may contribute with aromas like: banana, ester or solvent, while ethyl caproate and ethyl caprylate may confer a fruity-like aroma to the beer.

Also, higher alcohols that might confer hot, spicy and vinous aroma to the beers, as well as a warming sensation, presented a growing tendency. However, none of the alcohols have exceeded their odour thresholds.

The two most abundant acid compounds were caprylic and capric. Both compounds are responsible for conferring the caprylic flavour to the beer that resembles the odour of a goat. Although no specific trend was found during the first months of storage, the results showed an important increase on their concentration at the end of this study. However, despite being abundant, none of these acids exceeded their flavours thresholds, 15 mg/L to caprylic acid and 10 mg/L for capric acid, so it may be concluded that they should not have a great contribution to the global aroma of this lager beer, even after 12 months of storage.

Nonanal, a compound that may impart different scents to beer, namely tallowy and fruity odours, presented a growing tendency during the first five months, after that its trend was not clear. Taking into account its odour thresholds in water (35 µg/L) this compound may have no influence in the aroma of this lager beer.

This study revealed an increase of β -damascenone. This carotenoid-derived compound, usually associated with red fruits or strawberries flavours, may affect beer flavour during storage, since even at low concentrations its role in beer aroma could be preponderant due to its very low odour threshold. The fact that most ketones disappeared during storage may indicate that the beer aroma might be losing some fresh scents, those usually related with fresh beers.

The results showed that furfural, the most abundant furan in this lager beer, appeared after 7 months of storage. This compound is a sensitive indicator of beer staling, which increases during storage providing a caramel-like and nutty flavour to beer. Furthermore, furfural is a dehydration product of pentoses and an important indicator of beer flavour deterioration. Furfuryl alcohol was found only in the first 3 months.

From the class of terpenes, only linalool showed a clear trend during storage which was always crescent. Linalool levels have indeed reached 2-fold its initial value at the end of the monitoring.

In the current study, other compounds were found that may be relevant in the definition of the beer global aroma. One is 4-vinylguaiacol, a volatile phenol usually considered as an off-flavour in lager beers since it imparts phenolic, medicinal, or clove-like scents to beer. It was found that this compound increased during storage, and may be related to the presence of wild yeast strains in the final beer. Its formation is associated with the ferulic enzymatic hydrolysis during fermentation and chemical hydrolysis during long-term storage.

HMF may be considered an ageing marker for this lager beer, since it showed a linear increase ($R^2 = 0.9802$) during the 12 months of storage, at regular conditions. The usefulness of HMF as a potential ageing marker of this complex food mixture is that it opens new insights about the chemistry of beer ageing. Moreover, the results of the beer submitted to forced ageing also showed a similar trend.

The colour study was performed to better understand the ageing beer process. The analysis of beer colour, expressed in terms of EBC colour units, indicated changes during storage, for natural ageing and for forced ageing. This fact is shown by the consistent increase of the EBC units. The chromatic characteristics also reflected changes in beer colour along the storage at regular conditions, with yellow tones becoming predominant, the green's soft loss and also a decrease of luminosity. In general, the forced ageing assay revealed similar trends.

The quality parameters values determined during the twelve months of storage showed an increase of VDK, namely 2,3-butanedione (diacetyl) and 2,3-pentanedione, which may give undesirable flavours to the beer. There was also an increase of haze, turbidity and colloidal stability during the storage period. On the contrary, foam stability decrease over time.

Another relevant compound, namely acetaldehyde, was quantified in this study. There was some variability in the obtained results. At first, this compound remained practically constant, but then an increase

after the 6th month. This variability may be attributed to some variables that were not taken into account in the apparatus management, which were not identified at the time. Also, this fact may be related with different oxygen levels in the bottled beers.

Finally, the ageing process was also followed by sensory analysis, in order to establish a connection between the organoleptic and the compositional changes occurring during the storage, so as to achieve a deeper understanding of the beer stability process and how the compositional changes may affect the organoleptic properties of beer. It was shown that only trained consumers might detect the differences between the naturally aged beer and the fresh beer. Moreover, in spite of some variations in the sensory attributes of the lager beer in study, it seems that this beer was still organoleptically acceptable for consumption after 12 months, since most defects detected in the chemical analysis did not have great impact, i.e., the average score given by the panel to stale beer attributes was always less than 2, corresponding to a moderate expression.

Several possible paths may be planned for future developments in the area of beer analysis. With the advances in technology, it is imperious to explore new analysis techniques of beer that allow a more effective control of the beer quality and stability. It should be interesting to continue this study for several batches of the same lager beer or even follow the whole process of production of a beer and identify and control the volatiles changes that occur, and relate them to the sensory characteristics of the final beer. This study might provide a more effective control of the brewing process, allowing the optimization of production conditions, regarding the quality of the final product and the development of new products.

APPENDIX A – Average results of the descriptive test.

	STORAGE TIME														
	ATTRIBUTES	0	1	2	3	4	5	6	7	8	9	10	11	12	
VISUAL	Foam	2	2	1	2	2	2	2	2	2	1	2	2	2	
	Haze	0	0	0	0	0	0	1	0	0	0	0	0	0	
ODOR	Isoamyl acetate	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Ethyl caproate	0	1	1	0	0	1	0	1	0	1	0	0	0	
	Geraniol	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Acetaldehyde	0	1	1	0	0	0	0	0	0	0	0	0	0	
	Grainy	1	0	0	0	0	0	0	0	0	0	0	0	0	
	Paper	0	0	0	1	1	1	1	2	1	1	1	1	2	
	Dimethyl sulphide	1	1	0	0	0	2	1	1	1	1	0	0	1	
	Diacetyl	1	0	1	0	1	0	1	1	0	0	0	1	0	
	Lightstruck	0	0	0	0	1	0	1	1	1	1	1	1	0	
	Isovaleric acid	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Caramel	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Roasted	0	0	0	0	0	0	0	0	0	0	0	0	0	
	Phenolic	0	0	0	0	0	0	0	0	0	0	0	0	0	
	TASTE	Acid	1	1	1	1	1	1	0	0	1	1	0	1	1
Acetic		0	0	1	0	0	0	0	0	0	0	0	0	0	
Sweet		0	0	0	0	0	0	0	0	0	0	0	0	0	
Salty		0	0	0	0	0	0	0	0	0	0	0	0	0	
Bitter		2	1	2	1	1	1	2	1	2	1	2	1	1	
Body		1	1	1	1	1	1	1	1	1	1	1	1	1	
Diluted		2	1	1	2	1	1	1	1	1	1	2	1	0	
Carbonation		2	2	2	2	1	2	2	1	2	1	2	2	1	

APPENDIX B – Volatile levels in lager beer during the twelve months of storage.

Concentration (µg/L)																		
#	0 m	±SD	1 m	±SD	2 m	±SD	3 m	±SD	4 m	±SD	5 m	±SD	6 m	±SD	7 m	±SD	8 m	±SD
1	188.4	2.1	183.1	6.9	178.1	3.6	172.1	13.6	172.6	14.2	139.9	4.2	153.3	8.1	230.3	22.3	319.2	16.2
2	12.1	0.1	12.6	0.3	11.6	0.1	10.8	0.5	10.7	0.6	9.6	0.4	10.2	0.7	16.8	0.9	18.8	1.6
3	31.0	0.0	29.9	0.1	29.9	0.5	27.2	1.3	27.6	1.8	23.4	2.1	25.4	0.9	40.6	1.6	52.4	3.1
4	35.3	0.4	33.6	0.8	34.3	1.3	35.3	3.2	34.8	3.1	24.2	2.2	27.8	5.9	61.7	3.3	85.2	6.9
5	768.7	0.4	671.2	0.5	695.7	23.2	723.5	17.0	651.4	11.1	796.4	71.1	865.0	57.2	1400.0	164.3	1684.6	153.6
6	2.7	0.0	2.4	0.1	2.4	0.1	2.0	0.2	1.9	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	2.6	0.4	1.5	0.2	1.8	0.2	1.4	0.3	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	439.8	7.0	423.3	1.8	411.4	12.1	473.9	1.2	442.2	6.0	508.5	20.6	535.0	22.3	924.5	56.3	1430.5	168.3
9	417.4	2.9	363.2	4.1	393.5	13.2	383.4	3.9	379.6	5.2	416.8	22.3	441.2	2.8	657.1	34.3	745.5	71.2
10	1.5	0.1	1.6	0.1	1.7	0.1	2.4	0.1	2.6	0.2	1.8	0.2	0.0	0.0	0.0	0.0	0.0	0.0
11	0.9	0.1	0.8	0.0	0.7	0.0	1.1	0.1	1.2	0.1	0.4	0.1	0.0	0.0	0.0	0.0	0.0	0.0
12	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.1	1.9	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13	13.2	0.0	13.0	0.5	12.5	0.6	10.1	0.6	11.0	0.8	8.7	0.7	9.6	0.4	12.9	0.8	17.0	0.7
14	1.3	0.0	0.8	0.2	1.1	0.1	0.9	0.1	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	1.2	0.0	1.0	0.0	1.1	0.1	0.8	0.0	0.8	0.0	0.9	0.2	0.0	0.0	0.0	0.0	0.0	0.0
16	1.3	0.0	1.5	0.3	1.7	0.0	1.6	0.0	1.8	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17	1.5	0.0	1.3	0.0	1.4	0.1	1.2	0.1	1.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	1.5	0.0	1.2	0.0	1.3	0.0	1.1	0.0	1.2	0.1	0.8	0.2	0.0	0.0	0.0	0.0	0.0	0.0
19	9.9	0.2	7.5	0.2	8.7	0.7	6.4	0.4	7.2	0.3	5.3	0.4	6.0	0.2	7.7	1.0	12.0	0.9
20	2.4	0.0	2.1	0.0	2.2	0.1	1.9	0.1	2.0	0.1	1.5	0.1	0.0	0.0	0.0	0.0	0.0	0.0
21	1.8	0.0	1.8	0.0	1.8	0.1	1.6	0.1	1.6	0.1	1.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0
22	16.1	0.2	12.3	0.4	13.9	1.0	10.2	1.1	11.3	0.6	8.2	0.6	9.6	0.7	15.3	1.8	18.2	1.1

APPENDIX B – Volatile levels in lager beer during the twelve months of storage (Continuation).

Concentration (µg/L)																										
#	0 m	±SD	1 m	±SD	2 m	±SD	3 m	±SD	4 m	±SD	5 m	±SD	6 m	±SD	7 m	±SD	8 m	±SD	9 m	±SD	10 m	±SD	11 m	±SD	12 m	±SD
23	2.2	0.1	2.0	0.1	1.9	0.5	3.0	0.2	2.9	1.2	2.7	1.0	0.0	0.0	0.0	0.0	8.0	0.5	0.0	0.0	0.0	0.0	6.1	1.4	6.5	2.0
24	4.0	0.0	3.2	0.1	3.4	0.2	4.5	0.3	3.8	0.3	2.0	0.1	2.4	0.4	3.5	0.1	5.8	0.4	4.3	1.6	0.0	0.0	5.5	0.6	5.7	0.5
25	931.4	28.4	601.2	27.3	688.4	60.4	719.5	16.8	647.6	34.9	622.5	39.4	712.2	105.7	1210.4	204.9	1886.3	215.4	2023.5	614.4	2217.3	249.2	1787.6	355.5	1885.4	138.3
26	1.8	0.0	1.9	0.0	1.9	0.1	1.7	0.0	1.9	0.0	1.5	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	7.9	0.1	7.9	0.3	7.7	0.5	8.8	0.7	7.3	0.1	6.5	0.4	8.0	0.4	15.8	1.6	15.5	1.0	18.5	5.8	28.0	10.9	22.9	3.6	31.0	9.1
28	28.8	1.2	16.4	1.2	19.1	2.2	17.1	0.3	16.9	0.6	10.2	0.8	14.9	2.9	0.0	0.0	35.0	2.6	25.7	9.6	29.0	8.5	19.5	4.5	29.4	5.2
29	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	36.4	6.1	0.0	0.0	0.0	0.0	39.6	0.7	38.1	1.2	19.4	4.5
30	10.1	0.1	8.8	0.8	9.2	0.7	9.4	0.2	10.7	1.7	5.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	2.2	0.2	1.1	0.1	1.3	0.3	2.6	0.4	3.1	0.9	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32	4.7	0.5	2.1	0.2	2.6	0.1	2.2	0.0	2.0	0.3	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
33	14.0	0.3	13.9	0.2	13.4	0.4	13.5	0.5	13.7	0.4	12.1	0.6	14.8	0.6	22.0	0.7	33.8	2.0	36.0	4.3	35.6	3.6	29.9	1.4	48.6	2.5
34	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
35	19.8	0.2	18.5	0.2	17.9	0.4	19.5	0.5	18.8	0.4	15.5	1.6	16.8	1.3	30.1	1.1	40.2	3.6	43.2	8.9	42.5	3.2	32.7	0.7	37.3	1.2
36	1.6	0.1	0.9	0.0	1.1	0.2	0.9	0.0	0.9	0.1	1.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
37	1.5	0.0	1.3	0.0	1.2	0.0	1.5	0.0	1.4	0.1	1.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
38	1.9	0.0	1.2	0.0	1.2	0.1	1.8	0.0	1.8	0.1	0.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.3
39	0.9	0.0	1.0	0.1	0.8	0.0	0.3	0.0	0.4	0.1	13.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
40	111.7	15.2	38.9	6.5	34.4	5.1	25.5	1.5	24.8	3.5	3.1	0.1	16.7	5.6	41.0	11.0	77.4	7.2	44.6	17.3	54.7	9.2	69.6	21.3	29.3	1.3
41	6.4	0.2	4.0	0.2	4.3	0.3	4.4	0.1	4.6	0.3	2.4	0.1	0.0	0.0	0.0	0.0	11.7	0.6	0.0	0.0	10.4	1.6	0.0	0.0	7.6	0.7
42	12.1	0.5	6.4	0.5	6.7	0.9	6.6	0.1	6.2	0.5	3.6	0.5	0.0	0.0	0.0	0.0	10.8	0.6	8.1	1.8	0.0	0.0	0.0	0.0	15.3	6.0
43	7.9	0.3	3.5	0.1	3.3	0.3	7.0	0.4	0.0	0.0	3.6	0.5	0.0	0.0	0.0	0.0	0.0	0.0	14.9	5.5	0.0	0.0	0.0	0.0	0.0	0.0
44	11.5	0.8	8.7	0.2	8.1	0.3	10.1	0.3	10.0	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

APPENDIX B – Volatile levels in lager beer during the twelve months of storage (Continuation).

Concentration (µg/L)																										
#	0 m	±SD	1 m	±SD	2 m	±SD	3 m	±SD	4 m	±SD	5 m	±SD	6 m	±SD	7 m	±SD	8 m	±SD	9 m	±SD	10 m	±SD	11 m	±SD	12 m	±SD
45	108.3	7.0	47.6	4.2	47.4	6.7	42.9	1.6	41.4	4.9	23.7	1.0	30.3	9.5	70.8	15.4	108.8	9.4	82.1	31.2	99.6	11.9	87.6	27.8	59.9	0.9
46	6.0	0.1	3.5	0.3	3.6	0.5	3.2	0.1	3.2	0.2	2.1	0.2	2.4	0.4	9.4	1.9	7.3	0.4	6.2	2.3	7.8	0.7	4.4	1.1	5.0	0.4
47	24.1	0.6	18.1	0.2	22.6	1.3	23.2	1.0	15.7	0.9	6.6	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
48	27.5	0.5	17.0	0.5	16.6	1.4	20.5	1.6	19.0	2.2	12.7	0.7	14.8	5.0	38.0	1.1	66.3	3.5	46.7	18.0	49.4	5.9	34.2	10.5	44.7	11.6
49	7.0	0.1	4.7	0.1	4.4	0.3	5.0	1.6	6.3	1.1	3.8	0.3	4.7	1.2	9.3	0.9	18.2	1.3	14.5	5.4	0.0	0.0	12.5	3.3	0.0	0.0
50	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.5	4.5	13.2	4.7	14.3	5.6	0.0	0.0	0.0	0.0
51	3.9	0.4	2.8	0.1	2.9	0.0	3.8	0.3	1.7	0.2	1.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.3	0.6
52	364.5	0.8	260.2	2.2	248.6	9.6	322.2	8.9	284.0	17.9	264.9	27.4	297.6	65.0	606.6	106.7	1268.5	175.1	951.3	370.8	1139.2	156.2	836.5	226.8	861.7	57.7
53	11.4	0.1	9.5	0.1	9.7	0.6	14.1	1.4	13.8	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	43.0	6.1
54	89.0	2.0	72.7	0.5	68.5	1.5	81.5	3.5	76.2	10.1	71.0	4.9	76.2	13.8	159.1	18.8	139.4	12.2	218.0	62.6	131.3	20.2	99.6	37.5	0.0	0.0
55	3.1	0.2	1.7	0.0	1.7	0.0	3.5	0.4	2.5	0.7	1.6	0.3	1.6	0.6	3.9	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
56	2.9	0.1	2.1	0.3	2.0	0.3	4.9	0.6	4.0	0.1	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
57	3.0	0.0	1.9	0.0	1.7	0.1	2.3	0.2	2.3	0.2	1.4	0.3	1.9	0.7	5.3	0.8	9.8	2.3	0.0	0.0	0.0	0.0	0.0	0.0	5.3	0.1
58	1.4	0.0	0.6	0.0	0.6	0.0	0.9	0.1	1.0	0.3	0.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
59	458.8	3.5	356.9	1.2	348.1	12.3	427.2	7.0	374.7	18.9	376.8	34.1	413.8	57.4	749.2	113.3	2025.5	301.5	1400.0	500.9	1565.7	207.3	1322.8	229.9	1253.7	66.1
60	8.6	0.9	6.4	0.1	5.8	0.2	11.4	1.9	3.8	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
61	11.2	0.1	6.9	0.1	5.9	0.5	8.7	0.5	8.4	0.9	5.6	0.9	6.4	2.3	18.3	2.9	30.6	0.8	15.3	5.9	25.3	3.1	16.7	5.4	13.1	0.9
62	2.6	0.2	2.2	0.0	2.0	0.3	2.7	0.2	2.7	0.2	1.9	0.1	0.0	0.0	8.0	1.3	26.1	1.5	13.0	5.0	16.0	2.3	7.4	2.7	7.2	0.6
63	597.9	1.4	408.4	3.3	394.0	10.1	665.0	22.8	579.3	46.8	557.6	69.7	634.5	184.8	1527.9	337.9	4181.3	452.6	2617.1	1010.4	3059.9	467.8	2404.4	895.5	1849.5	42.1
64	5.9	0.4	3.8	0.4	3.5	0.0	2.4	0.2	0.0	0.0	0.6	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
65	7.3	0.4	5.0	0.1	4.3	0.1	7.8	0.5	6.9	0.4	2.6	0.2	0.0	0.0	0.0	0.0	50.7	6.6	2449.2	956.0	0.0	0.0	0.0	0.0	0.0	0.0
66	4.0	0.1	3.1	0.2	2.3	0.3	3.1	0.4	3.3	0.5	2.1	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

APPENDIX B – Volatile levels in lager beer during the twelve months of storage (Continuation).

Concentration (µg/L)																										
#	0 m	±SD	1 m	±SD	2 m	±SD	3 m	±SD	4 m	±SD	5 m	±SD	6 m	±SD	7 m	±SD	8 m	±SD	9 m	±SD	10 m	±SD	11 m	±SD	12 m	±SD
67	1.9	0.1	1.2	0.1	1.0	0.1	1.2	0.0	1.2	0.1	0.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
68	9.0	0.1	5.8	1.2	4.4	0.3	7.0	0.8	6.9	0.9	5.2	0.8	0.0	0.0	18.9	7.3	41.6	6.2	18.2	7.1	28.3	5.0	0.0	0.0	0.0	0.0
69	192.7	5.5	113.6	2.0	99.6	6.8	176.4	11.7	162.5	22.5	98.7	15.4	119.8	45.1	333.8	131.2	1646.8	89.6	365.0	143.1	606.0	138.8	373.1	146.8	267.8	48.1
70	45.6	0.8	27.1	0.2	22.0	1.6	49.0	12.1	39.1	5.4	24.9	4.2	28.9	9.9	76.9	30.3	348.0	19.8	55.6	21.6	147.3	57.9	66.2	26.0	31.6	7.8

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