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Vicinal Diketones Monitoring During Lager Beer Fermentation
The Importance of Nitrogenous Compounds
Ana Carolina Leal Vieira

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MASTER DISSERTATION

Ana Carolina Leal Vieira
MASTER IN APPLIED BIOCHEMISTRY

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June | 2017

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SUPERVISOR
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CO-SUPERVISOR
Ana Cristina Rebola Pereira

Dedico esta tese ao David, às minhas irmãs, aos meus sobrinhos e em especial aos meus pais.

ACKNOWLEGEMENTS

To my parents and sisters, who always encouraged me to achieve my academic and life goals, for their unconditional support in all my choices, I would like to express my sincere gratitude, for contributing to my professional success and happiness, for the love and patience that they always demonstrate. To my nephews and niece, thank you for bringing joy and love every day and for expecting me to be successful.

David, for your support, love and affection during all these years, I am truly grateful and I wish to thank you for being always present and for sharing your joy and good humour in the most stressful occasions and in moments of tiredness and also for celebrating with me mine, yours and our achievements.

To my supervisor, Professor José Carlos Marques, I express my gratitude for allowing me to develop new tools and knowledge, for guiding me through my professional journey and for encouraging me during this period.

Cristina, I am truly grateful for your support, patience and guidance during all these years and I would like to thank you for sharing your knowledge with me and for recognizing my effort and my qualities, as well for helping me to improve my competences. Thank you for your friendship and for being such a great professional and co-worker.

I would also like to extend my gratitude to Vanda, for her friendship, help and for always being available to share her knowledge with me.

To my co-workers Isabel, Maria João and specially Andreia and Anísia, I would like to thank you for your support and help in this work. Also, I would like to thank you for your friendship and for bringing joy and good mood to our everyday conversations.

I also wish to thank to Ana Paula Vieira and Ana Paula Andrade for their sympathy and assistance during this period.

At last, I would like to acknowledge Empresa de Cervejas da Madeira for this opportunity and to express my sincere gratitude to Eng. Nuno Branco, Doctor Luísa Vieira and specially to Eng. Tomé Mendes, for sharing with me their knowledge, sympathy and enthusiasm about this work. Also, I would like to express my gratitude to all their colleagues that work on wort production, beer fermentation and in the chemistry laboratory, who were very patient and kind with me, allowing me to follow their work and for sharing their knowledge with me.

RESUMO

As dicetonas vicinais, o diacetil e a 2,3-pentanediona, são um sub-resultado da biossíntese de valina, leucina e isoleucina por parte da levedura. Estes compostos conferem um aroma desagradável à cerveja e por isso a monitorização dos mesmos é imprescindível durante a fermentação.

Uma nova metodologia para a determinação de compostos orgânicos voláteis em amostras de fermentação de cerveja por GC-MS foi desenvolvida, após a optimização da extração destes compostos por HS-SPME. As condições óptimas consistiram numa extração a 40ºC durante 20 minutos, através da exposição de uma fibra Car-PDMS num vial de 20 mL, contendo 10 mL de amostra e 3,3 g de NaCl. Adicionalmente, desenvolveu-se uma metodologia para a determinação de aminoácidos por HPLC-fluorescência.

As metodologias desenvolvidas apresentam boa linearidade nas gamas de concentração em estudo e valores de recuperação entre os 89,88-133,80% (voláteis) e os 77,60-112,39% (aminoácidos). A precisão, avaliada em termos de repetibilidade e reprodutibilidade, caracteriza-se por desvios inferiores a 10 e 15%, para a determinação dos compostos orgânicos voláteis e dos aminoácidos, respectivamente. Os valores de LOD e LOQ obtidos confirmam a alta sensibilidade dos métodos desenvolvidos.

As metodologias acima referidas, bem como a metodologia standard para a determinação do FAN, foram aplicadas para monitorizar 21 aminoácidos e 22 compostos orgânicos voláteis em três cubas de fermentação lager. Diferentes métodos de conservação foram testados e a adição de cloreto de cálcio associada ao armazenamento a -26°C por 2 dias foi o tratamento escolhido para evitar a evolução das amostras até à análise das mesmas.

Identicamente ao que se verificou em relação às substâncias nitrogenadas, as principais variações a nível dos compostos orgânicos voláteis ocorrem também nas primeiras 150 horas de fermentação. A análise das amostras em estudo permitiu relacionar a abundância dos compostos nitrogenados com as baixas taxas de fermentação observadas, justificando assim a formação/redução tardia das dicetonas vicinais. Efectivamente, associou-se o aparecimento de um segundo pico de diacetil à exaustão dos aminoácidos do grupo A, da leucina e da isoleucina.

Palavras-chave: Cerveja lager; Fermentação; Compostos orgânicos voláteis; Dicetonas vicinais; Aminoácidos.

SUMMARY

Vicinal diketones, diacetyl and 2,3-pentanedione, impart unpleasant aromas to beer, when their concentrations are higher than their odour threshold limits. The formation of these off-flavours is related to valine, leucine and isoleucine biosynthesis. Therefore, vicinal diketones as well as amino acids determination is a major concern during beer fermentation.

A new methodology for the determination of volatile organic compounds in fermentation samples by GC-MS, including a design of experiments for compounds extraction optimization by HS-SPME, was successfully developed. A 20-minute extraction at 40°C, by exposing a Car-PDMS fibre in a 20 mL vial containing 10 mL of sample and 3.3 g of NaCl was adopted. A methodology for the determination of amino acids by HPLC-fluorescence, including on-line derivatization was also developed.

The developed methodologies present a good linearity in the concentration ranges in study. Recovery mean values ranged from 89.88 to 133.80% and from 77.60 to 112.39%, while methods precision, evaluated in terms of repeatability and reproducibility, showed variations lower than, respectively, 10% and 15% for volatile organic compounds and amino acids determination. Also, LOD and LOQ values confirm the high sensitivity of these methodologies.

The methodologies mentioned above and standard FAN methodology were applied to monitor 21 amino acids and 22 volatile organic compounds in three batches during the fermentation process. Conservation tests were performed and chloride salts addition and storage at -26°C for two days was the most suitable treatment for sample preservation.

Volatile organic compounds evolution during the analysed lager fermentations showed that, similarly to nitrogenous compounds, major changes occur in the first 150 hours of fermentation. Low free amino nitrogen content may explain low fermentation rates and be the cause of delayed vicinal diketones formation/reduction. In addition to group A amino acids complete assimilation, leucine and isoleucine exhaustion leads to the occurrence of two diacetyl peaks and to increased fermentation time.

Keywords: Lager beer; Fermentation; Volatile organic compounds; Vicinal diketones; Amino acids.

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ABBREVIATIONS

ACN - Acetonitrile

ADP - Adenine diphosphate

AQC - 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

ATP - Adenine triphosphate

Car - Carboxen

CEOC - 2-(9-carbazole)-ethyl chloroformate

DEEMM - Diethyl ethoxymethylenemalonate

DNA - Deoxyribonucleic acid

DoE - Design of experiments

DVB - Divinylbenzene

EBC - European Brewery Convention

ECM - Empresa de Cervejas da Madeira

EDTA - Ethylenediamine tetraacetic acid

FAN - Free amino nitrogen

GC - Gas chromatography

HPLC - High pressure liquid chromatography

HS - Head space

IBLC - N-isobutyryl-L-cysteine

IDA - Iodoacetic acid

IoB - Institute of Brewing

ISO - International organization for standardization

LC - Liquid chromatography

LOD - Limit of detection

LOQ - Limit of quantification

MCE - 2-mercaptoethanol

MPA - Mercaptopropionic acid

4M1P - 4-methyl-1-pentanol

MS - Mass spectrometry

NAC - N-acetyl-L-cysteine

NAD - Nicotinamide adenine dinucleotide

NIOSH - National Institute of Occupational Safety and Health

OPA - Ortho-phthaldialdehyde

PA – Polyacrylate

PDMS - Polydimethylsiloxane

PTFE - Polytetrafluoroethylene

RP - Reverse phase

SBSE - Stir Bar Sorptive Extraction

SPE - Solid-phase extraction

UV - Ultra-violet

Vis - Visible

VDK - Vicinal diketones

VOC - Volatile organic compound

SHORT *curriculum vitae*

Ana Carolina Leal Vieira was born in 1993, in Santa-Cruz, Portugal and is graduated in Biochemistry (2014) by University of Madeira. In this institution, Carolina followed several scientific works in order to gain abilities related to chromatography, spectroscopy (methodologies implementation and validation) and fermentation. Later, in 2015, she joined a local brewery for a one month internship to learn more about beer fermentation and the main analytical procedures used in beer industry.

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Publications

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PART I: Scope and goals

SCOPE AND GOALS

Data concerning beer global production have shown an increasing trend during the last decade, keeping beer on the top rank of the most consumed and popular alcoholic beverages. Together with consumption and production volume increase, the market demands and consumer's preferences have also undergone changes which call for innovative technologies and a more comprehensive beer production knowledge (1). In the last years, scientific community together with beer industry have been focusing on the study of the chemical processes behind the formation of metabolites during the production process, namely during the fermentation phase, which have great impact in beer quality and stability (2, 3).

For that reason, advanced analytical methods are continuously being developed in order to identify and quantify beer compounds formed in each brewing stage, so that producers can understand how to eliminate undesirable substances and promote those that contribute to a superior quality beer (2). This is of particular importance for the comprehension of beer off-flavours formation, such as vicinal diketones, which impart a negative impact to beer appreciation, with a butter-like aroma (3).

The present study keeps in line with the previous considerations and looks for the development and validation of analytical characterization techniques which allow to monitor key metabolites during the fermentation phase, probably the most important phase during the production process. The present study is carried out in strict collaboration with a local brewery, the Empresa de Cervejas da Madeira (ECM). To be precise, the present study intends to develop, optimize and apply a methodology to determine vicinal diketones (VDKs) and other important volatile organic compounds (VOCs) produced during beer fermentation. Amino acids concentration influence (total and in the individual terms) on vicinal diketones formation will be also evaluated. The developed methodologies will be used to characterize different fermentation lots, in order to analyse the importance of raw materials, some processes parameters, as well as the process variability. In this regard, the production of three different beers lots were followed, during 12 days.

The present work is organized in four parts. This first part aims to established the scope and goals of the present study. Part II covers the background on the thesis theme, namely the main biochemical reactions involved in beer production. Special attention is devoted to metabolites produced by the brewing yeast during fermentation and their impact in the final beer perception. The process parameters which can also influence the final beer quality are also

presented. Due to the relevance for the present study, Part III is dedicated to vicinal diketones review, once the main goal of this work is to understand these metabolites evolution during beer fermentation. Part V gathers all the laboratorial methodologies implemented during this study and Part VI is dedicated to the presentation of the obtained results and to their discussion. Finally, Part VII includes the most important conclusions that result from this study and also adds some suggestions concerning improvements in future experiments.

PART II: Beer

BACKGROUND

Several centuries ago, right after the end of ice age, human began to plant cereals and other cultures in order to obtain food and learning how to preserve these for an extended period. Consequently, experiments have been carried out and soon early societies were able to germinate, mill and store cereals. Later, the first attempts of cereal fermentation were accomplished with the addition of fruits containing natural yeast to the wort and so, with no scientific knowledge, primordial beers were made. The dissemination of beer production culture and the different brewing preferences from region to region contributed to the diversity of modern beer styles, which vary according to malt type, adjuncts, yeast strains, alcohol content and taste. Simultaneously, the knowledge about fermentation and brewing related technologies also evolved (1).

During the 18th and 19th centuries, new scientific principles about brewing were developed to explain the chemical reactions involved in fermentation, such as the concepts of substrate and enzyme and the importance of controlling the temperature of malting and brewing processes. Considering the pertinence of these discoveries, new equipment and technologies were developed as well and the progress in optics, for example, allowed the study of microorganisms in beer production process for the first time. Pasteur, studying the role of these organisms in the conversion of sugars in alcohol, the differences between aerobic and anaerobic metabolisms and the different yeast strains, gave one of the greatest contributions to the science of fermentation (1).

In the latest years of the 19th century, several institutions were created worldwide and soon brewing became an accepted branch of natural sciences. Consequently, the industrialization of beer production created the modern market as we know today (1). In the 21st century, beer is still one of the most popular beverages in the world and is actually the most consumed alcoholic beverage (4, 5). Besides that, and although the excessive intake of any alcoholic spirit is not recommended, several benefits have been associated to the moderate consumption of beer. Scientific studies revealed that many chemical compounds found in beer show different biological effects, as a title of example, antioxidant, antiviral, anti-carcinogenic and anti-inflammatory activities. Additionally, polyphenols from malt and hops and the lipidic constitution of beer can also have a great influence in the coagulation system and may explain the decrease of the incidence of cardiovascular diseases (6).

In the early 1800's, Madeira Island (Portugal) began to import malt to produce beer and similar beverages and, in 1854, regulations started being applied to small breweries. Later, the increased consumption and production resulted in new local beer producers and in 1934, Empresa de Cervejas da Madeira (ECM) was founded by merging two pre-existing breweries. As the demand increased, new installations and equipment were acquired and nowadays ECM is the largest producer and distributor of beverages in Madeira, with more than 140 years of industrial experience. Up to 2013, ECM was internationally awarded several times and this company's management system became certified by international standards ISO 9001 and ISO 14001 (7).

ECM has been made a continuous effort for upgrading its production techniques and has also participated in several collaboratives studies to acquire deeper product knowledge which can lead to process improvement.

In a partnership between ECM and University of Madeira a study has been carried out in order to understand more extensively the processes inherent to beer stability during storage and their influence on the standard quality parameters and volatile fraction (7). The further data analysis study allows to detect statistically meaningful deviation from the desired scenario (month 0, fresh sample) after a period of 7 months of ageing in bottle, indicating that changes in terms of aromatic profile during this period can be perceived (8). Volatile metabolomics patterns of beer raw materials (9), terpenoid metabolomic pattern of hop-essential oil (10) were studied and a new methodology for the determination of prenylflavonoids in beers was also developed (11). Later, another study was carried out and a new optimized methodology was developed to determine two important beer off-flavours, diacetyl and 2,3-pentanedione (12).

INTRODUCTION

Beer is a fermented alcoholic beverage (3-5%, v/v) (5) produced from malt, cereals and hop, which are the raw materials used by yeast as a substrate to transform simple sugars in alcohol and carbon dioxide, in an aqueous and anaerobic environment (3). Besides the primary products of alcoholic fermentation, secondary metabolites are also produced and some of these impart a particular flavour to the final beer and have great relevance in beer colloidal and organoleptic stability. Contrarily, others induce certain off-flavours formation and contribute to beer degradation (4). The deepen understanding of all the raw materials properties, different

yeast strains metabolisms, the influence of adjuncts and brewing parameters is the modern industrial breweries main concern (2).

Beer properties depend not only on the type and quantity of raw material used but are also significantly different according to all the processes involved in its production (13). Consequently and besides of contributing to beer appreciation, when high quality raw materials are used and production processes are optimized, beers can also provide a significant source of vitamins, proteins, organic acids, minerals and polyphenols to consumers (14).

BEER RAW MATERIALS

Water

All chemical reactions related to beer production occur in an aqueous medium and water represents about 90% of the final composition of beer (15). The origin of the water used in wort manufacture is an important parameter in brewing because the amount and type of dissolved inorganic salts affects all the chemical reactions (5), influence reaction media's pH value and consequently affects hop compounds extraction during wort boiling. A lower water pH value increases some enzymes activity and a higher value may contribute to undesirable polyphenols extraction from grains, thus affecting beer flavour (15).

Some of the most important minerals found in the brewing water are the phosphor, which allows ATP production, integrates yeast's phospholipidic membranes and acts as a buffering ion, the sulphur, used in amino acid metabolism and potassium and sodium, necessary to hydrogen ion bomb activation. Iron and magnesium are two important ions in respiration metabolism and in cell multiplication and growth. Magnesium is an important coenzyme in yeast fermentation process and finally, calcium is very useful in brewing once it protects α-amylase from thermal degradation, contributes to a desirable wort colour and also helps controlling yeast flocculation (2).

At last, zinc is another important coenzyme to yeast metabolism (16) and optimal amounts of this ion, specifically when its concentration is up to 0.08 mg/L, increases fermentation rates (17). Zinc also affects protein production. However, even if some inorganic salts contain these metallic ions in brewing water, usually some of them are added during wort production to achieve an optimal yield later in fermentation (2).

Malt

Malt is the major raw material used in brewing and is obtained by the germination followed by the desiccation of cereal grains. This is the main starch source for yeast metabolism during fermentation (5). Barley is the most common cereal used as malt in the brewing industry due to its superior starch content and moderate protein, enzymes and nitrogen levels (15). Barley husks also contain a relatively high amount of polyphenols comparing to other cereals, which impart astringency to beer flavour (5) and its content in amylose and amylopectin is 24% and 76%, respectively (15). Different type of malt (Figure 1) can be added to wort manufacture to impart special character to final beer. Special malts, which can be, for example, smoked, acidified, organic or toasted, contribute from 10 to 25% of total malt and enrich beer colour, flavour and stability but base malts englobe almost the total enzymatic capacity (15).

Malting is a treatment applied to barley grains and is constituted by three important steps: steeping, germination and kilning (5). First of all, during steeping, selected cereal grains are immersed in water during 3 to 5 days, to increase their moisture content to approximately 40% (15), which induces germination and inherent chemical reactions, such as enzyme production and the degradation of complex sugars and proteins in soluble and smaller molecules (5). This process lasts for about 8 days (15).

Figure 1: Barley grains with different malting treatments (18)

After this period, grain physiologic changes are blocked when germination is stopped by increasing temperature to 60-70°C during a few minutes, which decreases water content to 2-5% and stopsthe germination process (15). At this stage, Maillard-type reactions and caramelization of sugars caused by heating contribute to beer colour and flavour formation. This is an absolutely decisive step in the malting process, once it allows brewers to obtain different types of malts, according to kilning temperature and duration (5).

Consequently, the evaluated malt parameters by beer producers are barley variety, sugar extract yield, nitrogen content, grain size and modification, moisture, colour and enzyme capacity (19). At the end of malting, controlled germination was achieved and interrupted, enzymes were produced, starch was modified and malt grains are softer and more soluble (19).

Hops

Since ancient times, herbal species have been added to fermented products to enhance its durability during storage but, nowadays, hop (Figure 2) is the only specie industrially used in the brewing process. Precipitation, temperature, pesticides and other parameters can immensely affect hop aromas and beer quality (20). There are about 200 compounds in hop essential oils and most of them are volatile substances (15). These are mainly monoterpenes, terpenes and sesquiterpenes. Therefore, hop enhances beer flavour and may impart spicy, herbal, floral, fruity, citrus and pine aromas to final beer (21).

Hops contain resins, essential oils, minerals and tannins as well (15) and some of these compounds are useful on colloidal and oxidative stability (21). Hop resins impart a characteristic and intense bitter flavour to beer and also contributes to microbiological stability of beer. These resins can be divided in insoluble α-acids and β-acids that can be isomerized and converted into respective iso-α/β-acids, that are dissolved in finished beer (5). Hop resins and isomers also showed activity against common beer spoilage microorganisms such as lactic acid bacteria. However, some microorganism strains seem to show resistance to these compounds (22).

Figure 2: Hops flowers used in beer production (23)

Hops are also the most important source of polyphenols found in beer. Besides their importance in terms of antioxidant activity (14), they also affect physical stability of beer during storage, since some of these polyphenols tend to react with proteins, leading to the formation of insoluble complexes. Consequently, these colloidal particles may precipitate and affect the beer transparency and thereby reduce the shelf-life of this beverage (24).

Adjuncts

Besides malt, other cereals are used in beer production and malt enzymatic potential is usually enough to catabolize additional starch from adjuncts. For this reason, 15 to 20% of the cereals used in wort production are raw grains that have not been submitted to the malting process, that have a higher carbohydrate content, such as rice, wheat, corn, sorghum and even barley (2). These cereals have a lower production cost and add peculiar characteristics to beer or attenuate other flavours (15). Adjuncts are processed before being added to wort, so that grains become softer and gelatinized (25).

In alternative to non-malted cereals, other adjuncts may be added during beer production like sugar, caramel and syrups. A higher content of adjuncts can affect beer quality due to off-flavours formation (15). Consequently, according to the cereals or additives chosen, adjuncts and their amount in wort production impart special characteristics to beer and also affect beer fermentation and quality (2).

WORT PRODUCTION

Wort is a complex and equilibrated solution that contains fermentable carbohydrates, amino acids and minerals and is a source of nutrients for yeast to perform alcoholic fermentation. In this solution, yeast can find all beer taste and aroma precursors that will define the organoleptic properties of final beer (15). During this process, several enzymes transform malt and other cereals grains in fermentable substances and other compounds that yeast can metabolize and that are necessary to yeast multiplication and growth. Thus, the main goal of wort production is to prepare everything that is needed for the fermentation (2). Wort production steps are summarized in Figure 3.

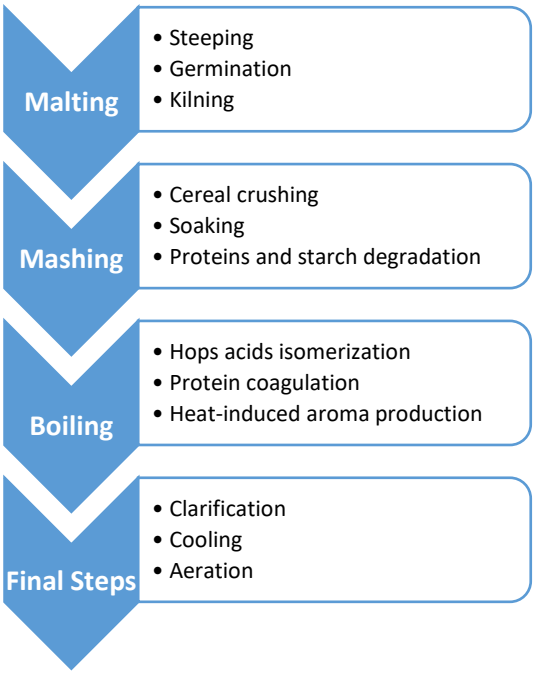


Figure 3: Wort production steps in brewing, adapted from Kunze (2)

Mashing

Prior to wort manufacture, adjuncts and malt are selected, processed and weighted and then are separately submitted to a mashing treatment (3). Right after the cereals are crushed, warm water is added to malt so that starch from grains can be hydrolysed and gelatinized (7). At this stage, several reactions are performed at increasing temperatures, so that insoluble starch can be transformed into soluble and fermentable sugars (mainly glucose and maltose) by amylolitic enzymes and other simpler sugars are also produced, like branched dextrins (7). At each temperature level, different malt enzymes (amylases, phosphatases and proteases) are activated (15) and so complex proteins are also transformed in soluble peptides and amino acids (5).

Adjuncts are similarly dissolved in warm water for a long period of time and are treated with phosphoric acid and calcium sulphate and chloride, so that a slightly acidic pH value is achieved, as usually required to a proper fermentation (2). Controlling the pH level is as important as setting the adequate temperature to optimal enzymatic activity in wort manufacture (15). Calcium chloride addition will also be indispensable later in fermentation, helping lager yeast to form calcium bridges and consequently flocculate, so that yeast can be easily removed (2).

Then, soaked malt and solid adjuncts are combined in one vessel and malt enzymes, specifically α and β-amylases, at approximately 65°C, degrade starch from malt and adjuncts in simpler sugars (7). This process is known as saccharification (15). Finally, the mixture is submitted to a higher temperature, so that enzyme denaturation occurs (2). At the end, the mixture is filtered in order to separate the solution were nutrients are dissolved from spent grains, through a process called lautering (7).

Boiling

When wort separation is complete, the wort solution is transferred to a kettle and is boiled for 1 to 2 hours (20). At this stage, zinc chloride is added to ensure a good fermentation (2) and hop is also added and its resins are isomerized into bitter acids, important to beer flavour. Due to high temperature, some of these volatile compounds may evaporate (15). During boiling, caramelization and Maillard reaction also occur between reducing sugars and primary amines (7), leading to the formation of compounds that are important to beer flavour (26). These reactions and other phenomena, such as polyphenols oxidation and wort concentration impart a darker colour to wort (15).

At last, protein coagulation is also provoked by increased temperature (7), which prevents beer haze that may be produced by the interaction between proteins and tannins (27), some of these prevenient from hops (24).

Wort clarification, cooling and aeration

After boiling, wort is transferred into a decantation vessel where suspense particles are removed and the wort becomes clarified (7) and, right after this, it is cooled until it reaches an adequate temperature for fermentation, which optimal values differ between bottom and top fermenting yeasts (2). Then, before yeast is inoculated, the wort is aerated so that fermentation media contains a proper oxygen level, usually from 4 to 14 mg/L, depending on yeast strain (28), required for a fast yeast cell multiplication and growth (2), although oxygenation needs to be restricted to avoid too vigorous fermentation (28).

As mentioned before, beer quality and specifications are affected by raw materials (type and proportion) used in wort production. Nevertheless, wort production and fermentation

parameters are evenly important to define the produced beer type and quality (15). For example, when the employed malting and mashing methods lead to an excessive content of proteolytic enzymes, increased ester formation occurs (2), due to the higher availability of yeast assimilable nitrogen compounds in beer (28). On the other hand, turbulence in wort production tanks increases fatty acid formation and, consequently, inhibits ester formation (2).

FERMENTATION

After the wort is cooled and enriched with oxygen, it is transferred into a fermentation vessel and, simultaneously, yeast is inoculated in wort. The fermentation main goal is to produce alcohol and carbon dioxide, as fermentable sugars are consumed by the yeast. However, while primary fermentation occurs, secondary metabolites are also produced such as esters, alcohol, fatty acids, aldehydes and ketones (7). In the fermentation vessel, all the parameters affect yeast physiologic and metabolic state and will influence beer final quality. Temperature, pH, pressure, reduction-oxidation potential, oxygen level and the type and concentration of nitrogenous compounds, sugar and minerals dissolved in wort will affect beer style and quality (2).

One important consideration to have in mind is that, in fermentation vessels, yeasts are not in their preferential state, once the increasing ethanol and carbon dioxide levels are prejudicial to them and they need to get adapted to these hostile conditions (2). For this reason, a good yeast cell vitality is the key to have a sufficient metabolic activity so that a high quality beer can be produced (29). Therefore, studying the influence of fermentation parameters on the yeast metabolism is a common concern of modern breweries. The deepen knowledge of fermentation derived compounds, of their precursors and respective formation pathways is culminant to avoid or reduce off-flavours formation that affect beer stability, quality and appreciation (2).

Together with raw materials, the yeast used in the fermentation process defines the beer style. The two major beer styles are the lager and ale beers.

Brewing yeast and beer types

Yeasts are non-photosynthetic unicellular fungi, widely used in food and beverages fermentation, that reproduce asexually by budding or fission. Brewer's yeast belongs to the genus *Saccharomyces* and its main metabolic products, ethanol and carbon dioxide, are

produced from glucose and other similar sugars (30). Simultaneously, other chemical reactions occur and secondary metabolites are also produced. Some of these substances impart pleasant flavours to the beer, while others are considered as off-flavours and negatively affect beer taste and aroma (2).

Yeast cells contain approximately 80% water and carbon is the main element found in these cells, followed by oxygen, nitrogen, hydrogen and phosphor. Additionally, 5 to 10% of yeast dry weight represents mineral elements. The most abundant macromolecule classes found in yeast cells, considering their dry weight, are proteins (40-45%), carbohydrates (30-35%), nucleic acids (6-8%) and lipids (4-5%) (17). Yeast also contains vitamins and even spent yeasts are often used as a nutritional supplement (31).

In a general way, beers can be classified as ale and lager, according to the type of yeast added to wort, that produce typical and distinct beer flavour profiles (15). These differences result from the morphologic and physiologic characteristics of the yeasts and from their performance in fermentation. Systematic yeast classification follows parameters like fermentation and flocculation behaviour, fermentation performance, yeast propagation extension, fermentation by products formation and removal (2) and attenuation limits (32).

An ale or top fermenting beer ferments at higher temperatures (14-15°C) for a shorter period of time, the active yeast settles on the top of fermentation vessel and, usually, *Saccharomyces cerevisiae* strains are used (2). These specifications lead to a highly fruity beer, due to an increased esters formation (7, 15). On the other side, a lager or bottom fermenting beer may be obtained by using *Saccharomyces uvarum*, also known as *S. carlsbergensis*, which ferments at lower temperatures (4-12°C) for a longer period of time and that flocculates at the bottom of the fermentation vessel. Bottom fermenting yeast shows a smaller number of enzymes (2). Therefore, lager beers are lighter than the previous, once yeasts have a lower influence in beer flavour (7). The main differences between top and bottom fermentation are illustrated in Figure 4.

Nowadays, modern breweries acquire pure yeast cultures and propagate them at brewery facilities in sterile wort (25). However, attention should be given to the fact that malt, adjuncts, industrial surfaces and equipment can be sources of wild yeast strains contamination, which may compete for nutrients with the brewing yeast and impart undesirable unpleasant flavours and turbidity to the beer (2). Also, bacteria like *Lactobacillus* and *Pediococcus* species and other microorganisms also contribute to malolactic fermentation and other chemical reactions, which affect beer quality (33).

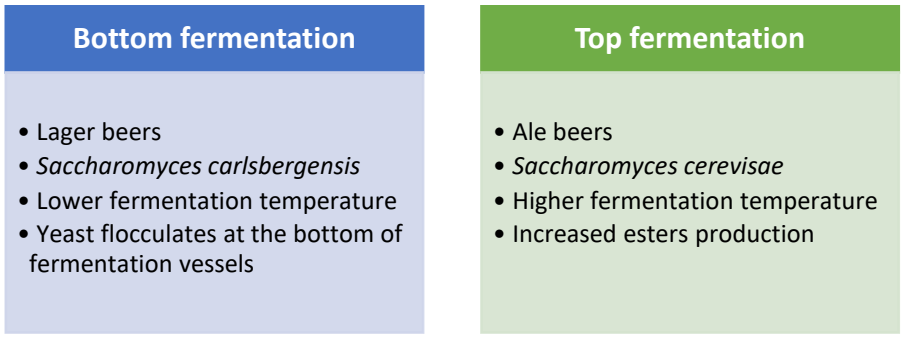


Figure 4: Bottom and top fermentation characteristics, adapted from Kunze (2)

In lager fermentation systems, vertical cylindroconical fermenters are used and the yeast is pitched between 7 and 8°C, and after a couple of days the temperature is increased to 10 to 11°C. Then, 3 to 4 days later, at peak fermentation, temperature is allowed to ramp up in order to facilitate a rapid reduction of diacetyl (32).

Yeast growth and multiplication

When yeasts are transferred into fermentation vessels, six different growth phases can be distinguished. Firstly, during lag phase and depending on yeast strain, age and conditions, yeast metabolism is activated and this stage is over with the first cellular division (2). This process can be divided in bud initiation, DNA synthesis, nuclear division and, at last, cell separation (30). Next, the culture experiences an acceleration phase, where continuous and increased cell multiplication have place, until exponential or logarithmic phase is achieved. At this moment, growth rate is maximal and constant, generation time is minimal and yeast shows the highest vitality (2).

Consequently, due to the low availability of nutrients and increased growth inhibitor metabolic products, a deceleration phase occurs and growth rate slows down gradually until a stationary stage is reached. At this moment, the living and dead cells number is similar and so there is a constant yeast cells number. Finally, yeast population is significantly reduced when the cell death rate increases, as a consequence of nutrient sources being almost fully drained and once ethanol and carbon dioxide contents increase, which starts inducing cell autolysis. This stage is known as decline phase. At this point, yeast should be removed in order to avoid intrinsic metabolites release into beer, which could cause major damages in beer quality (2).

Effectively, the duration and intensity of these stages is highly influenced by the substrate, water and oxygen content, by solution temperature and acidity and also by the physiologic state of the yeast (2). Yeast pitching, defined as the amount of inoculated brewing yeast, is another important parameter and must be adapted according to yeast viability, vitality, strain, flocculation capacity and according to the desired beer characteristics. For example, highly flocculent yeast strains tend to settle prematurely and additional yeast may need to be added to the fermenting wort. Wort composition, gravity, aeration and fermentation temperature also influence the pitching rate. (32).

In beer industry, yeasts can be reused several times, depending on strain, wort gravity and other factors (34). However, re-pitching can originate microbiological contamination and yeast deterioration can also occur (35). Repeated re-pitching causes subtle changes in yeast flocculation and a decline in its viability and vitality (31). Thus, yeast re-pitching must be limited in order to avoid problems during fermentation (35).

Free amino nitrogen (FAN) from wort is a group of low-molecular-weighted nitrogen compounds (36) that include amino acids, small peptides and ammonium ions (37), resultant from malt protein proteolysis during malting and mashing. When adapting to the new environment, yeasts use the available nitrogen to produce cellular proteins and other components. FAN is a good indicator of an appropriate cell growth and an efficient fermentation performance. Similarly to sugars, amino acids are absorbed by the brewing yeast by a preferential order (38).

Amino acids from group A are the first being totally consumed by yeasts, while amino acids from group B are gradually consumed at a slower rate and usually are not completely absorbed. Then, amino acids from group C are assimilated even more slowly and incompletely, after groups A and B amino acids were exhausted. A fourth group englobes the un-preferred amino acids, which are almost never consumed (39). Valine, leucine and isoleucine are important compounds related to some beer off-flavours formation (3). This will be addressed in part II of the present thesis.

Yeast metabolism

Yeasts are able to use two different processes to produce energy: respiration and fermentation. Even if respiration is energetically more efficient under aerobic conditions, surprisingly, some yeast species use both respiration and fermentation in order to obtain energy

in environments with sufficiently high glucose and oxygen levels. This phenomenon is known as Crabtree effect (40). Most of brewing yeasts belonging to the *Saccharomyces* genus are Crabtree-positive. Energy obtained during fermentation in anaerobic environments by yeasts is used to produce cellular substances, to absorb and assimilate surrounding compounds, to break and excrete unneeded or hazardous molecules and to transport substances inside the cells (2).

During alcoholic fermentation, glycolysis occurs and, from each glucose molecule, two ethanol and two carbon dioxide molecules are produced by yeasts and energy is also released during this process. This is the basic principle of beer fermentation chemistry. Other fermentable sugars can also be used as substrate but glucose is preferentially assimilated by the brewing yeast (15). Carbon dioxide concentration in beer is typically around 5 g/L for lager beers, but in certain specific beer styles can be as high as 10 g/L. The ethanol content of beer depends on the amount of fermentable sugars available in wort and usually oscillates between 3 to 5% (v/v) (5). The detailed metabolic pathway of glycolysis and alcoholic fermentation will be described below and is illustrated in Figure 5.

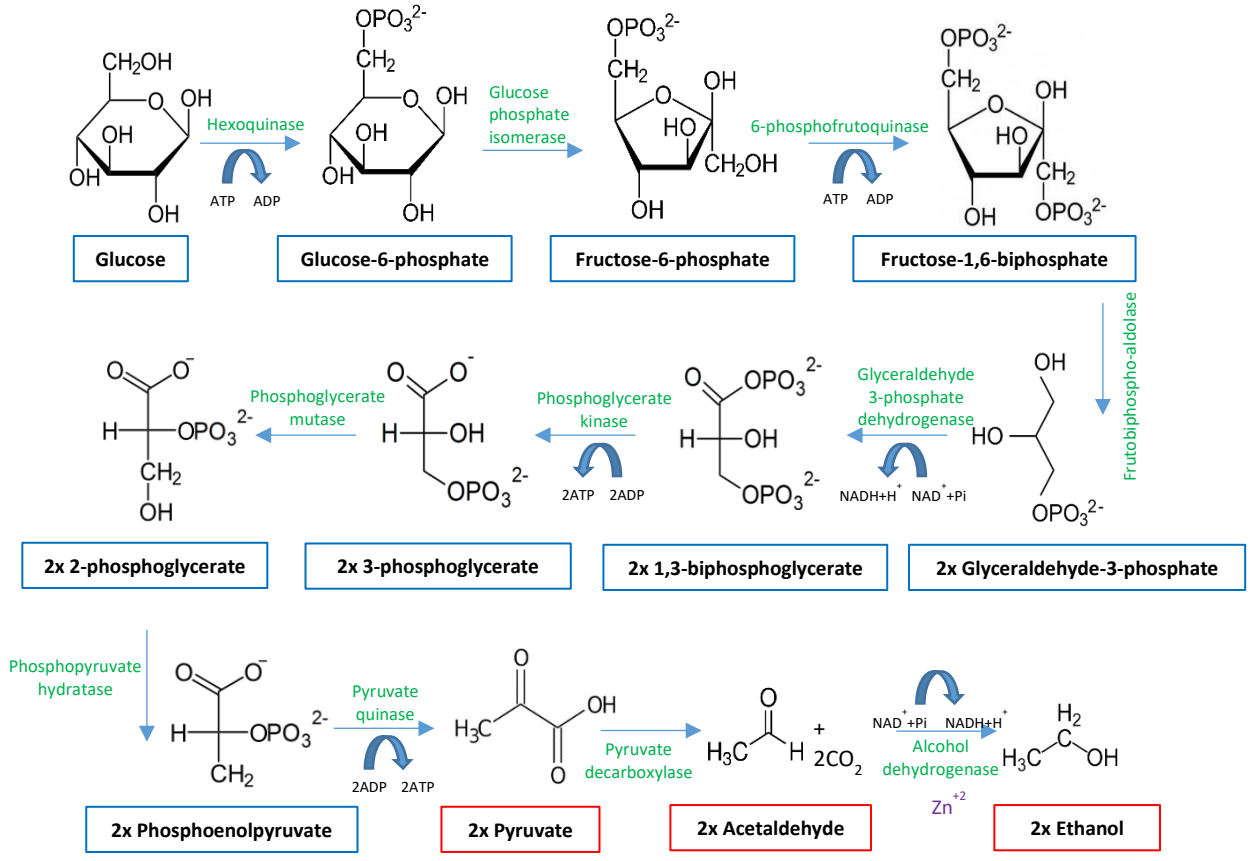


Figure 5: Chemical transformations that occur during glycolysis (blue text boxes) and alcoholic fermentation (red text boxes), adapted from Kunze (2)

Firstly, during glycolysis, each glucose molecule is phosphorylated and glucose-6-phosphate is produced and transformed into fructose-6-phosphate, which is also phosphorylated, originating fructose-1,6-biphosphate. This molecule ring is then split into two triose sugars that suffer several chemical rearrangements until two pyruvate molecules are formed. Subsequently, alcoholic fermentation begins and pyruvate decarboxylase transforms each pyruvate molecule into one carbon dioxide molecule and one acetaldehyde molecule and this last one is then converted to ethanol by alcohol dehydrogenase (2).

During fermentation, yeasts are constantly absorbing and releasing substances. Some compounds are transported passively through the phospholipidic membrane, while others are subjected to active transportation, where specific transport proteins intervene. Nitrogen compounds, for example, are absorbed by yeast to produce cellular substances, phosphate ions become part of indispensable nucleotides and constitute intern and extern phospholipidic cell membranes, while carbohydrates are stored as energy reserves. Oxygen molecules are useful in fatty acids and sterols synthesis, which also integrate cell membranes (2).

Minerals, like zinc, and other vestigial elements are also important to yeast and to fermentation (2). Zinc plays an important role in the function of many enzymes, including alcohol dehydrogenase (17). Some of these compounds can be found in wort, while others are produced by yeast from other complex molecules, but insufficient levels of any of them may cause serious problems during fermentation (2).

As a result of these compounds intake and transformation, important beer flavours are formed. Fusel alcohols are by-products of the Ehrlich mechanism and are produced in parallel with protein metabolism. These compounds result from amino acids, after suffering deamination and subsequent decarboxylation and reduction. When excreted from the cell, these compounds impart pleasant aromas to beer. Lipid metabolism is a secondary process, which is only activated when nitrogen sources used in protein metabolism are exhausted. This is an oxygen dependent mechanism that affects yeasts cellular membrane malleability. Lipids are negligible components in beer but can influence its organoleptic and physicochemical properties (41).

Secondary metabolites formation

The concentration of fermentation by-products in beer varies according to yeast cell growth patterns, that depend on wort composition and fermentation extension (2). Pitched

yeast strain and condition as well as pitching rate are key pre-requisites to obtain high quality fermentation products. Aeration, by rousing or stirring, the size and geometry of fermentation vessels, wort pH and fermentation temperature and pressure will also influence the compounds produced during fermentation. Depending on yeast strain, the order of importance of these parameters may be different (20).

The most important families of secondary metabolites produced during beer fermentation are the following: organic acids, higher alcohols, esters, aldehydes, sulphur compounds and vicinal diketones (2, 5, 15). These and other compounds concentrations are affected by different parameters that will be presented in the following paragraphs.

Higher alcohols formation is related to nitrogen compounds assimilation, by the deamination, decarboxylation and reduction of wort amino acids (15) and they may also be formed through carbohydrates metabolism (28). About 85% of beer higher alcohols are produced from three amino acids: L-leucine, L-isoleucine and L-valine (15). Most of these compounds are formed during primary fermentation and their formation is slightly increased at lager phase. Increased temperatures, higher wort extracts, intense aeration and lower amino acids levels increase higher alcohols formation, while lower temperatures, higher pitching rates and pressure lead to the opposite scenario (2).

Of about 40 alcohols found in beer, n-propanol (13), 2-methylpropanol, 2 and 3-methylbutanol and 2-phenylethanol are the most important alcohols that contribute to a positive beer flavour (5). These compounds contribute to a warming and alcoholic flavour (13). Concentrations higher than 100 mg/L negatively affect beer taste and acceptation. However, these compounds are characteristic of a finished beer (2). Higher alcohols are also the direct precursors of beer esters (5), through the reaction with acylated enzymes (13).

During fermentation, yeasts assimilate nitrogen compounds such as wort amino acids and, after removing amine groups in protein synthesis, the remaining parts are released into beer as organic acids (2). These compounds are responsible for lowering beer pH (15) and, consequently, impart astringency to beer (5) and also contribute to beer microbiological stability (15). Vigorous fermentations lead to a higher level of organic acids (5). Some of the most important organic acids in beer are acetic acid, formic acid, succinic acid and malic acid (15).

Unsaturated short-chain fatty acids, essentially from C_6 to C_{10}, are also produced during beer fermentation through the breakdown of longer-chain ones. They impart unpleasant flavours to beer and are also responsible for the inhibition of beer foam formation (17). Unsaturated fatty acid synthesis is promoted by oxygen and is associated with the inhibition of

ester formation (17). Usually, in finished beers, octanoic acid is the most abundant one, followed by hexanoic and decanoic (42-44) and total fatty acid content ranges from 15 to 30 mg/L (45). Sometimes, additive fatty acids flavour results in a perceptible rancid or goaty off-flavour (43).

Esters are an important family of compounds in beer, resulting from chemical rearrangements between alcohols and organic acids (15) and are the most important aromas produced during fermentation (2). These compounds impart fruity (5) and floral (15) notes to beer aroma and ethyl acetate, isoamyl acetate and isobutyl acetate are the most important ones, once they are usually above their flavour threshold in this beverage (5). These compounds are produced by alcohols and fatty acids esterification and a typical beer has an ester content of 10 to 30 mg/L. Esters formation is affected by almost all technological parameters. Higher aeration, wort concentration and temperature induces ester production, while pressure and insufficient aeration and wort concentration inhibits this process (2).

Aldehydes are carbonyl compounds derived from alcohols oxidation, malt fatty acids and lipids (5) or from organic acids decarboxylation (15). Acetaldehyde is the most important aldehyde found in beer and is excreted into beer at the beginning of fermentation and it is a normal fermentation intermediate compound. As fermentation goes on, acetaldehyde concentration decreases (2), either by reduction to ethanol or oxidation to acetate. When acetaldehyde is above its threshold limit, it imparts a green apple aroma to beer (5, 15) which is not desirable in beer and for this reason its concentration must be controlled during fermentation (15). Aldehydes are much more flavour-active than their corresponding alcohols (13). Acetaldehyde formation is induced by high fermentation temperatures, pitching rates, pressure, low aeration and by wort microbiological contamination (15).

Sulphur compounds have a great impact in beer volatile profile, imparting important notes to the overall beer appreciation at low concentrations. However, when these are found above their threshold limits, they negatively affect beer flavour and quality (5). Beer sulphur compounds include hydrogen sulphide, mercaptans, sulphur dioxide and dimethyl sulphide. Once these compounds have high threshold limits and very unpleasant aroma, their chemical and biochemical removal is an important step in beer production. Sulphur compounds may also be result of beer microbiological contamination (2).

Vinyl guaiacol, also known as 2-methoxy-4-vinylphenol, is produced through the decarboxylation of ferulic acid (46), either during barley roasting, wort boiling or fermentation (47). It presents a low odour threshold and a phenolic or medicinal aroma (46) and besides being appreciated in some ale beers, vinyl guaiacol aroma is often considered as an undesirable flavour

in lager beers (48). For that reason, the comprehension of its formation and possible strategies for controlling vinyl guaiacol during fermentation are important subjects in beer industry. Also, some yeasts are genetically manipulated so they cannot assimilate ferulic acid and in that case, vinyl guaiacol formation is strictly related to the presence of microbiologic contamination (49).

Vicinal diketones, diacetyl (2,3-butanedione) and 2,3-pentanedione are other problematic compounds that can be found in beer. They impart an unpleasant butter-like aroma to this beverage and are produced extracellularly by the spontaneous oxidative decarboxylation of α-acetohydroxy-acids. Amino acids such as valine, leucine and isoleucine are important compounds in vicinal diketones synthesis (50). These compounds will be carefully discussed in the next chapter.

Yeast flocculation and removal

Flocculation is a reversible process that involves yeast cells aggregation into large masses when sugars become less abundant and yeasts either sediment (bottom-fermenting) or remains suspended (top-fermenting) in the medium, forming a thick layer (17, 31). There are evidences that calcium ions and yeast membrane proteins interact to form salt-bridges between yeast cells, allowing them to stick together (20). This phenomena is very important to beer producers once it helps yeast removal at the end of primary fermentation (35). Flocculation must happen at the right stage of fermentation to avoid either insufficient yeast for repitching and a green beer with high residual yeast levels or a premature yeast mass that does not leave sufficient suspended yeast cells, which are required for beer maturation and inherent biochemical reactions (17).

MATURATION AND FINAL STEPS

Maturation or lagering starts right after most of the yeasts have been removed from beer. During this process, beer rests at very low temperatures for several weeks, in a highly carbonated atmosphere (20). Low temperatures are responsible for the precipitation of insoluble complexes, like those formed between polyphenols and proteins which, if not removed, may cause beer haze (15, 17). On the other side, residual yeast cells start a secondary fermentation by reducing some beer off-flavours, like vicinal diketones, into less flavour-active compounds (20). Diacetyl removal is one of the main purposes of beer maturation (51).

Simultaneously, beer flavour attenuation occurs once carbon dioxide, recently produced by the remaining yeast, promotes volatile organic compounds evaporation (45).

When maturation is achieved, beer may suffer some treatments such as the addition of specific flavours and beer physical properties stabilizers (13). Then, beer filtration process begins, by using diatomaceous earth to remove precipitated compounds and all the remaining yeasts. Finally, beer must be pasteurized to insure its microbiological stability (15).

PART III: Vicinal diketones

INTRODUCTION

Vicinal diketones, diacetyl and 2,3-pentanedione, are spontaneously produced as intermediates of amino acids synthesis by yeast during beer fermentation (50). These compounds impart an unpleasant buttery aroma (2) to beer even when present in low concentrations, due to their low threshold limit (52). Therefore, vicinal diketones affect beer organoleptic characteristics and their formation must be controlled during fermentation in order to guarantee final beer quality (17). Diacetyl and 2,3-pentanedione can also appear in beer as a consequence of the presence of some bacteria (3). However, after vicinal diketones formation, brewing yeasts are able to reduce these compounds into less flavour-active compounds that affect beer quality in less extent (52).

The knowledge of vicinal diketones formation pathways is a major concern for the brewing industry, and its main goal is to reduce these compounds during fermentation and maturation processes. In order to achieve that, yeast metabolism and consequently fermentation parameters influence on it must be deeply understood (2). It is known that yeast strain (17), vitality (29), wort composition, temperature and pH are some of the most important factors in vicinal diketones formation (50). Additionally to controlling these parameters, some brewers also implement other methods, such as enzymatic digestion and the use of immobilized yeasts (50, 53, 54).

General description

Diacetyl (2,3-butanedione) and 2,3-pentanedione are natural by-products of alcoholic fermentation. These two compounds are vicinal diketones (55), containing two ketone groups connected to adjacent carbon atoms (56) and impart either pleasant or undesirable flavours, depending on the beverage. VDKs are undesirable in lager beers, once this type of beverage requires a clean flavour profile (51), but sometimes are appreciated in some ale beers (57). Diacetyl and 2,3-pentanedione threshold limits are substantially different and for that reason their impact in beer flavour also differs (58).

Diacetyl, also known as 2,3-butanedione, is a simple α-dicarbonyl compound, a yellow liquid at room temperature, that is soluble in most organic solvents (59). This polar and hydrophobic diketone has a relatively simple structure of CH3-CO-CO-CH3 (60), showed in Figure 6. In beers, excessive diacetyl concentrations are considered as one of the most critical sensory defects (61). Diacetyl imparts a butterscotch (62) or buttery (56) flavour to beer and its threshold

limit is 0.10-0.15 mg/L, approximately 10 times higher than in 2,3-pentanedione (58). By default, modern breweries need to reduce diacetyl to below 100 µg/L, so that undesirable flavours are not detected in beers by consumers (62).

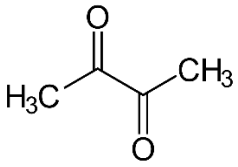


Figure 6: Diacetyl chemical structure (63)

Another ketone similar to diacetyl, 2,3-pentanedione (Figure 7), can also be produced during the brewing process (52). This is an intensively yellow compound (64), with the structure CH3-CH2-CO-CO-CH3 (3), that gives beer a honey-like (31) or buttery flavour (65) and its threshold limit is 1 mg/L (66). Due to this value, much higher than in diacetyl, 2,3-pentanedione has a lower influence in beer flavour (62).

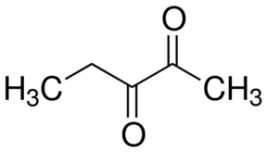


Figure 7: 2,3-pentanedione chemical structure (67)

Vicinal diketones can be naturally found in wine, brandy, balsamic vinegar, roasted coffee, honey, ensilage and in many other fermented foods (60). Diacetyl is also the principal flavour and colourant found in butter and therefore is added to margarines to improve their organoleptic properties (64). This compound is also used as a flavouring agent in the production of coffee, flour, chocolate, cooking oils, popcorn, other snack foods, dairy products and baked goods, in order to impart a buttery aroma and taste (68). Diacetyl is also found in cigarettes smoke (69). On the other hand, 2,3-pentanedione is used as an artificial flavour in alcoholic and non-alcoholic beverages (64) and, sometimes, as a substitute for diacetyl (68).

Toxicology

For centuries, diacetyl exposure to humans through fermented foods, has been generally recognized as safe by the Food and Drug Administration (60). In 1979, researchers of

the Department of Nutritional Sciences in California showed that diacetyl exhibits a dose-related mutagenicity in *Salmonella typhimurium* (70). Later, however, concerns have been raised that the inhalation of diacetyl vapours may be related to respiratory disorders in factory workers that were daily exposed to this compound (71). Consequently, substitute compounds with a similar chemical structures to diacetyl, such as 2,3-pentanedione, were also targets of investigation (72).

In 2000, a group of eight workers from a microwave popcorn plant in Missouri showed moderate to severe respiratory airways obstruction (71). The same injuries were registered in long-term employees in similar facilities in Illinois, Ohio and Montana (60). After a careful medical examination, high-resolution computer tomographies from patients confirmed a thickening of their bronchial walls (71). Investigations were carried out in the factory and revealed increasing rates and the prevalence of airway obstruction related with the increased exposure to diacetyl (72).

After analysing numerous microwave popcorns and flavouring production facilities, the National Institute for Occupational Safety and Health (NIOSH) concluded that diacetyl was the cause of these disorders and that workers were suffering from a rare disease, resembling bronchiolitis obliterans (68). Since that moment, this pathology became vulgarly known as popcorn worker's lung disease (73). Exposure to concentrations as low as 0.02 mg/L showed to affect workers from 5 different production plants (74).

Several experimental studies reported that the inhalation of diacetyl vapours cause dose-dependent irritations in the respiratory tract and necrosis of airways epithelial cells in rats and mice (59, 71, 72). Studies were also performed in human cultured epithelial cells exposed to diacetyl (75) and sensory irritation was estimated to occur above 20μg/g (59). As mentioned before, 2,3-pentanedione is used as a flavouring substitute for diacetyl. Toxicological studies showed that the exposure to this compound causes damages to epithelial cells, similar to those that were caused by diacetyl (76) and that a vascular endothelial growth factor may also be affected (72).

Following these findings, NIOSH determined the exposure-response relationship for diacetyl and some limits were stablished. NIOSH suggests a exposure to diacetyl below 5 parts per billion as a time-weighted average during a 40-hour work week and a short-term exposure of 25 parts per billion for a 15-minute time period. Also, for 2,3-pentanedione, NIOSH recommends an occupational exposure comparable to the one that were recommended for diacetyl and a short-term exposure limit of 31 parts per billion during a 15-minute period. The

use of personal protective equipment and engineering control should be adopted as well in order to control workplace exposures (76).

Other experimental studies showed that diacetyl can possibly mediate long-term neurological toxicity (77) and that adducts between diacetyl and DNA nucleobases cause its ternary structure disruption, which leads to cellular death (78). Additional experiments have been carried out and, fortunately, it was reported that dietary exposure to diacetyl it is not toxicologically significant and does not represents a risk to consumers, even if they are exposed to abnormal high concentrations of this substance. Therefore, lung disease in microwave popcorn workers is not related to diacetyl levels found in food and beverages but, instead, is correlated to the exposure of this compound in food production facilities (60).

CHEMICAL TRANSFORMATIONS

Diacetyl and 2,3-pentanedione are spontaneously produced by non-enzymatic oxidative decarboxylation of α-acetohydroxy acids, intermediates in the biosynthesis of particular amino acids (51). Later in fermentation and during maturation, the brewing yeast is able to reduce these compounds into molecules with a higher flavour threshold (52), that have lower impact in beer flavour (58). These mechanisms, involving vicinal diketones formation and reduction, need to be deeply understood by beer producers so that these off-flavours do not affect beer quality and appreciation (17). Detailed formation and reduction mechanisms of VDKs will be carefully studied in this section.

Formation

Brewing yeasts are the indirect producers of diacetyl and 2,3-pentanedione in beer, once these compounds are synthetized from excreted compounds resultant from yeasts metabolism (79). Additionally, when considering diacetyl, other mechanisms may also lead to its formation, such as by the chemical transformation of sugars, lipids and through the combination of the degradation products of these molecules. Maillard reaction (51) and bacterial presence, specially *Pediococcus* species, are other probable causes of diacetyl formation (57). Effectively, the mechanism explained below regarding vicinal diketones synthesis only approaches these compounds formation, after their precursors are excreted by the brewing yeast.

Concerning beer, diacetyl and 2,3-pentanedione are by-products of amino acids synthesis, through the spontaneous and non-enzymatic oxidative decarboxylation by metal ions or dissolved oxygen (57) of α-acetohydroxy acids, excreted by yeasts during fermentation (79). These compounds are intermediates resultant from amino acids biosynthesis in the brewing yeast and are excreted before turning into vicinal diketones. Their formation rate is influenced by temperature, pH, oxygen content and metal ions composition (66). Acetohydroxy acids concentration in beer depends on yeast strain and is enhanced by rapid yeast growth (17). Non-oxidative decarboxylation of α-acetolactate can also occur, resulting in the formation of acetoin, a less active flavour when compared with diacetyl.

Diacetyl is produced from α-acetolactate, as a by-product of valine and leucine synthesis and 2,3-pentanedione derives from α-acetohydroxybutyrate (Figure 8), an intermediate of isoleucine formation pathway (79). These α-acetohydroxy acids are produced from pyruvate and α-acetobutyrate, respectively (3) and their excretion is not yet fully understood, although it seems to be related with yeast protection against carbonyl stress (50). Conversion of α-acetolactate into diacetyl and the equivalent transformation of α-acetohydroxybutyrate into 2,3-pentanedione occur extracellularly and seem to be slow chemical reactions (62). This transformation is considered as a rate-limiting step in vicinal diketones formation (80).

One of the most important factors affecting vicinal diketones formation is the FAN content present in the wort and, as mentioned before, some amino acids formation pathways are, inclusively, directly related to diacetyl and 2,3-pentanedione synthesis (3). During fermentation, amino acids uptake by yeast seems to follow a particular absorption priority, according to four distinct groups (65). Group B includes leucine, isoleucine and valine and these amino acids are usually transported into yeast cells 12 to 24 hours after the beginning of fermentation. These compounds seem to be produced by yeasts, while amino acids from group A are being consumed (3).

Therefore, the regulation of these amino acids and consequently of vicinal diketones precursors is intimately related to enzyme activity and synthesis (80). For example, when valine uptake is sufficient in yeast cells, lower diacetyl levels are observed due to the feedback inhibition in valine biosynthesis pathway. Similarly, an increased uptake of isoleucine results into reduced levels of 2,3-pentanedione (56). Whether a higher FAN content in wort may be translated in a higher abundance of group A amino acids, group B amino acids do not need to be produced and α-acetohydroxy acids can be excreted to wort, resulting in intensified vicinal diketones formation (3).

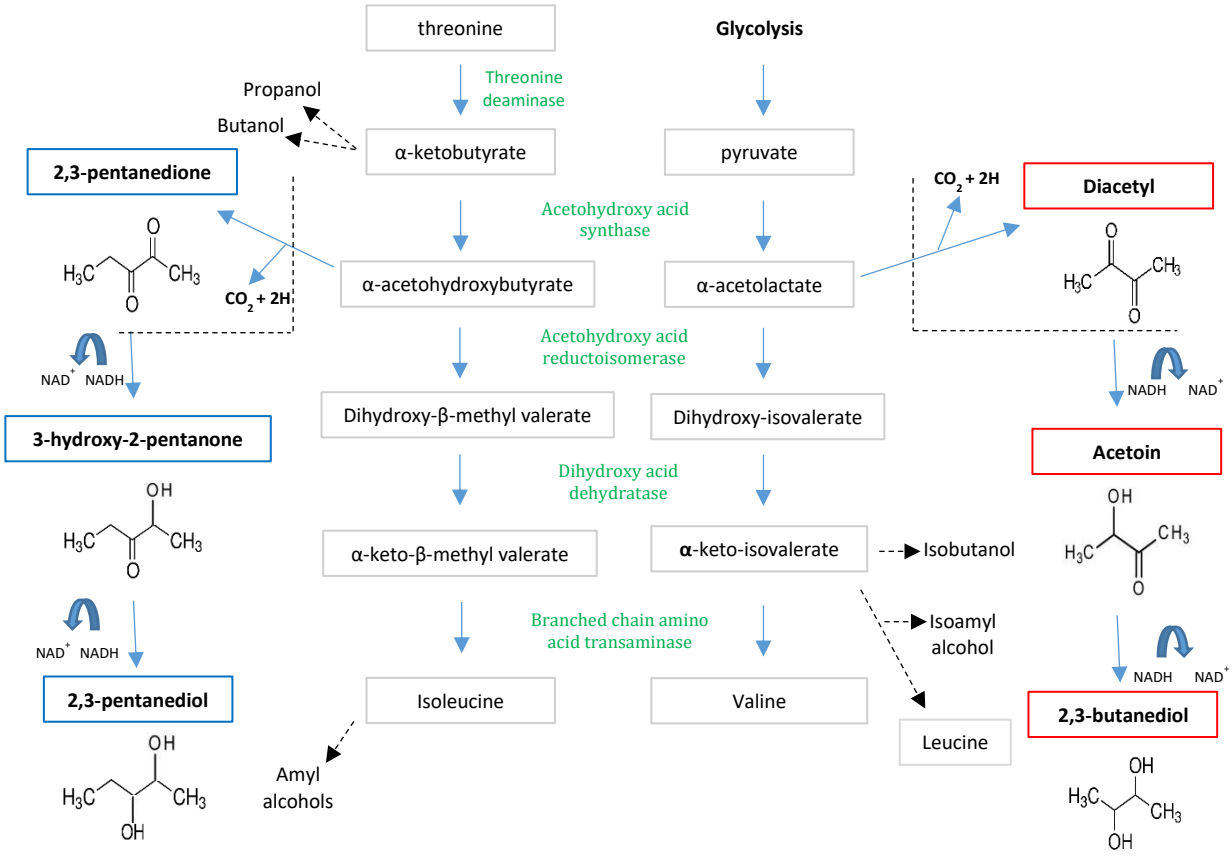


Figure 8: Metabolic pathway of vicinal diketones formation and reduction in the brewing yeast, adapted from Krogerus and Gibson (50)

Reduction

Contrarily to vicinal diketones formation, which occurs extracellularly, the brewing yeast is able to actively reduce these compounds into molecules with a lower impact in beer flavour. This process starts in beer fermentation but is completed later during the maturation process (51). Therefore, the VDKs produced in wort are reabsorbed by yeasts and then are enzymatically reduced (3) into molecules with a higher flavour threshold (51). In the case of diacetyl, this compound is firstly reduced to acetoin by diacetyl reductase, which is further reduced to 2,3-butanediol by alcohol dehydrogenase (62). Concerning 2,3-pentanedione, a similar process to the previous also occurs and this compound is also enzymatically reduced to 3-hydroxy-2-pentanone in a first step and then to 2,3-pentanediol (50).

FERMENTATION MANAGEMENT AND VICINAL DIKETONES IN BREWING INDUSTRY

Vicinal diketones are a major concern to beer industry and controlling these compounds reduction, efficiently and reproducibly, is one of the most important steps during fermentation and maturation (15). The first parameter that needs to be fulfilled in order to a complete reduction of vicinal diketones is the physiological state of the yeast (29) and so, selecting an appropriate yeast strain (17), with adequate viability and vitality (29), so that active yeasts can reduce these compounds (17). Vicinal diketones can be controlled as well through the regulation of fermentation conditions, wort composition and yeast improvement and modification. Minimizing oxygen sources and the addition of selected amino acids in wort are effective measures in VDKs reduction (79).

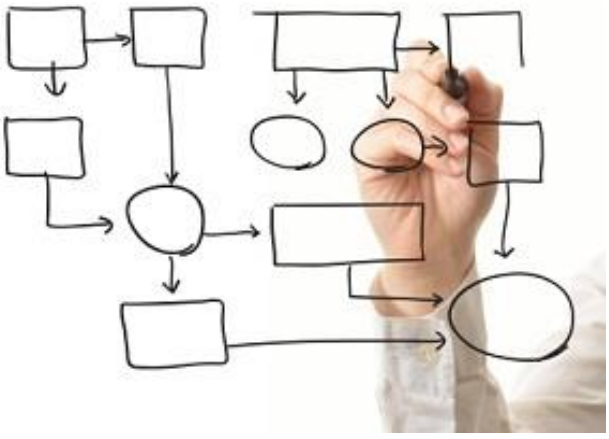
The final concentration of diacetyl is conditioned by the level of α-acetolactate and the stage when it is excreted (3, 50, 61). Fermentation temperature influences yeast growth (62) and consequently fermentation rates. Thus, as this temperature decreases, although maximum diacetyl levels are lower, diacetyl formation and reduction is delayed and fermentation or maturation period need to be extended (3, 39). On the other hand, worts with high levels of FAN are intimately related to high fermentation rates and, although VDKs formation is enhanced, their reduction also occurs more efficiently and one diacetyl peak is observed. Therefore, if low levels of FAN are available, when group A and B amino acids depletion occurs, diacetyl synthesis is induced once again and fermentation shows the occurrence of two diacetyl peaks (3, 61, 81, 82). Specifically, the increase of diacetyl in the fermenting wort is caused by valine depletion (82).

An increased temperature at the end of fermentation process (diacetyl rest), adding a small amount of fresh wort with healthy yeasts (Krausening), the use of immobilized yeasts and the addition of reductases are some of the alternatives to achieve a sufficient vicinal diketones reduction (55). Increased contact time with yeast favours diacetyl reduction but is an inviable method, once it immensely increases production time. Other factors such as changing pH, wort content, temperature and, as mentioned above, the use of immobilized yeasts or enzymes seem to be either too expensive processes or tend to affect beer organoleptic characteristics. Even if FAN content is optimized, other parameters can induce increased production of esters and superior alcohols (50), which is usually undesirable in lager beers (62).

One promising method of decreasing diacetyl formation during fermentation is through the control of the valine content of the wort (51). Authors report that modifying other branched-

chain amino acids concentrations in wort can also result in decreased diacetyl levels and faster beer production periods, without affecting fermentation performance (37, 51). Instead of using proteinases during wort production (83) or genetic modified yeast strains in fermentation (51), altering mashing conditions can also result in positive changes in FAN content, which is also helpful on diacetyl control. Additionally, increasing yeast pitching rate, wort initial oxygen content and temperature may also accelerate VDKs formation and, therefore, their reduction will be positively affected as well. Another important aspect to have in mind is to avoid bacterial contamination, specially of diacetyl producing species (3).

PART V: Methodologies



METHODOLOGIES REVISION

EBC routine methods

According to standard reference methods defined by the European Brewery Convention (EBC), there are several well documented procedures that are indispensable for controlling beer fermentation. The fermenting beer temperature and density are important parameters, once these allow brewers to follow the fermentation evolution by determining the extension of carbohydrates consume and the amount of ethanol produced by the brewing yeast (2).

Free amino nitrogen determination

The determination of free amino nitrogen (FAN) is a reliable way of following yeast cell growth and fermentation performance (38). Relatively to the measurement of FAN in wort and beer, the EBC recommends a ninhydrin method based in the spectrophotometric measurement of sample colour at 570 nm. Ninhydrin acts as an oxidizing agent, causing the oxidative decarboxylation of α-amino acids which, consequently, causes the loss of a NH_3 group and the formation of an aldehyde with less one carbon atom than the original amino acid. Simultaneously, fructose acts as a reducing agent and then, reduced ninhydrin molecules react with the unreduced ones, forming a blue complex. Potassium iodate from a diluting solution keeps the ninhydrin oxidized, avoiding that further colour reaction occurs (84). This method determines not only amino acids but also ammonia and terminal α-amino nitrogen groups of peptides and proteins (85).

Amino acids determination in beer

For years, amino acids in beer have been determined by methods based in cation-exchange chromatography and in a subsequence derivatization step using ninhydrin, which allows detecting amino acid derivatives in the visible region. However, due to these methods limitations, methods based in reversed-phase high performance liquid chromatographic columns with pre-derivatization systems have been developed (86). Ion exchange and ligand exchange chromatography were also tested by other authors (87).

Recently, automated pre-column derivatization and on-line reversed-phase LC separation with MS detection has been used for determining amino acids in the same matrix

and results showed good reproducibility of the derivatization process with a commercial amino acid analysis reagent (88). Ortho-phthaldialdehyde combined with 2-mercaptoethanol (MCE) is one of the most commonly used derivatization agent when HPLC reverse-phase (RP) column systems are chosen. This reagent mixture reacts with primary amino acids, forming substituted isoindole products, which are highly fluorescent (89). Additionally, carboxymethylation by using iodoacetic acid (IDA) can also be used to detection cysteine derivatives by fluorescence (90).

During the last decade, amino acids have been determined by HPLC in combination with a pre- or post-column derivatization, using fluorescent derivatization reagents such as OPA, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and other compounds that induce UV-visible absorption, like ninhydrin or dansyl chloride (91). Currently, RP-HPLC systems and pre-column derivatization with OPA/2-mercaptoethanol are preferentially chosen for amino acid determination in various matrixes (92). In Table 1, some of the most recent methods and the analytical parameters used for amino acids determination in beers and other matrixes are summed up. The methodology presented later in this chapter includes a IDA/OPA/MCE derivatization based in previous works (90, 92).

Table 1: Review of sample preparation, separation and detection parameters used in amino acids determination in beer samples

			SAMPLE PREPARATION			SEPARATION AND DETECTION				
Reference		Matrix	Degass	Volume/ mass	Derivatization agent	Column	Detector	Temperature	pH	Eluents
1	(93)	beer and wool**	yes	150 mg	CEOC	C18 column	fluorescence and UV/MS	25°C	8.8-10	A: 20% ACN in 0.02M acetate buffer pH4; B: ACN/water 60:40; C: ACN/water 60:40 with 0.1M borate buffer pH 8.9; D: ACN/water 95:5
2	(94)	various*	yes	10 mL	OPA-IBLC	Hypersil ODS2	fluorescence	25°C	10.4	A: sodium acetate buffer pH 5.95; B: methanol/ACN
3	(95)	wine, beer and vinegar*	no	-	OPA–MPA(NAC)	Hypersil ODS2 bonded phase	fluorescence and DAD	-	9.3	A: 23mmol sodium acetate trihydrate pH 5.95; B: Metanol/ACN
4	(92)	culture media, wine and beer*	no	100 µl	OPA/MCE	Reversed phase Xtimate C18	fluorescence	32°C	6	A: 0.05 M sodium acetate, pH 7.2; B: 0.1 M sodium acetate–ACN–Methanol (40:45:15) pH 7.2; C: methanol; D: ACN
5	(96)	beer*	yes	10 mL	AccQ.fluor	Nova-Pak C18	fluorescence	37°C	-	A: AccQ.Tag; B: ACN; C: methanol
6	(97)	malt and beer*	yes	5 µl	6-AQC	C18 Hypersil	fluorescence and UV	40°C	5.05	A: 140 mM sodium acetate, 17 mM triethanolamine, pH 5.05, with 1 mM EDTA; B: Acetonitrile; C: Water
7	(91)	wine and beer*	no	1 mL	DEEMM	ACE 5 C18-HL	DAD	16°C	5.8	A: 25 mM acetate buffer pH 5.8 with 0.02% sodium azide; B: 80:20 acetonitrile/methanol
	*HPLC, **reverse phase HPLC and LC-MS									

Volatile organic compounds determination in beer

According to previous publications, there are several different extraction, separation and detection methods that have been applied to beer flavours determination in the past. Considering extraction techniques, head-space (HS), solid-phase (SPE) and solid-phase micro extraction (SPME), as well as stir bar sorptive (SBSE) and liquid-liquid extraction are the most commonly used for this purpose. On the other hand, the separation of these compounds has been carried out mainly by chromatographic methods, such as gas (GC), liquid (LC) and high-performance liquid chromatography (HPLC), while flavour compounds detection has been achieved by nuclear magnetic resonance and mass spectrometry in most of the cases (98).

However, GC is considered the most appropriate separation technique used in the determination of beer flavour compounds (98). Considering extraction techniques, HS-SPME is the most successful extraction method used for the analysis of volatiles in beverages (99), due to the possibility of full automation, simplicity, speed, sensitivity and because it is a solvent-free technique (100). Finally, mass spectrometers, which allows a feasible flavour identification and quantification, are the most widely used detectors for this purpose. Innovative techniques, such as electronic nose and tongue, have shown to be valuable tools for the evaluation of beer aroma and flavours (98).

Additionally, concerning vicinal diketones determination, high performance liquid chromatography (101) and liquid chromatography (3), including derivatization steps, as well as gas chromatography (12, 51, 102-104) have been used before. Effectively, gas chromatography coupled with mass spectrometry detection (GC-MS) presents higher levels of sensitivity for the quantification of diacetyl and 2,3-pentanedione (12). For years, the EBC and the Institute of Brewing (IoB) have been studying the suitability of reference methods assigned for vicinal diketones determination, which include either a derivatization process, followed by ultraviolet spectrophotometric measurement or a time-consuming GC method by using a capillary column (105-108).

In the present work, a methodology to simultaneously determine diacetyl and 2,3-pentanedione, the acetoin and 2,3-butanediol was develop, as well as other volatile compounds with a known influence in beer aroma profile and which are produced during beer fermentation. The methodology developed started with HS-SPME optimization and evaluates the importance and the optimum levels of several extraction variables (factors). The factors and the levels to be tested were chosen after a preliminary screening of information available in the literature (Table 2).

Table 2: Review of treatments applied to samples and SPME conditions used in volatile organic compounds found in beer, by GC-MS

			SAMPLE				SPME					
Reference		**Main analyte**	**Optimization**	**Degas**	**Volume**	**+Salt**	**Fibre**	**Stirring**	**Extraction temperature**	**Incubation time**	**Extraction time**	**Derivatization**
1	(109)	beer volatiles	-	yes	5 mL	27g/100 mL	PDMS/DVB	1200 rpm	50°C	5 min	30 min	-
2	(101)	beer volatiles	-	no	10/20 mL	-	-	-	85°C	-	30 min	-
3	(99)	beer volatiles	yes	no	10 mL	3.5g	DVB-Car-PDMS	500/250rpm	40°C	10 min	30 min	-
4	(100)	beer volatiles	yes	no	5g/15 mL	2g	Car-PDMS	-	20°C	30 min	30 min	-
5	(110)	beer volatiles	-	no	15/20 mL	5g	PDMS	-	50°C	10 min	30 min	-
6	(111)	beer volatiles	-	no	10 mL	-	PA	-	37°C	30 min	60 min	-
7	(112)	carbonyl compounds	yes	no	10/20 mL	-	PDMS-DVB	-	60°C	10 min	40 min	-
8	(102)	beer volatile	yes	no	10/40 mL	3g	DVB/CAR/PDMS	-	60°C	1h (70°C)	30 min	-
9	(113)	beer volatiles	-	no	5/20 mL	-	PDMS/DVB	-	50°C	26°C	2 min	yes
10	(114)	beer volatiles	-	yes	10/20 mL	-	PDMS/DVB	500rpm	40°C	-	10 min	-
11	(115)	volatiles	-	no	5/20 mL	1.75g	DVB–CAR–PDMS	-	45°C	20 min	40 min	-
12	(116)	alcohols and esters	-	yes	10 mL	-	PA	-	60°C	-	50 min	-
13	(117)	volatile phenols	yes	no	6 mL	0.4g/ mL	DVB/CAR/PDMS	250rpm	80°C	5 min	55 min	-
14	(118)	beer volatiles	yes	no	5/15 mL	2g	PDMS	870rpm	24°C	-	45 min	-
15	(119)	carbonyl compounds	yes	no	5/20 mL	-	PDMS/DVB	250rpm	45°C	7 min	20 min	-
16	(120)	beer volatiles	-	no	5 mL	-	PDMS/DVB	-	40°C	1 min	5 min	-
17	(121)	beer volatiles	-	no	5/10 mL	-	Car-PDMS	-	45°C	2 min	15 min	-
18	(122)	aldehydes	-	no	10/20 mL	-	PDMS	-	20°C	-	20 min	yes
19	(123)	sulphur compounds	yes	no	10/15 mL	-	Car-PDMS	-	45°C	-	32 min	-
20	(124)	beer volatiles	yes	yes	10/20 mL	2g	DVB/Car/PDMS	400rpm	40°C	10 min	30 min	-
21	(12)	carbonyl compounds	yes	no	5/20 mL	-	Car-PDMS	yes	30°C	5 min	25 min	-

CHEMICALS AND SOLUTIONS PREPARATION

Free amino nitrogen determination

Derivatization solution was prepared by dissolving 10g of $Na_2HPO_4.12H_2O$ (99.0-102.0%, José M. G. dos Santos, Portugal), 6g of KH_2PO_4 (99.0%, Panreac Quimica SA, Spain), 0.5g of ninhydrin (laboratory reagent grade, Fisher Scientific, UK) and 0.3g of D(-)-fructose (99%, Acros Organics, Belgium) in distilled water. After homogenizing, the solution pH was adjusted until a value of 6.6 to 6.8 was achieved and then mixture was diluted to a final volume of 100 mL. This solution was used for a maximum period of two weeks, stored at 2°C in an amber bottle. A dilution solution used for FAN determination was prepared by dissolving 2g of KIO_3 (99.5%, Merck, Germany) into 600 mL of distilled water, followed by the addition of 400 mL of ethanol 96%. The mixture was then homogenized and stored at 2°C. Glycine standard solution was prepared by dissolving 0.1072g of L-glycine (Sigma-Aldrich,USA) in distilled water, to a final volume of 100 mL. This solution was then diluted by transferring 1 mL of the concentrated glycine solution for a final volume of 100 mL, in distilled water.

Amino acids determination

Borate buffer solution was prepared by adding 2.47g of boric acid (99.8%, Chem-Lab, Belgium) into 80 mL of ultrapure water. pH was then adjusted to 10.5 by adding NaOH (12.5g/100 mL) and the solution was diluted to 100 mL. OPA-MCE was prepared by dissolving 50 mg of 1,2-phthalic dicarboxaldehyde (≥98%, Acros Organics, Belgium) into 1.5 mL of absolute ethanol (>99.8%, Sigma-Aldrich, Germany), followed by the addition of borate buffer until a final volume of 10 mL is achieved. Then, 200µl of 2-mercaptoethanol (99%, Sigma Aldrich, Germany) were added to this mixture and, after homogenization, the solution was settled down for 90 minutes before filtering and using. This solution was stored for 9 days at 2°C and re-filtered before reusing. IDA solution was then prepared by dissolving 0.583g of iodoacetic acid (99%, Panreac Quimica SA, Spain) in borate buffer, to a final volume of 10 mL.

Individual amino acids solutions (10g/L) were prepared by dissolving 0.05g of each L-amino acid with a minimum assay of 98% (L- arginine, phenylalanine, asparagine, aspartic acid, isoleucine, leucine, valine, lysine, serine, threonine, glutamine, glutamic acid, cysteine, alanine, tryptophan, citruline, glycine, ornithine, histidine, (Sigma-Aldrich,USA) and L-gamma-aminobutyric acid (Fluka BioChemika AG, Switzerland) in HCl 0.1M to a final volume of 5 mL,

except for tyrosine (Sigma-Aldrich,USA), which was dissolved in HCl 1M to a final volume of 5 mL. Then, a stock solution (300 mg/L) was prepared by dissolving all the individual amino acids solutions in synthetic beer and ethanol content was also adjusted in this step. From this solution, successive dilutions (0.1, 0.25, 0.5, 1, 2.5, 5, 20, 50, 100 and 300 mg/L), were prepared in order to use in methodology calibration, validation and for sample spiking.

For chromatographic elution, mobile phase A (aqueous potassium di-hydrogen phosphate (99.0%, Panreac Quimica SA, Spain) buffer 10mM: HPLC-grade methanol (≥99.8%, Chem-Lab, Belgium): tetrahydrofuran (99.98%, Fisher Scientific, UK)) – 91:8:1, v/v) and B (100% HPLC-grade methanol) were prepared. A 20% HPLC-grade methanol aqueous solution was also prepared, for HPLC system cleaning purposes. The aqueous potassium phosphate buffer 10mM was prepared from a stock solution (200mM) and pH was adjusted to 7.3 with a potassium hydroxide (85%, Panreac Quimica, Spain) solution (1M).

Volatile organic compounds determination

Synthetic beer was prepared by dissolving 22g of tartaric acid (99.5%, Merck, Germany) and 80 mL of absolute ethanol (>99.8%, Sigma-Aldrich, Germany) in ultrapure water and by adjusting mixture pH to 4.0 with NaOH (12.5g/100 mL), before diluting this to a final volume of 2 L. This solution was used for the preparation of standards used both in GC and HPLC methodologies calibration and validation. A calcium chloride ($CaCl_2.2H_2Os$, ≥99.5%, Chem-Lab, Belgium) solution (50 g/L) was also prepared for sample conservation purposes. A stock solution of 4-methyl-1-pentanol (97%, Sigma-Aldrich, Germany) was prepared by dissolving 30.5 µl of this compound in synthetic beer, to a final volume of 10 mL and then a diluted solution (100 mg/L) was prepared to use as an internal standard in volatile organic compounds determination.

Several volatile organic compounds stock solutions and respective dilutions were prepared in synthetic beer: Solution A – diacetyl (99%, Acros Organics Belgium) and 2,3-pentanedione (97%, Acros Organics, Belgium) (5 g/L and 25 mg/L); Solution B - ethyl butyrate (99%, Acros Organics, Belgium), isobutyl acetate (98%, Acros Organics, Belgium)(2.5 g/L and 50 mg/L); Solution C – hexanoic acid (≥99.5%, Sigma-Aldrich, Germany) and octanoic acid (≥98%, SAFC, Malaysia) (250 mg/L), Solution D – acetoin (≥97%.0, Fluka, Germany), acetaldehyde (99%, Panreac Quimica SA, Spain), (5g/L and 500 mg/L); Solution E – ethyl acetate (99.97%, Fisher Scientific, UK), isobutyl alcohol and isoamyl alcohol (99%, Acros Organics, Belgium) (5 g/L); Solution F –acetic acid gacial (99.7%, Panreac Quimica SA, Spain), 2,3-butanediol R (≥98%, Acros Organics, Belgium), and phenylethyl alcohol (99%, Acros Organics, Belgium) (5g/L); Solution G -

isoamyl acetate (≥99%, Acros Organics, Belgium) and ethyl hexanoate (≥99%, Sigma Aldrich, Germany) (250 mg/L). These solutions were diluted in synthetic beer to different final concentrations, in order to be used in methodology calibration and validation.

CONSERVATION TESTS

Due to the impossibility to perform all the chromatographic analysis at the same day of sample collection, three different sample conservation strategies were evaluated, by testing lager beer fermentation samples obtained from a local brewery. Parameters used during these tests were selected in accordance to previous works, in order to ensure yeast immobilization: i) storage at different temperatures (2°C and -26°C), ii) calcium chloride addition and iii) sample pasteurization. The temperature treatment was also evaluated when combined with pasteurization and addition of CaCl2 (Figure 9). A set of samples without any treatment was also analysed, in order to evaluate samples evolution when fermentation is not interrupted.

Storage conditions effect was evaluated 2 days later, after obtaining and analysing fresh samples. This was the period required to store samples in order to complete the analysis when samples were collected on consecutive days. Additionally, fresh samples were also collected from two additional points of fermentation, to ensure that storage influence is equal since the first day of fermentation. Each sample was obtained in triplicate and analysis of each triplicate was analysed 3 times. This procedure was also adopted for all analysis carried out in HPLC-fluorescence, GC-MS and by UV-spectrometry.

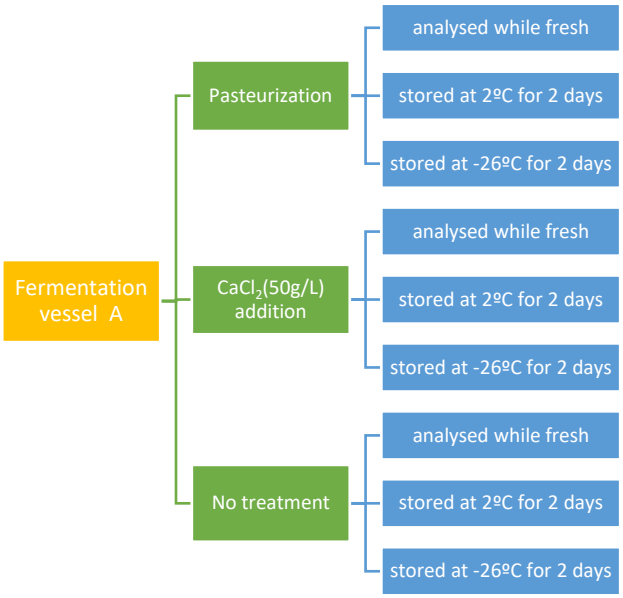


Figure 9: Different treatments applied during conservation tests to fermentation samples

SAMPLES

Initially, pale lager beer samples from the same batch were used during the design of experiments (DoE) process, carried out for volatile organic compounds extraction. These beers were also previously used for scanning the retention time of compounds of interest and then for methodology calibration and validation. Absolute ethanol was also use for alcohol content correction in early fermentation samples.

Pale lager beer fermentation samples were obtained from ECM and several samples were collected during the fermentation process, from three different fermentation vessels, all resultant from wort produced with the same raw materials and all of them were inoculated with a third-generation yeast (Figure 10). From each fermentation sample, triplicates with an approximate volume of 50 mL were collected and, at that time, 1 mL of calcium chloride (50 g/L), concentrated enough to stop fermentation (125), was immediately added to each flask. Then, replicates were homogenized and kept in ice during transportation. At the laboratory, aliquots from each replicate were obtained for FAN determination and for volatile organic compounds and amino acids analysis.

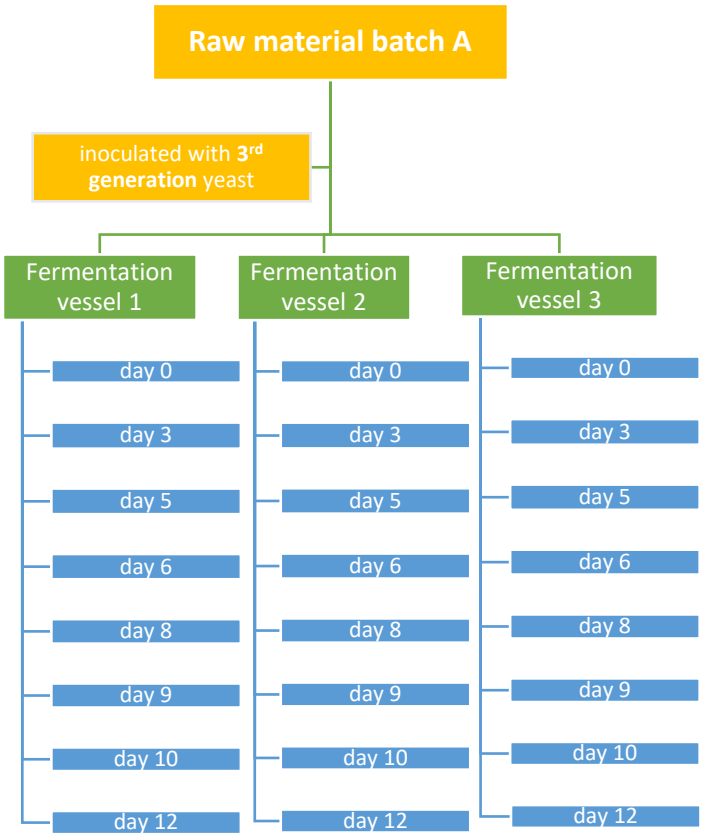


Figure 10: Samples collected for analysis during lager beer fermentation, using a 3rd generation yeast

EBC ROUTINE METHODS

As a complement of the academic laboratorial work, temperature and extract were determined in ECM. Analysis were performed according to the EBC standard reference methods, by using a thermometer and a standard densimeter, respectively. The results were used as a complement of other analytical data obtained during fermentation follow up period.

FAN

According to EBC standard method for FAN determination, 100µl from each fermentation replicate were diluted to a final volume of 10 mL in distilled water and kept at -26°C, if not immediately analysed. Then, for the derivatization process, 1 mL of ninhydrin was added to 2 mL of diluted sample and, after homogenizing, the mixture was sealed and placed in a boiling water bath for 16 minutes, followed by a 20-minute period of cooling at 20°C. After that, 5 mL of the dilution solution were added to the cooled mixture and, right after, mixtures were homogenized and samples absorbance at 570 nm was measured in a dual beam spectrophotometer Shimadzu UV-Vis 2600 (Kyoto, Japan).

Derivatization procedures were done in triplicate and absorbance of each replicate was measured in triplicate, as well. For each set of samples, in two test tubes, diluted sample was replaced by a glycine standard (10 mg/L) and in another tube, distilled water was used instead of sample, as a derivatization reaction control.

After analytical determinations, absorbance data were converted into FAN values (mg/L), following the formula described below, where *A1* is the sample absorbance at 570 nm, *A2* is the mean absorbance value of glycine standard and *d* is the sample dilution factor (100). FAN results were then presented as mean and standard deviation values of measure.

$$\text{FAN (mg N/L)} = \frac{\text{A1x2xd}}{\text{A2}}$$

AMINOACIDS BY HPLC-FLUORESCENCE

Methodology validation

For amino acids method calibration, 200µl of each standard solution were added to 1.5 mL of a 0.4M borate buffer solution and, after homogenization, samples were filtered using a

hydrophilic PTFE syringe filter (13mm 0.22um) obtained from Specanalítica, into a 300µl vial. This procedure was conducted in triplicate for each calibration solution. The repeatability (intra-day) was determined by the quantification of 10 successive replicates of beer with three different standard solutions (low, intermediate and high concentrations of each analyte) and reproducibility (inter-day) was assessed by performing the same analysis in 3 different days over a week. The values are expressed in terms of relative standard deviation. Additionally, 5 vials were also prepared by replacing sample with synthetic beer, analytes-free. These samples were used for determining compounds recovery, by comparing them with samples used for the determination of this method repeatability. The concentration ranges in wort, fermentation and beer samples found in literature for each analyte (Table 3), together with some preliminary tests, were used to define the work range in the present work.

Table 3: Review of amino acids typical concentration range in wort and beer

Compounds	Concentration range (mg/L) in literature	References
alanine	2.6-273.5	(93), (126), (95), (37), (92), (96), (127), (128), (91), (129), (130)
arginine	3.8-100.4	(93), (95), (37), (92), (96), (127), (91), (129), (130)
asparagine	0.38-206.0	(95), (37), (92), (127), (91), (130), (131)
aspartic acid	0.34-89.9	(95), (37), (92), (96), (127), (91), (130), (131)
citruline	4.2	(92), (131)
cysteine	1.2- 13.5	(93), (132)
leucine	0.1-205.8	(93), (126), (95), (37), (92), (91), (130), (131), (3), (133)
isoleucine	0.17-82.9	(93), (126), (95), (37), (92), (96), (128), (91), (129), (130), (131), (3)
GABA	7.72-193.6	(126), (95), (92), (91)
glutamic acid	0.3-62.5	(93), (95), (37), (92), (96), (127), (91), (129), (130), (131)
glutamine	0.90-26.0	(95), (92), (127), (91), (130), (131)
glycine	0.8-145.5	(93), (126), (95), (37), (92), (96), (127), (130), (131)
histamine	0.34-5.69	(92), (91), (134)
histidine	2.2-54.9	(93), (37), (92), (96), (127), (91), (129), (130)
lysine	0.137-77.8	(93), (126), (95), (37), (92), (96), (127), (91), (129), (130), (131), (134)
methionine	0.46-28.6	(126), (95), (37), (92), (96), (127), (91), (130), (131)
ornithine	1.3-10.2	(93), (126), (91), (131)
phenylalanine	1.66-131.7	(93), (126), (95), (37), (92), (127), (91), (129), (130), (131), (133)
serine	0.92-57.7	(126), (95), (37), (92), (96), (91), (130), (131)
threonine	0.3-86.4	(93), (126), (95), (37), (92), (96), (127), (130), (131)
tryptophan	3.84-27.5	(93), (95), (37), (92), (91), (129), (130), (131)
tyrosine	1.19-205	(93), (126), (95), (37), (92), (96), (127), (91), (129), (130), (135)
valine	0.8-188	(93), (126), (95), (37), (92), (96), (91), (129), (130), (131)

Sample preparation

For amino acids analysis, due to high analyte concentration in beer fermentation samples, 100µl of each sample replicate was added to 1 mL of a 0.4M borate buffer solution and, after homogenization, samples were filtered using a hydrophilic PTFE syringe filter (13mm 0.22um) from Specanalítica, into a 300µl vial. The different dilution used for samples was taken into account when converting fluorescence response into concentration values. Sporadically, samples were spiked with a standard solution in order to facilitate peaks identification confirmation. When not promptly analysed, samples were kept at 2°C, to avoid substances precipitation.

Derivatization

Amino acids derivatization follow the procedure described by Pereira *et al* (2015) (90). Briefly, the derivatization reaction was performed in the sample injection loop. Firstly, 5µl of buffered sample were aspired, followed by 5µl of IDA solution and 10µl of OPA/MCE solution. Then, the mixture was kept in the loop for 2 minutes, allowing the occurrence of the derivatization chemical reaction. During this period, flow was maintained at 0 mL/min and, after this time, mobile phase flow was set to 1 mL/min, forcing the loop content to enter in the chromatographic column.

Chromatographic optimal conditions and detection

Amino acids were separated in a HPLC system using Waters (Milford, USA) liquid chromatograph connected to an Empower Pro Software and equipped with an auto-injector (Waters 2695, separation module) and a Multi λ Fluorescence detector (Waters 2475). Chromatographic analysis was performed using an analytical scale silica-based, reversed-phase C18 column (Atlantis T3, 4.6mm×250 mm), with a particle size of 5µm, purchased from Waters (Ireland) and a C18 pre-column. Two mobile phases, A (1% of tetrahydrofuran, 8% methanol and 91% phosphate buffer 10 mM) and B (100% methanol) were used, according to the gradient shown in Table 4. The flow rate was set to 1 mL/min, the column temperature was kept at 35°C and total analysis time rounded 45 minutes. Fluorescence excitation and emission wavelengths were fixed at 335 and 440 nm, respectively.

Table 4: Elution parameters used for beer amino acids separation by HPLC

Time (min)	Flow (mL/min)	Eluent A (%)	Eluent B (%)	Curve
0	1	85	15	6
9	1	71.5	28.5	6
16	1	47	53	6
18	1	47	53	6
28	1	19	81	6
35	1	0	100	1
40	1	85	15	1

VOLATILE ORGANIC COMPOUNDS BY GC-MS

Extraction DoE and optimal conditions

For the optimization of volatile organic compounds extraction, three different solid-phase micro extraction fibres were tested, after a preliminary screening of factors and definition of experimental conditions. Several qualitative and quantitative parameters were evaluated, including sample degasification, volume and agitation, salt addition and pre-incubation and extraction time, as well as extraction temperature (Table 5), according to the literature reviewed, as previously presented in Table 2. Extraction conditions were chosen after a careful statistical analysis of experimental design results (Definitive Screening Design), from 36 aleatory assays, which was followed by a complete validation of the analytical method, conducted at optimal factor levels.

Table 5: Parameters tested during the optimization of volatile organic compounds extraction in beer by HS-SPME

Factor	Qualitative/Quantitative	Levels
Degass	Qualitative	L1 - No; L2 - Yes (15min)
Sample volume	Quantitative	L1 - 5 mL; L2 - 10 mL
Adition of salt	Qualitative	L1 - No, L2 - Yes
Type of Fibre	Qualitative	L1 - PDMS/DVB; L2 -CAR/PDMS; L3 - CAR/PDMS/DVB
Agitation	Qualitative	L1 - No; L2 - Yes
Pre-incubation time (min)	Quantitative	L1 - 0; L2 - 5; L3 - 10
Extraction time (min)	Quantitative	L1 - 20; L2 - 30; L3 - 40
Extraction temperature (°C)	Quantitative	L1 - 40; L2 - 50; L3 - 70

Methodology validation

During calibration, and following the selected and optimal parameters from the design of experiments for VOCs extraction, 10 mL of standard solutions with different analytes concentration, prepared in synthetic beer, were added into a 20 mL vial, containing 3.3g of sodium chloride, previously added. Right after, 5µl of 4-methyl-1-pentanol were added to the prepared sample and the vial was sealed and homogenized in a vortex. This procedure was conducted in triplicate for each calibration solution. The repeatability (intra-day) was determined by the quantification of 10 successive replicates of beer with three different standard solutions (low, intermediate and high concentrations of each analyte) and reproducibility (inter-day) was assessed by performing the same analysis in 3 different days over a week. The values were expressed in terms of relative standard deviation. Additionally, 5 vials were also prepared with no-spiked beer, in order to evaluate compounds recovery, by comparing them with samples used for the determination of this method repeatability.

Sample preparation

For organic volatile compounds analysis and by following the selected and optimal parameters from the design of experiments for VOCs extraction, 10 mL of fermentation aliquots were added into a 20 mL vial, containing 3.3g of sodium chloride, previously added. For samples corresponding to day 0, 3 and 5 of the fermentation process, 400, 200 and 100 µl, of absolute ethanol were added, respectively, replacing exactly the same amount of sample, so that ethanol content is equal. Right after, 5 µl of 4-methyl-1-pentanol were added to the prepared sample and the vial was sealed and homogenized in a vortex. This procedure was conducted in duplicate for each sample replicate.

Compounds extraction, chromatographic method and detection

After sample preparation, VOCs extraction was achieved by exposing the selected SPME fibre, 85 mm carboxenepolydimethylsiloxane (Car/PDMS), into the vial for 20 min at 40°C, under no stirring. Then, by inserting the fibre into the GC injection port of a Trace GC Ultra, equipped with a TriPlus autosampler (SPME mode) and a mass spectrometer detector (single quadrupole - electronic impact ionization mode) from Thermo Scientific (Hudson, NH, USA), the analytes were desorbed for 3 min at 250°C. The column used was a 60 m × 0.250 mm DB-WAXetr with 0.50µm of film thickness, from Agilent J&W (Folsom, CA, USA). Helium was used as carrier gas and a constant flow of 1 mL per minute was established. The transfer line and ion source

temperatures were both kept at 240°C. During the run time, the oven temperature was kept at 40°C for 2 min, then increased up to 250°C at 4°C/min and it was finally kept at 250°C for 5 min. Total run time rounded about 60 minutes.

The ions used for compounds quantification, as well as concentration range of each analyte in wort, fermentation and beer samples found in literature are indicated in Table 6. This ramp was defined after the injection of individual standards, in order to check their retention time and to confirm that there were no coeluted compounds. Concentration ranges of each analyte in wort, fermentation and beer samples, as well as their odour threshold and aroma descriptor found in literature are indicated in Table 7.

Table 6: Ions mass list used for volatile organic compounds detection by mass-spectrometry.

Time (min)	Mass list or range (amu)
4.50-7.00	42, 43, 44, 45
7.00-9.00	43, 45, 61, 70, 88
9.00-10.50	86
10.50-11.50	42, 43, 86
11.50-12.30	43, 56, 73, 86
12.30-13.10	43, 60, 71, 89, 101
13.10-14.20	43, 57, 85, 88, 100, 115
14.20-21.50	30-300
21.50-25.00	43, 45, 55, 56, 69, 75, 88
25.00-34.50	30-300
34.50-36.00	43, 60, 87
36.00-48.00	30-300
48.00-49.50	77, 107, 135, 150
49.50-60.00	30-300

RESULTS TREATMENT

The generation of the Definitive Screening Design and most of the computations (model estimation and optimization) were conducted in JMP-PRO ver. 12.1.0 (64-bit) (SAS Institute Inc.). Regular statistical analyses used for methodologies validation were performed with Microsoft Office Excel 2013, while statistical tests were performed in Minitab® ver. 17.1.0 (64-bit) (Minitab Inc). Tukey test was used to analyse variance between conservation tests samples, using a significance level of 0.05.

Table 7: Review of typical concentration range, odour threshold, aroma descriptors of the volatile organic compounds found in wort and beer samples

Compounds	Concentration range in literature (mg/L)	Odour threshold (mg/L)	Aroma descriptor
acetaldehyde	2-36 (17, 45, 55, 79)	5-50 (136, 137, 138, 139)	Green apple, solvent-like (136)
acetoin	1-86 (2, 28, 55)	8-50 (2, 28, 55, 139)	Buttery, sweet, fatty (140)
acetic acid	1-200 (2, 15, 45, 136)	90-130 (136, 137)	Vinegar, acidic (136)
2,3-butanediol	20-150 (2, 45)	≥100 (141)	Fruity, creamy, buttery (140)
decanoic acid	0.01-70 (2, 28, 115, 116 142)	10 (2, 28)	Fatty, rancid, sour (140)
diacetyl	0.008-1.18 (2, 28, 80, 136)	0.01-0.15 (2, 3, 28, 65, 136, 143, 144)	Buttery, milky butterscotch (136)
ethyl acetate	0.29-60.9 (2, 80, 110, 116, 142, 145, 146, 147)	3.9-33 (2, 28, 110,137, 138)	Nail varnish, solvent-like (136)
ethyl butyrate	0.0005-8.64 (2, 116, 136, 148)	0.3-0.4 (2, 28, 136, 137)	Tropical fruits, mango, pineapple (136)
ethyl caprylate	0.003-4 (45, 110, 145, 147, 149)	0.3-0.9 (58, 108, 145)	Apple, aniseed (147)
ethyl hexanoate	0.05-1.5 (2, 28, 45, 116, 136, 145, 146)	0.12-0.23 (2, 28, 136, 137, 146, 147)	Estery, apple, aniseed (136)
ethyl laurate	0.01-0.46 (2, 145, 150)	2-3.5 (2, 151)	Oily, fruity, floral, fatty (151)
hexanoic acid	0.6-6.5 (43, 115, 125, 142, 152)	5-10 (43)	Rancid, goaty (43)
isoamyl acetate	0.01-59.16 (2, 80, 99, 110, 116, 118, 136, 142, 146, 147)	0.6-1.6 (58, 80, 110, 136, 137, 138, 146, 147)	Estery, fruity, banana (136)
isoamyl alcohol	2.5-70 (2, 15, 45, 118, 142, 145)	30-70 (2, 58, 80, 138, 146)	Alcohol, solvent, banana (2)
isobutyl acetate	0.03-10.12 (2, 116, 145, 146)	0.4-1.6 (2, 146)	Banana, fruity (147)
isobutyl alcohol	0.3-59.92 (2, 15, 116, 117, 142, 146)	80-200 (58, 146)	Alcohol (2)
octanoic acid	2-85.38 (2, 115, 125, 136, 142, 152)	4-13 (136, 137)	Goaty, waxy, tallow (136)
2,3-pentanedione	0.001-1 (12, 28, 143)	0.9-1.5 (3, 28, 65, 154)	Buttery, rancid, sweet (137)
phenylacetate	0.2-1.7 (2)	0.2-1.7 (2)	-
phenylethyl alchocol	8-138 (2, 15, 45, 115, 118, 146)	28-125 (2, 138, 146)	Roses (2)

PART VI: Results and discussion

METHODOLOGIES VALIDATION

Amino acids determination by HPLC-fluorescence

The determination of individual amino acids in beer was based in Pereira, Pereira (90). All the data related to calibration curves of each amino acid and to methodology validation were obtained from assays in synthetic beer. Results regarding this method calibration curve and validation can be found in Table 8.

For all the amino acids, calibration curves showed a good linearity. Method accuracy was studied by analysing recovery mean values, varying from 77.60 (ornithine) and 112.39% (valine), except for cysteine which shows a punctual low recovery for one of the concentrations in study. The methods precision was evaluated in terms of repeatability and reproducibility, where variations were expressed as curve residual standard deviation values, which were lower than 10% and 15%, respectively, except for a punctual lysine value, for one of the three analysed concentrations.

The LOD and LOQ values determined showed to be very appropriate for the concentration ranges in study. In fact, asparagine was the only amino acid that showed concentrations lower than this compound LOQ in an intermediate phase of fermentation, whereas all the other amino acids were quantifiable during the fermentation. A chromatogram resultant from calibration standards injection is shown in Figure 11.

Concerning key amino acids, valine, leucine and isoleucine present, LOD values of 3.58, 0.27 and 0.64 mg/L, LOQ values of 10.86, 0.81 and 1.95 mg/L, respectively. The recoveries mean values of 112.39, 96.99 and 81.57%. Linear range of isoleucine (R^2=0.9999) covers concentrations from 0.10 to 50.34 mg/L, while for valine (R^2=0.9998) and leucine (R^2=0.9999) these values range from 0.10 to approximately 100 mg/L. These compounds precision parameters showed variations lower than 10%.

Table 8: Validation parameters for the determination of individual amino acids in beer by HPLC-fluorescence. C1, C2 and C3 concentrations correspond approximately to 0.25, 5 and 50 mg/L, respectively.

Compounds	RT (min)	Linear range (mg/L)	R^2	LOD (mg/L)	LOQ (mg/L)	Recovery (%)			Repeatability (%)			Reproducibility (%)		
						C1	**C2**	**C3**	**C1**	**C2**	**C3**	**C1**	**C2**	**C3**
Aspartic acid	4.30	0.10-100.22	0.9999	0.29	0.88	107.84	88.36	92.26	2.66	2.95	1.07	11.42	4.65	10.39
Glutamic acid	5.90	0.10-100.02	0.9999	0.79	2.39	91.17	88.80	97.13	2.68	3.79	2.12	5.06	6.38	3.19
Cysteine	6.86	0.10-4.99	0.9999	0.08	0.24	48.22	64.32	85.46	3.23	8.60	4.42	13.58	6.78	5.48
Asparagine	10.15	0.10-20.02	0.9997	0.47	1.42	86.21	81.92	102.01	3.00	6.75	2.49	7.71	3.69	9.72
Serine	12.07	0.10-50.04	0.9999	0.16	0.49	84.68	88.27	90.20	9.41	9.83	5.23	8.55	11.60	7.43
Glutamine	12.66	0.10-50.24	0.9999	0.13	0.40	80.98	67.30	86.16	3.46	3.50	1.87	5.50	7.31	11.73
Histidine	13.01	0.10-50.04	0.9999	0.07	0.20	-	73.78	93.07	-	5.36	2.90	-	6.43	4.58
Citruline	14.75	0.10-50.04	0.9999	0.21	0.64	66.65	86.07	97.65	3.44	5.00	1.47	1.90	3.04	7.75
Glycine	15.28	0.10-100.22	0.9998	1.57	4.77	82.24	88.46	95.90	5.25	2.92	0.79	12.01	5.91	5.40
Threonine	15.52	0.10-100.22	0.9999	1.33	4.04	85.72	92.97	100.35	2.82	2.37	1.11	1.89	7.67	3.67
Arginine	16.16	0.10-100.22	0.9999	0.43	1.31	62.76	85.86	99.85	6.00	3.12	1.54	15.09	1.86	1.99
Tyrosine	17.20	0.10-100.22	0.9999	1.42	4.31	129.34	103.62	101.79	3.57	2.39	2.40	4.01	3.34	2.06
Tryptophan	20.71	0.10-50.14	0.9999	0.59	1.80	69.70	85.64	96.35	2.31	6.06	3.98	3.12	3.06	8.30
Valine	22.07	0.10-100.02	0.9998	3.58	10.86	81.13	79.44	176.61	1.74	2.06	1.74	3.00	4.22	5.36
Isoleucine	24.36	0.10-50.34	0.9999	0.64	1.95	80.81	74.80	89.09	3.70	1.79	0.50	7.93	2.24	8.65
Leucine	24.95	0.10-100.82	0.9999	0.27	0.81	94.35	95.68	100.94	1.07	2.39	0.66	6.08	3.87	4.27
Ornithine	26.50	0.10-20.10	0.9999	0.07	0.21	74.47	67.43	90.90	4.08	4.98	2.09	7.83	7.94	5.96
Lysine	27.38	0.10-4.99	0.9999	0.04	0.13	70.93	76.91	91.02	7.02	3.82	1.28	23.98	8.25	5.74
		0.10-100.22	0.9999	0.32	0.98									
Phenylalanine	32.66	0.10-100.02	0.9999	0.68	2.06	76.53	75.53	80.88	2.54	1.71	2.20	3.94	1.75	6.25

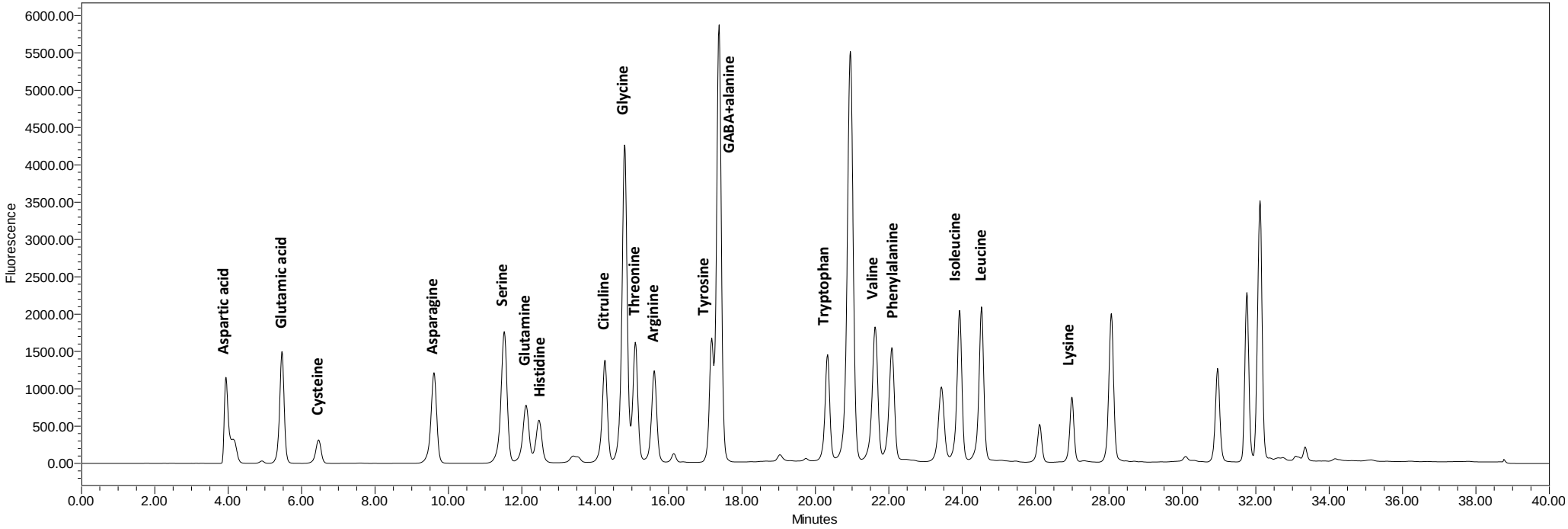


Figure 11: Chromatogram resultant from HPLC separation of amino acids calibration standard

Volatile organic compounds by GC-MS

The optimal conditions for the extraction of volatile organic compounds were established from a Definitive Screening Design based on 36 assays, analysed in a random order. The response variables analysed were the peak area of each compound and the optimization task was done to maximize the peak area of each compound under study. According to the obtained results, the choosen conditions for the extraction of volatile organic compounds from beer are presented in Table 9.

Table 9: Optimal conditions for the extraction of volatile organic compounds found in beer by HS-SPME

Factor	Optimal level
Degas	no
Sample volume	10 mL
Addition of salt	Yes (3.3g)
Type of Fibre	Car-PDMS
Agitation	no
Pre-incubation time (min)	no
Extraction time (min)	20
Extraction temperature (°C)	40

The selected fibre was the Car-PDMS. This was the fibre also used by Leça, Pereira (12) to study carbonyl compounds in beer. It was also found that carbonation does not significantly influence SPME sampling, in accordance with previous results reported by Pizarro, Pérez-del-Notario (117). Concerning the sample volume, the results suggest that 10 mL in a vial with 20 mL of capacity is the best option, since the extraction was not favoured when smaller sample amounts were used. Regarding the extraction time and temperature, the results indicate that these factors have a significant impact for almost every chemical families that were analysed and their settings were established according to VDKs best results. The agitation and incubation time factors were found to be relevant only for VDKs and acetoin, and therefore their settings were established based on the results of these compounds.

Following the extraction conditions described above, injections of standard solutions prepared in beer and in synthetic beer were analysed and calibration curves for all the analytes in study were determined in both cases. From these results, the matrix effect was studied by comparing the deviations between the slopes of the calibration curves resultant from beer and synthetic beer. It was observed that this parameter was higher than 15% for the majority of the compounds analysed, confirming that matrix effect occurs in this case. For that reason, beer was used for the quantification of VOCs and for the validation of this methodology. The parameters

of each analyte calibration curve, as well as validation results can be found in Table 10. A chromatogram resultant from a standard solution injection is shown in Figure 12.

Considering the samples in study, the developed quantification method shows a good linearity in the concentration range in study and recovery mean values ranging from 89.88 (ethyl isovalerate) and 133.80% (phenylethyl alcohol) confirm the method accuracy. The methods precision was evaluated in terms of repeatability and reproducibility, where variations were expressed as curve residual standard deviation values, which were generally lower than 10%. Also, the determined LOD and LOQ values were appropriate for the concentration ranges in study.

Concerning VDKs, diacetyl and 2,3-pentanedione presented LOD values of 17.22 µg/L and 0.90 µg/L and LOQ values of 57.41 µg/L and 3.01 µg/L, respectively. Their recoveries mean values were 125.21 and 107.86%. Linear ranges of diacetyl (R^2=0.9994) and 2,3-pentanedione (R^2=0.9999) cover concentrations from 5.00 to 499.95 µg/L and 5.02 to 1256.10 µg/L, respectively. Concerning diacetyl reduction products, acetoin and 2,3-butanediol, these compounds present LOD values of 0.94 and 6.76 mg/L, LOQ values of 3.13 mg/L and 22.54 mg/L and their recovery mean values are 114.62 and 108.79%, respectively. Acetoin (R^2=0.9999) and 2,3-butanediol (R^2=0.9987) linear ranges cover concentration from 10 to 200.38 mg/L and from 1.00 to 146.40 mg/L. These compounds precision parameters showed variations lower than 10%, except for 2,3-butanediol, which variations were lower than 20%.

Table 10: Validation parameters for the determination of volatile organic compounds in beer by GC-MS

Compounds	Kovats index	Identification/ **quantification** mode (m/z)	Linear range	R^2	LOD	LOQ	Recovery (%)			Repeatability (%)			Reproducibility (%)		
							C1	**C2**	**C3**	**C1**	**C2**	**C3**	**C1**	**C2**	**C3**
Acetaldehyde (mg/L)	483	42, 43, **44**, 45	2.12-206.40	0.9999	1.44	4.79	100.72	103.33	101.89	6.45	4.36	3.23	10.03	3.22	7.14
Ethyl acetate (mg/L)	881	**43**, 45, 61, 70, 88	1.00-50.06	0.9992	1.70	5.65	119.57	167.69	105.45	3.74	2.74	1.54	9.25	2.36	8.66
Diacetyl (µg/L)	991	**42**, 43, 86	4.99-499.95	0.9993	17.22	57.41	116.68	142.12	116.82	4.06	1.44	2.26	6.31	6.36	11.03
Ethyl butyrate (µg/L)	1050	43, 60, **71**, 89, 101	75.16-10021	0.9999	145.72	485.75	98.47	98.71	83.96	2.90	2.34	2.53	2.25	3.76	10.22
2,3-pentanedione (µg/L)	1075	43, 57, 85, 88, **100**, 115	5.02-100.49	0.9999	0.90	3.01	93.28	121.25	109.04	2.70	1.77	2.14	10.37	7.94	12.32
Isobutyl alcohol (mg/L)	1101	30-300	1.00-100.25	0.9998	1.65	5.51	125.29	137.80	118.66	4.75	2.43	1.42	10.35	6.54	8.97
Isoamyl acetate (µg/L)	1139	30-300	15.24-2540.40	0.9999	9.44	31.47	120.10	110.56	65.91	5.99	3.70	3.42	4.93	0.52	10.12
Isoamyl alcohol (mg/L)	1218	30-300	1.00-100.08	0.9999	0.69	2.29	-	-	-	5.46	2.67	2.21	13.79	10.26	9.97
Ethyl hexanoate (µg/L)	1256	30-300	5.04-1008.04	0.9999	10.99	36.63	100.10	107.57	73.61	7.92	4.27	2.97	5.37	7.56	10.60
			5.04-5040.20	0.9999	45.97	153.22									
Acetoin (mg/L)	1324	43, **45**, 55, 56, 69, 75, 88	0.10-200.38	0.9999	0.94	3.13	120.95	118.54	104.38	5.75	1.96	6.52	4.48	5.62	9.34
Acetic acid (mg/L)	1480	30-300	2.50-200.15	0.9996	6.20	20.66	107.06	110.35	115.61	4.41	7.74	5.00	4.60	6.20	8.12
2,3-Butanediol R (mg/L)	1556	30-300 (**45**)	1.00-146.40	0.9987	6.76	22.54	116.03	115.73	94.60	13.19	7.49	10.28	19.53	3.81	8.34
Hexanoic acid (µg/L)	1866	30-300	260.12-10404.80	0.9999	125.40	418.01	115.11	113.16	107.44	5.99	5.09	2.27	5.06	8.57	1.87
Phenylethyl alcohol (mg/L)	1944	30-300	5.01-200.15	0.9999	1.99	6.65	131.80	142.80	126.79	5.14	10.35	2.78	13.27	8.58	3.69
Octanoic acid (µg/L)	2076	30-300	49.14-50960	0.9999	508.78	1695.94	141.41	136.11	112.55	6.62	5.97	4.31	9.36	9.24	2.22

Note: Validation parameters for ethyl hexanoate (Table 10) were applied for the determination of ethyl caprylate, ethyl decanoate and ethyl laurate, while phenylacetate concentrations were extrapolated from ethyl acetate calibration curve. Also, the values presented for isoamyl alcohol include both isomers.

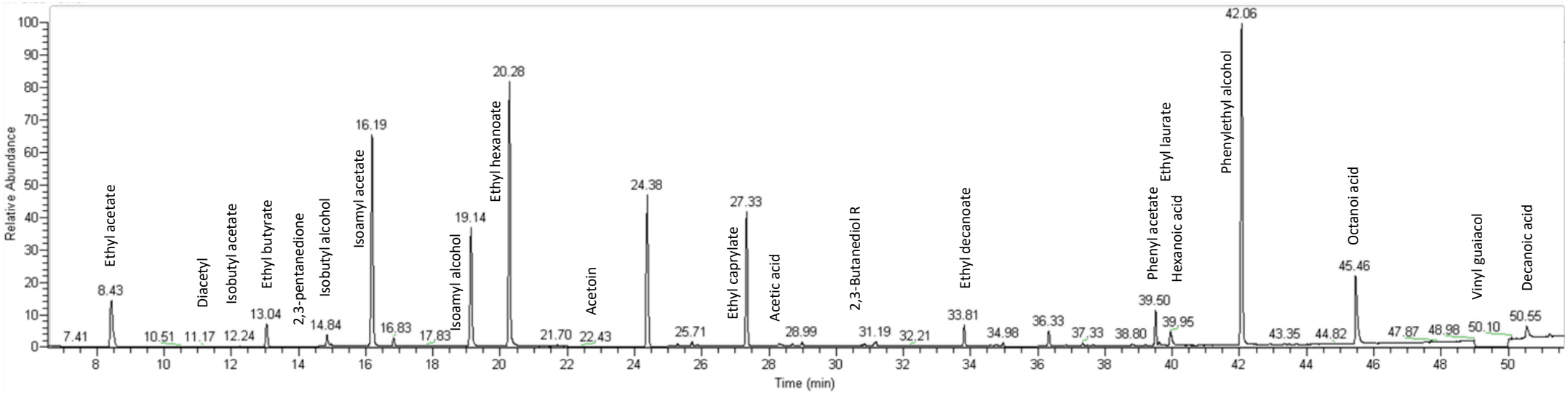


Figure 12: Chromatogram resultant from GC separation of volatile organic compounds calibration standard

CONSERVATION TESTS

The main goal of testing different conservation options for beer fermentation samples was to immobilize yeast cells dissolved in the inoculated wort solution and to, consequently, interrupt brewing yeast metabolic activity, so that the sample could remain chemically stable during transportation and storage in the laboratory, until it was finally analysed. Data from the performed conservation tests compare the efficiency of each treatment and also show differences between samples analysed fresh and after two days in distinct storage conditions.

The treatments applied to samples collected in the middle of fermentation were the addition of calcium chloride and thermal pasteurization, known to be effective in yeast immobilization. Chloride has a toxic effect on the brewing yeast at concentrations higher than 300 mg/L (125) and stops fermentation when above 600 mg/L (125, 136). On the other hand, pasteurization is usually used as a common method to destroy microbiologic contaminants and yeast cells found in beer (20). Additionally, storage for two days at 2°C and -26°C and its effect as a co-treatment for calcium chloride addition and pasteurization was also tested.

For that purpose, using a Tukey statistical test with a significance level of 0.05, all treatments data were compared. The chosen treatment was selected after balancing the effects of the different conservation tests on free amino nitrogen content (Figure 13), vicinal diketones (Figure 14) and on the three amino acids related with VDKs formation (Figure 15). Treatments that show no significant differences in analytes concentration share identical characters. In this study, only treatments which result in small differences between fresh samples and samples stored during 2 days (at 2 and -26^{o}C) were considered. Besides that, possible side effects that may occur as a consequence of calcium chloride addition or that may be caused by pasteurization were also taken into account.

Starting with the conservation tests labelled as 'no treatment, (…)', the obtained results show that this treatment effect is different according with the chemical nature of the analysed compound. The treatment seems to be effective to avoid leucine and isoleucine concentration variations, since the samples stored at 2^{o}C and -26^{o} C show no significant differences comparing to the fresh sample. Regarding FAN and valine, concentration of samples stored for 2 days at 2°C was also similar to the fresh one and differences were only found in samples stored at -26^{o}C. When observing VDKs concentration, both the samples stored at 2°C and at -26°C, that are similar to each other, are significantly different when comparing to the sample analysed while fresh.

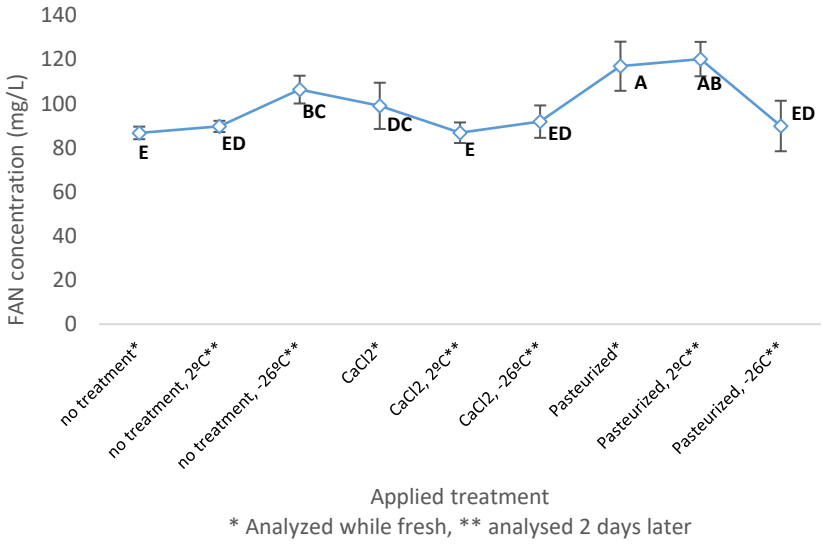


Figure 13: FAN concentrations obtained for each conservation test using a 95% confidence interval; Identical characters show no significant differences

According to literature, storage at 2°C may not be enough to inhibit yeast metabolism during beer fermentation, once yeast is capable of presenting a minimum development between 1 and 3°C (137), which in this study is more evident for VDKs in this study. Blanchette (3), when comparing fresh and samples kept at -50ºC for at least a month, showed that concentration variations during storage are evident for 2,3-pentanedione. This author also concludes that, at this temperature, diacetyl concentrations do not vary significantly. Results found in Figure 14 reveal differences between fresh samples and those stored at -26ºC, suggesting that a storage temperature lower than -26°C is needed to avoid diacetyl evolution when no other treatment is applied during sampling. Concerning amino acids evolution during beer fermentation samples storage, no conclusions were found in previous studies.

Concerning the pasteurized samples, the FAN and amino acids concentrations show no significant differences between fresh samples and samples stored at 2ºC. On the other hand, for VDKS, neither the concentrations of samples stored at 2°C or at -26°C are similar to the those observed in fresh ones. These results are in agreement with previous publications, where pasteurized and unpasteurized samples are compared when fresh and after storage at 22°C for almost an year (138) and at 40°C during 41 days (139). Analytical data from these studies show that pasteurization induces the formation of heat-induced volatile staling compounds, such as VDKs (2). Additionally, these studies also show that the content of volatile esters is higher in

unpasteurized beers, possibly due to active remaining yeast in these samples or as a consequence of the heat treatment suffered during pasteurization, which may cause the loss of these volatile compounds (138). This information is useful to predict the effects of storage on the VOCs that were not tested at this stage.

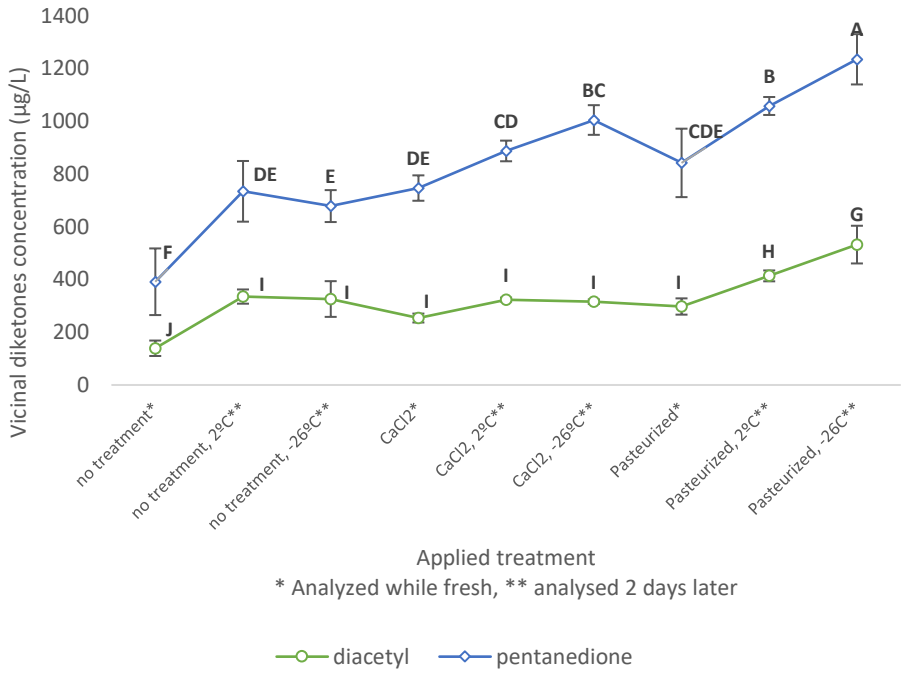


Figure 14: VDKs concentrations obtained for each conservation test using a 95% confidence interval; Identical characters show no significant differences

At last, diacetyl and amino acids concentrations from fresh samples treated with calcium chloride show no significant differences when compared with both samples stored for 2 days at 2 and -26°C. Moreover, the results of FAN concentrations reveal that samples stored at -26ºC are also similar to fresh samples, contrarily to those stored at 2ºC. 2,3-pentanedione concentration is the only compound that shows significant differences between fresh and samples kept at -26ºC.

After considering the observations mentioned above, it is possible to conclude that VDKs are the tested compounds that suffer more changes during storage, once their concentration is greatly affected both when beer fermentation samples are pasteurized and also when controlled storage temperature is the only treatment applied. When undergoing these treatments, FAN and amino acids contents are either not affected by storage temperature or their concentrations are only significantly different from fresh samples when they are kept at -26°C.

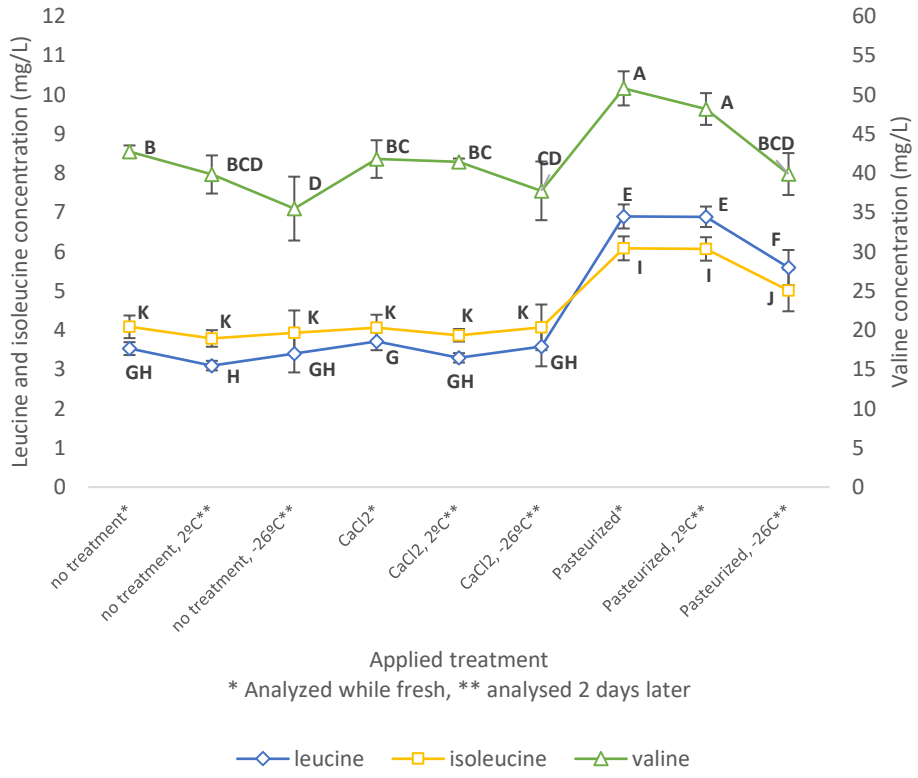


Figure 15: Amino acids concentrations obtained for each conservation test using a 95% confidence interval; Identical characters show no significant differences

Effectively, when calcium chloride is added during sampling, concentrations from fresh samples show no significant differences from those kept either at 2 and -26°C, in the case of diacetyl and the analysed amino acids. For FAN and 2,3-pentanedione, data show that only one of the storage temperature, 2°C and -26°C, respectively, greatly influences their concentration, when compared with samples analysed while fresh. When compared with the remaining evaluated treatments applied to beer fermentation samples, it is possible to conclude that the implementation of chloride salts addition is an important step to avoid sample evolution during storage, once it does not induce major variations in the analysed compounds in this assay. Besides salts addition, when evaluating possible storage temperatures, storage at -26°C seems to be more adequate to avoid sample evolution.

Consequently, the addition of calcium chloride during sampling, associated with storage at -26°C for 2 days, is the most efficient treatment to ensure proper sample preservation.

FERMENTATIONS GENERAL CHARACTERIZATION

Samples from three lager fermentation vessels, all resultant from wort produced with one malt batch and inoculated with a third-generation yeast, were obtained from a local brewery. Since the beginning of fermentation and until the beginning of maturation, several aliquots were collected at fermentation days 0, 3, 5, 6, 8, 9, 10 and 12. Once wort batches for each fermentation vessel were produced sequentially (the beginning of the F1 and F3 differs about 12h) and samples were daily collected all at the same time, a deviation of a few hours between the beginning of each fermentation must be considered in samples from the same day. Therefore, when possible and necessary, fermentation time is always expressed in hours, instead of days.

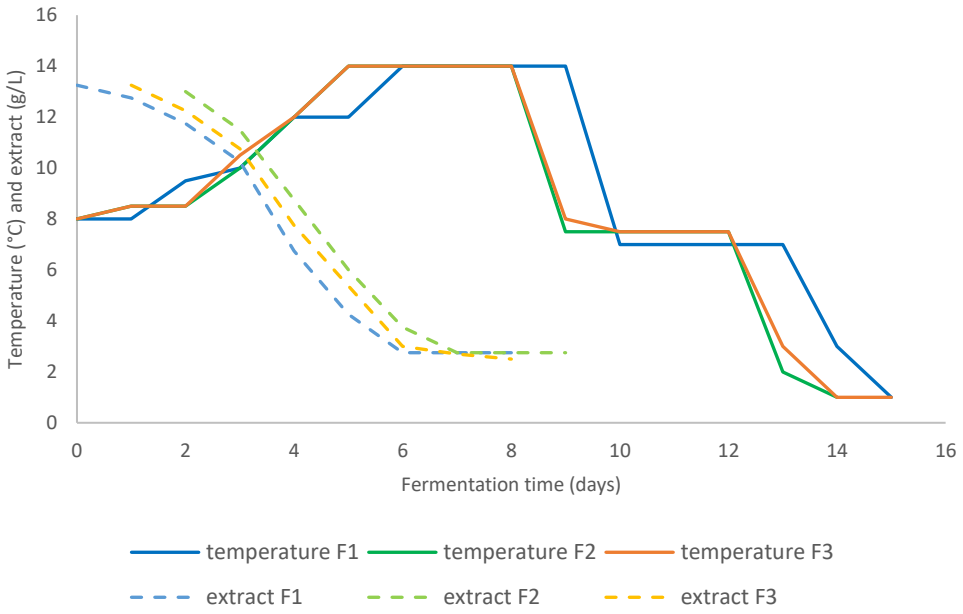


Figure 16: Temperature and extract during the analysed lager fermentations

Figure 16 shows the values of temperature and extract obtained from fermentations 1, 2 and 3 at the beginning of each day. Succinctly, for all the vessels, fermentation started at 8°C and followed a heating ramp until reaching a maximum temperature of 14°C, between day 5 or 6, which was kept for 4 days. Then, another ramp was applied until the temperature was reduced to 7°C. Finally, at the end of primary fermentation, solution temperature was set to 1°C, so that beer maturation could occur.

In terms of extract, fermentations 1 to 3 follow a similar evolution. At the beginning of fermentation, extract values are slightly lower than 14g/L and, as alcoholic fermentation occurs, sugars are consumed until day 6 or 7, when extract values stabilize between 3 and 2g/L. At this point, sugars transformation into ethanol and carbon dioxide ceases but still, secondary metabolites are being produced and ethanol attenuation may occur until the end of maturation (2). Little deviations observed between the three extract curves can be explained by the moment when sample was collected in relation to fermentation beginning in each vessel.

Effectively, when looking to FAN consumption (Figure 17), concentrations also decrease very quickly at the beginning of fermentation, reaching minimum values somewhere between 125 and 150 hours, suffering only minor variations until primary fermentation is over. Both sugar and FAN consumption curves demonstrate that cell growth exponential phase (125) occurred until the 6th or 7th day of fermentation, followed by the stationary growth phase. These results are similar to those obtained by Landaud, Latrille (140) although generally, nutritional components cease earlier in fermentation, as demonstrated before (3, 38, 61, 125, 141).

Results show that FAN initial contents vary between 186.69 (F1) and 197.78 mg/L (F2), while final concentrations go from 104.05 (F1) to 111.67 mg/L (F2). These variations are consistent with values observed for bottom flocculent yeasts (Cheong, Wackerbauer et al. 2007, Lei, Li et al. 2013). Although the measured initial FAN contents are in accordance with several studies (38, 140, 142), another publication shows that an initial FAN concentration lower than 300 mg/L can lead to an obligatory extended fermentation time to achieve a proper diacetyl reduction (61).

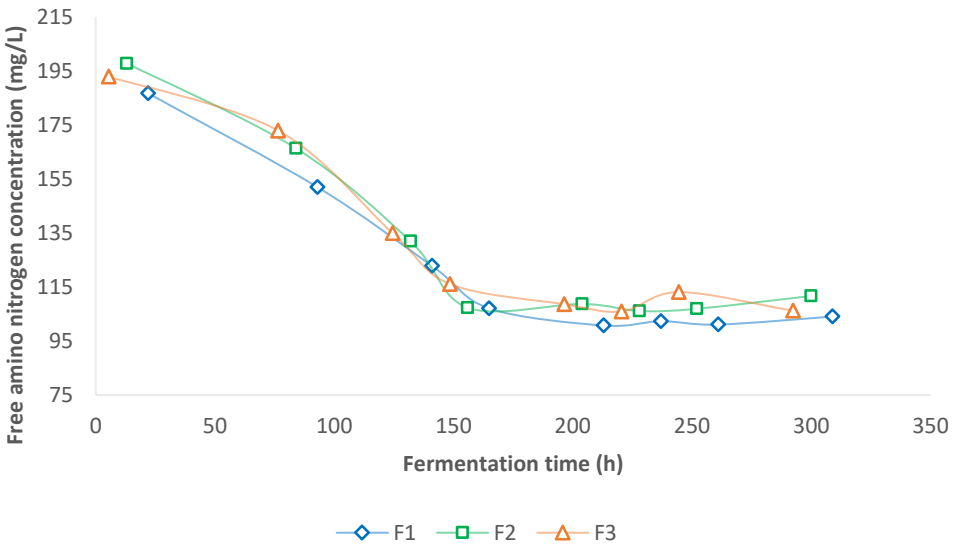


Figure 17: FAN consumption during the analysed lager fermentations; %RSD lower than 10

AMINO ACIDS UPTAKE DURING LAGER FERMENTATION

Individual amino acids concentrations were determined by implementing the developed HPLC-fluorescence methodology to lager beer fermentation samples. The obtained results are listed in Appendix I. Total amino acids evolution in the lager fermentations in study are shown in figure 18 and a preliminary observation of amino acids uptake shows that these curves are very similar to those observed for FAN consumption (Figure 17).

At the beginning of fermentation, total amino acids concentrations (Figure 18) achieve values close to 1000 mg/L, which are lower than those observed by other authors (81, 126, 143). This discrepancy may be related with the addition of nitrogen non-rich adjuncts during wort production of the analysed beer fermentations (2, 144). Different yeast strain, technological procedures used (96) and yeast vitality (3) may also explain the differences between the observed values and those found in literature. Similarly to what was observed in FAN uptake (Figure 17), a relatively fast decrease of total amino acids concentration takes place during yeast exponential growth phase (125, 144), until 125 to 150 hours of fermentation.

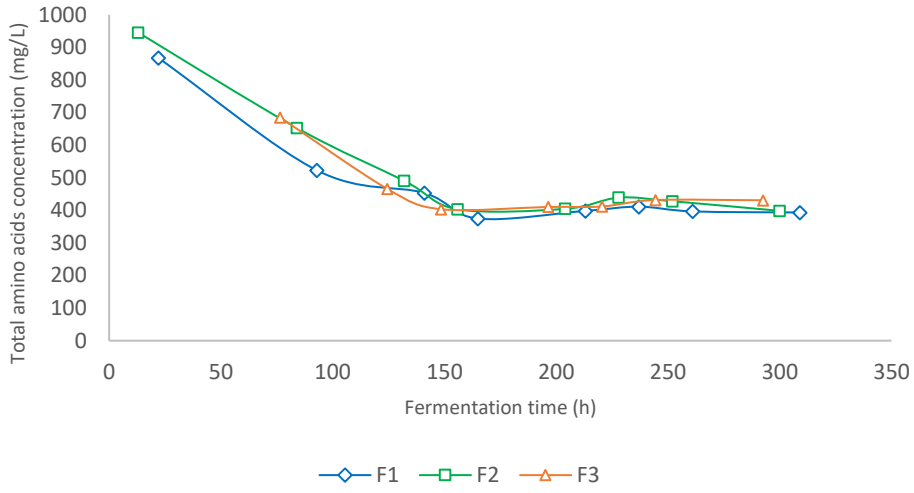


Figure 18: Total amino acids evolution during the analysed lager fermentations; Results from day 0 for F3 were not considered, once an unexpected error occurred during online derivatization for these samples replicates and because there was no fresh sample available for repeating the analysis.

Observed assimilation rates were lower than those from static lager fermentations studied before (145). However, in another study, lager fermentations also showed lower amino acids absorption rates, taking more than 72 hours until no more changes in amino acids were

observed and this author showed that yeast metabolism slows down intensively with decreasing fermentation temperatures (3). From this point forward, total amino acids concentration is basically constant in all fermentations, as yeast multiplication stops (144), except for a slightly increase, which occurs more or less 225 hours after the beginning of fermentation. This may be a consequence of the biosynthesis of an exhausted preferred amino acid by the brewing yeast (61).

At the end of fermentation, total amino acids concentrations round about 400 mg/L, which means that a bit more than 50% of the amino acids were consumed. These concentrations are consistent with those described for Czech lager beers (96) but are higher than values determined in Hungarian beers (95). Considering the amino acids assimilation ratio determined in previous publications, about 35% of the amino acids are consumed in normal gravity wort (37, 45), while in high gravity wort this ratio is as high as 45% (37).

For a better understanding of amino acids uptake during beer fermentation, these compounds are distributed in four distinct groups, according to the metabolic preferences of the brewing yeast. In lager fermentations, amino acids belonging to group A are exhausted from the medium early at the logarithmic phase of yeast growth. At the same time, amino acids from group B are consumed at a slower rate but usually are not completely absorbed. Then, amino acids from group C are assimilated even more slowly and incompletely, after those from group A were already exhausted (39, 146). A fourth group englobes the un-preferred amino acids, which are almost never consumed, unless all the others amino acids have ceased (39). This classification is summarized in Table 11.

Table 11: Amino acids uptake according to lager yeast metabolic preferences, adapted from García et al. (1994) (39) and Palmqvist and Äyräpää (1969) (146)

Group A	Group B	Group C	Group D
glutamine	histidine	glycine	proline
asparagine	valine	phenylalanine	γ-Aminobutyric acid
serine	methionine	tyrosine	
threonine	leucine	tryptophan	
lysine	isoleucine	alanine	
arginine	aspartic acid		
	glutamic acid		

Following this classification, amino acids were distributed in three groups, according their uptake curves observed during lager fermentation. Figure 19 shows amino acids uptake

patterns of each individual amino acids from groups A, B and C in lager fermentations 1 (F1), while Figure 20 summarizes the behaviour of each group of amino acids, elucidating the occurrence of different assimilation rates and concentrations at the end of fermentation. Data relative to fermentations 2 and 3 are not shown, once identic amino acid uptake behaviour was observed and these fermentations data would not add any other useful information to results discussion. GABA and alanine (coeluted amino acids in the applied methodology) were not considered here.

Comparatively to the classification established in other studies, results obtained for these lager fermentations differ relatively to some amino acids uptake patterns. Threonine, for example, was classified as a group B amino acid, instead of belonging to group A, once this amino acid was not totally consumed during fermentation, contrarily to what was demonstrated in other studies (39, 146). Glutamine (Appendix I), also usually placed in group A, shows an unusual uptake curve as well and for that reason it was not included in Figures 19 and 20.

There is a chance that these amino acids concentrations sharply decreased from day 0 to day 1 or 2 and that, subsequently, yeast cells synthetized this preferred amino acid according to their metabolic requirements. In the future, in order to confirm this hypothesis and presuming that important changes in amino acids concentration may have occurred during the first 72 hours of fermentation, samples should be collected more frequently during this period. Additionally, collecting a wort sample before pitching may add useful information to this study.

Besides that, other amino acids from group A, asparagine, serine and lysine, show initial concentrations lower than expected (39) but present typical uptake behaviours and were almost completely exhausted between the 3rd and the 5th day of fermentation. This is in accordance to previously presented results concerning lager static fermentations (39), although the same author shows that in fermentations with external agitation, these amino acids are already exhausted right after the second day of fermentation. Similar results were also verified in another static lager fermentation (38), while another study shows a lager fermentation in which group A amino acids are only fully exhausted after 100 hours of fermentation (147).

Secondly, amino acids from group B (Figure 19B) show a slower assimilation than those from group A, once they stop being consumed after about 150 hours of fermentation, much later than showed in other studies (38, 39), where amino acids from groups A and B stop being assimilated at the same time, though those from group A are exhausted, contrarily to those from group B. Nonetheless, in agreement with other publications (38, 39, 147), assimilation ratio values for these amino acids are always higher than 50%.

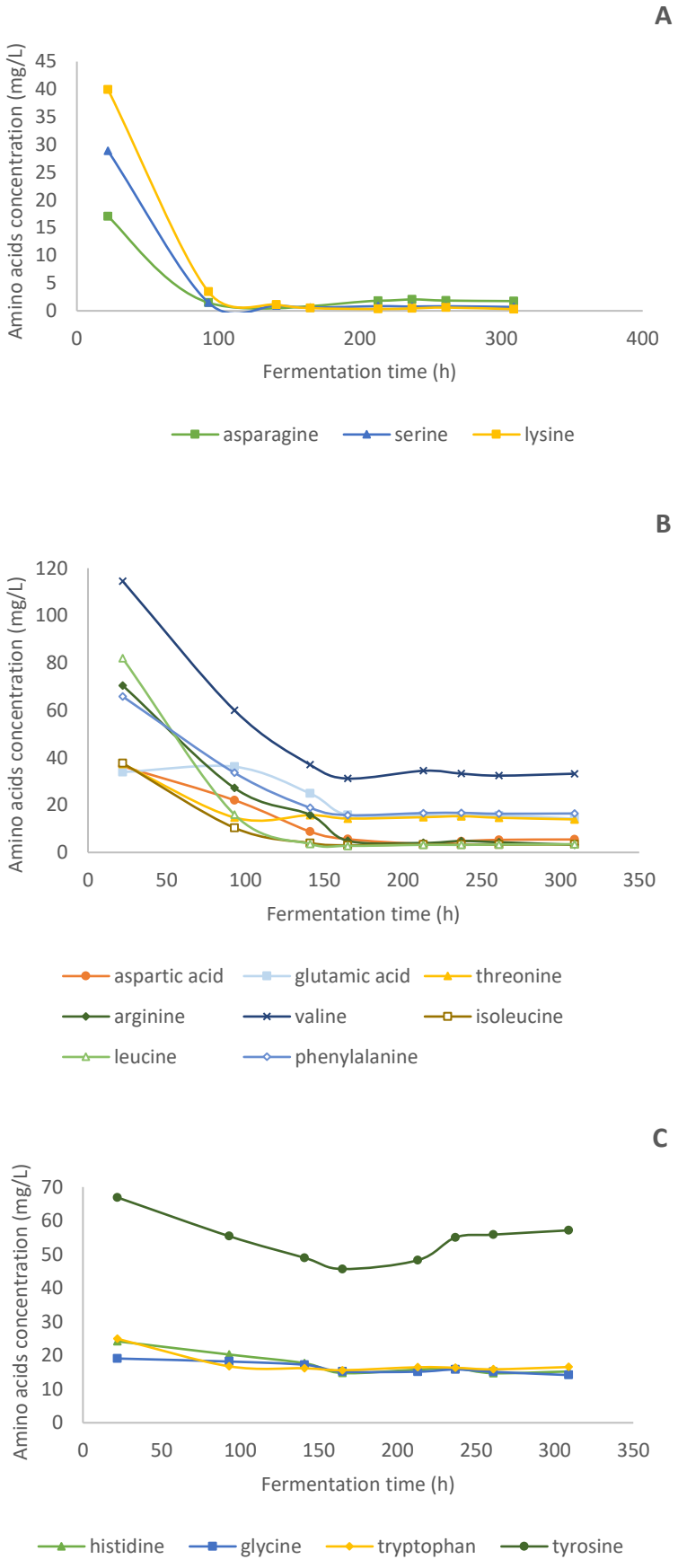


Figure 19: Group A (A), B (B) and C (C) individual amino acids uptake in lager fermentation 1; %RSD lower than 15

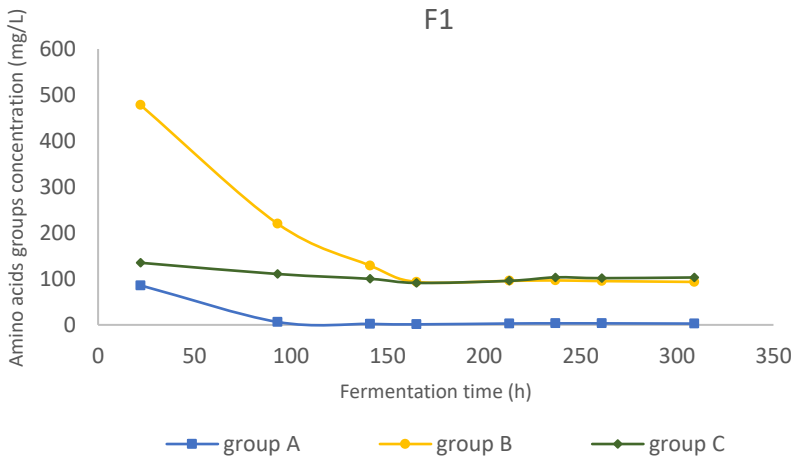


Figure 20: Group A, B and C amino acids uptake in lager fermentations 1

Finally, when analysing group C amino acids (Figure 19C), it is clear that histidine, glycine, tyrosine and tryptophan are barely consumed during fermentation, as reported by other authors (39, 146). Effectively, phenylalanine uptake curve (Figure 19B) is the only one that does not follow a typical uptake by this lager yeast, disagreeing with results found in literature (39, 146). Thus, instead of showing a very low to inexistent assimilation rate, its uptake pattern was similar to those from group B amino acids. For that reason, phenylalanine was characterized as a group B amino acid.

Concerning individual amino acids bioavailability in the beginning of fermentation, valine (about 120 mg/L) and glutamine (about 5 mg/L) are, respectively, the most and the least abundant amino acids found in the analysed lager fermentations. Then, at the end of primary fermentation, tyrosine and valine are the most abundant amino acids, presenting about 60 and 40 mg/L, respectively. Despite the two exceptions observed in amino acids uptake, the overall amino acids uptake pattern is in accordance with lager yeast metabolic preferences studied in other fermentations. Additionally, important points related to nitrogen quality and availability in wort and its impact on beer VOCs will be discussed later in this chapter.

VOLATILE ORGANIC COMPOUNDS EVOLUTION DURING LAGER FERMENTATION

In this section, volatile organic compounds evolution is studied in detail and compared with amino acids assimilation during lager fermentations. After implementing the new GC-MS methodology to lager fermentation samples, individual VOCs concentrations from fermentation days 0 to 12 were determined. Respective results are presented in appendix II. After a careful

analysis of individual concentrations obtained, all the volatile organic compounds were organized in groups according to their chemical nature. The evolution of these compounds and total amino acids concentration during fermentation time in vessels 1, 2 and 3 are represented in Figure 21.

Firstly, when analysing VOCs evolution, it is possible to conclude that each chemical family follows a characteristic evolution in the course of primary fermentation. However, it is clear that compounds start being produced at an early stage of fermentation, when amino acids are continuously assimilated by yeast, at its most active growth phase (125, 144). Then, after 150 hours of fermentation have passed, most of the chemical families show no significant differences in concentration values until fermentation is over, except for higher alcohols, which concentrations suffer oscillations during this period. This observation elucidates the importance of amino acids in beer volatile organic compounds formation, as demonstrated before (3, 45, 125). Indeed, the final concentration of higher alcohols in beer depends on the uptake efficiency of amino acids and sugars assimilation rate (80).

Contrarily to other families, higher alcohols show three peaks at, approximately, 125, 150 and 200 hours after yeast inoculation. These oscillations may be related to higher alcohols transformation into esters, once they are their direct precursors (5, 148) and to amino acids synthesis (61). After the third concentration peak, higher alcohol content decreases until reaching, more or less, 60 mg/L, in accordance to concentrations determined at the end of fermentation in other studies (37, 142), although pilsner finished beers may present higher alcohols content as high as 100 mg/L or more (142, 149). Phenylethyl alcohol, isoamyl and isobutyl alcohol approximate concentrations determined at the end of fermentations are, respectively, 30, 20 and 10 mg/L. These compounds are all below their odour threshold, in accordance to data found in literature (5).

Esters are usually considered as the most important family of volatile organic compounds in beer, once they impart characteristic aromas to this beverage (2, 144). These compounds are produced during fermentation mainly by the esterification of fatty acids and alcohols (2). At the end of primary fermentation, total esters concentration in the analysed samples sums about 30 mg/L. These values are in accordance with results obtained by other authors (37, 142). Considering individual esters, ethyl acetate is the most abundant at the end of primary fermentation, ranging between 20 to 25 mg/L, approximately, followed by ethyl caprylate with about 3.5 mg/L and isobutyl and isoamyl acetate, both showing concentrations close to 2 mg/L. In turn, ethyl laurate and phenylacetate were found at residual concentrations.

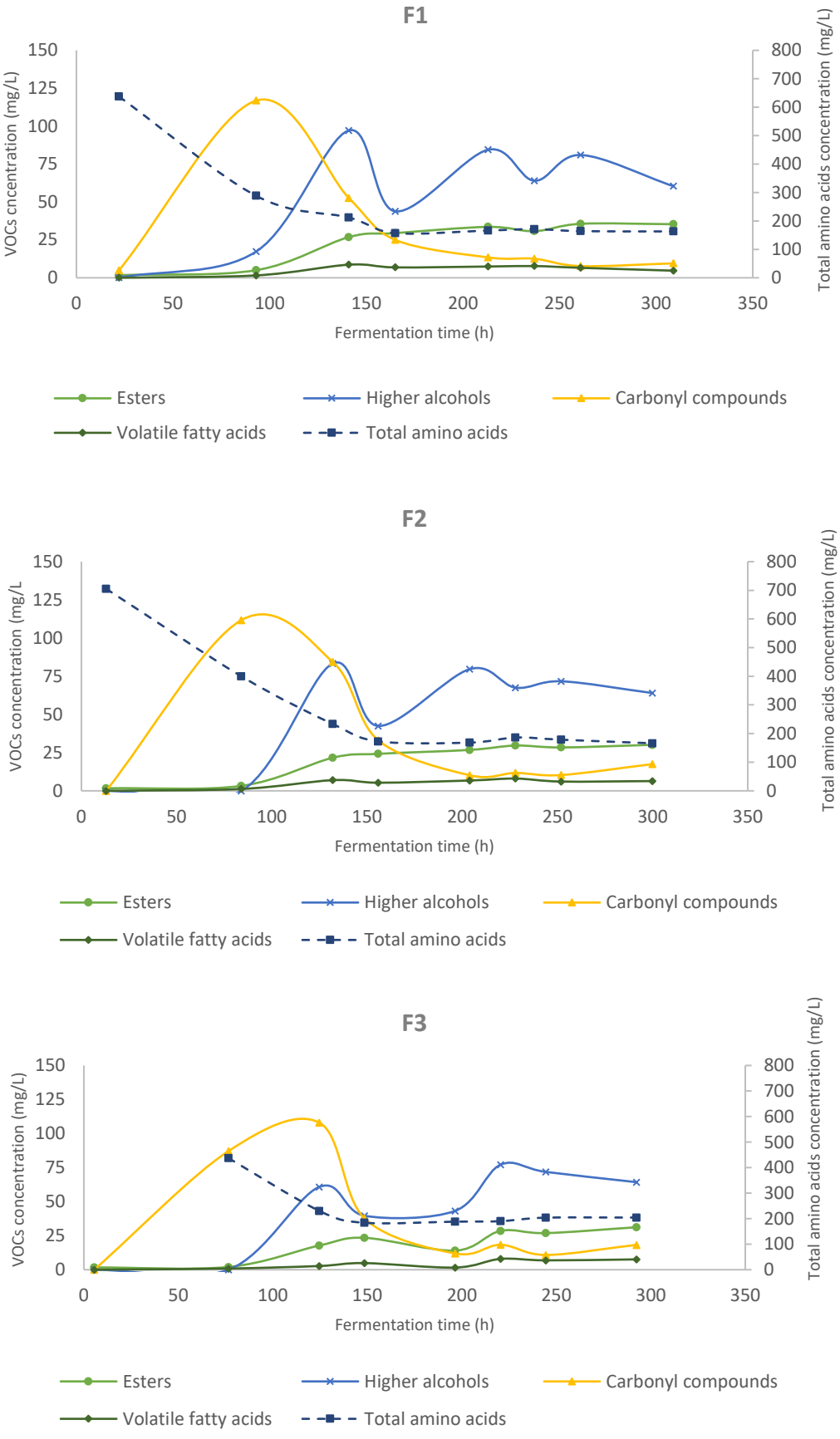


Figure 21: Volatile organic compounds (chemical families) evolution and total amino acids uptake during the analysed lager fermentations

According to other publications (37, 150), ethyl acetate is also the most abundant ester found in lager beers, and although isoamyl acetate concentrations from fermentation 1, 2 and 3 are similar to those determined in these studies (37, 116, 142), ethyl caprylate amounts in lager beers are usually lower than 0.5 mg/L (37). Besides this, most of the remaining esters show concentrations similar to the obtained results. Considering these compounds odour threshold limits, all esters except for phenylacetate, ethyl butyrate and ethyl laurate present concentrations higher than their threshold. In all the analysed fermentations, ethyl acetate final concentrations are slightly below this compound odour threshold but, once esters content may double during beer maturation (2), it is expected that ethyl acetate aroma will be perceptible in the final beer. Synergistic effects between esters odours were also suggested previously (81).

The volatile fatty acids were the least abundant family during the entire course of fermentation, showing concentrations of approximately 6 mg/L at the end of this stage. Decanoic acid presents individual concentrations of 3 to 4 mg/L, while octanoic and hexanoic approximate concentrations are 1.45 and 1 to 2 mg/L, respectively. These values are in the same order of magnitude of those reported by other authors, although usually octanoic acid is the most abundant one, followed by hexanoic and decanoic (42-44). All the analysed fatty acids concentrations are below their threshold limit.

Acetic acid concentrations (appendix II) were also evaluated. At day 0, these values are close to 40 mg/l and then, after 150 hours of fermentation a significant concentration decrease occurs, follow by an increase of acetic acid until maximum values of approximately 100 mg/L are reached at the end of fermentation. These values are in agreement with those observed in other beers. However, other authors affirm that acetic acid in beer may achieve concentrations as high as 1000 mg/L. Although at 100 mg/L the acetic acid flavour is perceptible, possible attenuation during maturation may contribute to lower this content bellow acetic acid threshold limit (151).

Vinyl guaiacol relative areas (appendix II) were also measured during the course of fermentation and its concentration increased about 5 to 6 times in the first 150 hours. Then, vinyl guaiacol content declines to about half and then remains constant when yeast growth stationary phase starts and until the end of primary fermentation. This compound presents a low odour threshold described as phenolic or medicinal (46) and, besides being appreciated in some ale beers, vinyl guaiacol aroma is often considered as an undesirable flavour in lager beers (48). The quantification of this compound was not a primary goal of the present work, therefore, a more accurate determination of this compound in local beer samples will be necessary to understand its influence in beer aroma.

Finally, carbonyl compounds, which include diacetyl, 2,3-pentanedione, acetoin and acetaldehyde, are intensively produced until the fifth day of fermentation. Then, this content decreases drastically and, at 225 hours of fermentation, another (less intense) peak appears. Acetaldehyde (appendix II) was only quantifiable from day 0 to day 6. After the 6th day, this compound concentration was always lower than its limit of quantification and, consequently, below its odour threshold. Vicinal diketones and acetoin evolution during fermentation, as well as related compounds such as 2,3-butanediol, will be carefully studied in the next section.

DISCUSSION

This final section is dedicated to the study of vicinal diketones evolution during the lager fermentations in study. Therefore, the analysed compounds that are inherent to vicinal diketones formation in fermentations 1, 2 and 3 were all brought together, as shown in Figure 22, 23 and 24, respectively, including secondary metabolites resultant from diacetyl reduction and the amino acids which formation paths are known to induce diacetyl and 2,3-pentanedione synthesis. Additionally, free amino nitrogen evolution during this period was also considered, once it represents a good indicator of yeast cell growth and fermentation efficiency (38).

Starting with nitrogenous compounds uptake, as mentioned before, FAN evolution is characterized by an accelerated decline in the first 150 hours of fermentation, correspondent to yeast most active growth and multiplication stage. At the beginning of fermentation, group A amino acids are transported into the cell and assimilated by yeast. Group B amino acids uptake is also taking place at this stage (3), although at a lower rate (146). This last group includes the amino acids valine, leucine and isoleucine, which are related to VDKs formation. Then, from that point forward, these compounds also stop being assimilated by the yeast and their concentrations remain constant until the end of fermentation.

The analysed fermentations (Figure 24, Figure 23 and Figure 24) show that valine is not completely removed from fermentation medium while, contrarily, isoleucine and leucine are almost consumed to exhaustion, after approximately 125 hour of fermentation, slightly earlier than valine. Actually, it was already demonstrated that sometimes these two amino acids can be classified as belonging to group A (146). Also, much faster and more complete wort amino acids absorption was verified in ale beers, while lager beer show slower amino acids absorption rates and a higher residual content of amino acids at the end of primary fermentation (3), in agreement to the observed results.

When looking to initial amino acids concentration, valine is the most abundant one in fermentations 1 and 2 with, approximately 250 mg/L, followed by leucine with about 200 mg/L and isoleucine, which shows concentrations rounding 100 mg/L, in fermentations 1 and 2. The same order is observed in fermentation 3 concentrations at the third day of fermentation. Other authors show that initial leucine concentrations range from approximately 150 to 300 mg/L and that this amino acid is more abundant than valine (37, 83, 146), which content usually ranges from 100 to 170 mg/L (37, 39, 83, 146). These values are much lower than those observed in the samples analysed in the present study. In these publications, isoleucine is always the least abundant amino acid and its concentration varies between 60 and 100 mg/L. Also, other author showed that high valine contents are usually associated to delayed diacetyl formation (152).

Then, at the end of fermentation, valine concentration is, approximately 30 mg/L and leucine and isoleucine contents are lower than 5 mg/L. Similar concentrations of valine were found in other experiments, although the same authors had shown higher final concentrations of leucine and isoleucine (146). As mentioned before, high residual content of amino acids is typical of lager beers, contrarily to ale beers, in which amino acids exhaustion usually occurs. This behaviour also showed to be intensified when fermentation temperatures lower than 16°C are used, once yeast metabolism is dramatically slowed down at lower temperatures (3). These observations may justify the observed valine concentration but not leucine and isoleucine contents at the end of fermentation which, usually, are not typical of lager fermentations.

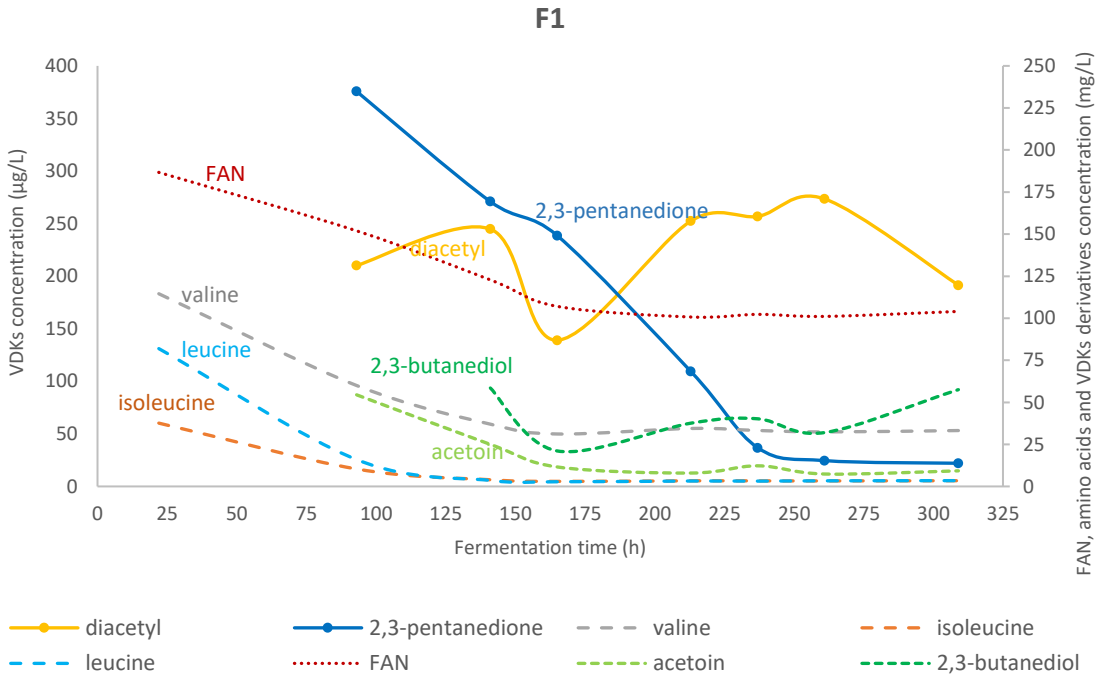


Figure 22: VDKs related compounds evolution during lager fermentation 1; % RSD lower than 20 for acetoin and 2,3-butanediol and lower than 15 for the other compounds

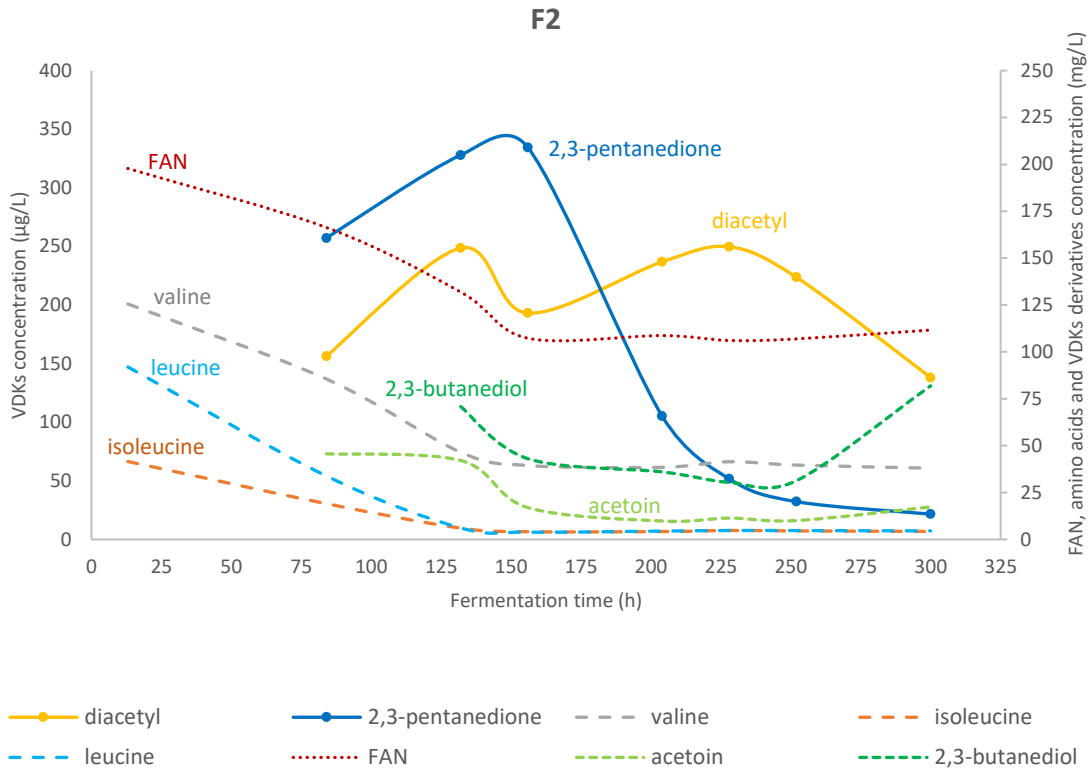


Figure 23: VDKs related compounds evolution during lager fermentation 2; % RSD lower than 20 for acetoin and 2,3-butanediol and lower than 15 for the other compounds

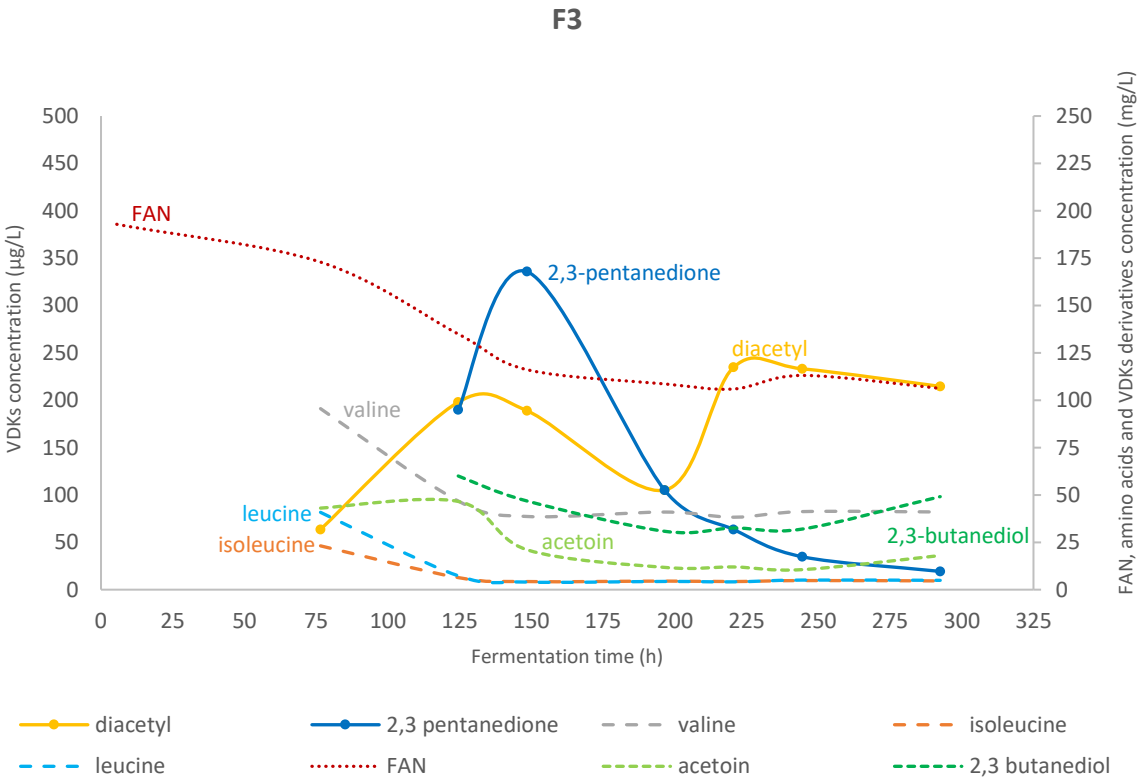


Figure 24: VDKs related compounds evolution during lager fermentation 3; % RSD lower than 20 for acetoin and 2,3-butanediol and lower than 15 for the other compounds

When considering diacetyl evolution during fermentations, valine, leucine, 2,3-butanediol and acetoin concentrations are intimately related to the synthesis of this vicinal diketone (2). Diacetyl, due to its extremely low odour threshold, is the most critical off-flavour produced during beer fermentation (3). Firstly, the referred amino acids biosynthesis by the brewing yeast is the metabolic process where an important intermediate, α-acetolactate, is also produced. This compound is then excreted to the wort and converted to diacetyl, through a spontaneous oxidative decarboxylation process (50). However, the brewing yeast has the ability of absorbing and enzymatically reducing diacetyl into less odour-active compounds, acetoin and then 2,3-butanediol, which have no negative influence on beer flavour (62).

In the analysed lager fermentations, two diacetyl concentration peaks are observed (Figure 232, 23 and 24). The first peak occurs at approximately 130 hours after pitching, which maximum registered concentration varied between 200 (fermentation 3) and 250 µg/L (fermentation 1 and 2). Previous studies dedicated to VDKs formation report the occurrence of a single diacetyl peak with concentration values higher than 500 µg/L in the first two days (48h) of fermentation (3, 57), which period was not monitored in the analysed fermentations. Therefore, a more frequent sample collection in the first days of fermentation will be necessary, in order to properly control diacetyl peak formation during this period. The second diacetyl concentration peak shows concentrations ranging from 250 to 300 µg/L and occurs approximately, at 230 hours after pitching. In fermentation 1, a slight decrease of diacetyl concentration occurs at this stage, probably due to an unbalanced diacetyl formation and reduction ratio caused by the activation/deactivation of amino acids biosynthesis pathway (3).

At the end of primary fermentation, diacetyl concentrations are slightly above its odour threshold limit, ranging approximately from 150 µg/L, in fermentation 1, to 200 µg/L, in the third fermentation vessel. It is expected that the remaining yeasts in solution are able to reduce diacetyl to values below this threshold limit during the maturation process (2, 51). According to results found in literature, the final concentration of diacetyl is conditioned by the level of α-acetolactate and the stage when it is excreted, but also by the yeast ability to enzymatically reduce diacetyl at certain fermentation conditions (3, 50, 61). Fermentation temperature influences yeast growth (62) and consequently fermentation rates . Thus, as this temperature decreases, although maximum diacetyl levels are lower, diacetyl formation and reduction is delayed and fermentation or maturation period are extended (3, 39).

As mentioned before, as diacetyl concentrations oscillate, other compounds levels also are affected. In a matter of fact, the increase of diacetyl in the fermenting wort is caused by

valine depletion (50), which can occur in two different extensions. Worts with high levels of FAN are intimately related to high fermentation rates and, for that reason, VDKs formation is enhanced, although their reduction also occurs more efficiently. In this case, group A amino acids are abundant and while these are being consumed, group B amino acids synthesis also occurs and, consequently, a single peak of diacetyl is observed. On the other hand, similarly to the results obtained in this study, when low levels of FAN are available and as soon as group A amino acids are exhausted, if group B amino acids depletion occurs, diacetyl synthesis is induced once again (3, 61, 81, 82).

Therefore, the existence of two diacetyl peaks is consistent with the FAN levels determined, which are lower than those in fermentations with only one diacetyl peak. Two diacetyl peaks were also detected in lager fermentations with external agitation (39). This author also confirms that each round of yeast propagation reduces its metabolic activity, which may explain delayed diacetyl formation as well. The use of coloured malts and adjuncts that yield little nitrogen also leads to amino acids concentration decrease in the wort, which is usually associated with enhanced levels of diacetyl (152).

The same way that group B amino acids exhaustion is accompanied by diacetyl formation, this compound is also reabsorbed and simultaneously reduced to acetoin by yeast, which is then reduced to 2,3-butanediol (51). Diacetyl transformation into acetoin is represented in the second half of diacetyl peak. At this stage, diacetyl formation occurs at a slower rate than diacetyl reduction and for that reason, when diacetyl concentration decreases, acetoin levels increase. Minor variations are observed in acetoin concentrations, once this compound is immediately transformed into 2,3-butanediol. This trend is more obvious at the end of fermentation. Final concentrations of acetoin and 2,3-butanediol range, respectively, from 10 to 20 mg/L and from 50 to 80 mg/L. Acetoin concentration values are slightly above respective threshold limits but this should not represent a problem, once further acetoin reduction into 2,3-butanediol, which presents a high odour threshold, will also occur later during maturation (51).

Concerning 2,3-pentanedione, a maximum concentration peak of, approximately, 400 to 450 mg/L can be observed 150 hours after pitching, both in fermentations 2 and 3. This occurs after the almost complete exhaustion of isoleucine, when yeast no longer needs to produce this amino acid and VDKs reduction rate is higher than the respective α-acetohydroxy acid formation rate, as demonstrated before (3). Then, contrarily to diacetyl, 2,3-pentanedione concentrations gradually decrease from this point over, achieving contents rounding the 15 µg/L at the end of

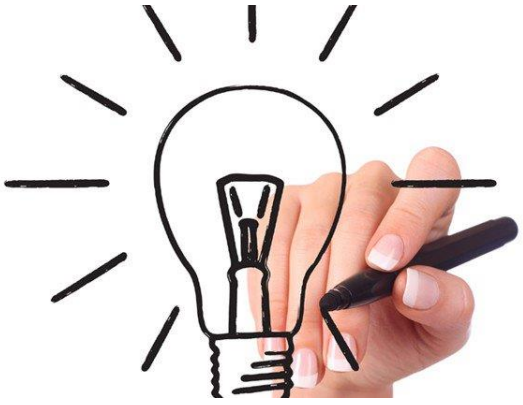
fermentation, once yeast is no longer producing VDKs precursors and it is now available to absorb 2,3-pentanedione and reduce it to its respective diol (3).

On the other hand, when analysing 2,3-pentanedione in fermentations 1, this concentration curve suggests the existence of an eventual higher concentration peak somewhere between the 1st and the 3rd day of fermentation, similarly to diacetyl's situation. This behaviour could eventually be verified in the remaining fermentations, though, more frequently collected samples will be needed in future studies, in order to confirm this. In fermentation 1, a slower reduction rate of this compound also occurs after 150 hours, when isoleucine stops being assimilated by the brewing yeast.

Previous experiments report maximum 2,3-pentanedione concentrations similar to those obtained, although in an earlier stage of lager fermentations performed at 12°C, namely between 40 to 75 hours after pitching (3). According to this author, this peak presents higher values with increased fermentation temperatures and occurs later at lower fermentation temperatures. Another study shows that 2,3-pentanedione peak occurs in a similar stage of fermentation, although maximum values are slightly lower than those observed in this study (153). The final concentrations of 2,3-pentanedione are also similar to those determined before by Blanchette (3) and Tian (153). Final concentrations of this VDK determined during the present study are much lower than this compound odour threshold (2, 12), and for that reason, 2,3-pentanedione aroma should be imperceptible in the finished beer.

According to the results presented above, it is suggested that high quality and sufficient FAN and amino acids content should be ensured at the beginning of fermentation by, for example, supplementing wort with key amino acids, in order to accelerate fermentation rates and to avoid the simultaneous exhaustion of groups A and B amino acids. Additionally, changing fermentation temperature can also positively affect fermentation rates, avoiding yeast metabolic activity slow down. By following these practices, it would be possible to avoid the formation of two diacetyl peaks and to greatly reduce the fermentation time, without affecting beer quality.

PART VII: Conclusion and future perspectives



CONCLUSION

Beer quality depends not only on the chemical characteristics of the raw materials used but is also greatly affected by the processes involved in its production. The availability and quality of nitrogenous compounds is intimately related with fermentation rates and volatile organic compounds formation by the brewing yeast, including beer off-flavours, such as vicinal diketones. Two new methodologies were developed, one for the determination of amino acids by HPLC-fluorescence and another for the determination of volatile organic compounds by GC-MS in beer fermentation samples. The second methodology development included a design of experiments for the optimization of analytes extraction by HS-SPME. Also, the EBC standard methodology for the determination of free amino nitrogen was also implemented.

Firstly, the methodologies mentioned above were applied to beer fermentation samples in order to determine the most adequate conservation procedure to avoid their evolution during transportation and storage. It was possible to conclude that the implementation of chloride salts addition and storage at -26°C are important steps for avoiding beer fermentation samples evolution during storage, once this treatment does not induce significant variations in the analysed compounds and interrupts brewing yeast metabolism. When no storage treatment was applied to samples, vicinal diketones were the most affected compounds, when compared to nitrogenous compounds. In agreement to literature, sample pasteurization affected sample composition, including VDKs, which formation can be induced by heat.

The developed methodologies and optimal conservation parameters were applied to three wort batches produced from the same raw materials, which were inoculated with a 3rd generation yeast. Eight samples were collected during the fermentation process, which lasted for 12 days. For each sample replicate, 21 amino acids, 22 volatile organic compounds and FAN levels were determined. Temperature and extract evolution were also monitored during this period. All the obtained data were analysed and relations between the analysed parameters, namely vicinal diketones formation and amino acids uptake, were established.

Considering the uptake of free amino nitrogen and amino acids during lager fermentations, concentrations remained stable approximately at, approximately, 150 hours after pitching, later than what was shown in previous studies. This period is associated with exponential yeast growth, which ceases when nutritional compounds stop being assimilated by the brewing yeast and stationary stage takes place. Concerning yeast uptake preferences, most

of the amino acids consumption patterns showed to be in accordance with results found in literature for lager fermentations. Therefore, group A amino acids were completely exhausted in the first 100 hours of fermentation, while those from group B were assimilated at a slower rate, showing high and constant residual concentrations (about 100 mg/L) approximately 150 hours after pitching. Finally, group C amino acids were barely consumed by the brewing yeast and also high concentrations were measured at the end of fermentation (about 100 mg/L).

A careful observation of volatile organic compounds evolution during the 12 days of fermentation allowed to conclude that, similarly to nitrogenous compounds, major changes also occur in the first 150 hours of fermentation. After this period, only subtle concentration changes take place, except for higher alcohols, once these are eventually produced as vicinal diketones synthesis occurs and are also assimilated during ester formation by the brewing yeast. At the end of fermentation, higher alcohols were the most abundant family of compounds (ranging from 35 to 65%), followed by esters (about 9%), which were generally above their odour threshold limit. Carbonyl compounds were the third most abundant family (about 2-6%), while only residual fatty acids concentrations (about 1-3%) were found at this stage.

At last, by interrelating all the obtained results, low free amino nitrogen (about 200 mg/L) levels together with insufficient amino acids concentrations seem to result in low fermentation rates, caused by a slow yeast metabolic activity. Indeed, once group A amino acids are consumed until exhaustion, complete absorption of some group B amino acids, namely leucine and isoleucine, are the cause of delayed vicinal diketones formation and reduction by the brewing yeast. Thus, this leads to the synthesis of group B amino acids in the middle of fermentation and consequently to diacetyl and 2,3-pentanedione formation, causing the abnormal occurrence of a second diacetyl peak, 230 hours after pitching. Contrarily to 2,3-pentanedione, diacetyl concentrations at the end of fermentation are slightly above their odour threshold limits (ranging from 150 to 200 µg/L) but it is possible to presume that no vicinal diketones related off-flavours will be detected in the final product, once their reduction to less flavour-active compounds will be certainly achieved during maturation.

Effectively, high quality and sufficient FAN and amino acids content ensures faster fermentation rates, avoiding yeast metabolic activity slow down and the simultaneous exhaustion of group A and B amino acids. Wort supplementation with key amino acids and changes in fermentation temperature are factors that also positively affect fermentation rates. Following these practices, it would be possible to avoid the formation of two diacetyl peaks and to greatly reduce the fermentation time.

FUTURE PERSPECTIVES

In addition to what was achieved in this study, suggestions for future work include:

- Collecting samples more frequently in the beginning of fermentation in order to detect a more detailed evolution pattern of nitrogenous and volatile organic compounds;
- Evaluating the effect of changing wort production and fermentation parameters (for instance, the temperature) on the occurrence of more than one diacetyl peak;
- Testing the influence of different brewing yeast generation in vicinal diketones reduction rate.

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APPENDIX

Appendix I: Individual amino acids concentration values during the analysed lager fermentations; nq: non-quantifiable

Fermentation time (days)	Fermentation vessel	Amino acids concentration (mg/L)																			
		aspartic acid			glutamic acid			cysteine			asparagine			serine			glutamine			histidine		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	F1	36.35	3.65	10.05	33.92	0.54	1.59	0.76	0.02	3.26	17.03	1.51	8.87	28.87	1.23	4.28	5.55	0.10	1.76	24.18	0.40	1.64
	F2	34.84	4.55	13.05	36.50	0.29	0.81	1.00	0.10	10.27	19.40	3.00	15.46	36.21	3.56	9.83	6.68	0.48	7.11	32.33	4.74	14.65
	F3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	F1	22.04	2.42	10.98	36.17	2.88	7.98	1.12	0.18	16.01	1.47	0.13	9.03	1.47	0.20	13.80	6.65	0.83	12.52	20.26	2.59	12.80
	F2	30.01	2.93	9.75	40.87	3.90	9.55	0.84	0.12	14.11	3.42	0.32	9.37	2.71	0.22	8.24	5.93	0.45	7.52	25.44	2.38	9.35
	F3	30.49	1.71	5.60	34.91	2.47	7.07	1.23	0.12	10.06	5.85	0.46	7.91	3.99	0.13	3.33	4.83	0.25	5.25	28.34	0.33	1.15
5	F1	8.81	0.53	6.04	24.91	2.05	8.21	1.40	0.09	6.23	nq	-	-	0.97	0.03	3.13	29.84	3.70	12.39	17.68	1.38	7.81
	F2	9.74	0.55	5.64	27.20	1.49	5.49	1.31	0.11	8.06	nq	-	-	1.11	0.08	7.47	26.25	2.07	7.90	19.06	0.54	2.86
	F3	8.66	0.85	9.82	24.72	2.74	11.08	1.23	0.13	10.52	nq	-	-	0.92	0.10	11.34	19.91	2.49	12.53	17.94	1.14	6.36
6	F1	5.60	0.53	9.53	15.84	0.31	1.98	1.13	0.15	13.25	nq	-	-	0.59	0.04	6.10	17.01	1.48	8.69	14.76	1.34	9.06
	F2	6.65	0.54	8.19	17.56	0.26	1.48	1.22	0.14	11.07	nq	-	-	0.60	0.02	3.60	15.49	0.26	1.69	16.32	0.30	1.86
	F3	6.52	0.58	8.87	17.88	1.05	5.88	1.19	0.13	10.99	nq	-	-	0.62	0.06	9.07	15.51	0.49	3.18	16.42	0.65	3.94
8	F1	3.62	0.24	6.70	15.54	1.02	6.53	1.14	0.12	10.75	1.82	0.09	5.17	0.84	0.07	8.81	20.02	0.30	1.51	15.81	0.12	0.75
	F2	3.33	0.31	9.35	15.12	1.31	8.68	1.01	0.14	14.03	1.57	0.13	8.38	0.74	0.03	4.18	16.59	1.08	6.51	15.55	1.30	8.36
	F3	3.59	0.35	9.74	15.19	0.67	4.38	1.38	0.10	6.96	1.64	0.07	4.35	0.75	0.04	5.91	17.06	0.11	0.65	16.47	0.22	1.32
9	F1	4.69	0.43	9.26	15.41	0.85	5.49	1.51	0.14	9.48	2.06	0.11	5.11	0.77	0.04	5.74	22.86	0.64	2.80	16.15	0.42	2.62
	F2	5.02	0.36	7.10	16.71	0.62	3.70	1.62	0.18	10.99	2.11	0.09	4.08	0.79	0.05	5.82	20.52	0.52	2.54	17.24	0.54	3.16
	F3	3.74	0.18	4.73	15.04	1.50	9.94	1.49	0.16	10.68	1.92	0.14	7.45	0.72	0.11	14.55	19.44	1.96	10.07	16.32	1.56	9.56
10	F1	5.31	0.57	10.72	15.58	1.07	6.85	1.29	0.11	8.35	1.85	0.11	6.09	0.83	0.05	5.45	19.72	0.84	4.25	14.73	0.77	5.22
	F2	5.89	0.65	11.12	16.34	1.59	9.71	1.31	0.19	14.49	1.81	0.19	10.22	0.84	0.06	7.47	17.74	1.04	5.87	15.76	1.06	6.70
	F3	6.37	0.68	10.72	16.57	0.53	3.19	1.38	0.20	14.84	1.87	0.09	4.61	0.94	0.06	6.31	17.97	0.63	3.50	16.54	0.41	2.48
12	F1	5.47	0.58	10.67	14.24	0.75	5.30	1.21	0.12	9.71	1.76	0.14	8.14	0.70	0.04	5.88	20.93	0.67	3.18	15.23	0.64	4.23
	F2	2.63	0.43	16.17	13.68	1.63	11.92	1.15	0.12	10.80	1.68	0.23	13.74	0.71	0.07	9.55	17.47	2.06	11.82	15.86	1.83	11.56
	F3	5.73	0.70	12.13	15.01	0.77	5.15	1.45	0.14	9.96	1.86	0.16	8.44	0.82	0.07	9.09	19.05	0.46	2.41	17.00	0.67	3.94

Appendix I: Individual amino acids concentration values during the analysed lager fermentations; nq: non-quantifiable (cont.)

Fermentation time (days)	Fermentation vessel	Amino acids concentration (mg/L)																				
		citruline			glycine			threonine			arginine			tyrosine			GABA + alanine			tryptophan		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	F1	4.29	0.16	3.66	19.07	0.40	2.07	37.32	0.60	1.62	70.50	2.32	3.28	66.90	1.26	1.89	162.42	3.27	2.01	24.92	1.36	5.46
	F2	5.20	0.35	6.73	20.75	0.78	3.74	42.80	1.03	2.40	79.30	2.67	3.37	71.30	3.24	4.54	167.98	19.65	11.70	24.51	1.51	6.17
	F3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	F1	4.00	0.36	9.09	18.19	0.24	1.35	14.74	0.41	2.80	27.19	2.67	9.80	55.41	5.75	10.38	177.64	10.16	5.72	16.77	0.58	3.45
	F2	4.51	0.52	11.53	19.68	0.30	1.51	14.84	0.34	2.27	42.14	2.48	5.87	62.95	3.85	6.12	188.88	5.16	2.73	19.17	0.75	3.90
	F3	4.89	0.22	4.49	19.08	0.08	0.43	14.55	0.28	1.90	47.27	3.01	6.38	67.63	2.80	4.15	185.03	0.90	0.49	20.11	0.82	4.07
5	F1	4.01	0.38	9.52	17.21	0.22	1.26	15.74	0.26	1.67	15.89	1.99	12.51	48.98	2.23	4.55	191.07	7.00	3.67	16.18	1.12	6.92
	F2	4.72	0.06	1.29	18.16	0.49	2.69	14.75	0.49	3.29	17.72	1.31	7.41	52.82	0.78	1.48	202.97	4.77	2.35	16.03	0.73	4.58
	F3	4.64	0.26	5.63	17.66	0.26	1.48	13.48	0.20	1.46	16.77	1.84	10.96	53.27	3.43	6.43	190.34	3.82	2.01	14.74	0.58	3.92
6	F1	3.47	0.51	14.62	15.22	0.65	4.29	14.31	0.29	2.05	4.89	0.19	3.98	45.66	3.56	7.80	170.66	6.46	3.78	15.61	0.58	3.73
	F2	4.14	0.16	3.90	15.26	0.31	2.02	13.24	0.24	1.78	5.77	0.24	4.19	49.39	1.68	3.39	179.80	4.15	2.31	15.68	0.61	3.86
	F3	4.34	0.10	2.20	15.13	0.17	1.10	12.74	0.13	1.02	7.09	0.35	4.98	51.07	2.93	5.73	179.15	2.45	1.37	14.44	0.68	4.68
8	F1	3.69	0.10	2.67	15.17	0.16	1.06	14.89	0.12	0.83	4.01	0.08	2.06	48.27	1.49	3.08	182.60	1.93	1.06	16.49	0.81	4.89
	F2	3.96	0.48	12.07	15.73	0.71	4.49	13.75	0.30	2.18	4.30	0.28	6.61	49.61	3.33	6.72	185.52	5.84	3.15	15.75	0.58	3.66
	F3	4.34	0.10	2.26	15.30	0.17	1.11	12.99	0.15	1.19	5.09	0.04	0.86	51.96	0.85	1.63	184.67	0.95	0.51	15.41	0.38	2.44
9	F1	2.73	0.16	5.98	15.83	0.17	1.05	15.29	0.27	1.77	4.83	0.15	3.05	55.04	1.96	3.55	183.58	3.81	2.08	16.34	0.81	4.98
	F2	3.52	0.11	3.12	16.42	0.55	3.35	14.27	0.52	3.62	5.71	0.27	4.70	54.37	2.05	3.77	196.90	7.56	3.84	16.32	0.52	3.20
	F3	3.72	0.52	14.02	15.48	1.27	8.24	12.92	1.20	9.27	6.21	0.83	13.40	54.24	4.99	9.21	183.88	15.17	8.25	14.91	1.35	9.08
10	F1	2.46	0.32	12.82	15.12	0.19	1.26	14.64	0.15	1.04	4.29	0.14	3.21	55.87	2.79	5.00	175.56	5.11	2.91	15.88	1.00	6.28
	F2	3.06	0.28	9.18	16.14	0.46	2.84	14.04	0.45	3.20	4.90	0.34	6.96	56.27	6.85	12.18	191.74	7.91	4.12	16.30	1.14	6.97
	F3	2.89	0.13	4.48	16.06	0.74	4.59	13.73	0.56	4.11	5.68	0.41	7.16	55.84	2.86	5.12	190.38	7.57	3.98	16.97	1.07	6.30
12	F1	2.52	0.08	3.04	14.17	0.45	3.19	13.94	0.49	3.48	3.22	0.14	4.31	57.15	3.91	6.85	171.98	4.88	2.84	16.55	0.75	4.53
	F2	2.63	0.29	11.12	15.45	0.87	5.64	13.27	0.33	2.48	3.70	0.23	6.29	53.24	4.97	9.33	178.03	8.09	4.54	15.74	0.75	4.77
	F3	2.99	0.35	11.57	16.00	0.35	2.20	13.35	0.13	0.96	4.54	0.14	3.15	60.90	3.85	6.33	188.14	4.54	2.41	16.49	0.83	5.03

Appendix I: Individual amino acids concentration values during the analysed lager fermentations; nq: non-quantifiable (cont.)

Fermentation time (days)	Fermentation vessel	Amino acids concentration (mg/L)																			
		unknown + methionine			valine			phenylalanine			isoleucine			leucine			ornithine			lysine		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	F1	6.64	0.06	0.96	114.55	2.16	1.89	65.87	1.21	1.84	37.64	1.82	4.83	81.93	1.44	1.76	1.43	0.10	6.87	39.94	1.43	11.12
	F2	6.55	0.61	9.27	125.56	2.32	1.85	66.69	8.82	13.23	41.55	1.59	3.84	91.97	2.28	2.48	1.51	0.20	12.93	46.24	6.50	14.06
	F3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	F1	5.89	0.43	7.22	60.00	7.95	13.26	33.62	1.74	5.18	10.39	0.98	9.48	15.97	1.06	6.61	2.63	0.37	14.00	3.43	0.32	7.14
	F2	6.51	0.24	3.74	85.55	9.65	11.28	46.62	1.08	2.31	19.08	1.31	6.89	33.84	1.30	3.84	2.55	0.21	8.11	10.65	0.50	4.73
	F3	7.01	0.18	2.60	95.54	0.94	0.99	49.41	0.48	0.97	23.12	0.91	3.94	40.80	0.49	1.19	2.39	0.27	11.07	13.05	0.93	9.40
5	F1	7.04	0.37	5.32	37.02	3.98	10.74	18.86	0.96	5.07	3.98	0.35	8.91	3.57	0.22	6.24	2.49	0.44	17.56	1.14	0.18	11.91
	F2	6.50	0.12	1.80	46.47	2.47	5.31	23.70	0.49	2.07	6.05	0.24	3.99	6.14	0.28	4.49	2.68	0.31	11.49	1.02	0.08	7.38
	F3	6.36	0.34	5.39	46.78	3.92	8.39	24.61	0.42	1.71	6.39	0.24	3.69	7.33	0.30	4.10	2.38	0.35	14.59	0.78	0.09	15.38
6	F1	6.73	0.36	5.37	31.19	3.49	11.20	15.76	0.69	4.35	3.02	0.27	8.80	2.78	0.25	9.16	1.93	0.18	9.50	0.49	0.06	10.66
	F2	6.31	0.22	3.56	39.44	1.36	3.45	18.30	0.33	1.83	4.14	0.13	3.16	3.91	0.46	11.78	2.13	0.22	10.31	0.36	0.02	6.28
	F3	6.07	0.35	5.81	38.63	1.43	3.71	18.42	0.35	1.90	4.28	0.32	7.57	4.01	0.36	8.93	1.82	0.26	14.09	0.28	0.03	12.41
8	F1	6.71	0.15	2.29	34.47	0.61	1.78	16.62	0.13	0.78	3.35	0.09	2.57	3.22	0.24	7.58	1.66	0.20	12.21	0.32	0.05	13.97
	F2	6.36	0.42	6.55	38.46	3.28	8.53	18.62	0.53	2.84	4.20	0.26	6.10	4.47	0.16	3.47	1.51	0.08	5.12	0.11	0.00	0.76
	F3	6.20	0.27	4.30	40.96	0.87	2.13	18.77	0.26	1.37	4.52	0.14	3.18	4.34	0.26	5.96	1.90	0.21	10.86	0.20	0.03	13.91
9	F1	6.97	0.32	4.62	33.26	1.41	4.25	16.69	0.36	2.13	3.32	0.15	4.41	3.17	0.13	3.95	1.33	0.06	4.24	0.44	0.04	11.49
	F2	6.71	0.21	3.13	41.44	0.88	2.13	19.67	0.47	2.40	4.78	0.14	2.98	4.78	0.07	1.53	1.56	0.15	9.93	0.24	0.02	8.03
	F3	6.01	0.38	6.25	38.24	2.60	6.80	18.54	1.55	8.37	4.29	0.45	10.52	4.13	0.55	13.34	1.55	0.19	12.36	0.29	0.03	8.82
10	F1	6.60	0.34	5.15	32.41	2.17	6.68	16.34	0.31	1.87	3.29	0.17	5.07	3.34	0.12	3.60	1.22	0.18	14.38	0.59	0.04	12.01
	F2	6.75	0.32	4.72	39.65	3.10	7.83	19.28	0.67	3.47	4.55	0.32	6.97	4.74	0.37	7.81	1.50	0.20	13.21	0.57	0.06	11.07
	F3	6.67	0.51	7.59	41.20	1.26	3.06	19.61	0.74	3.78	4.80	0.22	4.51	5.06	0.31	6.05	1.61	0.02	1.42	0.55	0.07	6.79
12	F1	6.43	0.24	3.73	33.24	1.34	4.02	16.44	0.46	2.80	3.41	0.20	5.75	3.44	0.15	4.25	1.27	0.09	6.91	0.30	0.04	14.97
	F2	6.25	0.48	7.73	38.01	3.77	9.92	18.62	0.94	5.03	4.29	0.47	10.90	4.58	0.25	5.55	1.19	0.17	14.59	0.21	0.02	11.27
	F3	6.47	0.35	5.38	41.00	1.79	4.37	19.70	0.35	1.76	4.62	0.13	2.89	4.92	0.13	2.72	1.73	0.13	7.51	0.22	0.03	13.43

Appendix II: Individual volatile organic compounds concentration values during the analysed lager fermentations; nq: non-quantifiable

Fermentation time (days)	Fermentation vessel	Volatile organic compounds																	
		acetaldehyde			**ethyl acetate**			**diacetyl**			**isobutyl acetate**			**ethyl butyrate**			**2,3-pentanedione**		
		Concentration (mg/L)			Concentration (mg/L)			Concentration (µg/L)			Concentration (µg/L)			Concentration (µg/L)			Concentration (µg/L)		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	**F1**	4.82	0.13	2.64	nq	-	-	nq	-	-	1667.62	2.63	0.16	nq	-	-	nq	-	-
	F2	nq	-	-	nq	-	-	nq	-	-	1670.40	7.32	0.44	nq	-	-	nq	-	-
	F3	nq	-	-	nq	-	-	nq	-	-	1666.37	1.67	0.10	nq	-	-	nq	-	-
3	**F1**	61.79	7.06	11.42	nq	-	-	210.21	22.55	10.73	1788.85	17.07	0.95	nq	-	-	375.77	38.12	10.14
	F2	65.67	12.95	19.72	nq	-	-	156.42	10.62	6.79	1717.44	5.00	0.29	nq	-	-	257.19	21.25	8.26
	F3	43.94	9.93	22.61	nq	-	-	63.55	3.46	5.44	1740.91	93.38	5.36	nq	-	-	-	-	-
5	**F1**	27.07	5.40	19.94	17.00	2.31	13.59	244.79	23.38	9.55	2117.00	42.98	2.03	97.55	13.42	13.76	270.97	28.96	10.69
	F2	41.69	6.90	16.56	12.12	1.84	15.14	248.61	26.00	10.46	2000.00	30.29	1.51	78.19	15.39	19.68	327.92	21.52	6.56
	F3	61.16	7.11	11.63	8.81	0.65	7.40	197.99	7.73	3.90	1932.36	20.40	1.06	56.90	7.60	13.36	189.62	38.81	20.47
6	**F1**	13.01	2.28	17.50	20.15	1.50	7.43	139.10	14.04	10.09	2155.64	49.82	2.31	133.42	7.05	5.28	238.61	19.82	8.31
	F2	15.49	2.78	17.97	16.41	0.84	5.11	193.07	6.03	3.12	2071.68	10.53	0.51	112.18	3.53	3.15	334.40	12.86	3.85
	F3	16.15	2.49	15.42	15.30	1.13	7.37	188.81	26.77	14.18	2035.21	27.61	1.36	105.44	6.37	6.04	335.69	20.60	6.14
8	**F1**	5.11	1.43	28.02	23.94	1.47	6.15	252.54	28.41	11.25	2077.44	65.76	3.17	116.56	16.44	14.11	109.28	11.78	10.78
	F2	nq	-	-	18.40	2.87	15.62	236.85	12.30	5.19	2000.15	62.26	3.11	105.16	13.15	12.50	105.26	4.34	4.12
	F3	nq	-	-	10.10	0.60	5.95	105.13	3.07	2.92	1903.36	19.31	1.01	75.39	8.60	11.41	104.86	7.96	7.60
9	**F1**	nq	-	-	22.23	1.50	6.74	256.85	26.94	10.49	2071.98	14.38	0.69	123.67	9.11	7.36	36.77	5.49	14.93
	F2	nq	-	-	20.96	1.90	9.06	249.70	19.00	7.61	2065.19	26.07	1.26	120.61	5.75	4.77	51.78	5.31	10.25
	F3	6.02	0.71	11.77	19.43	2.66	13.68	234.81	33.01	14.06	2053.58	47.12	2.29	122.29	16.44	13.44	63.55	6.51	10.24
10	**F1**	nq	-	-	26.25	1.44	5.50	273.48	10.79	3.95	2137.06	25.92	1.21	133.19	9.09	6.83	24.56	2.48	10.10
	F2	nq	-	-	20.92	1.07	5.12	223.72	9.64	4.31	2029.96	11.66	0.57	108.30	4.42	4.08	32.27	4.17	12.93
	F3	nq	-	-	19.42	2.05	10.54	233.13	10.39	4.46	2031.86	27.88	1.37	112.12	12.31	10.98	34.69	5.17	14.90
12	**F1**	nq	-	-	26.83	2.19	8.16	191.47	-	-	1774.45	270.57	15.25	138.89	10.13	7.29	22.06	0.28	1.25
	F2	nq	-	-	20.93	2.79	13.34	138.12	13.09	9.48	1875.19	163.50	8.72	115.06	20.02	17.40	21.63	2.12	9.79
	F3	nq	-	-	21.84	2.47	11.32	214.60	12.18	5.68	2106.50	91.91	4.36	123.81	24.55	19.83	19.28	1.01	5.26

Appendix II: Individual volatile organic compounds concentration values during the analysed lager fermentations; nq: non-quantifiable (cont.)

Fermentation time (days)	Fermentation vessel	Volatile organic compounds																	
		isobutyl alcohol			**isoamyl acetate**			**isoamyl alcohol**			**ethyl hexanoate**			**acetoin**			**ethyl caprylate**		
		Concentration (mg/L)			Concentration (µg/L)			Concentration (mg/L)			Concentration (µg/L)			Concentration (mg/L)			Concentration (µg/L)		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	**F1**	nq	-	-	nq	-	-	nq	-	-	nq	-	-	nq	-	-	nq	-	-
	F2	nq	-	-	nq	-	-	nq	-	-	nq	-	-	nq	-	-	nq	-	-
	F3	nq	-	-	nq	-	-	nq	-	-	nq	-	-	nq	-	-	nq	-	-
3	**F1**	nq	-	-	nq	-	-	5.26	0.79	15.10	nq	-	-	54.5	6.0	11.0	2023.3	394.8	19.5
	F2	nq	-	-	nq	-	-	nq	-	-	nq	-	-	45.6	8.8	19.2	717.1	161.2	22.5
	F3	nq	-	-	nq	-	-	nq	-		nq	-	-	43.1	7.3	17.0	194.2	39.5	20.3
5	**F1**	13.79	0.78	5.67	1397.49	145.78	10.43	25.96	2.62	10.09	288.45	37.39	12.96	25.00	1.87	7.47	2881.26	572.24	19.86
	F2	12.05	1.10	9.10	981.84	120.59	12.28	25.36	2.57	10.13	308.97	54.91	17.77	42.10	7.07	16.79	3369.51	389.23	11.55
	F3	9.70	0.59	6.13	540.31	105.34	19.50	20.53	0.98	4.78	239.79	41.97	17.50	46.56	4.41	9.48	3487.76	426.17	12.22
6	**F1**	14.15	2.51	17.71	1844.21	134.91	7.32	25.85	1.88	7.26	431.34	25.84	5.99	11.58	1.01	8.70	3602.30	526.91	14.63
	F2	13.65	0.56	4.12	1445.84	75.42	5.22	25.76	0.91	3.52	359.20	8.86	2.47	17.00	1.66	9.75	2660.58	237.53	8.93
	F3	13.07	0.84	6.46	1289.58	111.25	8.63	25.57	2.85	11.13	348.31	20.25	5.81	21.04	1.78	8.47	2755.65	240.25	8.72
8	**F1**	14.45	1.84	12.72	1754.99	137.70	7.85	28.20	1.26	4.45	417.27	31.07	7.45	7.96	0.97	12.13	3960.14	314.92	7.95
	F2	12.22	2.31	18.94	1333.40	155.80	11.68	27.33	2.36	8.62	360.80	23.95	6.64	9.84	0.65	6.60	3458.33	221.84	6.41
	F3	9.08	0.57	6.32	860.74	103.28	12.00	19.80	0.77	3.91	151.06	15.54	10.29	11.74	0.46	3.94	825.96	153.99	18.64
9	**F1**	10.73	1.74	16.20	1836.04	148.06	8.06	21.16	3.15	14.91	405.27	37.64	9.29	12.26	2.35	19.12	3039.60	300.47	9.89
	F2	11.69	0.70	5.96	1856.28	196.93	10.61	21.37	1.96	9.16	449.63	27.87	6.20	11.40	1.86	16.30	3069.15	204.32	6.66
	F3	12.33	2.54	20.62	1703.33	267.47	15.70	23.74	4.31	18.14	411.22	64.46	15.68	11.98	1.74	14.56	3605.52	608.68	16.88
10	**F1**	13.81	0.78	5.67	2133.24	126.17	5.91	26.70	1.86	6.98	482.56	25.26	5.23	7.38	0.51	6.89	3694.82	235.88	6.38
	F2	12.05	0.72	5.95	1568.49	60.89	3.88	24.97	1.38	5.52	389.63	40.73	10.45	10.08	0.98	9.68	2728.69	509.34	18.67
	F3	11.91	1.18	9.88	1618.08	214.74	13.27	24.01	1.58	6.57	408.21	34.51	8.45	10.58	1.71	16.19	2564.33	216.09	8.43
12	**F1**	11.32	0.03	0.24	2112.81	-	-	18.39	1.86	10.14	471.63	18.20	3.86	9.28	0.33	3.58	3072.64	212.33	6.91
	F2	10.90	0.85	7.80	1863.08	198.80	10.67	20.34	2.16	10.60	476.51	57.28	12.02	17.33	1.14	6.56	3750.00	739.30	19.71
	F3	10.96	1.25	11.39	2191.88	433.86	19.79	22.63	4.04	17.86	454.05	62.28	13.72	18.02	2.54	14.09	3214.75	642.97	20.00

Appendix II: Individual volatile organic compounds concentration values during the analysed lager fermentations; nq: non-quantifiable (cont.)

Fermentation time (days)	Fermentation vessel	Volatile organic compounds														
		acetic acid			**2,3-butanediol R**			**ethyl decanoate**			**phenylacetate**			**ethyl laurate**		
		Concentration (mg/L)			Concentration (mg/L)			Concentration (µg/L)			Concentration (µg/L)			Concentration (µg/L)		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	**F1**	34.46	6.19	17.96	nq	-	-	nq	-		nq	-	-	nq	-	-
	F2	51.34	9.70	18.89	nq	-	-	nq	-		nq	-	-	nq	-	-
	F3	nq	-	-	nq	-	-	nq	-		nq	-	-	nq	-	-
3	**F1**	41.67	7.87	18.89	nq	-	-	1176.30	186.50	15.85	nq	-	-	nq	-	-
	F2	61.56	8.21	13.34	nq	-	-	796.63	93.49	11.74	nq	-	-	nq	-	-
	F3	63.27	11.29	17.85	nq	-	-	79.14	17.68	22.34	nq	-	-	nq	-	-
5	**F1**	38.53	7.78	20.18	58.51	10.40	17.77	2726.50	587.95	21.56	78.41	7.72	9.84	235.25	50.54	21.48
	F2	76.13	15.66	20.56	70.85	15.42	21.76	2501.87	367.39	14.68	42.56	7.33	17.23	238.43	32.30	13.55
	F3	62.06	7.89	12.71	60.00	11.16	18.60	2462.32	366.05	14.87	22.49	3.71	16.48	208.17	46.63	22.40
6	**F1**	21.02	4.91	23.34	nq	-	-	989.30	154.55	15.62	62.51	5.95	9.52	76.06	14.60	19.20
	F2	34.49	5.00	14.50	43.06	6.60	15.33	1042.44	182.51	17.51	39.88	3.81	9.55	74.81	8.58	11.47
	F3	32.47	9.17	28.24	46.79	5.77	12.34	1367.11	174.84	12.79	40.20	3.79	9.42	183.30	23.47	12.81
8	**F1**	42.99	3.39	7.88	37.63	7.13	18.94	1227.60	189.21	15.41	63.71	1.25	1.96	nq	-	-
	F2	41.42	4.79	11.56	36.03	4.12	11.43	1072.69	187.60	17.49	47.36	6.95	14.68	nq	-	-
	F3	44.20	5.35	12.11	30.80	6.37	20.67	105.89	16.12	15.22	10.01	0.85	8.47	nq	-	-
9	**F1**	46.95	5.94	12.66	40.22	5.34	13.28	819.56	151.90	18.53	49.90	11.46	22.96	160.01	18.70	11.69
	F2	45.05	9.97	22.14	30.45	3.52	11.54	929.03	108.82	11.71	41.75	7.36	17.63	141.29	13.96	9.88
	F3	46.00	10.42	22.65	32.54	5.43	16.69	1103.74	214.85	19.47	43.81	7.95	18.16	51.77	10.23	19.76
10	**F1**	nq	-	-	32.05	6.34	19.79	701.10	72.03	10.27	61.34	1.95	3.17	nq	-	-
	F2	49.36	5.03	10.20	31.34	4.36	13.90	611.64	128.01	20.93	39.98	3.20	8.01	nq	-	-
	F3	23.35	4.36	18.67	32.01	4.78	14.92	683.59	114.80	16.79	40.71	3.38	8.30	nq	-	-
12	**F1**	91.35	7.91	8.65	57.54	-	-	834.14	-	-	43.24	12.49	28.88	68.23	-	-
	F2	177.48	15.81	8.91	81.88	12.54	15.31	1000.39	263.85	26.37	38.41	6.24	16.25	62.26	8.81	14.15
	F3	129.23	13.65	10.56	49.10	10.64	21.66	1185.76	50.16	4.23	38.95	8.13	20.86	42.33	2.01	4.74

Appendix II: Individual volatile organic compounds concentration values during the analysed lager fermentations; nq: non-quantifiable (cont.)

Fermentation time (days)	Fermentation vessel	Volatile organic compounds														
		hexanoic acid			phenylethyl alcohol			octanoic acid			vinyl guaiacol			decanoic acid		
		Concentration (µg/L)			Concentration (mg/L)			Concentration (mg/L)			Relative area			Concentration (µg/L)		
		mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD	mean	±sd	%RSD
0	F1	nq	-	-	nq	-	-	nq	-	-	0.94	0.08	8.89	nq	-	-
	F2	nq	-	-	nq	-	-	nq	-	-	1.12	0.16	14.57	nq	-	-
	F3	nq	-	-	nq	-	-	nq	-	-	0.24	0.05	20.17	nq	-	-
3	F1	nq	-	-	11.99	1.45	12.13	nq	-	-	2.25	0.28	12.37	1543.94	291.53	18.88
	F2	nq	-	-	nq	-	-	nq	-	-	1.80	0.37	20.86	1222.32	177.49	14.52
	F3	nq	-	-	nq	-	-	nq	-	-	1.18	0.05	4.06	864.18	136.94	15.85
5	F1	483.81	58.15	12.02	57.42	4.47	7.78	3052.77	298.29	9.77	5.59	0.55	9.77	5075.16	667.31	13.15
	F2	489.06	133.28	27.25	45.94	6.56	14.27	2396.81	486.42	20.29	4.66	0.72	15.50	4089.55	693.22	16.95
	F3	nq	-	-	30.54	3.08	10.08	nq	-	-	3.55	0.29	8.27	2719.89	370.52	13.62
6	F1	1238.87	136.52	11.02	42.75	3.93	9.20	2388.05	302.30	12.66	3.61	0.53	14.54	3209.90	295.69	9.21
	F2	834.84	151.76	18.18	35.53	3.41	9.59	1912.87	232.14	12.14	3.11	0.58	18.79	2569.70	474.05	18.45
	F3	nq	-	-	35.72	3.29	9.20	1956.27	258.73	13.23	3.28	0.49	14.94	2904.96	364.44	12.55
8	F1	1906.03	198.64	10.42	41.78	1.33	3.19	2102.63	52.29	2.49	2.82	0.19	6.67	3391.28	390.12	11.50
	F2	1790.63	229.58	12.82	40.11	4.43	11.05	2048.51	407.54	19.89	2.67	0.39	14.41	2920.37	387.43	13.27
	F3	688.41	33.09	4.81	14.37	0.47	3.24	nq	-	-	0.94	0.03	3.33	902.54	86.00	9.53
9	F1	1630.38	222.71	13.66	32.11	5.57	17.35	1771.38	335.52	18.94	2.72	0.53	19.53	4319.15	465.54	10.78
	F2	1570.10	136.62	8.70	34.49	4.28	12.41	1825.35	107.35	5.88	2.98	0.19	6.29	4636.06	382.16	8.24
	F3	1089.15	201.89	18.54	41.06	3.60	8.78	2107.00	331.77	15.75	2.95	0.57	19.18	4736.48	585.41	12.36
10	F1	2114.43	99.19	4.69	40.46	1.65	4.09	1972.32	195.85	9.93	3.31	0.16	4.93	2475.89	162.32	6.56
	F2	1755.26	131.76	7.51	34.68	2.72	7.85	1722.30	202.85	11.78	2.97	0.24	8.00	2593.99	153.02	5.90
	F3	1592.70	64.35	4.04	35.91	1.79	4.97	1832.71	236.86	12.92	3.10	0.14	4.40	3443.50	391.46	11.37
12	F1	1507.01	264.52	17.55	30.65	4.98	16.25	nq	-	-	2.52	0.31	12.38	3165.00	656.71	20.75
	F2	1406.96	261.42	18.58	32.78	2.84	8.67	1323.97	163.22	12.33	2.79	0.24	8.61	3648.32	87.26	2.39
	F3	1418.44	278.74	19.65	30.72	5.10	16.61	2343.47	488.83	20.86	3.15	0.57	17.95	3827.65	637.97	16.67