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Evolution of Staling Aldehydes on Lager Beer Stability
Impact of maritime transport and storage conditions

MASTER DISSERTATION

Dayana Nataly de Menezes Aguiar
MASTER IN APPLIED BIOCHEMISTRY

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Resumo

A estabilidade organolética da cerveja engarrafada é atualmente um dos principais desafios da indústria cervejeira. Não obstante à contribuição de inúmeras substâncias, os compostos carbonilos, em particular os aldeídos, são responsáveis por muitas das mudanças desfavoráveis e percetíveis ao consumidor. Estas modificações são favorecidas por temperaturas não refrigeradas, armazenamento durante períodos longos, vibrações induzidas pelo transporte, entre outros fatores. Atualmente, existem ainda poucos dados na literatura científica sobre o impacto das condições reais de transporte, nomeadamente impacto das vibrações e temperaturas não refrigeradas, na estabilidade organolética da cerveja.

O presente trabalho teve como objetivo avaliar a evolução de 10 aldeídos em cerveja Lager engarrafada durante exportação por via marítima e armazenamento no destino. Para tal, simulou-se as condições reais que a cerveja produzida localmente é submetida, nomeadamente temperatura (19-30ºC), vibração (1.7 Hz) e tempo (até 120 dias). A análise dos compostos em estudo foi realizada por micro-extração em fase sólida seguida por cromatografia gasosa acoplada a espetrometria de massa.

Os resultados obtidos revelaram que as condições de transporte (influência de tempo, temperatura e vibração) e armazenamento (tempo, temperatura) simuladas i) promoveram o aumento médio na concentração dos aldeídos de *Strecker*, de 65%, ii) enquanto os aldeídos formados a partir oxidação lipídica bem como o acetaldeído, regra geral, não apresentam variações significativas neste período. O aumento descrito em i) apresentou dois padrões: garrafas com abertura tradicional (carica) apresentavam valores médios de 131.6±9.9 e 190.3±9.4 µg/L enquanto garrafas com um sistema de abertura fácil 190.5±10.0 e 180.3±9.5 µg/L, após transporte e armazenamento respetivamente.

O fenilacetaldeído foi o composto com maior variação nas condições estudadas, aumentando de 94.7±7.3 (cerveja fresca) para 143.6±8.0 e 168.9±8.9 µg/L, após transporte e considerando um período adicional de armazenamento, respetivamente.

Adicionalmente, verificou-se que o procedimento de envelhecimento forçado tipicamente adotado, pode apresentar limitações a reproduzir as condições reais em alguns compostos. Em particular, destaca-se o benzaldeído, que em qualquer período de envelhecimento forçado, 7, 14

e 28 dias, apresentou concentrações, em média, inferiores, de 5.3±0.3 µg/L, 5.4±0.3 µg/L e 5.4±0.3 µg/L, respetivamente, em comparação com o teor real ao fim de 120 dias, de 6.4±0.4 µg/L.

Palavras-chave: exportação, vibrações, temperatura, tempo de armazenamento em garrafa, compostos carbonilos, envelhecimento forçado

Abstract

The flavour stability of bottled beer is the main challenge of the brewing industry. Carbonyl compounds, in particular aldehydes, are responsible for the unfavourable and perceptible changes detected by consumers. Those modifications are favoured by unrefrigerated temperatures, prolonged storage, transport-induced vibrations, among other factors. Currently, there are few data in the scientific literature on the impact of real transport conditions, namely vibrations and unrefrigerated temperatures, on beer stability.

The aim of this study was to evaluate the evolution of 10 aldehydes in bottled Lager beer during maritime exportation and storage at the destination. To this end, the real conditions that the locally produced beer is subjected to, namely temperature (19-30ºC), vibration (1.7 Hz) and time (up to 120 days) were simulated. The analysis of the compounds under study was performed by solid-phase microextraction followed by gas chromatography coupled with mass spectrometry.

The results revealed that the conditions of transport and storage simulated i) promoted an average increase in the concentration of Strecker aldehydes of 65%, ii) while the aldehydes formed from lipid oxidation as well as acetaldehyde, in general, do not present significant variations. The increase described in i) presented two patterns: bottles with traditional cap had mean values of 131.6±9.9 and 190.3±9.4 µg/L while bottles with a ring pull cap had 190.5±10.0 and 180.3±9.5 µg/L, after transport and storage respectively.

Phenylacetaldehyde was the compound with the greatest variation, increasing from 94.7±7.3 (fresh beer) to 143.6±8.0 and 168.9±8.9 µg/L, after transport and considering an additional storage period, respectively.

Additionally, it was found that the forced ageing procedure typically adopted may have limitations in reproducing the real conditions. Benzaldehyde stands out, which in any period of forced ageing, 7, 14 and 28 days, presented concentrations, on average, lower, of 5.3±0.3 µg/L, 5.4±0.3 µg/L and 5.4±0.3 µg/L, respectively, compared to the actual content after 120 days, of 6.4±0.4 µg/L.

Keywords: beer export, vibrations, temperature, storage time, off-flavours, forced ageing

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B – Batches

ECM – *Empresa de Cervejas da Madeira*

GC- Gas chromatography

GC-MS –Gas chromatography coupled to mass spectrometry

HS-SPME- Headspace solid-phase microextraction

LOD – Limit of detection

LOQ – Limit of quantification

MS - Mass Spectrometry

OThs - Odour thresholds

PRISMA - Preferred Reporting Items for Systematic reviews and Meta-Analyses

SB - Synthetic beer

SD – Strecker degradation

t_R– retention time

Short curriculum vitae

Dayana Nataly de Menezes Aguiar (ORCID ID: 0000-0002-3527-447X) was born in 1994, in Caracas, Venezuela. In 2008, Dayana started the 3rd cycle of basic education in *Escola Básica e Secundária Gonçalves Zarco*, in Funchal, Portugal. Later, she graduated in Biochemistry (2018) in University of Madeira. In this institution, Dayana followed several scientific works to gain abilities related to chromatography, spectroscopy (methodologies implementation and validation) and knowledge in food science and technology areas.

COLLABORATION IN PROJECTS

- Projecto **SUPERPRO** – I: Supervisão do processo fermentativo da cerveja Lager, (M1420-01-0247-FEDER-000007) – 2017-2019.
- Projecto **IMPACT III** - Impacto das Tecnologias de Produção na Qualidade do Vinho Madeira (M1420-01-0247-FEDER-000024) – 2018-2020.

LIST OF PUBLICATIONS

REFEREED JOURNAL ARTICLES:

- **D. Aguiar**, A. C. Pereira, J. C. Marques, Agricultural Rum of Madeira matured on the seafloor: improved physico-chemical changes induced by a pioneering seafloor ageing process, European Food Research and Technology (2021) (10.1007/s00217-021-03855-2).
- **D. Aguiar**, A. C. Pereira, J. C. Marques, The influence of transport and storage conditions on beer stability - A systematic review (under submission).

POSTER COMMUNICATIONS:

- **D. Aguiar**, A. C. Pereira, J. C. Marques, Agricultural Rum of Madeira matured on the seafloor: improved physico-chemical changes induced by a pioneering seafloor ageing process, *XV Encontro de Química dos Alimentos*, September 2021, Funchal, Portugal.

- **D. Aguiar**, A. C. Pereira, J. C. Marques, Influence of transport and storage conditions on oxidation markers in bottled beer, *XV Encontro de Química dos Alimentos*, September 2021, Funchal, Portugal.

1. GENERAL INTRODUCTION

1.1. Scope and motivation

Beer is the most consumed alcoholic beverage worldwide and is one of the oldest human achievements [1-3]. Beer can be defined as a natural beverage obtained from the alcoholic fermentation of essentially four ingredients (malt, hops, yeasts, and water). Thanks to the evolution and development of different brewing processes and the diversity of raw materials various beer types are produced worldwide [1, 4, 5]. Notwithstanding, two main styles can group this multiplicity: the lager and ale styles. The lager beers, the most popular all over the world [4, 6-8], are produced using *Saccharomyces uvarum*, also known as *S. carlsbergensis* or *S.pastorianus*, a yeast strain that ferments and maturates at lower temperatures (4–12°C) for a longer time (up to three weeks). On the other hand, the production of ale beers usually involves fermenting and maturating by *S. cerevisiae* yeast strains at higher temperatures (14–15°C) for shorter periods (7–10 days) [9, 10]. In the resulting beers, a variety of volatile compounds from several families define their organoleptic profile such as alcohols, esters, organic acids, terpenic compounds and aldehydes [4]. However, beer flavour starts to deteriorate almost immediately after production ends due to inappropriate storage conditions, presence of high oxygen levels or exposure to vibrations for example, leading to the formation of off-flavours and loss of the typical and fresh attributes [11, 12].

In 2019, the worldwide beer production amounted to about 1.91 billion hectolitres, up from 1.3 billion hectolitres in the last 21 years. In 2019, China was the leading producer (≈376.53 million hectolitres), followed by The United States (≈210.88 million hectolitres) [13].

In both regions, the total consumed volume is assured by imports, about 10% and 18%, respectively. In the European Union, in 2018, the production totalised approximately 405.94 million hectolitres, an increase of about 4.2% compared to the previous five years (389.46 million hectolitres). Regarding international trade, the European Union exported about 22% of their production, namely 88.72 million hectolitres, while the imports represented lower amounts [14]. Portugal has a total of 120 active breweries and approximately 25% of the total beer produced in 2019 was exported to several countries across the world [15]. Thus, the trade exchanges bring an additional concern to the sector: to ensure that beer maintains, as much as possible, its freshness and sensorial features until the final consumer after going through uncooled temperatures and being exposed to vibrations. Therefore, the attention of companies and the scientific community in recent years has focused on studying the impact of transport and storage conditions on beer flavour stability [16]. This is also the focus of the present study, to evaluate the influence of temperatures, vibrations and long-time of travels on bottled beer. Considering the prominent role of aldehyde compounds on beer flavour degradation, these were chosen to measure the impact of the previous described transport and storage variables.

Although the mechanisms of formation of these compounds have been quite studied, their presence in packaged beer and increase during storage or after transportation are still not so well explored. In particular, little is known about the impact of transport vibrations on those compounds, since the studies regarding the influence of vibrations that beer faces during road or maritime transport on their flavour stability are scarce.

1.2. General and specific objectives

The main aim of this study was to assess the impact of maritime transport conditions (warm temperature, vibrations and travel time) on beer flavour stability, namely through the identification and quantification of aldehyde compounds that promote undesirable flavour changes and that compromise the acceptability of the product by the consumer. The following operational objectives were put in place to accomplish the research:

- Preparation of a laboratory setup to simulate the maritime transportation and storage conditions.
- Implementation of a sensitive, simple and automatized analytical methodology for the determination and quantification of the target aldehydes.

- Definition and screening a representative lager beer sample dataset for assessing the impact of variables under study.
- Evaluation of temperature-dependent forced ageing procedure regarding the assessment of staling aldehydes.

1.3. Thesis Outline

This thesis is presented in four main parts.

The first part (Chapter 2) gives general background information about the topic of this thesis, particularly on beer stability and off-flavour compounds. In particular, the literature review on the impact of temperature, storage and transport conditions on beer flavour stability was accessed by PRISMA methodology. Regarding off-flavour compounds, special attention was given to beer staling aldehydes, their impact on beer flavour and their formation pathways.

The second part (Chapter 3) is devoted to the quantification of aldehydes compounds in beer. It is presented the analytical methodology applied and the results of its validation.

The third part comprises the Chapter 4 and 5. The first one is devoted to the study of impact of maritime transport and storage conditions on beer staling markers. The Chapter 4 presents the conditions adopted to simulate the maritime transport and storage conditions as well as the samples analysed. A detailed discussion on the evolution of aldehydes quantified is carried out in this chapter. The Chapter 5 presents and discusses a common forced ageing procedure used to evaluate the real beer ageing conditions.

The fourth part (Chapter 6) presents the final remarks and the future perspectives of the present study.

2. LITERATURE REVIEW

2.1. Beer stability: classification, causes and evaluation approaches

2.1.1. Biological and non-biological stability

Beer stability is defined by its biological stability, also known as microbiological stability, and non-biological stability, usually categorised into the following groups: flavour, and physical [17, 18].

Biological stability is currently a threat controlled by most of the breweries. Good hygiene practices, efficient filtration, and pasteurisation routines minimises the risk of microorganism's (bacteria, yeast or fungi) contamination and are widely implemented in the beer industry [18-20]. Additionally, the intrinsic beer properties, namely the low pH, alcohol concentration, antiseptic action of hop acids, anaerobic environment and carbonation do not favour microbial growth. Some of these points were discussed by Vaughan *et al* [21], who reviewed how antimicrobial properties of naturally occurring components of beer can be exploited to enhance the microbiological stability of beer. Hop compounds deserved special attention in this field. In particular, the α-iso-acids, the fraction obtained from α-acids isomerisation during boiling, inhibit Gram-positive bacteria. Hop addition is therefore optimised to maximise antibacterial activity and attain the bitterness desired without

compromising flavour, since excessive amounts of hop compounds can also lead to light-struck off-flavours [22].

Non-biological stability usually includes the flavour and the physical stability, the latter including the colloidal, foam, gushing and light stability. Colloidal stability is strictly related to beer haze development, which can be reversible, the chill haze can appear during cooling and dissolved at temperatures of about 20°C, or the permanent, a haze that no longer dissolves [18, 23, 24]. The latter occurs when beer is successively chilled and warmed or stored for longer times without cold. The most frequent source for haze is the complexes formed from the interaction between flavonoids and proteins, which can be favoured by high temperature, the presence of oxygen (an effect stimulated by light), heavy metal ions and movement [18, 25].

Like haze, the beer foam is another aesthetic aspect that consumer pays much attention. Together with the visual impact, foam also promotes the exchange of aromas towards the consumer's olfactory sensors [19]. The proteins, the hop iso-α-acids, the metal ions and polysaccharides play a vital role in foam formation and stability [18], as well as the brewing technique applied, the ethanol content and the type of beer packaging [18, 26]. The gushing effect is common in carbonated beverages such as beer, cider, lemonades, and sparkling wines. Beer gushing is defined as an over-foaming of beer that can be observed when a bottle is opened, leading to a significant volume loss. This phenomenon can happen immediately after filling or after several weeks and is promoted by the presence of microorganisms (fungi). Gushing can be divided into two classes: sporadic and epidemic. The factors that contribute to this phenomenon are the increased level of carbonation, prolonged low-temperature storage, and excessive levels of iron present in the beverage [18, 19, 25]. Flavour stability is probably the most critical quality challenge that brewers currently face. To attain and preserve a desired beer flavour profile for as long as possible is of the utmost importance for brewers. The beer flavour instability causes irreversible changes in the aroma and taste, leading to positive attribute loss and developing ageing characteristics [16]. Indeed, beer flavour starts to deteriorate almost immediately after the production ends, limiting beer's shelf life [27, 28], and more rapidly compared to other alcoholic beverages, such as wine and whiskey. Beer ageing is considered an unfavourable process due to the formation of off-flavours and loss of the typical and fresh attributes [11, 12].

Several pathways and mechanisms involved in the beer ageing process have been studied, describing the chemical changes regarding volatile and non-volatile fractions in beer

bottled [29-31]. Despite these reactions being beer style-dependent, strictly related to raw material, production process, packaging and storage conditions, in general, they comprise a decline in bitterness as well as in fresh notes (fruity and floral), and an increase in staling off-flavours, perceived by the consumer as cardboard, ribes, honey, caramel and sherry notes. Roughly, the loss of pleasant beer bitterness and fresh notes is mainly related to iso-α-acids degradation and loss in ester compounds, respectively, and the staling flavours are due to increased carbonyl compound concentrations [28, 32-35]. In the present work, attention will be directed to carbonyl compound, in particular for staling aldehydes.

Several routes have been proposed for the formation of staling aldehydes in beer (discussed in section 2.2.1). *Trans*-2-nonenal, the flavour related to the cardboard attribute and one of the first identified markers related to the beer staling manifestation, is mainly linked to linoleic acid autoxidation during the boiling process and subsequent release of free *trans*-2-nonenal in the bottled beer [30, 36]. The Strecker degradation of amino acids can contribute to the formation of 2-methylpropanal and 3-methylbutanal, from valine or leucine, catalysed by iron and copper ions in the presence of oxygen [30, 37]. This route can also form two other notable aldehydes, benzaldehyde and phenylacetaldehyde from phenylalanine [38]. Moreover, the oxidation of higher alcohols, such as 2-methyl-propanol, 2-methyl-butanol, 3-methyl-butanol and 2-phenylethanol can increase aldehyde levels during beer ageing when high oxygen concentrations are presented. The oxidation of higher alcohols has the additional effect of contributing to the decrease of alcoholic flavour and loss of the warming character that these usually confer or, at the limit, the loss of desirable flavour (e.g., 2-phenylethanol) in specific beers [39]. However, the latter two paths contribute less to staling aldehydes formation due to the low levels of amino acids in bottled beer and the high light exposure requirement, respectively.

Maillard reactions are also related to beer ageing [40]. Most of the time, the Maillard products and 5-hydroxymethylfurfural are present at low levels in freshly bottled beer, below their flavour threshold. However, other reactive intermediates of these reactions can be present in considerable quantities, such as 3-deoxyglucosone (3-DG), which can further react with typical beer constituents to produce furanic staling compounds like 5-hydroxymethylfurfural [8, 18, 34, 41, 42]. The bound aldehydes, known as bisulfite or cysteine adducts, are currently considered the primary sources of aldehydes in beer ageing. These substances are formed during

the wort and beer production phases and are released from their non-volatile adducts into the beer during ageing [43, 44].

Together with aldehydes, many other compounds, either individually or simultaneously, can influence the stale beer flavour in a synergistic or antagonistic sense [4, 34, 45]. For example, esters, together with higher alcohols, are the most abundant volatile groups, representing a well-known group of flavour active compounds, which generally confer a pleasant fruity-flowery aroma to beer. Some of these can be hydrolysed during ageing, such as 3-methylbutyl acetate and ethyl hexanoate, justifying the decrease in fruity flavours initially present in some beers and contributing to the increase in the perception of eventual stale flavours. On the other hand, others can be formed from ethanol and organic acids, such as 3-methylbutyrate and ethyl 2-methylbutyrate, resulting the winy ageing flavours in beer [8].

Apart from volatile compounds and their non-volatile precursors, changes in the concentration of non-volatile compounds may also induce significant alterations in flavour stability. In this regard, iso-α-acids and polyphenols have received the most attention. Both are readily oxidised beer constituents, sometimes appearing as staling markers. In particular, the *trans*-isomers of iso-α-acids have been indicated as suitable markers for the flavour deterioration of beer since they are more sensitive to degradation than *cis*-isomers [30]. This group of compounds is susceptible to light, compromising the flavour quality of the beer by a phenomenon that is typically referred to as "light-struck flavour" (LSF). In this situation, the light (350 – 500 nm) can penetrate clear and green glass bottles and promote off-flavour formation, due to iso-α-acid degradation. They are easily detected in the odour and taste of the beer, even when the loss of the total concentration of iso-α-acids is not significant. For example, 3-methyl-2-butene-1-thiol (MBT), has been attributed to the "skunky-like" aroma. This phenomenon occurs less in beer stored in brown bottles [18, 35, 46, 47]. Since changes in the polyphenol's contents are usually associated with the occurrence of astringent tastes, they are sometimes used as indicators of storage-related alterations [25, 48].

In the last decade, several studies demonstrated the negative impact that transportation vibrations have on beer flavour stability [12, 49-51]. In section 2.3.3, the results of these studies will be reviewed and discussed.

2.1.2. Factors affecting beer stability

In this section, the external factors and the intrinsic characteristics of the matrix that are commonly identified as factors that affect beer stability are briefly presented.

Temperature is the principal factor affecting the beer ageing. Usually, the Arrhenius equation is used to describe some beer ageing phenomenon. According to this model, the compound degradation is temperature-dependent, favoured when the matrix is exposed to higher temperatures [52]. The chemical compounds are unequally formed due to differences in the activation energy and according to the precursors available, and consequently different concentrations of staling compounds are obtained [11, 12]. When the Arrhenius equation describes the phenomenon, an increase of two to threefold can be observed in the reaction rate when the temperature is raised 10°C [19]. Hence, when a beer is stored under different temperatures, the same concentration of staling compounds are not produced [12, 19].

The chemical composition of beers varies depending on the raw materials and brewing technique. In terms of raw materials, the levels of amino acids, antioxidant activity and metal ions should be monitored since the content of these compounds differ. Fresh raw materials are preferred over aged ones, since the later can contain higher levels of aldehydes or their precursors. During malting and brewing the appearance of staling compounds can be prevented or minimised by controlling the heat load and limiting the oxygen content, factors that favour the occurrence of staling reactions [53, 54].

During the beer packaging process, the oxygen concentration in the bottled beer should be minimised (lower than 50 ppb). Additionally, the material of the applied packaging is fundamental since it has been proven that packaging beer in brown glass bottles inhibits the photodegradation of bitter hop acids. In the transportation and storage chain, beers should be kept at refrigerated temperature (under 7°C) to inhibit the staling reactions. Additionally, subjecting the beer to vibrations or shocks should be avoided since those can enhance the beer oxidation rate [35, 54, 55]. Supplementary information regarding potential strategies to prevent overall loss of beer stability can be found in Baert *et al* [54].

2.1.3. Forced ageing methodologies to evaluate beer flavour stability

Forced ageing methodologies aim to simulate and accelerate the natural ageing process to predict beer flavour stability. In these experiments, beers are usually submitted to different temperature regimes during specific period [12, 56-58].

As discussed in the previous section, the temperature is one of the most important factors that influence the flavour stability of beer. This parameter is very frequently considered in forced ageing methods. However, the range of temperatures vary significantly between 28°C and 60°C as well as the time that samples are submitted to the temperature regimes (for three days up to three weeks) [56]. This wide range of temperature and time, together with the other factors listed above lead to some limitations on drawing parallelism and conclusions between force aged and naturally aged beers, as have been demonstrated by several authors.

In 2018, Lehnhardt *et al* [59] studied the prediction power of forced ageing, using both sensorial and analytical approaches. They compared 20°C for up to 17 months (natural ageing) with forced ageing for up to nine days at 40°C, previously shaken samples for 24 hours. One of the main differences that the authors found was that forced ageing leads to the development of mainly cardboard and bready notes, whereas natural ageing leads to fruity/sweetish attributes. Another interesting result that the authors found was that some carbonyl compounds (e.g., 2-methylpropanal, 2-methylbutanal, phenylacetaldehyde, methional, and *trans*-2-nonenal) increase linearly with forced ageing, while the same was not observed during natural ageing. For example, no clear tendency to increase during ageing was found regarding *trans*-2-nonenal and other saturated and unsaturated linear aldehydes derived from lipid oxidation. The *trans*-2-nonenal has been chiefly found under extreme (heated or acidified) conditions [30], not presenting the same behaviour during natural ageing [59]. According to their results, the authors conclude that forced ageing methods should be assessed critically. Each brewery should be aware that the prediction of sensory stability by forced ageing will lead to significant differences in aroma profile and analytical indicators [59].

In another study, Suarez *et al* [60], tested colouring agents in different beers under forced and natural ageing conditions. The forced ageing conditions tested were 60°C for seven days, and the natural ageing was considered after 12 months. These authors evaluated 15 ageing-flavour compounds: 12 carbonyl and three ester compounds. They concluded that, regardless of

the colouring agent used, forced ageing underestimated the natural ageing in all beers analysed. They observed that the sum of all ageing compounds was always lower, on average 2.7 times lower, in forced aged beer compared to naturally aged beer. This observation indicates that forced ageing treatments have limitations when attempting to mimic the natural beer ageing process.

Lehnhardt *et al* [56] reviewed this topic in 2018 and compared different forced ageing regimes in terms of staling compound production. They concluded that forced ageing tests do not give a realistic picture of staleness, since they did not match with the results of the naturally aged tests. The authors also cited cases where study conclusions diverge. For example, force ageing pilsner beer at 60°C for three days created high concentrations of furaneol, phenylacetaldehyde and *trans*-2-nonenal, and lager beers naturally aged for one year at 22°C, only presented phenylacetaldehyde at quantifiable levels. Additionally, the authors reinforced the notion that forced ageing at elevated temperatures promotes reactions that might not occur at lower temperatures because of the higher activation energies of specific compounds. Indeed, it has been shown that the concentrations of methional and phenylacetaldehyde increase with the temperature.

Notwithstanding the results discussed above, forced ageing method based on temperature manipulation has also been used successfully to predict single markers' behaviour. Heuberger *et al* [58] reported that 5-methylthioadenosine (5-MTA), a non-volatile flavour stability marker of ageing for beer, can be predicted from three days at 37 °C to mimic 16 weeks of regular storage. 5-MTA is a by-product of the non-enzymatic degradation of methionine and beer oxidation and staling marker, together with other purines [61]. However, the mechanism by which 5-MTA it is associated with beer flavour stability still requires additional studies to be understood completely [59].

Based on the papers reviewed and discussed above, developing a forced ageing method with a greater predictive power of the actual sensory shelf-life of beer is a very complex task, especially when the goal is to generalise it as a universal method. It has been proposed that a forced ageing method should be designed and evaluated by each brewery or for each beer style according to their physicochemical properties. Additionally, variables such as the average temperature of a given region, moisture level, and the degree of vibrations that the beer

experiences post-packaging are important parameters that should be considered to create a more realistic and accurate forced ageing method.

2.2. Staling compounds in beer: a brief summary

Among volatile organic compounds, carbonyl compounds are widely found in alcoholic beverages and spirits such as wine, beer and vodka. They are usually found in low concentrations and have low flavour thresholds. In particular, the concentration increase of these compounds have been reported as the main contributors of beer off-flavours found during beer ageing [38, 62].

The carbonyl compounds can be formed from a wide range of chemical reactions or derive from the raw materials [33, 38]. Malt can directly provide staling aldehydes but also several precursors that contribute to beer staling. In terms of precursors, they can be divided in two groups: bound-state aldehydes and precursors for the *de novo* formation. The later comprises substrates (amino acids, reducing sugars, unsaturated fatty acids), intermediate products (Amadori compounds, α-dicarbonyls) and reaction catalysts (transition metal ions) [33]. Regarding the brewing process, aldehydes are formed by heat induced Maillard reactions, Strecker degradation and fatty acid oxidation in the malting, mashing and wort boiling processes. Also, during wort boiling aldehydes with high volatility are removed through evaporation, whereas in the fermentation step, aldehydes can be reduced to their respective alcohols throughout yeast metabolism, while others are produced. Finally, as beer ages a change in its flavour, known as ageing flavour, is linked to the increase of aldehyde levels, in their free form, in the bottled beer. These compounds can be present in fresh beer or can arise during beer ageing thought different chemical reactions, but elucidation of these pathways has not been quiet fully accomplished [63]. In Table 1, are listed the aldehydes that are considered as important in beer staling, as well as their corresponding aroma descriptor and thresholds.

In the following section (2.2.1), a brief reference to the formation pathways will be carried out. The Maillard reaction was not included since furan compounds evolution, in particular furfural and 5-hydroxymethylfurfural (5-HMF) results, were not described in the present thesis. The literature reviews by Mutz *et al* [64] and Baert *et al* [54] are suggested for an overview of this mechanism.

Table 1: Beer staling aldehydes: odour thresholds and common descriptors.

Aldehydes	Odour Threshold (µg/L)	Descriptors [a,d]
Acetaldehyde	10-25*[c] mg/L	green apple, fruity
Strecker degradation compounds		
2-methylpropanal	86[a, b]	Grainy, varnish, fruity
2-methylbutanal	45-1250 *[a,b]	Almond, apple-like, malty
3-methylbutanal	56 [a, b]	Malty, cherry, almond, chocolate
Phenylacetaldehyde	105-1600* [a,b]	Flowery, roses, hyacinth
Benzaldehyde	515-2000* [a,b]	Almond, Cherry stone, burnt sugar
Lipid Oxidation compounds		
Hexanal	88-350*[a,b]	Bitter, winey
Trans-2-nonenal	0.03-0.11*[a,b]	Papery, cardboard, cucumber
Nonanal	18*[a]	Astringent, bitter, fat, citrus, green
Maillard reactions products		
Furfural	15.157[a]	caramel, bready, cooked meat
5-hydroxymethylfurfural	35.784[a]	bready, caramel

*The indicated value corresponds to the flavour threshold in beer. a:Baert *et al* [54]. b:Gernat *et al* [63]. c:Liu *et al* [65]. d:Moreira *et al* [66].

2.2.1. Formation pathways

- **Strecker degradation of amino acids and related pathways**

One of the well-known pathways reported in literature is the Strecker degradation (SD) which originates the so called Strecker aldehydes: 2-methylbutanal, 3-methylbutanal, 2-methylpropanal, phenylacetaldehyde and benzaldehyde. These compounds are characterized by their low odour thresholds and consequently might affect beer flavour [67].

This pathway consists of a transamination between an amino acid and an α-dicarbonyl, resulting in an α-ketoamine and a Strecker aldehyde that contains one carbon atom less that the amino acid from which it is derived (Figure 1). The α-dicarbonyls used as substrate in this mechanism are formed during the Maillard reaction. This pathway is favoured by high temperatures, the presence of transition metal ions (copper and iron), reactive oxygen species and oxygen [33, 63, 64, 68]. Besides the direct reaction with amino acids, other pathway related to Strecker degradation has been reported. The reaction where the α-dicarbonyl is replaced for an α-unsaturated carbonyl compound such as *trans*-2-nonenal, furfural or benzaldehyde and that reacts with an amino acid is defined as “strecker-like” reaction [54]. Supplementary information

regarding detail mechanism description can be found in Baert *et al* [54] and Filipowska *et al* [33].

The Strecker aldehyde 2-methylbutanal derives from isoleucine, 3-methylbutanal from leucine, 2-methylpropanal from valine and phenylacetaldehyde from phenylalanine. Additionally, benzaldehyde is considered a Strecker aldehyde although it is formed by decarboxylation of phenylacetaldehyde [33, 38, 54].

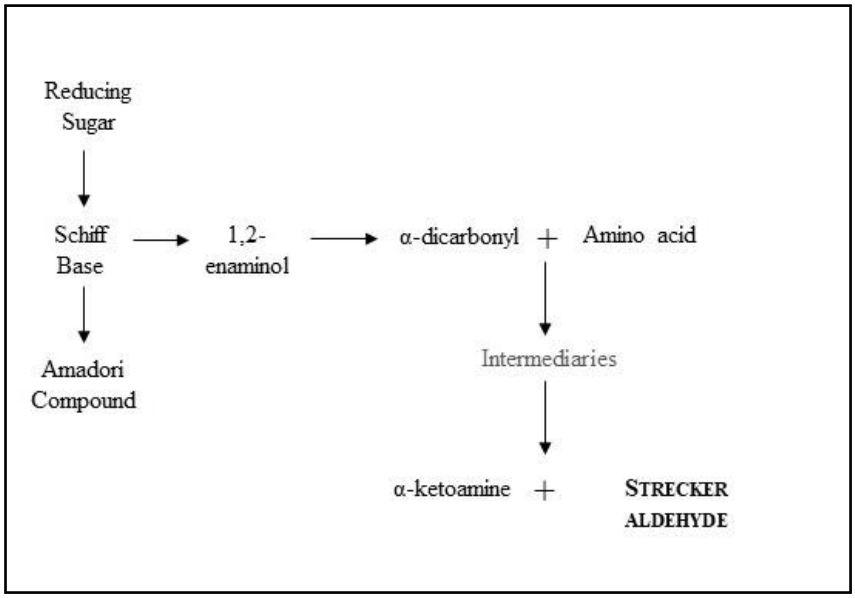

Figure 1: Overview of the main steps of the Strecker degradation.
Adapted from Filipowska *et al* [36].

- **Lipid oxidation**

The oxidative reactions that occur in beer are predominantly the oxidation of unsaturated fatty acids that came from the malt grains, mainly linoleic acid (18:2) and linolenic acid (18:3). These fatty acids can be oxidised via autoxidation, enzymatic oxidation, or photo-oxidation [33, 54].

The enzymatic oxidation or autoxidation reactions are the pathways that form the linear aldehydes hexanal and *trans*-2-nonenal (Figure 2). In the enzymatic pathway, the most abundant fatty acids in malt, the linoleic and linolenic acids, are oxidised by lipoxygenases (LOX-1 and LOX-2). The resulting hydroperoxy acids (13-LOOH and 9-LOOH) are degraded into carbonyl

compounds. Hexanal is produced through 13-LOOH pathway, whereas 9-LOOH yields *trans*-2-nonenal. This pathway is catalysed by enzymes, oxygen and high temperature [33, 54].

Another possible pathway for the formation of linear aldehydes is the autoxidation of unsaturated fatty acids promoted by reactive oxygen species to form lipid hydroperoxides (9-LOOH and 13-LOOH). This pathway is favoured at high temperatures, by oxygen and reactive oxygen species, enzymes and by the presence of oxidants such as transition metal ions (iron and copper) [33]. Furthermore, the secondary autoxidation of *trans*-2-nonenal forms shorter chain aldehydes such as hexanal, heptanal and octanal [54].

Finally, the photo-oxidation (activation of oxygen species by light radiation) of linolenic or oleic acid by photosensitizers like riboflavin (vitamin B2) to originate hydroperoxides and aldehydes is not a relevant issue nowadays since beer is packaged in green or brown bottles to limit the light passage [54].

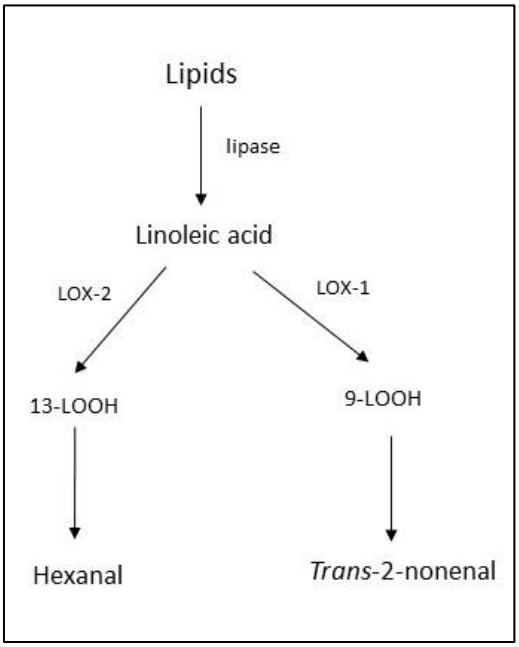

Figure 2: Main steps of enzymatic lipid oxidation leading to the formation of hexanal and *trans*-2-nonenal. Adapted from Filipowska *et al* [36].

- **Acetaldehyde pathway**

Ethanol is the major organic molecule present in beer and its oxidation involving the Fenton reaction forms acetaldehyde. The reaction of ethanol with a hydroxyl radical originates

the 1-hydroxyethyl radical. When this radical binds to oxygen forms acetaldehyde and a hydroxyperoxyl radical. Moreover, acetaldehyde can also result from the Strecker degradation of alanine or can be produced during the fermentation process [54, 67].

- **Other mechanisms**

Another possible path, is the Aldol condensation of unsaturated aldehydes with high flavour threshold that yields unsaturated aldehydes with lower flavour threshold. An example, is the formation of *trans*-2-nonenal from heptanal and acetaldehyde. Additionally, the higher alcohols present in beer, such as 2-methyl-propanol, 2-methyl-butanol, 3-methyl-butanol and 2-phenylethanol, can be oxidised to their corresponding aldehydes during ageing when high oxygen concentrations are present or at low pH by a process known as the melanoidin-catalysed oxidation. However, a minor importance is given to this path since is inhibited by iso-α-acids and polyphenols and also needs light irradiation [54].

One of the proposed mechanisms is the formation of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal as a result of the degradation of *trans*-iso-α-acids [69], however according to De Clippeleer *et al* [70] this path is not related to the increase of those aldehydes in beer during storage, since the beers hopped only with *trans*-iso-α-acids developed similar levels of aldehydes as the beers hopped with *cis*-iso-α-acids or isomerized extract and unhoped beers.

2.2.2. Free and bound-state aldehydes

The increase of Strecker aldehydes in bottled beer has been linked to the *de novo* formation as reported by Wietstock *et al* [55] and Gibson *et al* [71]. These authors observed an increase in Strecker aldehydes after supplementing fresh beers with amino acids upon beer ageing. Nowadays the theory that beer staling is only related to chemical compounds that are formed in bottle beer during storage is a misconception due to low levels of their precursors in packaged beer. The ageing indicators such as aldehydes are produced during the brewing process and end up in the final beer either in their free state or in reversible bound to an adduct. These bound-state aldehydes, known as bisulfite or cysteine adducts, are currently considered as one of the primary sources of aldehydes in ageing beer. Due to their non-volatile character these bound-state aldehydes are not removed by evaporation during wort boiling or be reduced during fermentation, and consequently they may be present in the final beer. In their bound-

state, they are undetectable in fresh beer and the same accounts for their sensory perception. Factors such as beer pH, storage temperature and vibrations during transport can promote the degradation of the adducts, realising aldehydes and causing an increase of stale flavour [28, 33, 54, 64]. According to the review of Baert *et al* [54] the appearance of *trans*-2-nonenal during beer ageing is explained by this process and the authors believe that other staling aldehydes are present in fresh beers in their bound-state. The formation of bisulfite and cysteine adducts are discussed below.

- **Bisulfite adduct formation**

Sulfur dioxide protects beer in two different ways. Firstly, acts as an antioxidant, inhibiting oxidation reactions and the formation of undesired compounds such as aldehydes, and consequently, increases beer flavour stability. Secondly, is a carbonyl-binding agent in the formation of aldehyde-bisulfite adducts, known as hydroxysulfonates. This sulfite produced by yeast during fermentation, can immediately form adducts with carbonyl compounds preventing them from being reduced into their corresponding alcohols. Consequently, in their bound state the ageing flavours that are developed during storage are masked [54, 72].

- **Cysteine adduct formation**

The reaction between the amino acid cysteine and an aldehyde results in a bound-state aldehyde. The capacity of an aldehyde to bind cysteine or to bind toward bisulfite is explained by the electrophilicity of the carbon atom of the carbonyl group of the specific aldehyde. Additionally, the nucleophilic -SH group of cysteine has high reactivity for staling aldehydes. The occurrence of cysteine adducts in beer model solutions was first proposed and reported in 2015 by Baert *et al* [73]. Further investigations in model solutions confirmed that at beer pH (4-5) the aldehydes 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, phenylacetaldehyde and hexanal have a strong interaction with cysteine and that the addition of a strong base like 4-vinylpyridine (releasing agent) dissociates those bounds liberating staling aldehydes [74]. Baert *et al* [32] later suggested cysteine as a potential agent to improve beer flavour stability after observing a significant reduction of free aldehyde content in fresh and in forced aged cysteine-spiked beers. Those fresh beers showed lower levels of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, hexanal and benzaldehyde whereas the forced aged ones showed less

concentration of the same aldehydes, along with *trans*-2-nonenal, furfural and phenylacetaldehyde. These reported studies confirm the presence of cysteinylated aldehyde adducts in beers. Notwithstanding the importance of this source of aldehydes in bottled beer, there is currently only a few papers in the scientific literature [28, 32, 73, 74].

2.3. Influence of transport and storage conditions on beer stability

2.3.1. Prisma methodology

- Information sources and search strategy

In order to discuss the results currently available regarding the transport and storage conditions on flavour stability, a systematic review was carried out according to the PRISMA 2015 guidelines [75]. The literature search was performed on three electronic databases: MEDLINE (PubMed), Web of Science and Scopus and included studies published from 2005 to September 2020 (date last searched). The search string included the term "Beer" together with "flavour stability" or "flavour instability" or "shelf-life" or "ageing compounds" or "forced ageing" or "natural ageing" or "packaging type" or "staling" or "storage conditions" or "transport vibrations". These terms were matched to the titles, abstracts and keywords of articles written in English between 2005–2020. Figure 3 depicts the process utilised for collecting, selecting and summarising the currently available data on the impact of transport and storage conditions on beer's organoleptic features and stability.

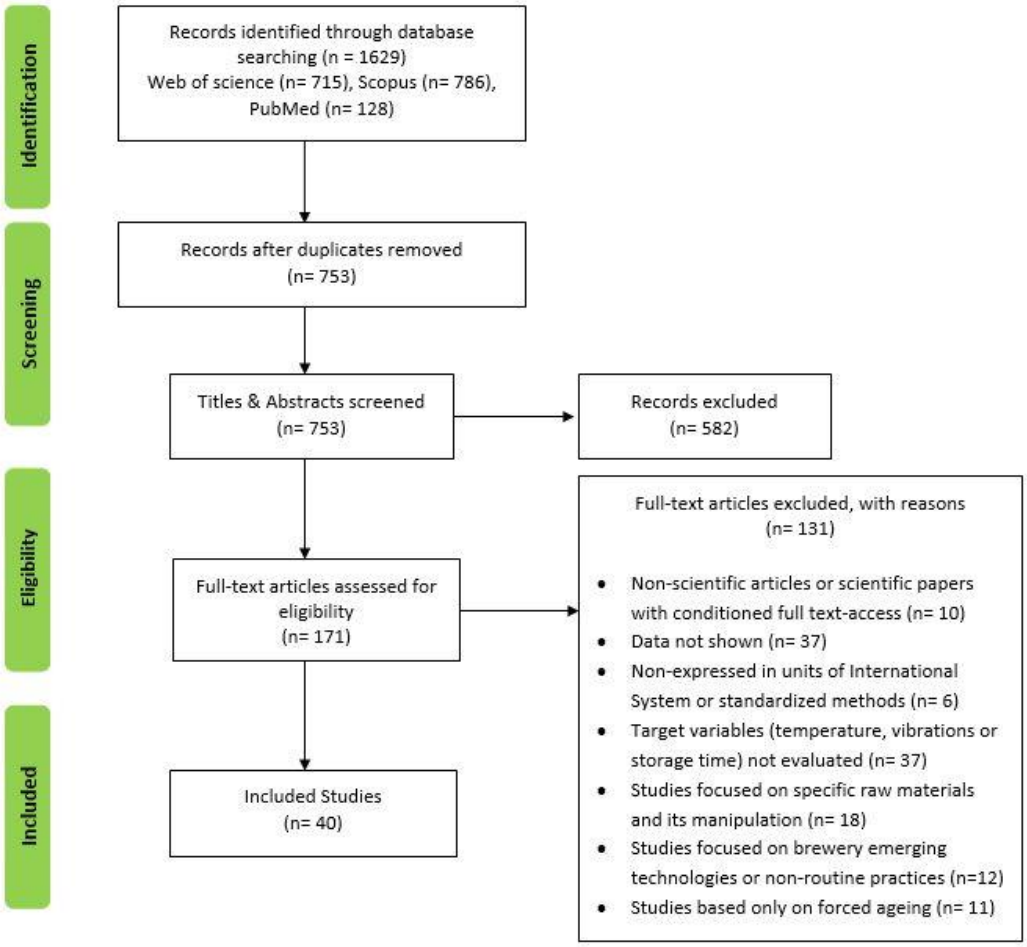

Figure 3: PRISMA Flowchart of studies included in current literature review.

- Eligibility criteria

The three databases retrieved a total of 1629 records. This list of publications was first screened based on the title, authors, and year. Duplicate articles were removed. Next, we screened the titles and abstracts, excluding studies not published in the chemistry field, reviews, books or book sections. The present review included and analysed only research papers.

The "Eligibility" of the articles to be considered in the present literature review was determined according to the following seven exclusion criteria: i) full-text not available; ii) data was not shown or iii) non-expressed in concentration units; iv) the impact of temperature, vibrations or storage time was not taken into account in the beer stability evaluation; v) research papers which focus was to evaluate the impact of raw materials changes on beer stability; vi)

non-routine brewery production practices or studies focused on emerging brewing technologies or vii) studies based only on the impact of forced ageing on beer stability (according to the discussion carried out in section 2.1.3).

After the identification, screening and eligibility steps, 40 publications were selected to assess the impact of temperature, vibrations and/or storage time on beer physico-chemical properties. Most of them (about 85%) studied the impact of beer exposure to ambient temperature (or higher) over time, while a few others evaluated the combined effect of temperature and stirring. Regarding beer properties, the volatile compounds (carbonyl compounds, higher alcohols, esters, ketones and terpenes), the polyphenols, beer colour, haze and alcohol content are beer features usually monitored. A summary of these study results is provided in Table 4 of appendix A. The research papers in which the data is only presented in graphical form will be discussed throughout the following sections, and it is not presented in the Table 4 of appendix A.

2.3.2. Effect of temperature and storage time on beer properties

Carbonyl compounds, typically associated with staling off-flavours or indicators of flavour deterioration, are the main characteristics evaluated to assess the impact of beer exposure to temperature. As previously discussed, in lager beers, these compounds present an unpleasant aroma and low flavour threshold [55, 56, 71].

In fresh beer, carbonyl compound concentrations are usually low, but the post-packaging conditions that the beer experiences, together with intrinsic beer characteristics, can trigger several temperature-dependent reactions and increase their concentration [76]. The main beer characteristics that can promote these reactions include the initial compound concentration in fresh beer (produced during the boiling phase, as discussed in section 2.1), the oxygen content on bottled beer, the metal content, the content of amino acids and of bound-state aldehydes, as will be discussed below.

Malfliet *et al* [77] evaluated the aldehyde profile of natural aged pale lager beers for 9 months at 22°C, as well as at 30°C for 60 days (forced aged). The authors observed that the concentration of all the Strecker degradation aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, benzaldehyde and phenylacetaldehyde) increased after natural and forced ageing (Table 4 of Appendix A), with a more significant increase observed in the

naturally aged samples. In particular, the authors reported 2-methylpropanal values up to 11 times higher after nine months at 22°C when compared with fresh beers. The same trend was observed for furfural and hexanal, with average values of five and 15 times higher, respectively, in naturally aged samples. Regarding *trans*-2-nonenal, the concentration of this compound remained constant under both natural and forced aged conditions. Another interesting result from this study, is the differences between the two regimens studied. For example, the average value of the sum of aldehyde concentrations studied on forced and natural aged was 206.0 and 506.9 µg/L, respectively, 4 and 10 times higher compared to the fresh beer (51.7 µg/L). This result suggests forced ageing may not fully recapitulate the natural ageing process. The same was observed in terms of beer colour and iso-α-acid evolution. Furthermore, the sensory analyses indicated that 2-methylpropanal, 2-methylbutanal, 3-methylbutanal and furfural correlated with beer ageing, at 20°C and 30°C.

Furfural and Strecker aldehydes were also analysed by Jaskula-Goiris *et al* [78] in pale lager beer stored in the dark at 30 °C up to 120 days. In this case, the authors reported that furfural concentration increased between 10 up to 30 times and aldehydes from Strecker degradation (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, benzaldehyde and phenylacetaldehyde) and lipid oxidation (hexanal and *trans*-2-nonenal) increased by 2.5 times on average (Table 4 of Appendix A). Moreover, an average decrease in the total concentration of iso-α-acids of about 12% was also reported (30°C for 60 days), and the colour and haze (both permanent and chill) increased significantly during the study period.

Despite not necessarily being off-flavours, furfural and 5-hydroxymethylfurfural (5-HMF) are indicators of flavour deterioration and are widely regarded as key compounds related to beer ageing. The concentrations of these compounds usually increase during pasteurisation and storage, especially at higher temperatures. Therefore, they are usually evaluated in beer ageing studies. Viegas *et al* [52] measured 5-HMF concentrations in several fresh commercial pilsner beers from different countries and reported values from 2.42 up to 7.22 mg/L. To evaluate 5-HMF evolution during storage, a particular group of these beers were stored for 40 days at (30°C, 40°C or 50°C). After 40 days at 30°C, the concentration of 5-HMF increased by 32%, whereas at 50°C, it increased almost five times compared to fresh beers (Table 4 of Appendix A). The authors concluded that 5-HMF formation is temperature-dependent in beer and forced ageing protocols may not reflect the 5-HMF evolution during storage.

A good correlation between furfural concentration and temperature was also reported by Cameiro *et al* [79]. The authors stored beers under different conditions (up to 20 weeks at 37°C) and found that furfural reached a maximum concentration of 380 µg/L (an increase of fifteenfold) in the forced aged beers at 37°C for 14 days. After 20 weeks of storage at 20°C, the levels of the studied aldehyde ranged from 220 to 296 µg/L. In beer samples stored at 4°C for 20 weeks an increase of twofold in the concentration of furfural was observed (from 20.2–24.2 µg/L to 38.4–50.2 µg/L). Additionally, it was concluded that oxygen promotes the increase in the concentration of furfural and that sulphur dioxide can retard its development. In general, the concentration of furfural increased in beers with an oxygen level of 3.4 mg/L, whereas furfural did not increase in beers with a high concentration of sulphur dioxide (9.0 mg/L) stored at 4°C or the first four weeks of storage at 20°C. Cejka *et al* [80] proposed that furfural is the best ageing indicator for predicting the stability of beer stored at 30°C.

Li *et al* [76] studied the evolution of antioxidant activity and ageing compounds during storage in several commercial lager beers. It was concluded that these types of beers should not be stored at 25°C for more than four months since the flavour stability is highly affected. They observed that the content of the Strecker aldehydes 2-methylbutanal, 3-methylbutanal, benzaldehyde and phenylacetaldehyde increased by more than 50%, on average, after six months of storage at 25°C (Table 4 of Appendix A). The authors also analysed other beer compounds, namely furfural, 2-acetylfuran, diethyl succinate, ethyl nicotinate and ϒ-nonalactone and observed the same trend. In terms of total ageing compounds, its content varied between 80.24 µg/L and 277.01 µg/L in the first month of storage and reached values between 296.37 µg/L and 697.03 µg/L after six months.

Acetaldehyde is a notable flavour component in beer. When present in acidic conditions, this compound reacts with ethanol producing diethylacetal, the bound state of acetaldehyde. Liu *et al* [65] studied the changes in acetaldehyde and diethylacetal concentrations during natural (six months at room temperature) and forced ageing (60°C for up to four days) storage. In naturally aged beers, diethylacetal content was reduced by 24.76%, on average, and acetaldehyde content increased by 29.76% (Table 4 of Appendix A). Similar behaviour was observed in the forced ageing samples; diethylacetal was reduced by 32.38% (on average), while acetaldehyde levels increased 45.54% on average.

Techakriengkrai *et al* [81] studied the evolution of four compounds (furfural, 5-hydroxymethyl furfural (5-HMF), *trans*-2-nonenal and hexanal) responsible for staling lager beers during storage at 7°C, 12°C, 30°C and 37°C for up to 28 days. A clear evolution in the content of each staling compound with increased storage temperature and time was reported. Beers stored at 7°C, 12°C, 30°C and 37°C developed higher levels of furans (furfural and 5-HMF), on average 1.6, 1.8, 2.4 and 2.9 times higher when compared to fresh beers, respectively. The same trend was observed for lipid oxidation compounds (hexanal and *trans*-2-nonenal), which increased on average 1.4, 1.6, 1.8 and 2.0 times at 7°C, 12°C, 30°C and 37°C, respectively. The increase of these four aldehydes was associated with the flavour changes identified in the lager beers. The sensory analysis results of those beers facilitated the division of the flavour attributes into two groups. The stale character of beers was defined by the descriptors cabbagy, cardboard, catty, leathery, musty, skunky and sour, whereas the descriptors graine and honey were correlated to taste [82].

According to the research of Mohammad *et al* [83] unpasteurised forced aged craft-beers (30 days, 35°C) display a slight increase (0.01%) in the alcohol content by volume and by weight due to the high-temperature exposure causing the residual live yeasts in the bottled beer to continue fermenting. The beer colour change to darker tones was visible, and the pH increased slightly (from 4.30 to 4.32). In terms of volatile compounds, acetaldehyde, an indicator of oxygen uptake and not detected in the control samples, arised in the aged beers (3.74 mg/L), possibly by the expansion of the crown cap enabling the entry of oxygen. Among the analysed esters, ethyl acetate, isoamyl acetate and ethyl butyrate concentrations were attenuated by 33%, 68%, and 52%, respectively. In contrast, ethyl octanoate content was 70% higher, and ethyl hexanoate was present at values higher than the flavour threshold, yielding an anise flavour and aroma. Additionally, aged beers lost 30% of isobutanol, 12% of isoamyl alcohol and 82% of 1-propanol. The average content of diacetyl after ageing was 50 mg/L, while 2,3-pentanedione rose to 4.0 mg/L. The authors also evaluated SO_2 and verified an increase of 0.39 mg/L, which was correlated with aged beer bitterness.

Sotolon, 4,5-Dimethyl-3-hydroxy-2(5H)-furanone, is a natural compound responsible for the curry-like and walnut aroma, in several beverages such as sweet wines, sake (Japanese rice wine), Madeira and old Porto wines. Its occurrence in aged beer was described by Scholtes *et al* [84]. The authors found sotolon concentrations on beer above its flavour threshold (2 µg/L),

namely 4.6 up to 42.1 ug/L in top-fermented beers stored 20°C between 6–24 months (Table 4 of Appendix A). The authors also observed lower values in lager beers, approximately 9 µg/L, after six months of natural ageing. Additionally, they estimated that aged beer for 30 days at 40°C might be the best forced conditions to estimate sotolon in the latter beer styles.

Several sensorial studies have also been performed to understand better the impact of temperature and storage in the organoleptic profile of the beer. Paternoster *et al* [16] compared fresh (stored at 5°C for 120 days) and aged beers (30°C for 120 days) in terms of consumer preference and drinkability, using paired sensorial comparison tests. The results demonstrated that in both cases, consumers detected differences, giving significantly higher scores to the fresh beer. Both beer groups, were also chemically characterised. While beer colour increased slightly (from 7.44 to 8.05 EBC), the concentration in evaluated aldehydes increased significantly. In particular, after 120 days at 30°C, the concentration of furfural increased by about 18 times, reaching concentrations of about 338 µg/L (Table 4 of Appendix A). Regarding Strecker aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional, benzaldehyde and phenylacetaldehyde), the 2-methylpropanal and 2-methylbutanal displayed significant increases.

In 2006, Callemien *et al* [85] described the role of 4-vinylsyringol in naturally aged lager beer (20°C for three and six months). They compared its presence in these beer styles with other known beer ageing off-flavours, such as *trans*-2-nonenal, dimethyltrisulfide and β-damascenone, which usually confer cardboard, onion and red fruit notes in lager beers, respectively. By gas chromatography-olfactometry, the authors associated the smoky-tobacco aroma with 4-vinylsyringol and hypothesised that it might be released in lagers during ageing.

Lehnhardt *et al* [59] evaluated the evolution of fresh lager beer aroma during ageing. The authors observed that the initial fruity aromas due to the high presence of esters compounds change after natural ageing for five months, at room temperature (≈20°C), resulting in period notes of berry and cardboard (associated with *trans*-2-nonenal) aromas. The continuous storage up to 17 months led to the gradual appearance of the bready, sherry, berry, sweetish and honey attributes. They also reported that the aromas identified in the forced aged beer samples (40°C for nine days) differed from the naturally aged beers, prevailing sweetish, dull, and cardboard notes. Accordingly, the authors call attention to the discrepancies between forced and natural

ageing processes and recommend that breweries should critically evaluate different prediction methods.

Saison *et al* [57] verified that the exposure of lager beers to different temperatures results in variations of the final flavour. This study analysed the following conditions: five days at 60°C, three weeks at 40°C, three months at 28°C, six months at 20°C and 10 years at 20°C. The authors noted a trend with the known off-flavour *trans*-2-nonenal. For example, the beer ageing at 60 °C for five days resulted in a strong cardboard flavour, correlated strongly with *trans*-2-nonenal. The high concentrations of this compound mask the impact of other compounds, namely Strecker aldehydes which are also favoured by temperature. When forced ageing for three weeks at 40°C was adopted, they observed that acetaldehyde, 3-methylbutanal and methional, together with *trans*-2-nonenal, contribute to characterise the beer flavour. At 20°C, both for six months and 10 years, Madeira-like flavours stand out (not detected in the remaining ageing conditions tested). The authors point out that this flavour seems to result from of a complex combination of many compounds, highlighting the possible contribution of the synergistic effect of 2-furfuryl ethyl ether, acetaldehyde, diacetyl, 5-HMF and some Strecker aldehydes.

The flavour-active volatile phenols 4-vinylguaiacol and 4-vinylphenol have been studied and reported as unstable during ageing. In two blond specialty beers aged at 20°C for 40 weeks, the concentration of both phenols decreased over time. In one of the specialty beers (6.9% v/v alcohol and 12 EBC colour), the content of 4-vinylguaiacol dropped from 1.37 mg/L to less than 1.00 mg/L, whereas in the second specialty beer (9.3% v/v alcohol and 14 EBC colour) decreased from 2.71 mg/L to less than 1.50 mg/L. This phenol was always present in concentrations higher than its flavour threshold (0.3 mg/L). Meanwhile, in both specialty beers the content of 4-vinylphenol decreased from around 0.61–0.65 mg/L to less than 0.50 mg/L. The authors also reported that exposing the beer to higher temperatures (60°C) promoted a fast degradation of 4-vinylguaiacol. Additionally, a more rapid loss of 4-vinylguaiacol occurred at elevated temperatures associated with the presence of oxygen or carbon dioxide in the bottle headspace (around 91.0% and 84.5%, respectively). Furthermore, the degradation of 4-vinylguaiacol leads to the formation of vanillin and apocynol (4-vinylguaiacol with the addition of a water molecule) in beers [86].

The effect of beer storage temperature on alcohol content has been evaluated in few studies. The alcohol content of an ale beer stored over 18 months at 4ºC showed a significant increase up to 0.3%, which can be associated with a short fermentation process in the bottled beer. In beers stored at -18°C and 4°C, the alcohol content remained nearly constant, and the authors concluded that the best temperature to store beer is -18°C [87]. However, the storage at this temperature has enormous practical and economic implications. Later, Zendeboodi *et al* [88] evaluated the influence of storage temperature in non-alcoholic beer. They observed that increased temperature during storage leads to a significant ethanol increase in non-alcoholic beers but never reached levels higher than 0.5% v/v (the legal limit for this type of beer). They also observed that beer packaging has an influence. For example, beer packaged in polyethylene terephthalate (PET) bottles had an alcohol content of 0.016% v/v after nine months at 4°C and 0.033% v/v when stored at 24°C. In contrast, the alcohol content in beers packaged in aluminium cans did not suffer significant variations during storage time.

Tran *et al* [89] studied the occurrence of 3-sulfanyl-3-methylbutyl formate with those of 2-sulfanyl-3-methylbutyl formate and their corresponding acetates in beer under natural (four weeks at 20°C) and forced ageing (four weeks at 40°C) conditions. Those compounds are responsible for the typical ribes flavour (similar to the odour of the stems and leaves of currant plants) found in beers. The authors concluded that the occurrence of those compounds is rare in both naturally and forced aged beers. Only 3-sulfanyl-3-methylbutyl formate was detected in lager beer, together with its corresponding acetate, at 629 ng/L and 187 ng/L, respectively. However, after spiking beers with oxygen, 3-sulfanyl-3-methylbutyl formate was detected after one month at 20°C or one week at 40°C of ageing, at 100 ng/L and 70 ng/L, respectively.

The hop variety used during the beer-making process has been proven to directly impact beer stability during ageing [90]. The iso-α-acids (hop bitter acids) are fundamental for the beer foam stability and cling, besides their antibacterial properties and contribution to bitterness. As mentioned in section 2.1, these compounds are sensitive to light but are also affected during beer ageing.

In pale lager beers, the time required to observe a degradation of 50% in the original content (known as half-life) of the *trans*-iso-α-acids was found to decrease from 471 to 12 days when the temperature increases tenfold (e.g., from 4°C to 40°C). Moreover, the average *trans*/*cis*-isomer ratio decreased from 0.60 at 4°C to 0.49 at 50°C [91].

The investigation of Karabín *et al* [92] showed that beers stored in the dark at 20 and 30°C for up to five months lost around 9% and 19%, respectively, of the *trans*/*cis* ratio due to the higher sensitivity of *trans*-isomers to auto-oxidation which corresponded to a loss of around one-third. Moreover, beers stored under the same conditions showed a significant decrease in their antioxidative potential, which was associated with a decrease of more than 70% in sulphur dioxide content. In contrast, Intelmann and Hofmann [93] observed a decrease of 32% in the sum of iso-α-acids and 59% in the *trans*/*cis* ratio after ageing pilsner beer at 28°C for eight months.

Rodriguez-Bencomo *et al* [94] monitored the evolution of volatile compounds in pilsner beers aged for up to five months at 4ºC, 20ºC and 40ºC. The authors observed that the volatile composition diminishes during storage time and with the increase of storage temperature. Furthermore, in beers stored for one month at room temperature (20°C) and 40°C, the concentration of some compounds such as terpenes and C13 norisoprenoids (β-citronellol and β-damascenone) was lower compared with beers stored at 4ºC (Table 4 of Appendix A).

The monitorisation of the sulphanylalkyl acetates in the first three months of storage of a lager beer showed a clear evolution in the content of 2-sulphanylethyl acetate (from less than 4 µg/L up to 9 µg/L) and 3-sulphanylpropyl acetate (from values below 0.5 µg/L up to 1 µg/L). In the remaining seven months, their concentrations gradually decreased to values below 2.1 and 0.2 µg/L, respectively [95].

Rettberg *et al* [96] verified that one of the most critical hop-derived volatiles in ale beers was 2-methylbutyl isobutyrate (2-MBIB) formed by the esterification of 2-methylbutanol and isobutyric acid. During storage at 4°C and 20°C for 24 weeks, more than 60% (on average) of the original content of 2-MBIB was lost, whereas a reduction of up to 50% was detected in three forced aged beer samples (first shaken at 20°C for 24 hours and then held at 40°C for 40 days). Additionally, the isoamyl acetate and phenethyl acetate concentrations decreased between 55–85% in beers stored at 20°C for 24 weeks. The authors concluded that 2-MBIB was less stable in unpasteurised beers due to enzymatic degradation and chemical hydrolysis and that the forced ageing regimen applied was not suitable to predict the natural evolution of the studied compound.

Several studies have been published concerning beer physical stability, namely the beer foam capacity and turbidity. Although this systematic review is focused on beer flavour

stability, the studies by Wu *et al* [97], Cai *et al* [98] and Jongberg *et al* [99] are recommended as supplementary information about beer physical stability.

According to the papers reviewed and discussed above, it can be concluded that in general, long-term storage and the exposition of beer to higher temperatures (>20°C) results in the loss of the overall visual quality of the beer as evidenced by a colour evolution to darker tones and the appearance of haze. Moreover, a significant degradation of the flavour stability due to the formation of off-flavours (such as staling aldehydes), reduced ester concentrations and bitter hop acid degradation have been reported.

2.3.3. Insights into the impact of vibrations on beer physicochemical properties

In the past few years, it was thought that the only parameters that negatively affect the quality and stability of food and beverages were temperature and storage duration. A few studies describing the impact vibrations and shocks have on food and beverages have been published, but further and in-depth research is required. For example, a simple search in the Web of Science database using the keywords Beer and transport vibrations resulted in eight entries within chemistry-related fields, and all were published in the last six years. This result demonstrates that the scientific community has started to study the effect of vibrations on beer. Table 4 of Appendix A shows an overview of the few research carried out up to today.

In 2014, the research carried out by Janssen *et al* [100] made it possible to discover that the vibrations and shaking that beer experiences during transport are related to the formation of turbidity. In this research paper, the authors highlight the importance of investigating the impact of vibration exposure on the beverages shelf-life.

More recently, studies focusing on identifying the vibration frequencies of different types of transportation and the impact on the beer' physicochemical and organoleptic properties have been published. It is estimated that during road transport (by truck), the beer is exposed to vibrations up to 100 Hz, compared with 0.1–5 Hz by ship and up to 500 Hz by plane [12].

The visual aspect of a beer is the first factor of acceptance by the consumer. In this sense, the few existing studies that associate vibration with temperature and storage time have evaluated the evolution of colour and colloidal stability. According to Paternoster *et al* [49], no

significant differences were observed in the colour of beer samples exposed to vibrations of 50 Hz and 15 m/s^2 at 5°C, 30°C and 45°C for 22, 38 and 90 hours. On the contrary, Jaskula-Goiris *et al* [12] analysed pilsner beers after transport by ship and observed significant differences compared to fresh beer. The pilsner beer transported from Belgium to Japan had an increase of 0.7 EBC, and the pale beer exported to the USA had an increase of 1.7 EBC. The higher increase found was 14 EBC in a dark specialty beer (Table 4 of Appendix A). Regarding haze formation, an almost two-fold increase in the permanent and chill haze was observed in the pilsner beer exported to Japan. Additionally, the laboratory simulation made by the same group showed a significant EBC increase in beer samples submitted to 30°C during 30 days without vibrations (from 7.85 to 8.38 EBC), and interestingly the combination of vibrations (1.7 Hz and 1.14 m/s^2) at the same temperature and duration reinforced the evolution of colour (from 7.85 to 8.56 EBC). The same pattern was observed in the evolution of permanent and chill haze.

As mentioned before, the oxygen content on bottled beer should be limited since it is responsible for the oxidation reactions that beer experiences immediately after production. Apart from the temperature and storage time, recent studies have demonstrated that exposing bottled beer to vibrations can promote oxygen uptake from the beer bottleneck into the beer, enhancing oxidation reactions that lead to the appearance of off- flavours or the degradation of original flavours.

Paternoster *et al* [49] evaluated the effects of temperature and vibrations on bottled beer oxygen evolution. First, they analysed the isolated effect of temperature and verified that the total packaged oxygen on beer samples with high initial oxygen content (1265 ppb) showed a decrease around 74% and 76% in beer samples kept at 5°C and 30°C for 90 hours, respectively, whereas the exposition to 45°C promoted an uptake around 91%. Next, they evaluated the combined effect of temperature and vibrations caused by truck transport (50 Hz and 15 m/s^2). Under these conditions, beer samples exposed to both 5°C and 30°C temperatures displayed an increase in the oxygen uptake of 80% and 87% (respectively), while oxygen uptake values at 45°C were around 94%.

The same authors conducted another study to evaluate the effect of vibrations that beer experiences during truck transport on beer oxygen levels. The authors found an oxygen uptake in beers after observing significant differences in both headspace and dissolved oxygen contents for all tested ranges of vibrations (5 Hz, 15 Hz, 30 Hz and 50 Hz and 15 m/s^2) during four days

at room temperature (20°C).In beer samples exposed to the vibration of 5 Hz and 15 m/s^2 the headspace oxygen decreased by around 8%, whereas the dissolved oxygen decreased between 70–84% [50].

The research of Jaskula-Goiris *et al* [12] also highlighted the additive effect of vibrations in combination with temperature on beer properties. The results showed a significant decrease of around 53.5% of the total packaged oxygen in beer samples shaken at 30°C for 30 days, whereas in beers only exposed to 30°C for 30 days, the uptake of total packaged oxygen was less than 10%.

In terms of the compounds associated with the characteristic taste or flavour of beers, some studies have also discussed the influence of vibrations on the evolution of those compounds (Table 4 of Appendix A). Transport by ship of pilsner and dark specialty beers promoted a decrease of around 10% of the initial iso-α-acid content, whereas more than 20% was lost in pale beers. Additionally, the laboratory simulation of transport (1.7 Hz and 1.14 m/s^2 for 30 days) showed a decrease of 3%, whereas beer samples kept at 30°C for 30 days only lost 1% [12]. However, temperature and vibrations during short storage time (e.g., 90 hours) do not affect the total iso-α-acids content [49].

Recent research suggests that carbonyl compounds (aldehydes) are also sensitive to vibrations, especially when combined with temperature. In 2018, the first research about this topic was published by Paternoster *et al* [50]. Their study suggested that aldehydes were sensitive to vibrations only in a dark beer with refermentation, due to the increased concentration of the individual and total aldehydes with increasing frequency. Exposition to 30 Hz and 50 Hz vibrations induced a higher impact on beer flavour stability since the total aldehyde content raised around 92% and 148%, respectively. Later, Paternoster *et al* [49] verified significant changes in the content of some Strecker degradation aldehydes and furans in beer samples exposed to high temperatures. The concentration of 2-methylpropanal increased by about 26% and 34% in beers exposed to 30°C and 45°C, respectively, whereas an increase of less than 20% was observed for 2-methylbutanal. Additionally, the furan furfural increased by around 50% at both tested temperatures. In terms of total aldehyde content, the authors observed a clear increase as a result of vibration (50 Hz and 15 m/s^2) at all tested temperatures and durations. Furthermore, in beer samples stored at 45°C for 90 hours, an increase up to 38% was observed, whereas an increase of around 67% was recorded in beer samples exposed to

30°C for 90 hours. Notably, no significant differences were observed in the aldehyde concentration after storing beers for 60 days at 30°C. The analysis performed in beers after ship transport also showed an apparent increase in the concentration of aldehydes resulting from Strecker degradation (2-methylpropanal, 2 and 3-methylbutanal, methional, benzaldehyde and phenylacetaldehyde), lipid oxidation (hexanal and *trans*-2-nonenal) and in furfural. The total aldehyde content of the exported pilsner, dark and pale beers increased from 30 µg/L to 160 µg/L, 58.3 µg/L to 252.3 µg/L and 97.1 µg/L to 176.7 µg/L, respectively. Moreover, laboratory simulations at 30°C without vibrations resulted in a significant increase in the levels of 2-methylpropanal (from 9.3 µg/L to 17.0 µg/L), 2-methylbutanal (from 1.4 µg/L to 2.4 µg/L), furfural (from 35.9 µg/L to 101.8 µg/L) and *trans*-2-nonenal (from 0.003 µg/L to 0.05 µg/L). Despite this result, the concentration of the aldehydes previously mentioned showed higher concentrations when beer samples were exposed to vibrations at 30°C, although no significant differences were detected in most compounds [12].

Sensorial studies were also performed by Jaskula-Goiris *et al* [12] to evaluate the changes in the aroma profile of beers after transport. The dark and pale beers were assessed as less fruity and bitter and with a characteristic cardboard aroma, while the pilsner beer was characterised as less sulphury and bitter but with higher musty and cardboard aromas.

In summary, beer can be defined as an extremely sensitive beverage since temperature variations promote significant and irreversible changes in its chemical composition, consequently impacting its overall flavour, especially during its commercialisation due to the combination of temperature with vibrations.

2.3.4. Final remarks of transport and storage conditions on flavour stability

This review carried out in the previous sections describe the possible changes on beer flavour due to the impact of storage time as well as the temperature and vibrations that bottled beer undergo during storage and transportation.

Most studies have only focused on assessing organoleptic properties evolution when beer is stored at cold or warm temperatures, at short or long storage time. However, recent studies have demonstrated that the vibration impact beer undergoes during transport is not

negligible. The currently available data demonstrate that vibrations negatively influence beer flavour properties. Due to the growing globalisation of beer, additional in-depth studies are required to clarify this research gap and help the brewery industry to adopt measures to minimise its effect. Furthermore, breweries and the scientific community should also be mindful that most forced ageing methods present several limitations in reproduce the natural ageing conditions.

3. QUANTIFICATION OF ALDEHYDES IN BEER

3.1. Introduction

The analysis of the several flavour compounds present in beverages and other foodstuffs requires analytical methods with good sensitivity, specificity and ideally taking low time-consumption. Among them, gas chromatography coupled to mass spectrometry (GC-MS) is diversely reported as the most appropriate and applied technique to identify and quantify volatile compounds in several matrixes [101]. Usually, the analysis of volatile compounds using gas chromatography (GC) requires a preconcentration and extraction step [102, 103]. The solid-phase microextraction (SPME) is probably the most commonly solvent-free extraction techniques used before GC methodologies [104]. This technique can be applied to analyse compounds present in levels of part per million (ppm) and is known as a fast and reliable technique due to its simplicity, sensitivity, selectivity, low cost and no need for solvents [68, 102].

The identification and quantification of carbonyl compounds, namely aldehydes, in foods and beverages has been extensively reported in literature. Before the application of chromatographic techniques their presence in beer was performed by spectrophotometric methods [105]. According to the literature, the first published paper reporting the identification of aldehydes in beer using GC-MS was in 1994 by Ojala *et al* [105], where sixteen carbonyl compounds were identified by using a liquid-liquid extraction with derivatization. The derivatization allows to detect aldehydes which are present in low concentrations, have low

volatility and that are very reactive owing to the polar carbonyl group [102]. Nowadays, other analytical methods are available, with and without derivatization, presenting good sensitivity and selectivity [106].

Nowadays, most of the available publications that focuses on the determination of aldehydes in several beer types, brewing raw materials or other alcoholic beverages for example are based on-fiber derivatisation in combination with headspace solid-phase microextraction and gas chromatography coupled to mass spectrometry (HS-SPME-GC-MS) [44, 62, 107-109]. More recently, Vieira *et al* [110] determined the optimal HS-SPME conditions without derivatisation for analysing carbonyl compounds in wort and beer fermentation samples, as well as finished beers. This methodology allowed the identification and quantification of several volatile compounds, including staling aldehydes such as *trans*-2-nonenal and 2-methylpropanal.

The objective of this part of the study was to implement the previous reported methodology for the analysis of aldehydes compounds under study. Thus, the optimal HS-SPME extraction conditions reported by Vieira *et al* [110] were adopted. The chromatographic conditions were adapted to reduce the time of the analysis. Additionally, the validation parameters such as precision, repeatability, recovery, linearity, and sensitivity of the implemented HS-SPME-GC-MS method were assessed.

3.2. Materials and methods

3.2.1. Chemicals

All chemicals used had a purity grade higher than 95%. Hexanal, benzaldehyde, 2-methylpropanal, nonanal, 2-methylbutanal, 3-methylbutanal and 4-fluorobenzaldehyde standards were purchased from Sigma-Aldrich (Steinheim, Germany). Acetaldehyde, phenylacetaldehyde and *trans*-2-nonenal were purchased from Acros Organics (Geel, Belgium). Absolute ethanol was from Sigma-Aldrich (Steinheim, Germany). Sodium chloride was obtained from Panreac (Barcelona, Spain). Ultra-pure water with a resistivity of >18 MΩ.cm (type 1) was obtained from a Millipore Simplicity® UV apparatus (Milford, MA). The alkane solution (C7-C30) was obtained from Supelco (Sigma Aldrich, St. Louis, MO, USA).

3.2.2. Aldehyde standard solutions

Standard solutions (250 mg/L) of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, hexanal, benzaldehyde, phenylacetaldehyde and nonanal were rigorously prepared in absolute ethanol. Similarly, standard solution of *trans*-2-nonenal (2 mg/L) and acetaldehyde (7 g/L) were also prepared in absolute ethanol. These solutions were used for the preparation of six calibration points by spiking the synthetic beer (SB), within the linear range presented in table 2. SB consisted in a solution prepared in ultra-pure water, containing 5.1% volume of ethanol/water and pH adjusted to 4.0 with an aqueous solution of 4.2 M chloridric acid, before adjusting this to a final volume of 2 litres.

The standard (10 g/L) and working (50 mg/L) solutions of the internal standard, the 4-Fluorobenzaldehyde, were prepared in absolute ethanol and SB, respectively.

3.2.3. Beer samples

The content of the aldehyde compounds was determined in 120 lager beer samples of several batches kindly donated by *Empresa de Cervejas da Madeira* (ECM) – Sociedade Unipessoal, Lda. This beer, known as *Coral Branca*, is a lager type beer with an alcohol content of 5.1% produced from barley malt, a lower percentage of unmalted cereals (corn) and hops of Czech origin, fermented and matured at low temperatures. It is characterized by its pale golden colour, light body, smooth flavour and pleasant hop flower aroma. This beer has a shelf life of 9 months in bottle and one month in barrel [111].

3.2.4. Extraction and chromatographic conditions

- **Sample preparation and Headspace Solid-phase microextraction**

The solid-phase microextraction conditions for analysing the oxidation compounds (aldehydes), as well as the sample preparation were previously optimized and reported by Vieira *et al* [110]. These authors optimised the headspace solid-phase microextraction (HS-SPME) conditions for volatile compound analysis on wort to beer fermentation samples, as well for finished beers using a multi-target design of experiment approach. The sample preparation consists in adding 3.3 g of sodium chloride and 10 mL of sample in a 20 mL capped glass vial.

Moreover, 5 µL of the internal standard 4-fluorobenzaldehyde at a concentration of 50 mg/L was added. The extraction consists in exposing the capped glass vial to the Carboxen/Polydimethylsiloxan (CAR/PDMS) fiber coating (85 µm film thickness) for 20 min at 40°C. The fiber used was purchased from Supelco (Bellefonte, PA, USA) In the present study, the extraction was performed in an automatic TriPlus autosampler, in SPME mode, from Thermo Scientific (Hudson, NH, USA) The extraction procedure was performed in triplicate for all samples under study.

- **Gas chromatography – Mass spectrometry conditions**

The gas chromatography–mass spectrometry (GC-MS) method reported by Vieira *et al* [110] was adapted to reduce the GC run time (60 minutes), in about 20 minutes. The oven temperature program used is described in the following paragraph.

GC-MS analyses were carried out using a TRACE GC Ultra gas chromatograph coupled to an ISQ single quadrupole from Thermo Scientific (Hudson, NH, USA). The employed capillary column was a TRB-WAX column (60 m × 0.25 mm) with 0.25 µm film thickness (Teknokroma, Spain). Helium was employed as the carrier gas and was injected at a constant flow rate of 1 mL/min. The injector port was kept at 260°C, in splitless mode, while the transfer line and the ion source were maintained at 240°C. The oven temperature program started at 50°C, was held for 2 min, initially increased up to 100°C at 3°C/min, then increased up to 159°C at 6°C/min and finally up to 230°C at 35°C/min and kept at this temperature for 7 minutes. The total GC run time was about 40 minutes. The first chromatograms were obtained spiking a mixture of standards solution in different beer samples, in order to obtain the retention time (t_R) of each target aldehyde and to confirm that there were no coeluted compounds, both in full scan (total ion count) and selective ion monitoring (SIM) mode. After the confirmation of the t_R, the analyses were always performed with the characteristic and major ions of each analyte and the characteristic ions were used for quantification purposes (Table 2).

The mass spectrometer was operated in the electron impact (EI) mode at 70 eV. The SIM operating mode was used with the characteristic ions for each analyte. Data were recorded and processed using the Thermo Xcalibur 2.2 software, compound identification was made by comparing the mass spectra with those in the NIST08 and Wiley 6.0 libraries, and by comparing the obtained Kovats indexes with those stated on NIST Chemistry WebBook. The comparison

between obtained mass spectra with those present in the MS library databases was only considered when a fair match was achieved (> 80%).

3.2.5. Method validation

- **Selectivity**

Selectivity was evaluated by the absence of chromatographic interferences at the retention times associated to each target aldehyde in a local lager beer, in several standard solutions and in lager beers with different ageing times.

- **Linearity**

An internal standard calibration method was implemented. A six-point concentration scale in SB was prepared for each aldehyde in study, except for heptanal that was quantified through hexanal calibration curve. The concentration range for each analyte are presented in Table 2 of this chapter. Each point was extracted and injected in triplicate, and the calibration curve was plotted.

- **Sensitivity**

The limit of detection (LOD) and limit of quantification (LOQ) were determined to evaluate the sensitivity of the method according to the linear regression approach [110]. The values considered were: LOD = 3.3 σ/b and LOQ = 10 σ/b, where σ stands for the standard deviation of the regression and b is the slope.

- **Precision**

Three standard solutions prepared in SB (low – C1, intermediate – C2, and high – C3 concentrations of each aldehyde) were used to determine the intra-day (repeatability) and inter-day (reproducibility) precision expressed in terms of relative standard deviation (%RSD). The repeatability was assessed by the quantification of 10 successive replicates and the reproducibility by performing the same analysis in three different days.

- **Accuracy**

The accuracy was evaluated thought recovery tests. A beer was spiked with known amounts of aldehydes under study covering three different concentrations points of the calibration range (low – C1, intermediate – C2, and high – C3 concentrations). Recovery was calculated by comparing the percentage of variation between the theoretical concentrations, of each concentration, and the mean values obtained for each one of the three tested levels.

- **Carry-over**

Carry-over was tested by running a SB solution after extracting the highest concentrated working standard solution of each aldehyde. This test allowed the adjustment of the fiber conditioning time.

3.3. Results and discussion

In this part of the study, it is presented the figures of merit of analytical methodology implemented for the following aldehyde compounds quantification in beer samples: acetaldehyde, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, hexanal, nonanal, benzaldehyde, *trans*-2-nonenal and phenylacetaldehyde.

3.3.1. Method performance

The implemented method showed a good selectivity, since the chromatograms of both beer and synthetic beer were free of interferents at the retention times of each aldehyde in study. There were also no coeluted analytes with target compounds. The validation results, as well as the parameters of each analyte calibration curve can be found in Table 2. Vieira *et al* [110] previously evaluated the matrix effect and verify that for the aldehydes studied, in the same type of beer samples, the matrix effect does not occur. The linearity of the method within the tested ranged was confirmed by the good correlation coefficient (R^2 = 0.999) found for all the compounds analysed.

The estimated LODs and LOQs of the method were adequate, similar or below to other reported methods and were below the OThs of aldehydes analysed, as well (excepted for *trans*-2-nonenal) [45-47]. Regarding the accuracy of the method, recovery mean values ranged from 72.86% (nonanal) and 124.96% (benzaldehyde), confirming that the method is accurate. The method also showed a good precision, the average value of repeatability and reproducibility regarding the 3 concentrations evaluated do not exceeded 5.48% (2-Methylbutanal) and 15.64% (Nonanal) of RSD, respectively.

The methodology previous presented was applied to the determination and quantification of 10 staling aldehyde compounds in 120 beer samples. These include beers stored at 2°C (15 samples), beer samples subject to a temperature profile and vibrations (total of 60 samples), and samples submitted to temperature-depended forced ageing test (45 samples). These results will be further discussed in Chapters 4 and 5 and confirm the applicability of the method presented.

Table 2: Performance results of the HS-SPME-GC-MS implemented methodology to quantify aldehydes in lager beer.

$t_{R\ (minutes)}$	Analyte (µg/L)	Kovats index	Identification/ quantification ion (in bold)	Concentration range in literature (µg/L)	Linear range (µg/L)	R^2	LOD (µg/L)	LOQ (µg/L)	Recovery (%)			Intra-day precision (%)			Inter-day precision (%)		
									C1	C2	C3	C1	C2	C3	C1	C2	C3
4.60	Acetaldehyde	630	41, 42, **43**, 44	0.6-40 [57, 110, 112, 113]	503.22 – 15061.3	0.999986	73.03	243.43	102.58	87.64	91.27	3.54	2.98	4.33	6.47	11.15	7.03
5.32	2-Methylpropanal	671	41, 42, 43, **72**	1-229 [12, 57, 62, 77, 107, 110, 114]	1.00-100.17	0.999998	0.17	0.57	87.92	99.38	97.49	2.12	6.52	3.07	20.89	5.42	18.18
6.50	2-Methylbutanal	724	**57**, 58, 86	0.7-60.41 [12, 57, 77, 107]	1.00-60.16	0.999994	0.21	0.71	104.25	98.87	91.67	4.48	5.80	6.17	11.43	12.09	15.59
6.60	3-Methylbutanal	727	44, **58**, 71, 86	0.97-57.20 [12, 57, 77, 107, 109, 112]	1.00-60.10	0.999995	0.19	0.62	93.35	99.41	98.39	6.55	1.17	6.49	12.50	9.25	10.79
10.45	Hexanal	1064	44, 56, **57**, 82	0.5-36.01 [12, 57, 77, 109, 112]	1.00-100.08	0.999999	0.16	0.52	82.67	95.90	99.25	1.47	6.11	4.04	13.11	6.81	7.43
22.26	Nonanal	1390	56, **57**, 70, 98	1.63-24.08 [66, 112, 115]	2.00-30.07	0.999880	0.41	1.35	72.86	84.01	98.94	3.96	5.68	5.48	17.92	17.78	11.24
26.40	Benzaldehyde	1520	77, 105, **106**	0.5-30.9 [12, 57, 66, 77, 107]	4.01-30.04	0.999072	1.01	3.38	115.79	112.9	124.96	1.59	2.33	3.94	5.06	13.41	6.20
26.62	*Trans*-2-nonenal	1536	70, **83**, 84	0.03-20.08 [12, 57, 109, 110]	0.05-8.00	0.999930	0.09	0.31	95.91	103.56	108.40	6.80	3.47	5.44	9.03	16.13	12.14
29.28	Phenylacetaldehyde	1638	**91**, 92, 120	3.01-132 [12, 57, 62, 77, 112]	5.01-150.29	0.999979	0.99	3.31	89.74	92.69	95.31	2.88	2.66	2.49	10.86	14.87	10.70

3.4. Conclusions

The analytical methodology implemented was based on Vieira *et al* [110] study, which optimized the SPME extraction conditions for key flavour compounds on lager beer samples. The HS-SMPE conditions adopted were reported by the previous authors, while the chromatographic conditions were adapted in order reduce the time of the analysis, from 60 to 40 minutes. Then, the proposed methodology was validated, showing good performance results in terms of linearity, sensitivity, selectivity, precision, and accuracy. These parameters were comparable or sometimes even better than other recent reported methods [62, 110, 114].

The implemented HS-SPME/GC-MS methodology was applied to identify and quantify 10 aldehydes: acetaldehyde, 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, hexanal, heptanal, nonanal, benzaldehyde, *trans*-2-nonenal and phenylacetaldehyde in 120 beer samples, related to the main goals of this thesis (the content of each aldehyde *per* batch is presented in Table 5 of Appendix B).

4. IMPACT OF MARITIME TRANSPORTATION ON BEER STALING ALDEHYDES.

4.1. Introduction

As discussed in section 2.3, maintaining the flavour stability of fresh beer depends not only on temperature and storage time, but also on the vibrations that beer experiences during transport. The data available have shown that vibrations have a negative impact on beer flavour properties, particularly with respect to aldehydes and that further research is required to better understand the increase of those staling compounds in packaged beer. This part of the thesis is focused on the study of the influence of maritime transport conditions, vibrations, and warm temperatures on the evolution of aldehydes.

4.2. Maritime transport and storage conditions simulation

This study simulated the longest common shipping route that beer is subjected to before reaching the consumer to study the impact of transport and storage conditions on beer flavour stability markers. In particular, the temperature, time, and vibrations which beer experience during maritime transport were simulated in the laboratory. Figure 4 shows the route, from Madeira to China (Shanghai), and the range of external temperatures that beer undergoes. The duration of the trip is approximately 45 days and the maximum time that beers stay in the distributor's warehouse is 75 days. Summer temperatures were considered (end of June to October). Online weather databases were consulted to determine the average temperature from 2018 to 2020 for all the transport route points [34, 116]. These monthly average temperatures were used to simulate the transportation and storage conditions. According to Jaskula-Goiris *et*

al [12], the vibrations that bottled beer experiences during maritime transport correspond to frequencies of 1.7 Hz (102 rpm). These vibrations and the journey temperature profile were simulated in a laboratory, using an incubator and orbital agitation (Comecta - Ivymen, Spain).

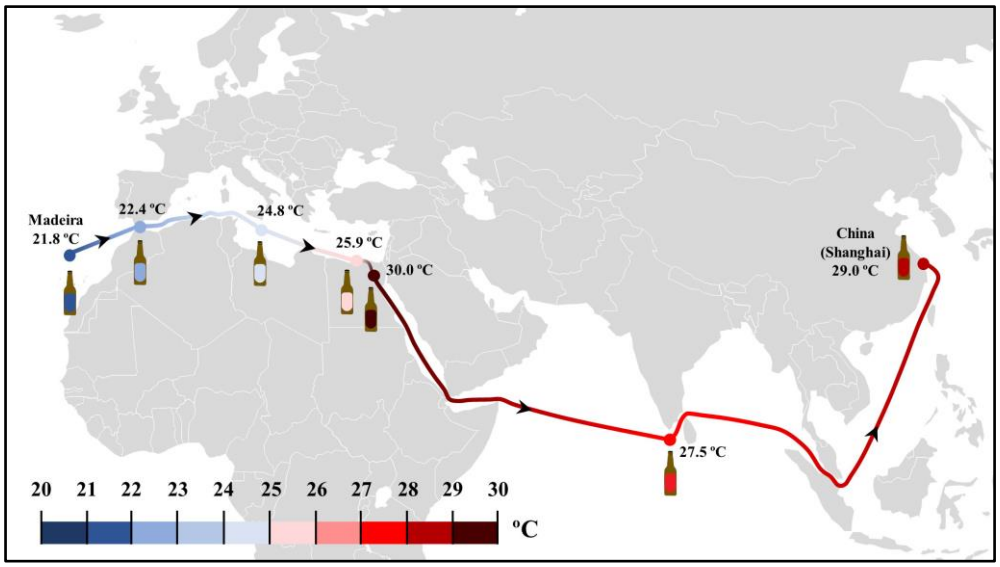

Figure 4: Overview of the maritime transportation route from Madeira Island to China with average temperatures.

Five sample groups were defined to evaluate the conditions previously described (Figure 4), namely: fresh samples, samples subjected to shipping conditions, samples subjected to shipping conditions and an additional storage time, and control samples for the last two groups. In the following sections, these sample groups of samples will be denoted as follows: i) Fresh (T0), ii) Transport simulation, iii) Transport&Storage simulation, iv) Transport Control, and v) Transport&Storage Control. Significant differences between these experimental groups were evaluated by the analysis of variance (One-Way ANOVA and Tukey test) with a significance level of $\alpha = 0.05$, using Minitab® 17 statistical software. Figure 5 summarises the number of samples analysed and the conditions that each went through.

Five different batches of beer samples were considered in this study (B1, B2, B3, B4 and B5). They differ in terms of bottle opening system and bottle volume.

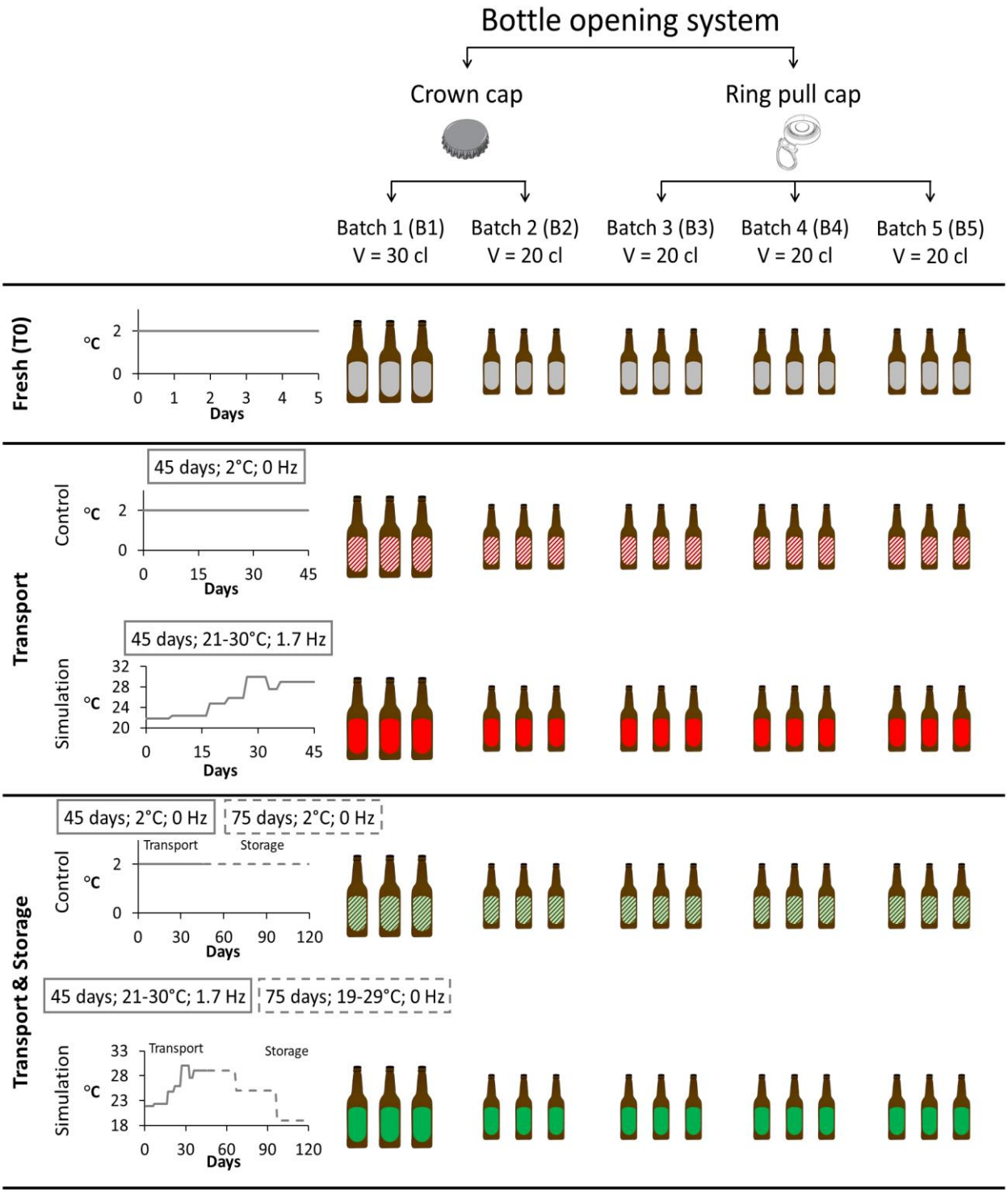

Figure 5: Overview of the experimental design to simulate maritime transport and storage.

4.3. Results and discussion

4.3.1. Characterization of fresh lager beer: aldehyde profile

As mentioned above, the aldehyde composition was studied in pale lager beers produced by a company settled on Madeira Island. This section presents the results of fresh beer samples analysed a few days after bottling according to the method described in Chapter 3. A total of 10 aldehydes were identified in all samples and the average concentration of each compound per batch is shown in Table 3.

Acetaldehyde, also known as ethanal, was the most abundant aldehyde found in the analysed fresh beers, varying in concentration from 593 µg/L to 1265 µg/L (Table 3). During the fermentation process, yeasts produce acetaldehyde as an intermediate compound in the conversion of glucose to ethanol. Higher levels of acetaldehyde in fresh beer may indicate an unideal fermentation and that the expected conversion of acetaldehyde to ethanol is less than optimal. Yeast viability, elevated wort oxygen concentration and fermentation temperatures may influence acetaldehyde accumulation in beer, compromising beer flavour. In fresh beer, the acetaldehyde concentration can vary from 600 µg/L up to 2400 µg/L [57, 117].

The Strecker aldehydes formed through the Strecker degradation, (see section 2.2.1) represent the largest fraction of aldehydes found in the beer samples analysed, with five compounds quantified. Among them, phenylacetaldehyde was the predominant, with concentrations ranging from 87.6 µg/L to 97.1 µg/L (Table 3). This compound is usually found in lower concentrations in pale lager beers, from 3.1 µg/L up to 22 µg/L [57, 77]. In the five batches analysed, the concentration of benzaldehyde ranged from 5.2 µg/L to 6.4 µg/L, values of the same order of magnitude of those found in fresh pale lagers analysed by Malfliet *et al* [77] (1.1–3.2 µg/L) and by Saison *et al* [57] (average values of 1.2 µg/L). The remaining Strecker aldehydes were found at lower concentrations and far from their OThs. Regarding the 2-methylpropanal and 3-methylbutanal, the concentrations varied between 2.5–5.6 µg/L and 3.3–9.5 µg/L, respectively. These values are also consistent with concentrations found in similar beers, with average values of 11.0 µg/L and 9.0 µg/L, respectively [57]. In all samples analysed, the 2-methylbutanal was not detected.

Regarding the oxidation compounds formed by lipid oxidation, four aldehydes were evaluated: hexanal, heptanal, nonanal, and *trans*-2-nonenal. The hexanal was only quantified in samples from two batches (B1 and B2), presenting values around 0.80 µg/L. Similar values were found by Malfliet *et al* [77] , while Techakriengkrai *et al* [81] found slightly higher values on other pale lager beer samples (1.6 µg/L). Nonanal was present in concentrations between 1.8–4.5 µg/L. Heptanal, a derivative of *trans*-2-nonenal (see section 2.2.1), was always found below its corresponding LOD (limit of detection) (0.16 µg/L). Regarding *trans*-2-nonenal, its concentration was higher than its flavour threshold (0.03 µg/L) in all samples analysed ranging between 0.5–0.7 µg/L. This compound is responsible by the cardboard/papery flavour of aged beers [29, 66]. Malfliet *et al* [77] and Saison *et al* [57] found lower concentration values in pale lager beers, 0.04 µg/L and 0.03, respectively.

Table 3: Concentration of aldehydes in fresh lager beers (three beer samples *per* batch). Values expressed in µg/L - mean value ± standard deviation.

	B1	**B2**	**B3**	**B4**	**B5**
Strecker degradation compounds					
2-methylpropanal	4.7±0.7	9.5±1.2	4.1±0.8	5.4±1.2	3.2±1.0
2-methylbutanal	nd	nd	nd	nd	nd
3-methylbutanal	4.2±1.1	5.6±0.8	2.5±0.6	3.9±0.6	3.9±1.6
Benzaldehyde	6.2±0.7	6.4±0.5	5.6±0.5	5.4±0.4	5.2±0.2
Phenylacetaldehyde	87.6±6.6	97.1±7.2	95.9±7.7	96.1±6.6	97.0±8.3
Lipid Oxidation compounds					
Hexanal	0.8±0.2	0.8±0.1	nd	nd	nd
Heptanal	nd	nd	nd	nd	nd
Nonanal	4.0±0.5	4.5±0.6	2.2±0.2	2.1±0.2	1.8±0.5
Trans-2-nonenal	0.5±0.1	0.7±0.1	0.5±0.1	0.7±0.1	0.5±0.1
Acetaldehyde	1040.7±134.1	1265.0±280.9	1114.6±193.5	593.0±292.5	858.0±250.3

nd, not detected

4.3.2. Impact of maritime transportation on beer: aldehyde evolution

4.3.2.1. Evolution of Strecker aldehydes

The four Strecker aldehydes evaluated in the fresh samples were also quantified after Transport and Transport&Storage simulations, as well as, in their corresponding control samples. Figure 6 illustrates the total concentration of Strecker degradation compounds (sum of the four aldehydes) *per* batch in each one of the five experimental sets.

In relation to fresh samples, the content of this group of aldehydes increased after the Transport&Storage simulation. This increase was faster in beer bottled samples with ring pull cap opening system (B3–B5), presenting values close to 200 µg/L right after sea transport simulations. In samples of B1 and B2, a continuous increase was observed until end of Transport&Storage simulation, reaching values of 198.4 µg/L and 184.7 µg/L, respectively. The differences found in samples with different bottle cap systems can be related to oxygen diffusion into the beer bottle. Exposure to variations and elevated temperatures can cause the bottle cap to expand, favouring the oxygen diffusion into the bottle, which may be greater in bottles with a ring pull cap opening system [83]. During transport, the oxygen present in the beer bottleneck can be incorporated into the beer due to vibrations. Therefore, the rate of beer oxidation can be increased, leading to the *de novo* formation of these Strecker aldehydes (on the assumption that the correspondent precursors are available) or the release of these aldehydes from their bound state [33, 49, 56]. These transport-triggered phenomena may continue during the storage period.

The analyses of control samples reveals that storage time is of greater importance for Strecker aldehyde content in bottled beer. In the transport simulation control set, only significant differences were detected in B1 and B5, namely 25% and 19% respectively. On the other hand, the Strecker aldehydes content was significantly different (increases ranging from 42% to 86%),

in all batches regarding transport&storage control sample sets when compared to the fresh samples.

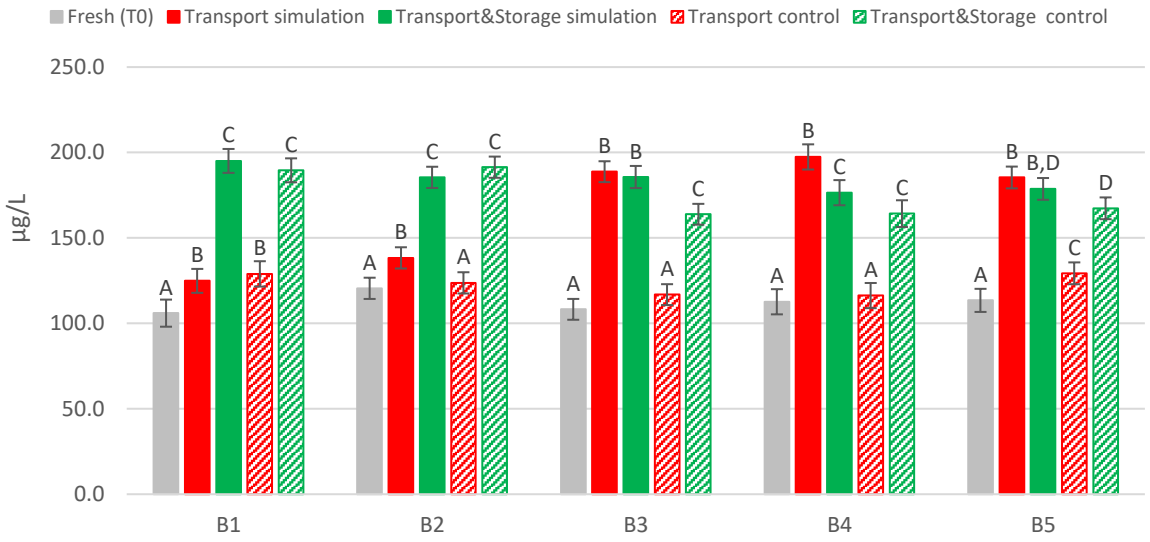

Figure 6: Evolution of the sum of Strecker aldehydes at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

The following paragraphs will discuss the evolution of each one of the identified Strecker aldehydes in detail.

- **Phenylacetaldehyde**

Phenylacetaldehyde was the most abundant aldehyde of this family in all sample sets, with concentrations ranging between 87.6 µg/L up to 178.3 µg/L. The concentration and evolution of this compound *per* batch in each one of the five experimental sets is represented in Figure 7.

For most control sets, a continuous and significant evolution was observed until end of the 120 days of cold storage, surpassing its flavour threshold (105 µg/L) [54]. Concentration of phenylacetaldehyde on control sets reached 176.4 µg/L. Thus, 45 days of cold storage was enough to detect a clear increase of 21%, on average, in three batches (B1, B2, B5), whereas in the remaining batches (B3 and B4) significant differences only occurred after almost four months of cold storage (increases around 55–101%, approximately). The availability of phenylalanine in bottled beer can lead to a continuous phenylacetaldehyde formation, a reaction that can be favoured by the oxygen present in the bottle [33].

As shown in Figure 7, after the maritime transport simulation, the content of phenylacetaldehyde clearly increased in all batches, exceeding its flavour threshold (105 µg/L) [54]. The most accentuated increase occurred in beer bottles with ring pull cap opening system, where the concentration reached almost 180 µg/L, whereas in the B1 and B2 it did not exceed 120 µg/L. Similar trends after transport simulation have been previously reported. The experimental maritime transport simulation conducted by Jaskula-Goiris *et al* [12] demonstrated an increase of almost 50% on phenylacetaldehyde concentrations (from 17.7 µg/L to 26.3 µg/L). The authors observed an increase of approximately 76% (from 10.8 µg/L up to 19.0 µg/L) in samples that underwent actual maritime transportation (≈30 days). Paternoster *et al* [49] found that phenylacetaldehyde content was two times higher (from 37.7 to 78.7 µg/L) after simulation of truck transport at 30ºC and 50 Hz when compared to beers only kept at 30ºC.

The concentration of phenylacetaldehyde continuously increased until the end of Transport&Storage simulation only in the first two batches (B1 and B2) where an average increase of 88% was observed, compared to fresh samples. In the remaining batches, the content was similar between simulation sets.

Therefore, storage time even at cold temperatures leads to significant increases. Additionally, higher concentrations were only observed when beers were exposed to vibrations and warm temperatures, particularly in bottles with ring pull cap opening system. Two hypotheses can explain these results. Vibrations were reported as the factor responsible for the absorption of the headspace oxygen into the beer, which favours the occurrence of oxidative reactions (dependent on the phenylalanine content) and consequently the appearance of this staling aldehyde in higher contents [49]. In fact, a higher diffusion of oxygen through the cap may have occurred in the last three batches due to the ring pull cap opening system. Further incorporation into the beer was magnified by vibrations, promoting a higher rate of oxidation. Second, the transport conditions (vibrations in combination with elevated temperatures) and

diffusion of oxygen through the bottle cap can promote the liberation of phenylacetaldehyde from its bound state to amino acids like cysteine at a higher rate.

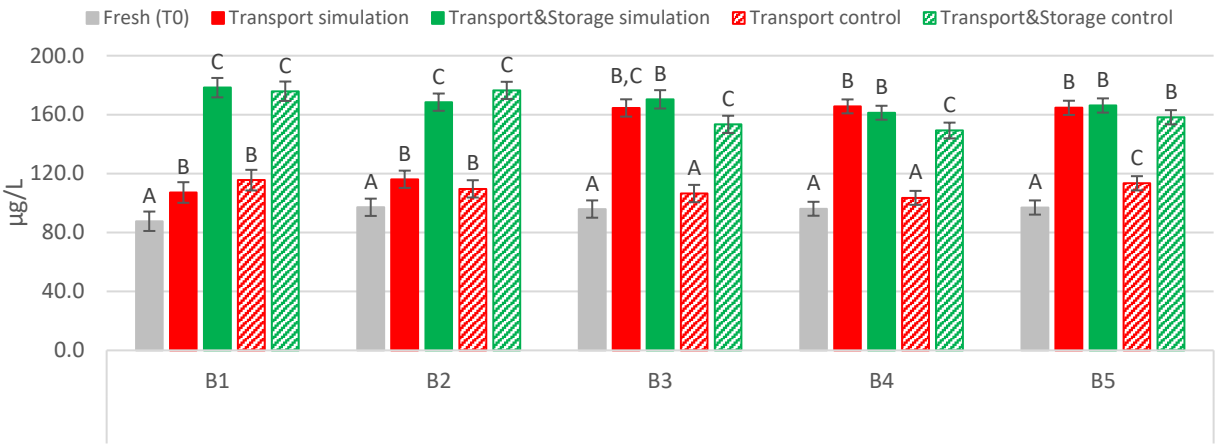

Figure 7: Evolution of phenylacetaldehyde aldehydes at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **Benzaldehyde**

In general, the storage time is not critical, except for B2 when an increase in concentration is observed in Transport&Storage control samples (Figure 8). However, this only represents 11%.

The simulated maritime transport conditions lead to a significant increase in bottled beers with ring pull cap opening system. The benzaldehyde concentration after the transport simulation showed an average increase from 5.4 µg/L up to 7.2 µg/L, whereas after further storage concentrations fell to an average of 6.4 µg/L compared to transport simulation samples. These results differ from the values found by Jaskula-Goiris *et al* [12]. In their study, no differences were reported in benzaldehyde content after maritime transport simulation. A less pronounced increase was recorded for beers that underwent the truck transport simulation at 30ºC and vibrations set at 50 Hz. Benzaldehyde concentrations varied from 1.5 µg/L up to 1.7 µg/L [49]. Similarly to phenylacetaldehyde, the diffusion of oxygen through the ring pull cap opening system of these samples can catalyse the formation of these aldehyde or in combination with the transport conditions can lead to its liberation from a bound state.

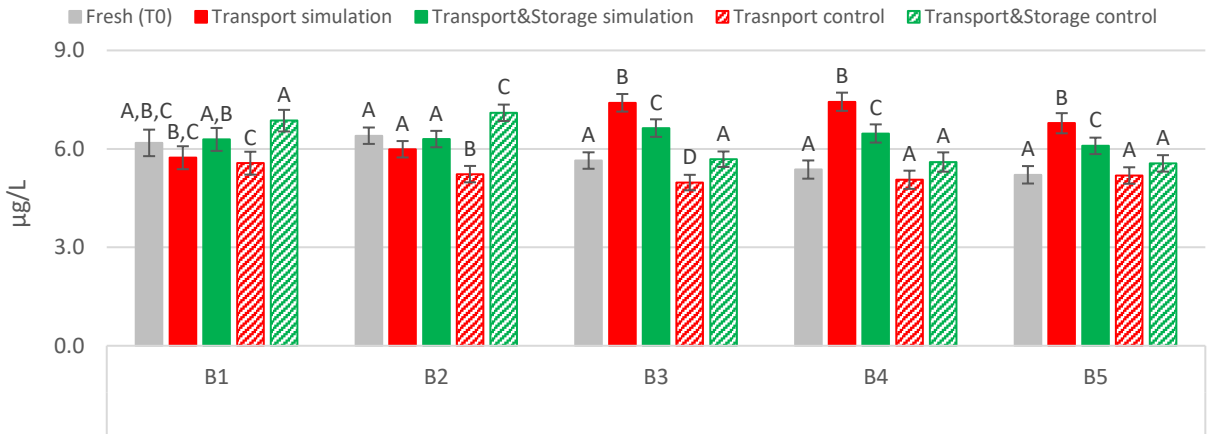

Figure 8: Evolution of benzaldehyde at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **2-Methylpropanal**

Storage time under low temperatures do not favour an increase of 2-methylpropanal (Figure 9). In terms of the transport simulation controls, only significant differences were registered in B2 and B5, corresponding to a decrease of about 53% and an increase of 87%, respectively. Conversely, the concentration of 2-methylpropanal was always significantly lower in the controls related to the simulation of Transport&Storage in batches B2 and B3. 2-methylpropanal was not detectable in the last two batches, B4 and B5, where it had values lower than its LOD (0.170 µg/L).

The transport conditions simulated (warm temperatures and vibrations) favoured the increase of 2-methylpropanal content in bottled beer. In general, the concentration of this aldehyde was always higher than 10 µg/L. These results are in accordance with the observed evolution in beers tested after real maritime transportation. After 51 days of maritime transport, 2-methylpropanal concentration was higher than 10 µg/L, reaching 28.5 µg/L from 4.1 µg/L in fresh beers. According to Jaskula-Goiris *et al* [12], beers submitted to the maritime transport simulation doubled their initial 2-methylpropanal concentration. Paternoster *et al* [49] also reported the critical effect of vibrations in combination with temperature and highlighted that the higher the exposure temperature, the greater the effect of vibrations. The authors observed an increase from 11.6 µg/L up to 23.7 µg/L after a truck transport simulation at 30ºC and 50 Hz of vibrations for 90 hours, whereas beers exposed to 30ºC for 90 hours developed lower levels

(18.8 µg/L). In contrast, the exposure to 45ºC instead of 30ºC in the transport simulation led to a total concentration of 79.6 µg/L. These differences may be related to the formation in bottled beer according to the availability of valine or by the presence of this aldehyde in its bound state in fresh beer. The greater the content of 2-methylpropanal in its bonded state, the greater the concentration of the compound after the bond is broken due to vibrations in combination with inappropriate temperatures.

Curiously, after further storage of the beer samples (at the end of 120 days), the concentration of this aldehyde significantly decreased (72% on average). The diminishment after further storage has been reported before by Paternoster *et al* [49]. Beers stored at 30ºC for 60 days after truck transport simulation (30ºC or 5ºC at 50 Hz for 90 hours) had 5% and 22% less 2-methylpropanal when compared to their corresponding non-vibrated beers only kept at 30ºC and 5ºC for 90 hours. These results suggest that since beers are no longer subject to vibrations, the aldehyde can bind again to free amino acids such as cysteine, consequently reducing the free content of 2-methylpropanal that can be detected.

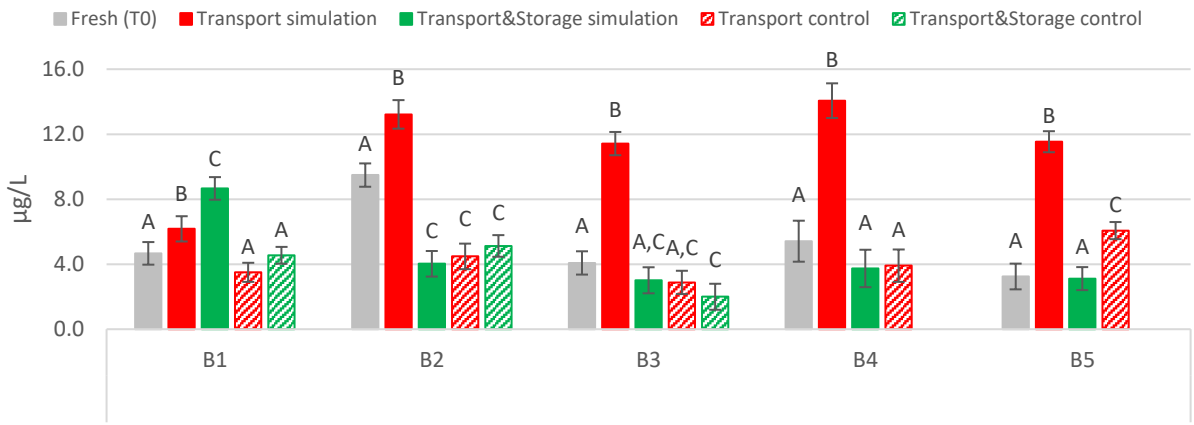

Figure 9: Evolution of 2-methylpropanal at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

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- **3-Methylbutanal**

Cold storage keeps the concentration of 3-methylbutanal in similar levels of freshly packaged beer during prolonged storage (Figure 10).

The concentration of 3-methylbutanal was not affected by transportation in the samples of the first two batches (B1 and B2). However, a clear evolution was observed after 45 days of transport simulation in the remaining batches made up of samples with a ring pull cap opening system, where its concentration increased between three to four times. These results are in accordance with what was previously reported by Jaskula-Goiris *et al* [12] in actually transported samples and maritime transport simulation samples. The authors quantified higher levels (6.8 µg/L) in beer shipped for 51 days compared to 2.1 µg/L in beer kept at 0ºC. In the transport simulation, the 3-methylbutanal content was two times higher (10.3 µg/L from 5.2 µg/L). In another study, the truck transport simulation conditions also promoted an increase of this aldehyde up to 9.9 µg/L and 10.9 µg/L from 8.8 µg/L according to the temperature tested (30ºC and 45ºC, respectively) [49]. The reported differences can be explained by the *de novo* formation according to the availability of its precursor (leucine) in beer, a reaction that can be catalysed by oxygen present in bottled beer. Also, the oxygen content in those three batches could be higher due to the possible oxygen diffusion through beer cap and further incorporation into beer from vibrations. Another possibility, since the levels of amino acids is limited in bottled beer, is the degradation of the adducts formed between 3-methylbutanal and the amino acid cysteine due to unfavourable conditions (vibrations, temperature, and oxygen levels) resulting in the detection of higher levels of this aldehyde.

Interestingly, the concentration of 3-methylbutanal after transport with further storage simulation reverted to levels similar to those found in fresh beers. There is no available data for this compound after further storage on the transport studies previously published. Similarly to 2-methylpropanal, this behaviour can result from the strong interaction between this aldehyde with an amino acid like cysteine, leading to a possible re-formation of a bound-state which reduces the volatility of the aldehyde and consequently lower levels of free 3-methylbutanal can be detected. This mechanism has only been proven in beer model solutions [32, 74].

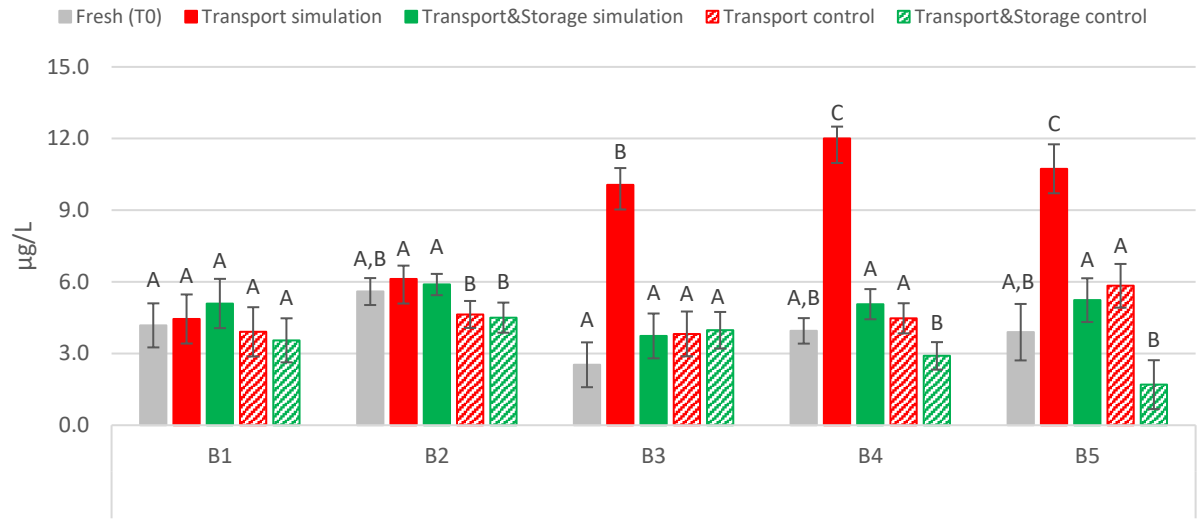

Figure 10: Evolution of 3-methylbutanal at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

4.3.2.2. Evolution of lipid oxidation aldehydes

Figure 11 shows the total concentration (sum of the three aldehydes) per batch in each of the five experimental sets: fresh, the transport and transport with storage simulations, as well as in their corresponding control samples.

The concentration of this group of aldehydes is interesting since there is a clear difference between the batches that may be related to the sample opening system. Beers after transport simulation with or without further storage contained lower or similar concentrations compared to the fresh samples. The same conclusions are drawn for the control sample sets. These results are only shared by Paternoster *et al* [49]. In their study, beers submitted to a truck transport simulation had a lower concentration of these aldehydes (0.5 µg/L) compared to referenced beers (0.6 µg/L). On contrary, beers that underwent maritime transport simulation or actual transportation had increased levels of these aldehydes, around 30% and 100%, respectively [12].

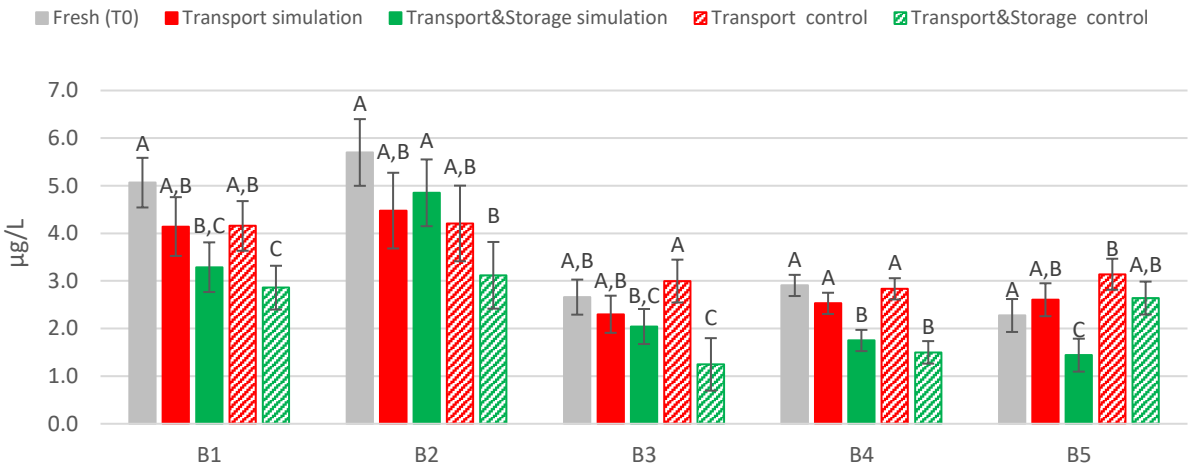

Figure 11: Evolution of lipid oxidation aldehydes at simulated conditions storage. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **Hexanal**

Hexanal was only quantified in the fresh samples B1 and B2, as represented in Figure 12. Regarding the control samples of these batches, hexanal was only quantified in Transport&Storage control group presenting an increase of 32% and 70%, respectively. In B3, B4 and B5 hexanal only quantified after transport and Transport&Storage simulation samples. The concentration in the latter sample group was always lower than those found in samples of transport simulation.

The results of the transport simulation indicated that vibrations combined with elevated temperatures favour the appearance of hexanal, especially in B3, B4, and B5 (samples with ring pull cap) where hexanal was only quantified in these samples at higher concentrations, between 1.9 to 2.4 µg/L. Additionally, when comparing the first two batches with fresh samples, significant differences were only detected in B2, where hexanal levels duplicated. Only the results of the first two batches are supported by a previously reported study, where authors found that hexanal content duplicated after maritime transport (0.4 to 0.8 µg/L), but in the performed simulation no variation on its concentration was observed [12]. After the truck transport simulation, the hexanal levels dropped from 0.6 µg/L to 0.4 µg/L [49].

Significant concentration differences were detected in the transport and storage simulation samples for all batches depending on the bottle opening system, with exception of B2, where hexanal levels were similar to the transport simulation. The batches made up of bottles with a crown cap, B1 and B2, developed higher hexanal concentrations or kept similar levels when compared to the transported beers. Hexanal concentrations dropped for all the batches with ring pull cap bottles (B3, B4, and B5) after further storage. There is no available data for this aldehyde in terms of transportation with further storage.

The behaviour of hexanal in the transported samples of B2 to B5 suggests that the vibrations, in combination with unstable temperature, can lead to the *de novo* formation. This may disrupt its bound state already in fresh beers, resulting in higher hexanal levels detected. The results of the transport with storage simulation for the last three batches indicate that the free hexanal liberated or formed during transport appears to bind again during storage. These results are supported by a previously published study of Baert *et al* [32], which reported that the hexanal increase showed a greater tendency to be transformed into a bound state than to remain free during beer ageing. Authors observed an increase of 58% in hexanal concentration after ageing, whereas an increase of only 20% was detected in beers spiked with cysteine after ageing due to the strong interaction of this aldehyde with cysteine at beer pH (≈4.4).

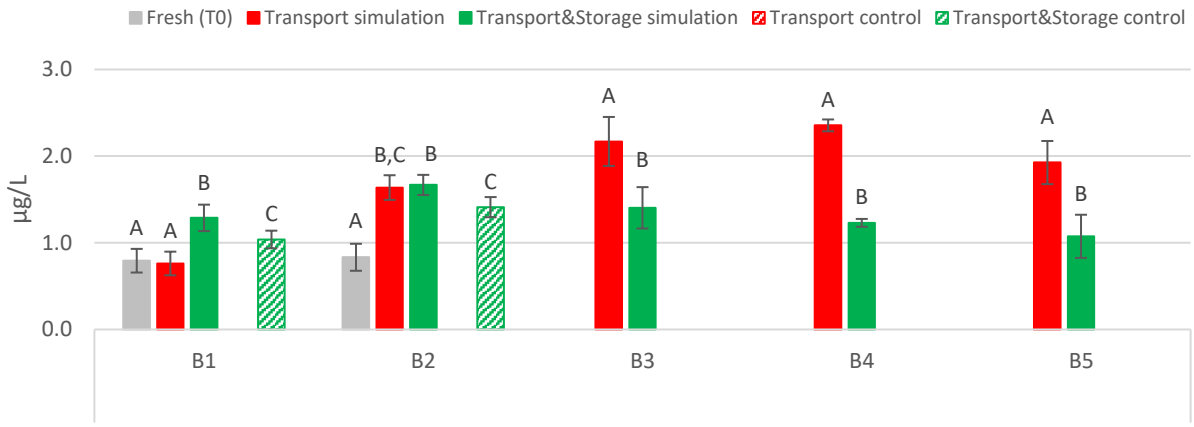

Figure 12: Evolution of hexanal at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- ***Trans*-2-nonenal**

Trans-2-nonenal was the first aldehyde linked to beer ageing, responsible for the appearance of cardboard off-flavour.

As observed in Figure 13, the evolution of this aldehyde was different between batches. In B1, no significant differences were found between the experimental sets. In B2, differences were only observed in the transport with storage set and its control group, whereas simulation sets (transport and transport with storage simulation) and the transport simulation with respective control were different in B4 and B5, respectively, compared to fresh samples. The

concentration of this compound was lower than its LOQ in the transport with storage simulation control for B4.

In this experimental setup, the *trans*-2-nonenal concentration varied from 0.3 µg/L to 0.8 µg/L across all experimental sets. Vieira *et al* [110] also quantified similar (or higher) values for lager beer. The significant differences observed after transport simulation were not consistent between the last two batches, since a reduction of 30% occurred in B4 whereas an increase of 30% was found in B5. Defining the impact of transport conditions for this aldehyde is not possible as a clear trend is not observed across the five analysed batches. However, increases in storage time yielded lower or similar concentrations detected in the control sets.

The sea transport simulation conducted by Jaskula-Goiris *et al* [12] was able to predict the same levels that the authors found in the actual transported samples, having found that the concentration doubled (0.03 µg/L to 0.06 µg/L). In contrast, no differences were found after truck transport simulation at 30ºC and 50 Hz [49].

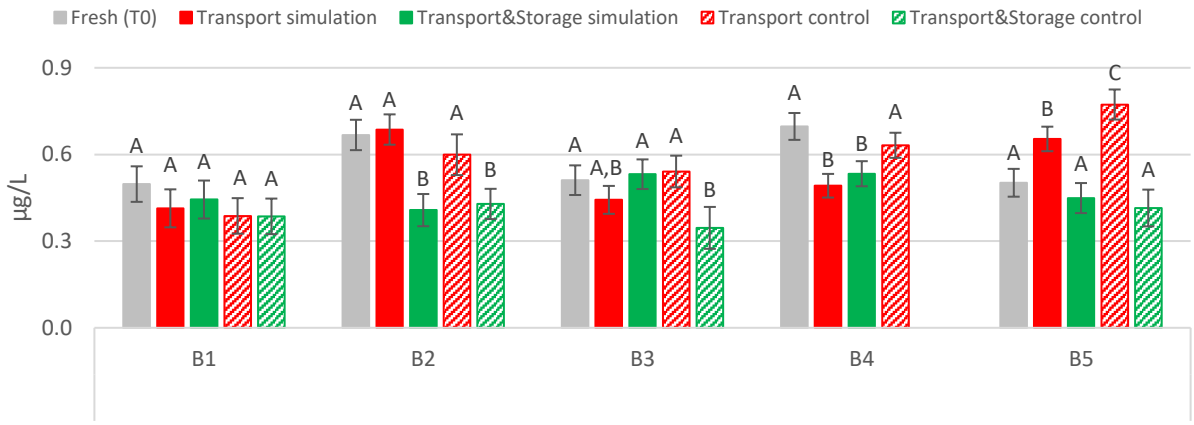

Figure 13: Evolution of *trans*-2-nonenal at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **Nonanal**

Nonanal showed a different evolution in relation to the other aldehydes originated by lipid oxidation, as represented in Figure 14. In general, its concentration in bottled beer tends to decrease or remain similar during storage at cold temperatures when compared to fresh beers.

Vibrations in combination with unstable temperatures promoted a significant reduction of nonanal of 32%, on average, in the transport simulation samples of B1 and B2 (crown cap opening system). No significant differences were recorded after subsequent storage in relation to samples subjected to the transport simulation. In the remaining batches, the presence of this aldehyde after transport simulations and storage dropped to values below its LOQ.

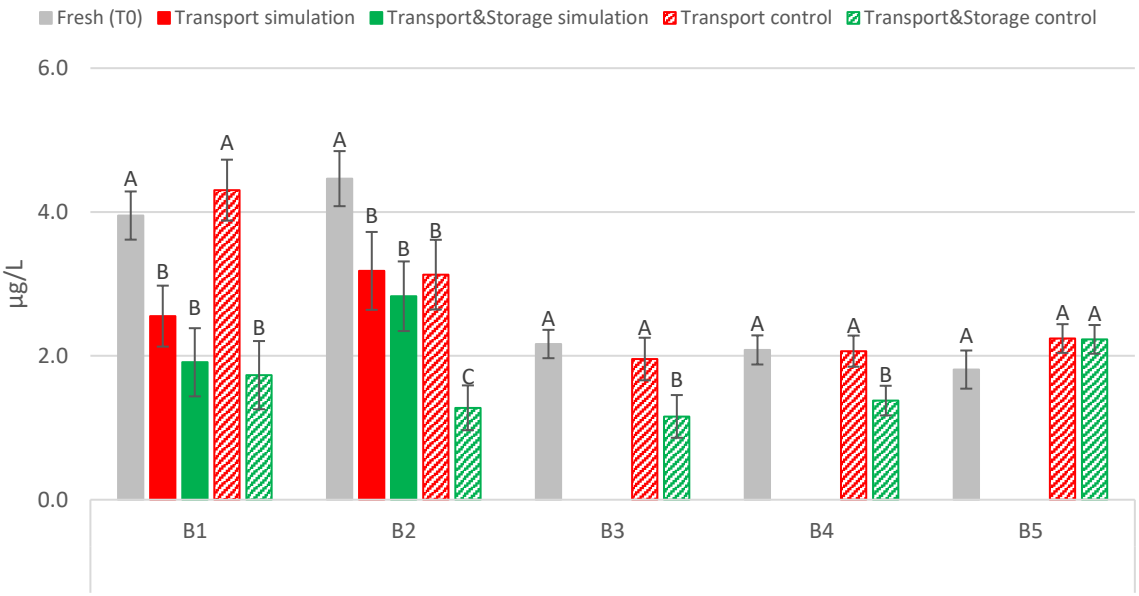

Figure 14: Evolution of nonanal at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

4.3.2.3. Acetaldehyde

No significant differences were observed in any sample set from B1 and B4. In terms of controls, there was a significant decrease in the transport with storage control of B3, while the acetaldehyde content in B1 and B2 was lower than LOD and LOQ, respectively (Figure 15).

The transport simulation had a significant impact on the second batch. The acetaldehyde concentration decreased by 44%. Transport samples from B5 stored for an additional 75 days

experienced a significant increase of 62%, while in the remaining batches there were no major variations in comparison with the transport simulation samples.

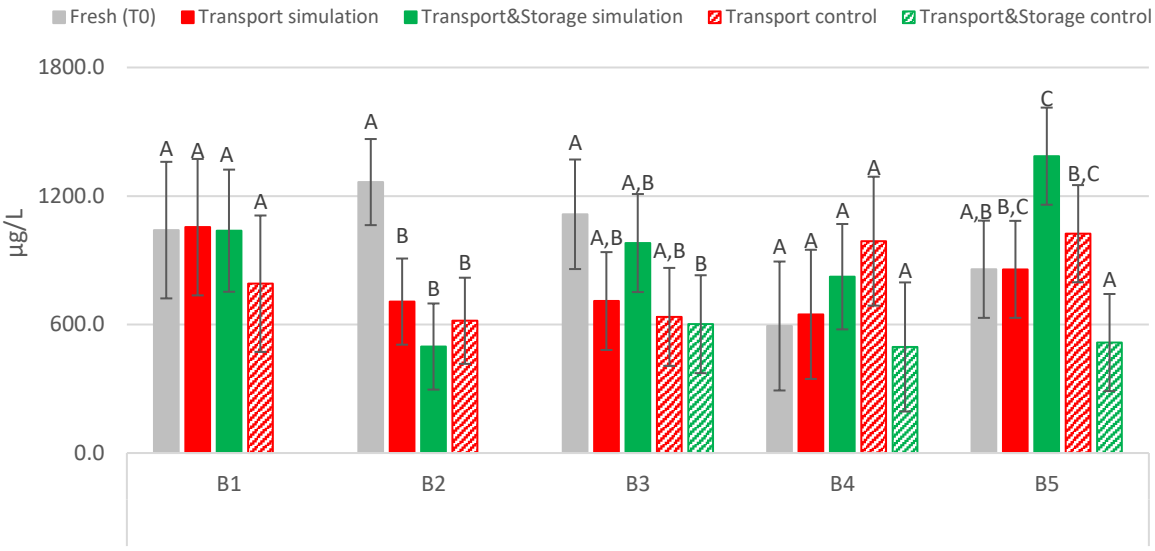

Figure 15: Evolution of acetaldehyde at simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

4.4. Conclusions

Storage time is of greater importance for Strecker aldehydes content in bottled beer. Additionally, the concentration of these aldehydes increases faster after Transport&Storage simulation in beer bottles with ring pull cap opening system than crown cap, due to the possible oxygen diffusion into the bottled beer that seems to be enhanced by the transport conditions (vibration and warm temperatures).

The maritime transport conditions simulated 45 days, vibrations of 1.7 Hz and warm temperatures (21-30ºC), favoured a pronounced increase in the content of the Strecker aldehydes 2-methylpropanal and 3-methylbutanal up to three times higher. The levels of their precursors on bottled beer, valine and leucine, may be related to this increase during this period. The liberation of these aldehydes from their bound state may also justify the increase.

Besides the clear impact of storage time in phenylacetaldehyde, the transport conditions accelerate an increase in its concentration when the beer opening system is ring pull cap. Benzaldehyde content present on bottled beer also increased significantly, 33% on average, only in beer samples with the referred cap. In terms of further storage after transport simulation, in beers with ring pull cap the content of phenylacetaldehyde remained the same or slightly decreased in the case of benzaldehyde, while the levels of 2-methylpropanal and 3-methylbutanal significantly dropped around 73% and 57%, respectively.

Higher content of the lipid oxidation aldehyde hexanal was registered after transport simulation in beer bottles with ring pull cap (besides being only present in those samples after simulations), whereas minor increases in its content were found in bottled beer with traditional cap. Moreover, a reduction of 43%, on average, on hexanal concentration were found in beer samples with ring pull cap stored for an additional time (75 days). On contrary, nonanal content continuously decreased until the end of the Transport&Storage simulation in bottled beers with traditional cap, not being quantified in samples with ring pull cap. Finally, a clear evolution trend was not possible to define regarding *trans*-2-nonenal and acetaldehyde.

5. EVALUATION OF ALDEHYDES THROUGH FORCED AGEING METHODOLOGY

5.1. Introduction

As reviewed in Chapter 2.1.3, forced ageing methodologies aim to simulate and accelerate the natural ageing process in order to predict the flavour stability during beer ageing. These procedures usually submit packaged beer to high temperatures during specific periods. However, several limitations on drawing parallelism and conclusions from these approaches have been reported, since forced ageing methodologies only consider the effect of one transport/storage variable – the temperature. Moreover, the high temperatures commonly used lead to significant differences in the chemical reaction rates as well as can lead to reactions that not occur during real beer ageing conditions [56, 59]. Thus, this part of the thesis is focused on the evaluation of the prediction power of a temperature-dependent forced ageing procedure, commonly used by breweries, to evaluate its applicability regarding aldehyde compound evolution on bottled beer, when this is submitted to real export conditions.

5.2. Setup of the forced ageing method

The prediction power of a temperature-dependent forced ageing procedure, commonly used by breweries, was evaluated. Forty-five lager beers (nine samples *per* batch) were stored in a dark oven at 37 ± 1°C for 7, 21 and 28 consecutive days. This corresponds to two, six and eight months of natural ageing, respectively. An overview of the experimental setup is presented in Figure 16.

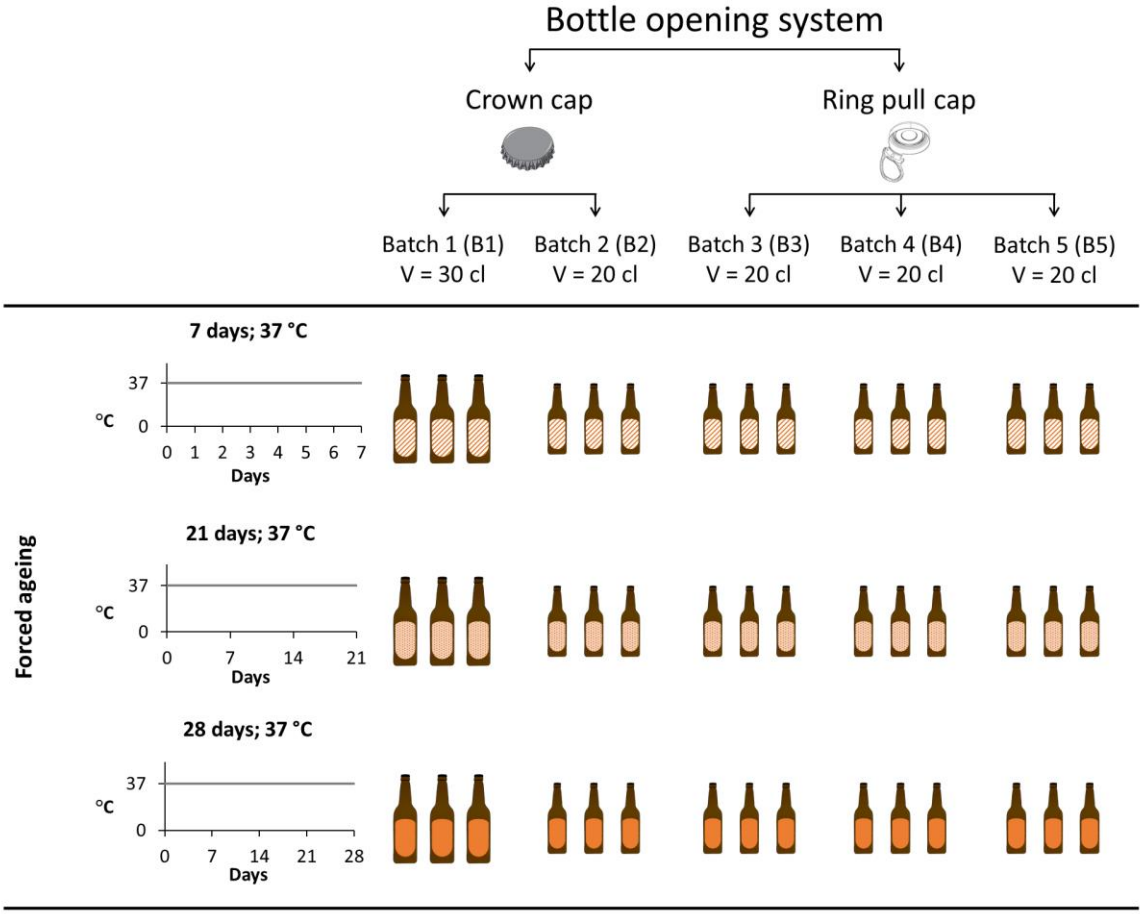

Figure 16: Overview of the forced ageing experimental design.

5.3. Results and discussion

In this section, the discussion compares the aged beer samples submitted to the transport with storage simulation (approximately 120 days) denoted as naturally or real aged beers with the results of three forced ageing periods previously described. Significant differences between these experimental groups were evaluated by the analysis of variance (One-Way ANOVA and Tukey tests) using Minitab® 17 statistical software.

5.3.1. Evolution of Strecker aldehydes

Due to the high concentration of phenylacetaldehyde when compared with the other Strecker aldehydes, in this section it was decided to analyse only this class of compounds individually rather than grouping them.

- **Phenylacetaldehyde**

The evolution of phenylacetaldehyde in samples exposed to forced ageing as well as the actual evolution of the compound after transport simulation with subsequent storage is shown in Figure 17. It is verified that the B3-B5 samples aged at 37ºC for a period equivalent to two months present lower levels to those obtained after transport simulation in the last three batches (153.2 µg/L on average), so the actual evolution of the compound was underestimated under these conditions. In contrast, B1 and B2 samples batches, at the same conditions does not present significant differences from real transport and storage simulation (169.6 µg/L, on average). Regarding forced ageing mimicking six months of ageing, the oppositive was observed, that is, significant differences were observed on B1-B2 and not on B3-B5 samples comparatively to transport with storage simulation. Malfliet *et al* [77] reported that lager beers naturally aged for nine months had a phenylacetaldehyde content between 8.2–31.2 µg/L, whereas forced aged beers at 30ºC for 60 days had a lower concentration (5.2–20.2 µg/L). Suarez *et al* [60] also concluded that the naturally aged beers developed higher levels than forced aged ones at 60ºC for seven days. After testing several beers, the authors verified increases from 1.3 to 3.0 times.

Finally, an ageing period equivalent to eight months was the period that better simulated the real evolution of this aldehyde during 120 days, except for the first batch, resulting in an average concentration of approximately 167.1 µg/L. Similarly to the linear increase verified in

the last three batches (B3, B4 and B5), forced ageing at 40ºC for 4 days also promoted a continuous increase of phenylacetaldehyde in lager beers [59].

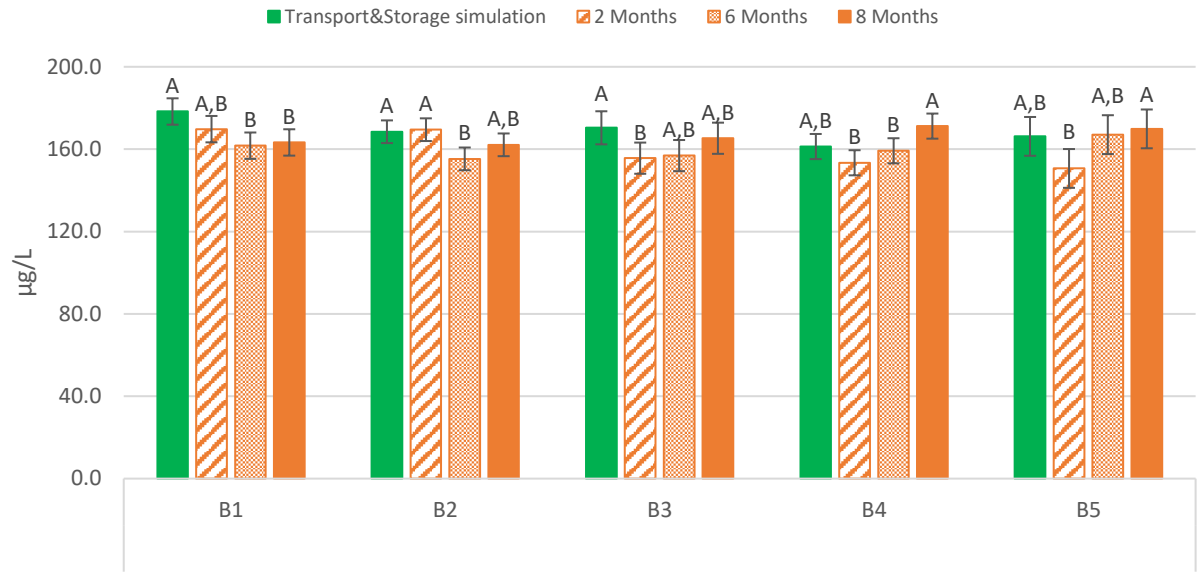

Figure 17: Comparison of phenylacetaldehyde evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **Benzaldehyde**

The concentration of benzaldehyde in forced aged samples was always lower than that found in samples simulating real transport with storage (Figure 18).

Comparing the forced ageing periods tested, two patterns were found. In B1 and B2 samples was observed a decrease with time while in B3 to B5 samples an increase was observed. However, in the later ones the concentrations were between 14% and 22%, on average, lower than real transport simulation. These results are similar to those from the reported study of Malfliet *et al* [77]. The authors found that naturally ageing pale lager beers for nine months developed higher levels (1.9–4.4 µg/L) than upon forced ageing at 30ºC for 60 days (1.5–3.3 µg/L). Additionally, Lehnhardt *et al* [59] reported that benzaldehyde did not continously increase during ageing. In another research, naturally aged beers had contents between two and 4.5 times higher than forced aged beers at 60ºC [60].

Based on the results discussed above, this kind of forced ageing procedure poorly predicts the true evolution of the compound, for any period tested, since the combination of the real conditions that beer faces, namely after exposing the samples vibrations in combination with unstable temperatures, higher concentrations of this aldehyde were found in naturally aged samples. Therefore, the application of this forced ageing test to simulate a certain ageing period underestimate the actual evolution of benzaldehyde.

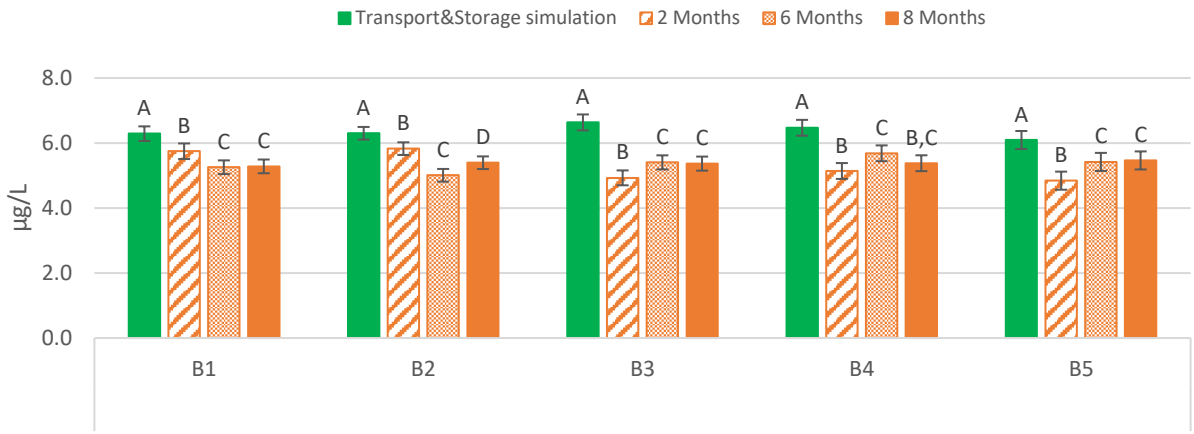

Figure 18: Comparison of benzaldehyde evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **2-Methylpropanal**

The maintenance of lager beers in an oven at 37ºC between 21 and 28 days (six and eight months, respectively) overestimated the actual content of 2-methylpropanal obtained after simulation of the exportation process, mainly in B2 to B5 (Figure 19). In these bacthes, the naturally aged beers had an average concentration of 3.5 µg/L while after an 21 and 28 days of forced ageing, their average content increase to 11.9 µg/L and 7.9 µg/L, respectively.Seven days (that brewers assume as two months of ageing) was the best suitable period to estimate the content of this aldehyde after natural ageing. Lehnhardt *et al* [59] verified a increase of this aldehyde during ageing at 40ºC for four days, a tendency that in general is verified in the tested conditions (37ºC). The increase in 2-methylpropanal may be strickly related to the valine cocentrations in this type of beer. Previous studies (results not published) indicate that this secondary amino acid is present at higher concentration in bottled beer, which together with temperatue effect (37ºC), can justify the increase observed in 2-methylpropanal.

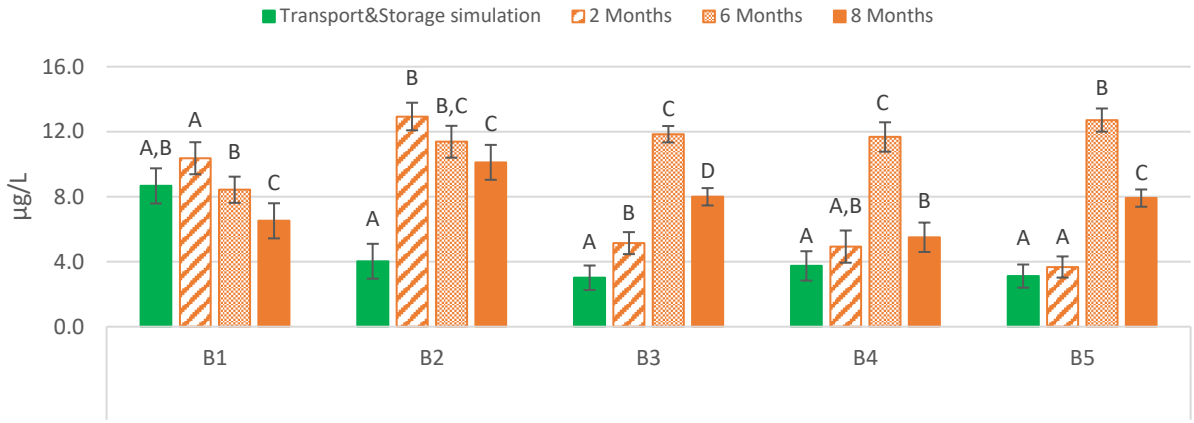

Figure 19: Comparison of 2-methylpropanal evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **3-Methylbutanal**

Contrary to what was observed for 2-methylpropanal, forced ageing corresponding to six and eight months was the best way to predict the natural evolution of 3-methylbutanal, as represented in Figure 20. In four batches, the period of six and eight months reproduced without major differences the actual content of the naturally aged beers for almost four months: 5.0 µg/L of 5.1 µg/L (B1), 5.0 µg/L of 5.1 µg/L (B4) and 5.3 µg/L of 5.2 µg/L (B5), respectively (values corresponding to the average of six with eight months). In the samples from the second batch, only the period of 21 days at 37ºC (six months) represented a similar concentration (5.5 µg/L) to the actual value of approximately 5.9 µg/L, while in B3 it was the eight months of ageing that simulated the reality, although the concentration was slightly higher (4.4 µg/L of 3.7 µg/L). Finally, for most batches (B2, B4, and B5) the exposure for seven days at 37ºC (two months) does not allow to predict the concentration of 3-methylbutanal after exportation.

Based on the description above, to estimate the content of this aldehyde in the studied lager beers after export, an ageing period of at least 21 days at 37ºC (six months) would be necessary. In most batches, naturally aged beers had a higher content of 3-methylbutanal than after some forced ageing periods, as also previously reported in the literature [60, 77].

The leucine is the precursor of 3-methylbutanal. Previous studies monitoring the amino acid content in these lager beer reveal that leucine was present at residual levels in bottled beer. Therefore, the formation of 3-methylbutanal through the amino acid seem to be limited.

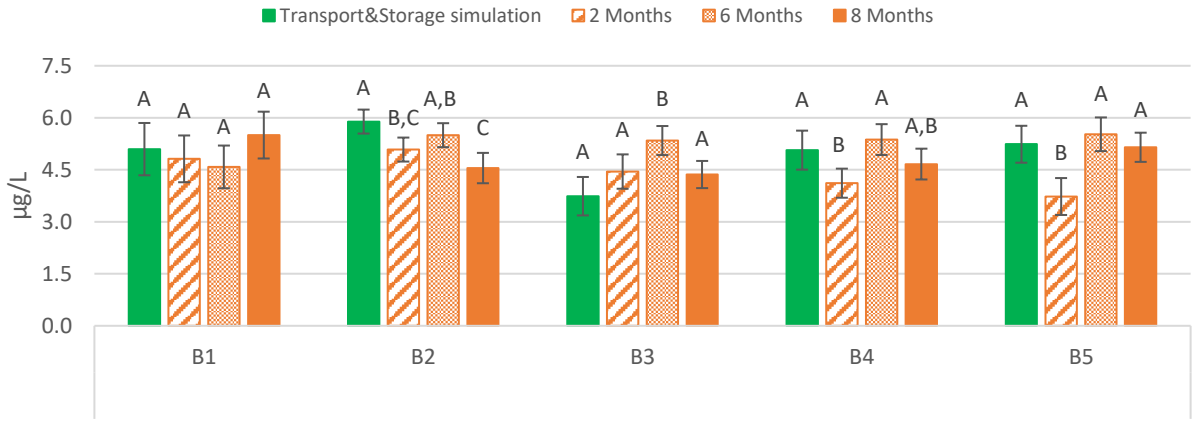

Figure 20: Comparison of 3-methylbutanal evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

5.3.2. Evolution of lipid oxidation aldehydes

In this section it was decided to analyse only this class of compounds individually rather than grouping them.

- **Hexanal**

The hexanal content in samples subjected to forced ageing rarely presented significant differences when compared to its natural evolution (Figure 21). The only differences found were in forced ageing, corresponding to six months of B2 and B5, and in B4, where significant differences were observed in the two-month and eight-month ageing periods. Despite that, it can be shown that beer storage at 37ºC for 28 days (equivalent to eight months) predicted the actual evolution of hexanal in most batches. These findings vary from the literature since the forced ageing conditions underestimate the normal evolution of hexanal during natural beer ageing. The hexanal content has been found to always be lower, on average 2.2 or 3.2 times lower, in forced ageing when compared to beer naturally aged [60, 77].

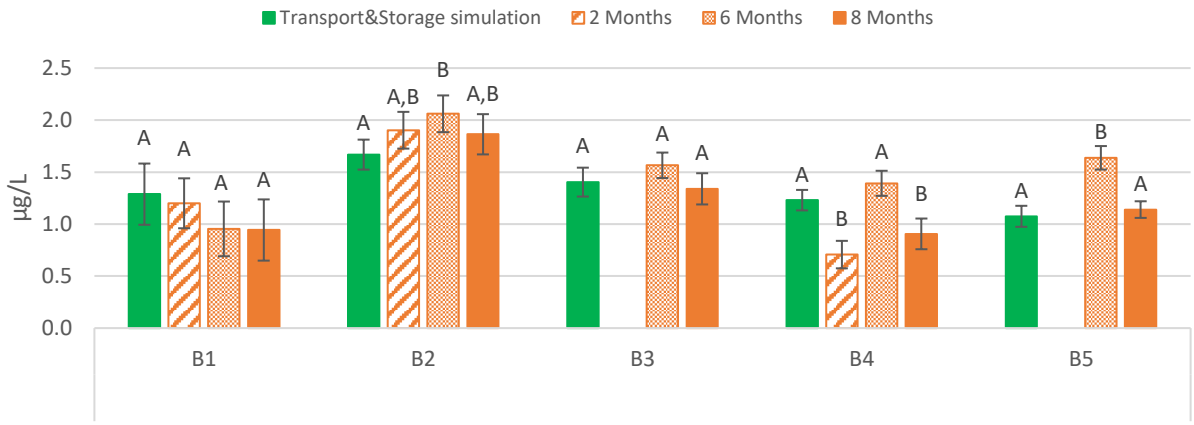

Figure 21: Comparison of hexanal evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- ***Trans*-2-nonenal**

According to the simulation performed, the actual content of *trans*-2-nonenal in these lager beers never exceeds 0.6 µg/L after approximately four months of natural ageing, as shown in Figure 22. However, after forced ageing at 37ºC, its concentration increased significantly, varying between 0.7–1.5 µg/L (except for eight months of B1). Therefore, the storage of beer at 37ºC significantly favors the increase of this compound for any simulated period. Consequently, this forced ageing procedure overestimates its actual evolution. Lehnhardt *et al* [59] also concluded that forced ageing techniques will lead to significant differences in aroma profile and analytical indicators, and also that *trans*-2-nonenal is mainly found under extreme (heated or acidified) conditions which results in significantly increases under forced ageing conditions than during natural ageing, being able to surpass its OTh and confer cardboard notes to beer flavour. On the contrary, Suarez *et al* [60] reported that forced ageing underestimates the natural ageing in all beers analysed. They observed that *trans*-2-nonenal was always lower, on average 7.9 times lower, in forced ageing when compared with beers naturally aged.

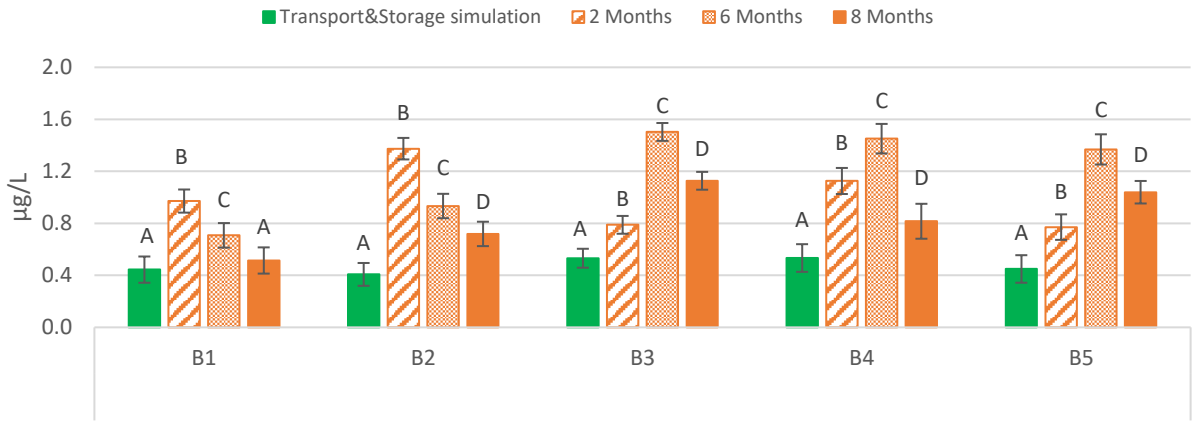

Figure 22: Comparison of *trans*-2-nonenal evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

- **Nonanal**

The nonanal variation is interesting since there is a clear division of batches that may be related to the sample opening system, B1 and B2 versus B3, B4 and B5 (Figure 23). This compound was quantified in all experimental sets of the first two batches, while in the remaining ones only in the forced aged samples. For the first two batches, it appears that an ageing time equivalent to at least six months is necessary to predict a concentration close to the concentrations present in the samples of the transport with storage simulation. For example, the actual concentration in the first batch is 1.9 µg/L and the mean value after forced ageing was approximately 2.0 µg/L (average of both six and eight months). Regarding the samples with a ring pull cap opening system (B3, B4, and B5), the nonanal content was below its respective LOQ in the transport with storage simulation samples (as mentioned in Chapter 4) and the same was observed in the samples with eight months of ageing in B3 and B4. Therefore, the results of the last three batches indicate that the conditions of forced ageing studied overestimate the natural ageing, as previously reported [59], favouring the formation of nonanal in these lager beers, with no significant differences being observed only in B5.

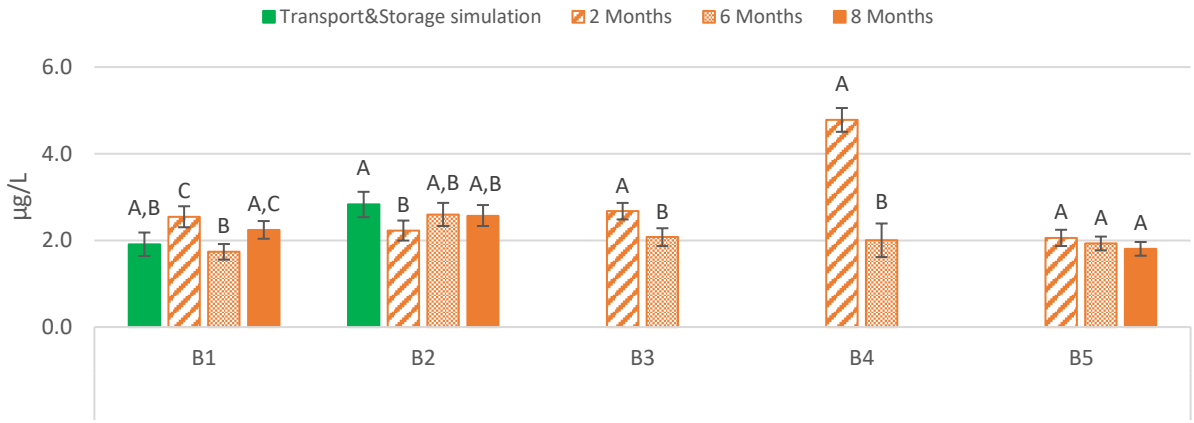

Figure 23: Comparison of nonanal evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed per each batch. Different letters represent statistically significant differences ($P < 0.05$).

5.3.2.1. Acetaldehyde

The comparison between the two experimental sets (natural ageing versus forced ageing) demonstrates that the actual evolution of acetaldehyde in the analysed lager beers is normally overestimated at 37ºC, regardless of the ageing period (Figure 24). The only exception were beers with two months of B1 and B5, where similar concentrations were predicted (1021 µg/L and 1425 µg/L from the actual 1038 µg/L and 1385.9 µg/L, respectively). Therefore, forced ageing at 37°C does not predict the acetaldehyde concentration of the natural ageing transport with storage simulation beer samples. These results are similar to the results reported by Liu *et al* [65]. Authors compared natural ageing up to six months with forced ageing at 60ºC for up to four days. The authors observed besides the continuous increase over time, that beers forced aged for four days had a concentration of 4.9 µg/L, which overestimate the acetaldehyde concentration in beers naturally aged for six months (4.3 µg/L).

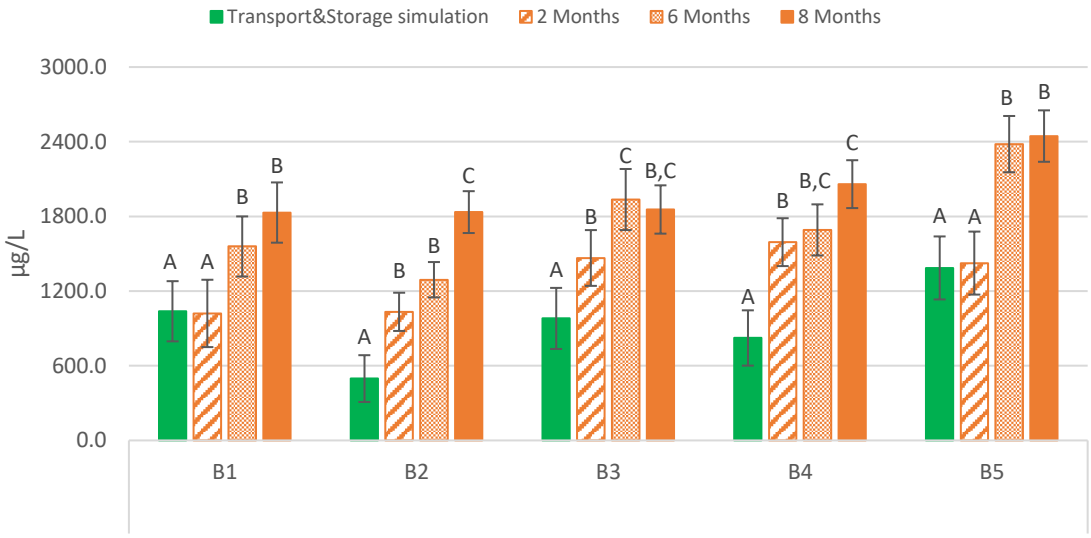

Figure 24: Comparison of acetaldehyde evolution during forced ageing *vs* real simulated conditions. The Tukey test was performed *per* each batch. Different letters represent statistically significant differences ($P < 0.05$).

5.4. Conclusions

The application of forced ageing procedure commonly used by breweries to simulate beer ageing, should be carefully analysed when the goal is to predict the real evolution of staling aldehydes on exported bottled beers.

The overall appreciation of the results, in terms of Strecker aldehydes, demonstrates that the forced ageing procedure applied allowed the prediction of similar natural ageing levels of phenylacetaldehyde and 3-methylbutanal found in Transport&Storage simulation beer samples. However, the actual levels developed after simulation experiment regarding benzaldehyde and 2-methylpropanal were not well predicted, being underestimated and overestimated, respectively.

The lipid oxidation aldehydes *trans*-2-nonenal and nonanal are favoured by the forced ageing conditions while, in general, the content of hexanal in the simulation samples was predicted. Finally, acetaldehyde content in forced aged beers continuously increased, being higher than in samples submitted to real export conditions.

Despite the adequate prediction for some aldehydes, the influence of other variables such as vibrations seem to limit the prediction power of the forced ageing method used. Moreover, it was found that the beer opening system appears to influence the content of some aldehydes in the forced ageing conditions evaluated. The content of phenylacetaldehyde, benzaldehyde and acetaldehyde continuously increase over time and nonanal only appeared in beer samples with a ring pull cap opening system.

6. FINAL REMARKS AND FUTURE PERSPECTIVES

6.1. Final Remarks

The main aim of the present study was to assess the impact of maritime transport conditions (warm temperature, vibrations and travel time) on beer flavour stability, namely through the identification and quantification of aldehyde compounds. The analytical methodology implemented was validated showing good performance results in terms of linearity, sensitivity, selectivity, precision and accuracy. The assessment of the evolution of staling aldehydes in the beer samples submitted to maritime transport simulation and forced ageing was also evaluated. The following results are the most important.

Regarding the impact of maritime transportation simulation on staling aldehydes:

- Storage time is of greater importance for Strecker aldehydes content in bottled beer.
- Transport and storage conditions do not favour increases in their concentration when beer bottles present crown cap opening system. On the other hand, transport conditions (vibrations and warm temperatures) may lead to oxygen diffusion during Transport&Storage on bottles with a ring pull cap opening system and consequently the Strecker aldehyde content increase.
- The Strecker aldehydes content increase after Transport&Storage simulation was faster in bottles with ring pull cap opening system than traditional crown cap.

- Transport simulation conditions (vibrations and warm temperatures) leads to a pronounced increase of up to three times in 2-methylpropanal and 3-methylbutanal.
- Transport simulation conditions leads to a significant increase of 33% (on average) in benzaldehyde content of beer bottles with ring pull cap.
- Phenylacetaldehyde increases during storage. Higher concentrations were only found when beers with ring pull cap were exposed to warm temperatures and vibrations.
- Transport conditions were determinant for hexanal, being only found in the exportation simulation groups of beer samples with ring pull cap.
- Transport&Storage simulation conditions leads to a continuous decrease of nonanal in bottled beers with traditional cap, not being quantified in samples with ring pull cap.
- Further storage after transport simulation significantly reduced the content of 2-methylpropanal, 3-methylbutanal and hexanal by 73%, 57% and 43%, respectively, suggesting the formation of a bound state with an amino acid available such as cysteine.
- The influence of maritime transport conditions was not clear for *trans*-2-nonenal and acetaldehyde.

Regarding of the application of forced ageing method to assess aldehyde evolution on bottled beer during the storage time:

- Power prediction of Forced ageing at 37ºC for 7, 21 and 28 consecutive days was evaluated.
- The prediction of similar natural ageing levels of phenylacetaldehyde and 3-methylbutanal found in Transport&Storage simulation beer samples was achieved, while benzaldehyde and 2-methylpropanal were not predicted.
- The formation of 2-methylpropanal was favoured under forced ageing conditions. The high levels of its precursor (valine) in bottled beer may be justify the increase when beer is submitted to higher temperatures

- In general, the hexanal content on bottled beer after transport&storage simulation can be estimated from forced ageing method applied.
- Forced ageing conditions favours the increase of *trans*-2-nonenal up to 1.5 µg/L, on average.
- A continuous increase over time during forced ageing was observed on phenylacetaldehyde and benzaldehyde in beer samples with ring pull cap.
- Acetaldehyde also increase over time during forced ageing, reaching higher levels in beer samples with ring pull cap.
- In beer bottles with ring pull cap opening system, nonanal was only quantified after forced ageing with an average content of 2.5 µg/L regarding the three simulated periods.
- The forced ageing methods exclusively dependent on temperature seem to present limitation to simulate the evolution of aldehydes in bottled beer at real transport and storage conditions. Vibrations and more realistic temperatures may explain the differences.

As discussed, previous studies have demonstrated that beer staling is mainly associated with the appearance of staling aldehydes in beer, being favoured not only by unfavourable storage conditions but also by transport conditions specially vibrations in combination with warm temperatures. Based on that principle, the focus here was to assess the impact of maritime transport and storage conditions on the evolution of 10 aldehydes. In summary, the results here demonstrated and confirmed that maritime transport conditions promote the development of higher levels of staling aldehydes on bottled lager beer, particularly when the beer opening system is ring pull cap. It was possible to verify that Strecker aldehydes are more affected by transport conditions than other aldehydes under study, namely the content of phenylacetaldehyde reached after maritime transport simulation remained stable after further storage up to 75 days. In contrast, the simulation of beer storage at the destination for a period of 75 days demonstrated a decline in the concentration developed after maritime transportation of 2-methylpropanal, 3-methylbutanal, and hexanal in beers with a ring pull cap raising questions about the mechanism's involved and the need to be further elucidated. These results also indicate that bottled beer with a traditional opening system (crown cap) are more suitable

for maritime exportation since a minor impact in beer flavour stability was observed and thus the acceptability by the consumer is not compromised. Furthermore, the comparison between the content of each aldehyde after Transport&Storage simulation with the results of forced aged beers at 37ºC demonstrated that this methodology has some limitations to mimic the real evolution of these particular family of compounds.

6.2. Future Perspectives

As future work, we consider important monitoring the amino acid levels, namely valine, leucine, isoleucine and cysteine, along with aldehyde content during transport simulation to better elucidate the mechanism behind the evolution of staling aldehydes in bottled beer. The content of this amino acid can be further correlated with 2-methylpropanal, 3-methylbutanal, 2-methylbutanal and bound state aldehydes respectively.

It will also be relevant monitoring the evolution of aldehydes during maritime transportation under milder temperatures. Moreover, we also consider important monitoring the impact of both bottle opening systems, crown cap versus ring pull cap, on beer flavour stability. Lastly, it would be important to develop a forced ageing method including a new variable – vibrations, to upgrade its power accuracy in simulating natural beer ageing.

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8. APPENDIX

Table 4: Summary of some studies discussed in Chapter 2 (literature review).

Beer style	T (°C)	Time	Other conditions	% ABV	Colour	Iso-α-acids		Haze		Total polyphenols (mg/L)	Flavanoids (mg/L)	Aldehydes (µg/L)								Esters (µg/L)			Alcohols (mg/L)	References
						Total	T/C ratio	Permanent	Chill			2MP	2MB	3MB	Methional	Benzal-dehyde	Phenyl-acetaldehyde	*Trans* -2-nonenal	Furfural	Total ageing compounds	Isoamyl acetate	Ethyl octanoate	Ethyl hexanoate	Isoamyl alcohol	
Pale Lager	-	0 d			6.7 - 7.2 IOB	9.9 - 24.3 mg/L	0.37 - 0.44 mg/L	0.1 - 0.8 EBC	0.3 - 1.8 EBC	96.4 - 234.1	20.4 - 35.3	3.8 - 11.2	1.3 - 1.8	2.7 - 5.5	1.1 - 5.3	1.1 - 3.2	3.1 - 7.1	0.02 - 0.04	14.7 - 34.0	33.3 - 63.3	392.8 - 2601.5	43.5 - 189.1	93.9 - 181.0	32.2 - 55.4	[77]
	22°C	9 m			7.6 - 10.8 IOB	5.9 - 16.3 mg/L	0.12 - 0.22 mg/L	1.4 - 15.9 EBC	5.9 - 57.3 EBC	96.4 - 227.6	18.1 - 29.8	22.5 - 146.8	5.1 - 7.0	11.8 - 20.9	4.7 - 29.2	1.9 - 4.4	8.2 - 31.2	0.03 - 0.05	237.2 - 524.1	294.8 - 719.1	227.4 - 1865.9	21.4 - 158.4	37.0 - 122.9	32.5 - 54.1	
	30°C (FA)	60 d			7.1 - 9.5 IOB	8.7 - 21.1 mg/L	0.26 - 0.34 mg/L	0.7 - 1.2 EBC	1.2 - 9.8 EBC	95.9 - 236.9	19.4 - 32.0	9.5 - 38.2	2.9 - 3.6	5.6 - 11.8	2.0 - 17.8	1.5 - 3.3	5.2 - 20.2	0.02 - 0.05	105.6 - 232.5	153.5 - 285.5	356.7 - 2317.3	24.9 - 184.6	61.8 - 155.6	34.0 - 55.8	

Beer style	T (°C)	Time	Aldehydes (µg/L)						Esters (µg/L)		Ketones (µg/L)		References
			2MB	3MB	Benzal-dehyde	Phenyl-acetaldehyde	Furfural	Total ageing compounds	Diethyl succinate	Ethyl nicotinate	2-acetylfuran	Y-nonalactone	
Lager	25°C	1 m	1.25 - 2.71	1.40 - 3.64	0.20 - 0.54	4.46 - 10.58	53.50 - 217.50	80.24 - 277.01	0.13 - 0.41	2.86 - 22.54	1.59 - 13.24	3.59 - 13.35	[76]
		2 m	1.26 - 3.31	1.42 - 4.33	0.34 - 0.70	4.32 - 8.29	87.25 - 285.00	126.17 - 358.49	0.14 - 0.61	7.03 - 35.39	2.70 - 13.84	7.22 -13.72	
		3 m	1.44 - 4.26	1.93 - 13.26	0.29 - 0.74	3.84 - 20.44	114.25 - 289.06	151.41 - 469.60	0.35 - 2.18	10.41 - 114.92	2.74 - 14.19	14.21 - 31.50	
		4 m	1.41 - 4.40	3.41 - 9.70	0.30 - 0.87	9.52 - 17.46	100.50 - 304.06	164.07 - 509.57	0.50 - 1.90	17.56 - 131.67	5.22 - 17.88	22.52 - 40.78	
		5 m	1.37 - 4.24	3.63 - 10.34	0.40 - 0.96	9.21 - 21.43	132.75 - 464.25	209.65 - 650.55	0.66 - 3.17	19.23 - 143.83	5.44 - 23.36	27.04 - 45.01	
		6 m	2.56 - 4.60	5.62 - 9.81	0.50 - 1.00	8.89 - 25.87	205.00 - 495.00	296.37 - 697.03	0.70 - 2.88	21.00 - 147.24	5.63 - 24.89	24.64 - 47.04	

Beer style	T (°C)	Time	Other conditions	% ABV	Colour	Iso-α-acids		Haze		Aldehydes (µg/L)								References
						Total	T/C ratio	Permanent	Chill	2MP	2MB	3MB	Methional	Benzal-dehyde	Phenyl-acetaldehyde	*Trans* -2-nonenal	Furfural	
Lager	5°C	120 d			7.25-7.63 EBC	19.66-19.84 mg/L	43.27/ 45.22 %	0.3-0.39	0.89-0.92	1.5 - 1.7	0.8 - 0.9	3.5-3.7	0.6-0.8	0.7-0.9	6.4-7.5	0.06-0.10	16.1-19.5	[16]
	30°C	120 d			8.02-8.08 EBC	17.56/ 17.59 mg/L	31.32/ 32.83 %	1.31	3.55-3.88	8.7	2.4-2.5	7.2-7.5	1.2-2.2	1.4-1.5	9.4-11.1	0.012-0.16	336.7-338.5	

Beer style	T (°C)	Time	Aldehydes (µg/L)							Esters (µg/L)	Ketones (µg/L)	References
			2MB	3MB	Methional	Phenylacetaldehyde	Benzaldehyde	Heptanal	Furfural	Ethyl nicotinate	Y-nonalactone	
Lager		0 w	2.4 ± 0.4	4.9 ± 0.7	2.8 ± 1.0	8.3 ± 1.5	1.2 ± 0.3	< 1.0	169.8 ± 11.9	46.8 ± 5.3	4.9 ± 1.6	[55]
	28°C	12 w	4.7 ± 0.4	7.5 ± 0.1	4.9 ± 1.0	14.2 ± 0.1	2.0 ± 0.1	< 1.0	474.5 ± 35.9	76.8 ± 5.9	160.2 ± 10.6	

Beer style	T (°C)	Time	Terpenes (mg/L)		Phenols (mg/L)			Esters (mg/L)			Alcohols (mg/L)	Ketones (mg/L)	References
			Nerol (cis-geraniol)	β-Damascenone	4-Ethylphenol	2-Methoxy-4-vinylphenol	4-Vinylphenol	Isobutyl acetate	Isoamyl acetate	Ethyl decanoate	1-Hexanol	Y-nonalactone	
Pilsner		0 m	0.001	0.002	0.002	0.703	0.089	0.067	1.908	0.01	0.019	0.032	[94]
	4°C	5 m	0.001	0.002	0.002	0.58	0.071	0.068	1.298	0.012	0.017	0.032	
	20°C	5 m	0	0.004	0.002	0.698	0.058	0.063	1.245	0.011	0.017	0.047	
	40°C	5 m	0	0.006	0.002	1.326	0.051	0.046	0.975	0.01	0.019	0.05	

Beer style	T (°C)	Time	Other conditions	% ABV	Colour	Aldehydes (mg/L)	Esters (mg/L)			Alcohols (mg/L)			Ketones (mg/L)			References
						Acetal-dehyde	Isoamyl acetate	Ethyl octanoate	Ethyl hexanoate	Isoamyl alcohol	1-Propanol		Acetone	Diacetyl	2,3 Pentanedione	
Craft	3°C	30 d	SO2: 2.45 (mg/L) SO2:2.84 (mg/L)	0.0523	7.08 SRM	ND	1.01	0.2	ND	44.33	32.69	14.22	ND	46.93	ND	[83]
	35°C	30 d		0.0524	7.81 SRM	3.74	0.32	0.34	0.3	39.09	5.96	9.89	ND	49.58	3.95	

Beer style	T (°C)	Time	Aldehydes (µg/L)	Esters (mg/L)	References
			Acetal-dehyde	Diethyl-acetal	
Light beer	Room temperature	0 m	3.38 ± 0.22	6.22 ± 0.03	[65]
		2 m	3.85 ± 0.42	5.89 ± 0.31	
		4 m	4.17 ± 0.34	5.13 ± 0.24	
		6 m	4.35 ± 0.28	4.86 ± 0.25	
	60°C	0 d	3.38 ± 0.22	6.22± 0.03	
		1 d	3.52 ± 0.17	6.05 ± 0.31	
		2 d	3.96 ± 0.24	5.77 ± 0.42	
		3 d	4.27 ± 0.31	4.53 ± 0.21	
		4 d	4.96 ± 0.28	4.02 ± 0.25	

Beer style	T (°C)	Time	Other conditions	% ABV	Colour	Iso-α-acids	Haze	Total polyphenols (mg/L)	Flavanoids (mg/L)	Aldehydes (µg/L)					Esters (µg/L)	Alcohols (mg/L)	References
										2MP	2MB	3MB	Phenyl-acetaldehyde	Furfural			
	0°C	0 m								4.54	2.34	6.16	46	11.1			
		6 m								4.75	2.62	6.61	7.61	5.2			
	8°C	2 m								5.36 - 5.55	2.50-2.57	8.54-6.81	4.28-19.7	13.4-10.7			
		4 m								9.24-9.64	3.65-3.96	11.2-9.82	21.2-20.5	35.3-31.2			
		6 m								18.8-16.7	7.54-6.69	21.7-15.3	36.5-21.2	205-121			
	20°C	2 m								6.48-8.11	2.89-3.67	9.51-9.34	7.38-17.7	13.7-12.6			[80]
		4 m								12.1-14.7	4.79-6.36	13.8-14.3	11.0-27.8	55.6-49.5			
		6 m								-	-	-	-	-			
	30°C	2 m								8.00-8.60	3.36-3.56	11.4-9.93	22.9-12.2	13.5-14.1			
		4 m								16.5-14.8	6.13-5.73	18.3-13.8	38.7-15.9	99.3-51.9			
		6 m								28.2	15.8	31.6	38	452			

								Total	T/C ratio	Permanent	Chill						
												5-HMF					
		0 d										3.73 ± 0.12					
		10 d										4.06 ± 0.29					
	30°C	20 d										4.16 ± 0.04					
		30 d										4.80 ± 0.07					
		40 d										4.91 ± 0.30					
Not specified		10 d										5.01 ± 0.16					[52]
	40°C	20 d										5.39 ± 0.22					
		30 d										7.70 ± 0.57					
		40 d										9.27 ± 0.57					
		10 d										7.22 ± 0.19					
	50°C	20 d										9.12 ± 0.62					
		30 d										14.9 ± 0.60					
		40 d										17.8 ± 1.3					

												Aldehydes					
										2MP (µg/L)	2MB (µg/L)	3MB (µg/L)	Methional (µg/L)	Benzaldehyde (µg/L)	Phenylacetaldehyde (µg/L)	*Trans*-2-nonenal	5-HMF (mg/L)	Furfural (µg/L)	Acetaldehyde (mg/L)
										11	2.9	9	1.6	1.2	22	0.03	5 ppm	19 ppb	0.6
Lager	60°C	5 d								72	16.7	17	3.6	1.6	48	0.16	38 ppm	916 ppb	1.2
	40°C	3 w								48	6.2	14	2.5	1.8	35	0.11	13 ppm	287 ppb	1
	28°C	3 m								29	3.6	10	2.2	1.5	29	0.05	8 ppm	171 ppb	1
	20°C	6 m								46	4.9	18	2.6	1.8	38	0.08	11 ppm	273 ppb	1.3

												Aldehydes (µg/L)					
												Hexanal	*Trans*-2-nonenal	5-HMF	Furfural		
		0 d										[1.60-1.66]	[0.0022-0.0084]	[5360-14732]	[12.96-37.30]		
		7 d										[1.56-2.01]	[0.0021-0.0101]	[7544-27897]	[13.89-27.45]		
	4°C	14 d										[1.59-2.63]	[0.002-0.0094]	[11646-15056]	[16.75-36.86]		
		21 d										[2.44-2.81]	[0.0024-0.0125]	[11179-31297]	[17.30-43.78]		
		28 d										[3.01-3.33]	[0.0032-0.0154]	[11688-29028]	[14.89-71.93]		
		7 d										[1.78-1.92]	[0.0023-0.0095]	[8662-14723]	[19.06-43.00]		
		14 d										[2.39-2.42]	[0.0092-0.0096]	[15092-23031]	[22.04-38.06]		
Lager	12°C	21 d										[1.85-2.75]	[0.0029-0.0146]	[12491-24166]	[33.06-43.72]		[81]
		28 d										[2.54-3.16]	[0.0037-0.0278]	[11814-30132]	[19.34-80.73]		
		7 d										[1.62-3.01]	[0.0025-0.0095]	[8739-15795]	[22.68-54.77]		
		14 d										[2.66-2.67]	[0.0071-0.0101]	[17517-22833]	[30.24-49.30]		
	30°C	21 d										[2.83-2.89]	[0.0036-0.0148]	[15262-21295]	[33.06-64.37]		
		28 d										[2.97-3.18]	[0.0043-0.0291]	[21728-30237]	[31.27-102.96]		
		7 d										[1.64-3.08]	[0.0032-0.0110]	[12736-22492]	[28.23-64.52]		
		14 d										[2.56-2.71]	[0.0071-0.0117]	[17077-30278]	[38.57-94.46]		
	37°C	21 d										[2.62-2.87]	[0.0038-0.0214]	[16417-32685]	[59.28-104.40]		
		28 d										[2.84-3.34]	[0.0054-0.0309]	[19626-36603]	[56.10-132.27]		

Beer style	T (°C)	Time	Other conditions	% ABV	Colour	Iso-α-acids	Haze	Total polyphenols (mg/L)	Flavanoids (mg/L)	Aldehydes (µg/L)	Esters (µg/L)	Alcohols (mg/L)	References
										Sotolon (µg/L)			
Blond	20°C	Fresh								nq < 1			[84]
		6 m								6.4 - 14.2			
		12 m								8.9 - 17.0			
		18 m								10.9 - 36.2			
		24 m								8.9 - 42.1			
Dry hopped trapist		6 m								7.8			
		12 m								12			
		18 m								16.2			
		24 m								29.1			
Amber		6 m								4.6			
		12 m								9.4			
		18 m								14.7			
		24 m								12.1			
Brown		6 m								6.8			
		12 m								13.7			
		18 m								13			
		24 m								16.5			

										Volatile Phenol (mg/L)			
										Vanillin	4-vinylguaiacol	Apocynol	
Pilsner	60°C (FA)	0 w	+ CO2 + 2mg/L 4VG								2.13		[86]
		2 w									1.41		
		6 w									0.66		
		12 w									0.33		
	60°C (FA)	0 w	+O2 + 2mg/L 4VG								2.13		
		2 w									0.46		
		6 w									0.33		
		12 w									0.2		
	20°C	0 m	+ CO2 + 2mg/L 4VG							ND	2.13	ND	
		3 m								ND	1.69	0.36	
		6 m								ND	1.46	0.44	
		12 m								0.04	1.04	0.83	
	20°C	0 m	+ O2 + 2mg/L 4VG							ND	2.13	ND	
		3 m								0.33	1.15	0.28	
		6 m								0.41	0.89	0.37	
		12 m								0.59	0.31	0.81	
	4°C	0 w	+ CO2 + 2mg/L 4VG								2.13		
		6 w									2.11		
		12 w									2.06		
	20°C	0 w	+ CO2 + 2mg/L 4VG								2.13		
		6 w									1.94		
		12 w									1.74		
	40°C (FA)	0 w	+ CO2 + 2mg/L 4VG								2.13		
		6 w									1.41		
		12 w									1.14		

Beer style	T (°C)	Time	Other conditions	% ABV	Colour	Iso-α-acids	Haze	Total polyphenols (mg/L)	Flavanoids (mg/L)	Aldehydes (µg/L)	Esters (µg/L)	Alcohols (mg/L)	References
Pilsner	20°C	0 d	Light			0.44 mg/L	5.53						[92]
		22 d	Light			0.43 mg/L	4.45						
		30 d	Light			0.42 mg/L	4.06						
		60 d	Light			0.40 mg/L	2.3						
		94 d	Light			0.39 mg/L	1.42						
		121 d	Light			0.39 mg/L	1.28						
		149 d	Light			0.38 mg/L	1.19						
		0 d	Dark			0.44 mg/L	5.23						
		22 d	Dark			0.43 mg/L	4.7						
		30 d	Dark			0.43 mg/L	4.39						
		60 d	Dark			0.42 mg/L	2.68						
		94 d	Dark			0.42 mg/L	1.8						
		121 d	Dark			0.41 mg/L	1.66						
		149 d	Dark			0.40 mg/L	1.57						
		0 d	Dark			0.44 mg/L	5.53						
		22 d	Dark			0.41 mg/L	4.01						
		30 d	Dark			0.40 mg/L	3.44						
		60 d	Dark			0.39 mg/L	1.51						
		94 d	Dark			0.38 mg/L	0.62						
		121 d	Dark			0.37 mg/L	0.4						
		149 d	Dark			0.35 mg/L	0.29						
Pilsner		-				102.4 µmol/L	0.37 µmol/L						[93]
	28°C	8 m				70.0 µmol/L	0.15 µmol/L						

Beer style	T (°C)	Vibrations	Time	Packaging type	Other conditions	Colour (EBC)	Oxygen content (µg/L)			Iso-α-acids		Haze (FU)		Aldehydes (µg/L)									References
							TPO	HSO	DO	Total (mg/L)	T/C ratio	Permanent	Chill	2MP	2MB	3MB	Methional	Benzaldehyde	Phenylacetaldehyde	Hexanal	*Trans* -2-nonenal	Furfural	Total ageing compounds	
Blond beer without refermentation			90 h			-	1265 ± 185	1207 ± 180	58 ± 44															[49]
	25 °C	Without				8.08 ± 0.77	289 ± 48	258 ± 48	31 ± 8	13.96 ± 0.33				17.75 ± 2.03	3.06 ± 0.26	9.72 ± 0.69	14.84 ± 6.77	1.29 ± 0.24	42.89 ± 12.02	0.80 ± 0.29	0.05 ± 0.02	24.86 ± 4.68	115.27 ± 22.85	
	5 °C	Without				8.27 ± 0.15	334 ± 25	286 ± 25	48 ± 6	14.04 ± 0.14				11.63 ± 0.45	2.65 ± 0.09	8.84 ± 0.04	12.25 ± 2.72	1.48 ± 0.14	37.64 ± 9.72	0.57 ± 0.10	0.02 ± 0.01	10.46 ± 0.59	85.57 ± 12.08	
		50 Hz; 15 m/s-2				8.35 ± 0.10	253 ± 46	230 ± 46	23 ± 4	14.24 ± 0.27				12.34 ± 0.88	2.63 ± 0.18	8.51 ± 0.39	22.40 ± 4.10	1.25 ± 0.19	61.25 ± 21.66	0.36 ± 0.03	0.02 ± 0.01	12.33 ± 1.40	121.09 ± 26.18	
	30 °C	Without				8.24 ± 0.17	295 ± 44	259 ± 43	36 ± 10	13.84 ± 0.21				18.83 ± 1.09	3.05 ± 0.19	9.57 ± 0.72	11.73 ± 2.43	1.17 ± 0.09	37.74 ± 5.48	0.58 ± 0.18	0.03 ± 0.01	26.73 ± 1.35	109.43 ± 7.86	
		50 Hz; 15 m/s-2				8.33 ± 0.10	169 ± 4	153 ± 4	17 ± 1	13.90 ± 0.33				23.74 ± 1.03	3.61 ± 0.32	9.90 ± 0.86	24.75 ± 5.30	1.70 ± 0.43	78.72 ± 13.21	0.43 ± 0.04	0.02 ± 0.00	39.93 ± 1.97	182.78 ± 19.01	
	45 °C	Without				8.40 ± 0.12	118 ± 42	102 ± 42	16 ± 6	13.46 ± 0.42				59.22 ± 6.85	4.35 ± 0.16	11.47 ± 0.62	20.10 ± 6.42	1.27 ± 0.14	49.62 ± 17.46	0.65 ± 0.07	0.04 ± 0.01	143.62 ± 17.77	290.35 ± 44.65	
		50 Hz; 15 m/s-2				8.43 ± 0.11	81 ± 28	72 ± 28	9 ± 2	13.53 ± 0.34				79.64 ± 7.69	4.89 ± 0.36	10.85 ± 1.09	25.47 ± 4.52	1.35 ± 0.29	62.68 ± 16.80	0.62 ± 0.06	0.03 ± 0.01	217.14 ± 15.04	402.66 ± 30.41	
	25 °C	Without								13.12 ± 0.80				73.39 ± 6.54	5.17 ± 0.64							189.07 ± 27.57	342.63 ± 57.96	
	5 °C	Without								13.52 ± 0.37				82.43 ± 12.85	5.47 ± 0.28							199.65 ± 14.22	387.60 ± 30.35	
		50 Hz and 15 m/s-2								12.86 ± 0.80				64.43 ± 1.90	4.74 ± 0.16							177.14 ± 13.32	309.36 ± 16.40	
	30 °C	Without			Storage at 30 °C during 60 days					12.80 ± 1.04				70.75 ± 2.45	4.74 ± 0.17							166.63 ± 5.74	301.22 ± 16.76	
		50 Hz and 15 m/s-2								12.53 ± 0.19				67.51 ± 2.54	4.96 ± 0.25							172.35 ± 23.40	310.61 ± 22.08	
	45 °C	Without								13.01 ± 0.46				92.84 ± 7.10	5.94 ± 0.52							253.36 ± 28.68	380.5 ± 27.95	
		50 Hz and 15 m/s-2								13.78 ± 2.08				95.15 ± 8.92	6.04 ± 0.61							314.14 ± 57.02	444.67 ± 39.69	

														Aldehydes									
														2MP (µg/L)	2MB (µg/L)	3MB (µg/L)	Methional (µg/L)	Benzaldehyde (µg/L)	Phenylacetaldehyde (µg/L)	Hexanal (µg/L)	Trans-2-nonenal	Furfural (µg/L)	Total ageing compounds (µg/L)	
Pilsner beer	0 °C		51 d			6.7 ± 0.0	118.0 ± 12.0			21.0 ± 0.1 mg/L		0.3 ± 0.0	0.4 ± 0.0	4.1 ± 0.0	1.0 ± 0.0	2.1 ± 0.0	2.1 ± 0.3	0.5 ± 0.0	10.8 ± 0.3	0.4 ± 0.2	0.03 ± 0.00	9.1 ± 0.6	30.0 ± 0.8	
	-					7.4 ± 0.1	-			19.4 ± 0.1 mg/L		0.5 ± 0.1	0.8 ± 0.1	28.5 ± 0.4	2.5 ± 0.1	6.8 ± 0.3	6.5 ± 0.7	0.7 ± 0.0	19.0 ± 0.1	0.8 ± 0.1	0.06 ± 0.01	95.4 ± 2.0	160.2 ± 3.5	
Dark specialty beer	0 °C		100 d			85.9 ± 1.2	218.0 ± 33.0			26.2 ± 0.1 mg/L				2.6 ± 0.1	0.7 ± 0.1	4.1 ± 0.2	18.4 ± 1.7	1.2 ± 0.4	27.1 ± 3.3	0.5 ± 0.2	0.05 ± 0.03	3.8 ± 0.5	58.3 ± 2.5	
	-					99.8 ± 1.6	-			24.1 ± 0.1 mg/L				79.1 ± 3.9	1.9 ± 0.1	8.4 ± 0.3	20.3 ± 2.2	1.5 ± 0.2	25.6 ± 3.0	0.6 ± 0.2	0.07 ± 0.02	114.9 ± 10.5	252.3 ± 13.6	
Pale specialty beer	0 °C					10.0 ± 0.2	562.0 ± 27.0			33.7 ± 0.2 mg/L				8.8 ± 0.0	2.1 ± 0.0	11.7 ± 0.2	17.0 ± 1.6	2.0 ± 0.2	32.7 ± 10.4	0.5 ± 0.1	0.05 ± 0.02	22.4 ± 0.3	97.1 ± 9.8	[12]
	-					11.7 ± 0.3	-			26.5 ± 0.2 mg/L				25.9 ± 0.9	3.3 ± 0.0	14.5 ± 0.1	21.9 ± 4.2	2.1 ± 0.2	35.4 ± 1.8	1.0 ± 0.0	0.06 ± 0.00	72.6 ± 2.9	176.7 ± 2.4	
Pilsner beer	0°C					7.85 ± 0.1	488.5 ± 14.8	432.5 ± 20.5	51.0 ± 0.0	19.3 ± 0.41 mg/L		0.3 ± 0.0	1.2 ± 0.1	9.3 ± 2.3	1.4 ± 0.1	5.2 ± 2.1	6.3 ± 2.9	0.6 ± 0.1	17.7 ± 5.0	0.4 ± 0.2	0.03 ± 0.01	35.9 ± 8.5	76.8 ± 6.3	
	30°C	without	30 d			8.38 ± 0.0	447.5 ± 12.0	392.5 ± 14.6	55.0 ± 2.8	19.2 ± 0.41 mg/L		0.7 ± 0.0	2.7 ± 0.1	17.0 ± 2.4	2.4 ± 0.0	9.1 ± 3.3	8.1 ± 2.5	0.8 ± 0.1	24.9 ± 5.2	0.5 ± 0.1	0.05 ± 0.00	101.8 ± 11.2	164.7 ± 9.1	
		1.7 Hz; 1.14 m/s2				8.56 ± 0.0	227.0 ± 5.7	213.5 ± 9.2	13.5 ± 3.5	18.7 ± 0.19 mg/L		1.7 ± 0.1	5.2 ± 0.0	19.0 ± 2.3	2.6 ± 0.0	10.3 ± 4.5	9.0 ± 1.7	0.7 ± 0.1	26.3 ± 8.1	0.5 ± 0.1	0.06 ± 0.00	123.4 ± 13.9	191.9 ± 10.6	

Beer style	T (°C)	Vibrations	Time	Packaging type	Other conditions	Colour (EBC)	Oxygen content (µg/L)			Iso-α-acids		Haze (FU)		Aldehydes (10-6 g/L)									References
							TPO	HSO	DO	Total (10-3 g/L)	T/C ratio	Permanent	Chill	2MP	2MB	3MB	Methional	Benzaldehyde	Phenylacetaldehyde	Hexanal	Trans-2-nonenal	Furfural	
Lager beer	20 °C		0 d				251 ± 66	232 ± 64	19.0 ± 9.4														
		5 Hz; 15 m/s2	4 d				75 - 219	69 - 214	2.3 - 5.7														
		15 Hz and 15 m/s2					27 - 240	23 - 237	3.1 - 3.4														
		30 Hz and 15 m/s2					51 - 323	48 - 319	3.2 - 4.6														
		50 Hz and 15 m/s2					ago/58	54 - 80	4.4 - 4.7														
Blonde Specialty beer without second refermentation	20 °C		0 d				169 ± 23	159 ± 23	10.4 ± 22.5														
		5 Hz and 15 m/s2	4 d				21 - 48	18 - 43	2.7 - 3.8														
		15 Hz and 15 m/s2					16 - 21	14 - 19	1.5 -2														
		30 Hz and 15 m/s2					25 - 28	22 - 26	2.1 - 2.4														
		50 Hz and 15 m/s2					33	29	3.5														[50]
Blonde specialty beer with second refermentation	20 °C		0 d				118 ± 21	110 ± 21	7.1 ± 1.0														
		5 Hz and 15 m/s2	4 d				21 - 62	19 - 59	2.7 - 3.8														
		15 Hz and 15 m/s2					16	14	1.7														
		30 Hz and 15 m/s2					23 - 24	21 - 22	1.9 - 2.4														
		50 Hz and 15 m/s2					13 - 17	nov/15	1.7 - 2.3														
Dark specialty beer with second refermentation	20 °C		0 d				700 ± 252	658 ± 235	41.5 ± 18.8					7.0 - 11.9	0.9 - 2.0	2.5 - 7.6	3.2 -15.6	0.8 - 1.4	8.4 - 48.3	0.2 - 0.5	0.02 - 0.04	2.8 - 13.3	
		5 Hz and 15 m/s2	4 d				28 - 32	24 - 30	2.3 - 3.1					6.3 - 6.7	1.5 - 1.6	6.0 - 6.1	5.0 - 6.7	0.9	14.6 - 15.3	0.3	0.02	11.4 - 12.1	
		15 Hz and 15 m/s2					14	13	1.3 - 1.5					8.9 - 9.0	2.2	8.9 - 9.0	9.7 - 10.0	1.5 - 1.6	27.5 - 29.2	0.6	0.03 - 0.04	14.1 - 15.2	
		30 Hz and 15 m/s2					18 - 23	15 - 20	2.1 - 2.9					9.9	2.4	8.7 - 9.0	14.3 - 20.5	1.4	54.1 - 57.0	0.4 - 0.5	0.04	17.6 - 18.7	
		50 Hz and 15 m/s2					26 - 34	23 - 31	2.6 - 2.8					14.1 - 14.4	3.3	11.5 - 11.6	15.9 - 21.4	2.0 - 2.2	69.8 - 70.5	0.7 - 0.8	0.06 - 0.07	23.2 - 23.8	

Beer style	T (°C)	Vibrations	Time	Packaging type	Other conditions	Colour (EBC)	Aldehydes (mg/L)	Esters (mg/L)			Ketones (µg/L)			Alcohols (mg/L)		Dimethyl Sulfide (µg/L)	References
							Acetaldehyde	Isoamyl acetate	Ethyl acetate	Esters	Diacetyl	2,3 Pentanedione	Isoamyl alcohol	1-Propanol	Higher Alcohols		
Pale Lager	20 ± 1°C		0 m	keg			5.2 ± 0.12	0.5 ± 0.01	8.9 ± 0.22	9.4 ± 0.09	7.0 ± 0.01	3.0 ± 0.01	59.0 ± 0.31	12.0 ± 0.34	81.0 ± 0.19	47.0 ± 0.01	[113]
				PET			2.5 ± 0.26	0.6 ± 0.03	9.6 ± 0.26	10.2 ± 0.16	7.0 ± 0.01	6.9 ± 0.01	57.1 ± 0.23	13.7 ± 0.23	79.5 ± 0.20	25.2 ± 0.01	
				Glass			2.4 ± 0.51	0.6 ± 0.04	9.6 ± 0.34	10.2 ± 0.17	7.0 ± 0.03	7.1 ± 0.01	56.2 ± 0.11	12.8 ± 0.12	77.9 ± 0.29	27.2 ± 0.03	
				Can			2.4 ± 0.31	0.6 ± 0.01	8.1 ± 0.42	8.7 ± 0.08	10.0 ± 0.01	11.6 ± 0.03	55.7 ± 0.18	14.9 ± 0.12	81.0 ± 0.17	24.9 ± 0.02	
			1 m	keg			5.3 ± 0.21	0.5 ± 0.03	8.6 ± 0.21	9.1 ± 0.06	10.0 ± 0.01	5.0 ± 0.02	60.0 ± 0.16	12.0 ± 0.14	80.0 ± 0.28	38.9 ± 0.02	
				PET			4.3 ± 0.21	0.6 ± 0.02	9.5 ± 0.12	10.0 ± 0.08	14.0 ± 0.03	15.0 ± 0.01	58.0 ± 0.62	12.0 ± 0.14	79.0 ± 0.34	40.8 ± 0.01	
				Glass			3.0 ± 0.24	0.5 ± 0.03	9.4 ± 0.13	9.9 ± 0.06	8.0 ± 0.01	9.0 ± 0.02	56.0 ± 0.34	12.0 ± 0.16	76.6 ± 0.16	48.8 ± 0.02	
				Can			2.5 ± 0.31	0.5 ± 0.01	8.2 ± 0.12	8.7 ± 0.07	8.0 ± 0.03	9.2 ± 0.01	55.0 ± 0.23	15.0 ± 0.15	80.0 ± 0.35	36.5 ± 0.01	
				keg			5.7 ± 0.12	0.6 ± 0.03	9.2 ± 0.12	9.8 ± 0.18	11.0 ± 0.02	5.0 ± 0.01	60.0 ± 0.45	12.0 ± 0.23	80.0 ± 0.35	37.4 ± 0.01	
				PET			5.3 ± 0.14	0.6 ± 0.01	9.4 ± 0.13	10.0 ± 0.13	10.0 ± 0.01	16.0 ± 0.01	58.0 ± 0.23	12.5 ± 0.12	79.0 ± 0.12	6.3 ± 0.01	
			2 m	Glass			3.0 ± 0.44	0.6 ± 0.02	9.4 ± 0.13	9.9 ± 0.14	9.0 ± 0.02	8.2 ± 0.02	57.0 ± 0.12	12.3 ± 0.23	78.0 ± 0.34	53.3 ± 0.02	
				Can			3.3 ± 0.24	0.6 ± 0.01	9.4 ± 0.12	10.0 ± 0.09	8.0 ± 0.02	10.5 ± 0.02	62.8 ± 0.12	12.8 ± 0.25	87.0 ± 0.14	37.2 ± 0.02	
				keg			6.0 ± 0.14	0.6 ± 0.03	9.2 ± 0.12	9.8 ± 0.12	11.0 ± 0.02	9.0 ± 0.03	61.0 ± 0.23	12.0 ± 0.32	83.0 ± 0.42	41.3 ± 0.03	
				PET			5.6 ± 0.41	0.5 ± 0.02	9.2 ± 0.23	9.7 ± 0.15	23.5 ± 0.01	18.0 ± 0.02	57.0 ± 0.24	12.0 ± 0.15	78.0 ± 0.14	9.9 ± 0.03	
			3 m	Glass			3.0 ± 0.23	0.5 ± 0.01	9.2 ± 0.26	9.7 ± 0.16	10.0 ± 0.03	9.0 ± 0.02	57.0 ± 0.36	12.2 ± 0.14	78.0 ± 0.16	52.4 ± 0.02	
				Can			3.4 ± 0.15	0.6 ± 0.03	9.4 ± 0.10	10.0 ± 0.17	8.0 ± 0.02	9.6 ± 0.02	62.0 ± 0.28	12.6 ± 0.34	86.0 ± 0.34	36.4 ± 0.01	
				keg			6.1 ± 0.23	0.6 ± 0.02	9.3 ± 0.16	9.9 ± 0.17	9.0 ± 0.01	5.9 ± 0.02	61.0 ± 0.43	12.2 ± 0.22	83.0 ± 0.39	35.2 ± 0.02	
				PET			6.2 ± 0.11	0.5 ± 0.03	9.4 ± 0.12	10.0 ± 0.08	26.0 ± 0.01	18.0 ± 0.01	59.0 ± 0.32	13.0 ± 0.21	82.0 ± 0.23	17.1± 0.01	
			4 m	Glass			3.5 ± 0.13	0.5 ± 0.01	9.3 ± 0.12	9.8 ± 0.06	7.0 ± 0.02	7.4 ± 0.01	60.0 ± 0.21	12.8 ± 0.23	81.0 ± 0.28	47.5 ± 0.01	
				Can			3.3 ± 0.11	0.6 ± 0.01	9.0 ± 0.15	9.6 ± 0.16	9.0 ± 0.02	9.0 ± 0.02	60.0 ± 0.27	12.0 ± 0.23	83.0 ± 0.28	35.5 ± 0.02	
				keg			5.8 ± 0.13	0.5 ± 0.02	9.0 ± 0.15	9.5 ± 0.17	10.0 ± 0.02	35.5 ± 0.02	59.7 ± 0.45	12.0 ± 0.15	82.0 ± 0.38	25.5 ± 0.01	
				PET			6.9 ± 0.31	0.6 ± 0.01	9.9 ± 0.14	10.5 ± 0.16	54.0 ± 0.01	28.8 ± 0.02	59.9 ± 0.38	12.9 ± 0.15	82.0 ± 0.24	25.4 ± 0.04	
			5 m	Glass			3.9 ± 0.14	0.6 ± 0.02	9.5 ± 0.14	10.1 ± 0.13	10.0 ± 0.02	13.5 ± 0.03	58.4 ± 0.18	12.5 ± 0.32	80.0 ± 0.23	63.6 ± 0.01	
				Can			3.2 ± 0.25	0.6 ± 0.01	9.3 ± 0.15	9.9 ± 0.12	9.0 ± 0.01	9.0 ± 0.02	62.0 ± 0.24	13.0 ± 0.17	86.0 ± 0.17	31.2 ± 0.01	
				keg			6.0 ± 0.24	0.6 ± 0.03	8.5 ± 0.14	9.0 ± 0.12	12.0 ± 0.01	22.8 ± 0.02	60.2 ± 0.32	12.3 ± 0.15	82.0 ± 0.23	25.4 ± 0.03	
				PET			6.4 ± 0.13	0.6 ± 0.02	9.7 ± 0.14	10.3 ± 0.09	50.0 ± 0.01	28.0 ± 0.01	60.0 ± 0.36	12.8 ± 0.14	82.0 ± 0.23	5.5 ± 0.01	
			6 m	Glass			3.6 ± 0.31	0.6 ± 0.01	9.3 ± 0.26	9.8 ± 0.07	12.0 ± 0.01	12.0 ± 0.02	58.0 ± 0.29	12.2 ± 0.23	79.0 ± 0.34	48.6 ± 0.03	
				Can			3.4 ± 0.21	0.6 ± 0.02	9.7 ± 0.21	10.3 ± 0.05	11.0 ± 0.01	12.0 ± 0.02	65.0 ± 0.34	13.0 ± 0.27	89.0 ± 0.34	29.7 ± 0.02	

Supplementary material regarding the quantification of the target aldehydes in the maritime transport simulation sets and forced ageing sets.

Table 5: Detailed quantification results of the 10 aldehydes (three samples *per* batch). Concentration expressed in µg/L as mean value ± standard deviation.

Compound	Batch	Fresh (T0)	Transport simulation	Transport&Storage simulation	Transport simulation control	Transport&Storage simulation control	2 months	6 months	8 months
Acetaldehyde	1	1040.7±134.1	1055.0±428.8	1038.0±130.2	791.0±391.2	nd	1021.0±218.0	1559.0±288.5	1831.0±324.5
	2	1265.0±280.9	707.0±88.4	497.1±172.0	617.4±141.4	nq	1033.4±149.1	1291.2±203.8	1834.6±178.3
	3	1114.6±193.5	710.0±311.8	980.3±205.2	635.5±165.2	602.0±298.4	1466.0±297.0	1935.0±384.8	1855.6±161.1
	4	593.0±292.5	647.0±284.1	823.3±138.2	989.0±483.3	495.2±174.5	1593.1±150.0	1692.0±410.3	2059.3±262.3
	5	858.0±250.3	857.3±267.8	1385.9±188.0	1024.2±169.9	515.8±166.6	1425.0±241.9	2381.0±271.9	2445.6±231.6
Strecker degradation aldehydes									
2-methylpropanal	1	4.7±0.7	6.2±0.1	8.7±1.4	3.5±0.5	4.5±0.6	10.4±1.2	8.4±1.2	6.5±0.7
	2	9.5±1.2	13.2±1.1	4.0±0.6	4.5±0.6	5.1±0.6	12.9±1.2	11.4±1.4	10.1±1.2
	3	4.1±0.8	11.4±1.1	3.0±0.6	2.9±0.4	2.0±0.7	5.1±0.5	11.8±0.7	8.0±0.9
	4	5.4±1.2	14.1±2.3	3.7±0.4	3.9±0.6	nd	4.9±0.5	11.7±1.1	5.5±1.6
	5	3.2±1.0	11.5±0.6	3.1±0.4	6.1±0.9	nd	3.7±0.5	12.7±1.2	7.9±0.8
2-methylbutanal	1	nd	nd	nd	nd	nd	nd	nd	nd
	2	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd
	4	nd	nd	nd	nd	nd	nd	nd	nd
	5	nd	nd	nd	nd	nd	nd	nd	nd
3-methylbutanal	1	4.2±1.1	4.4±1.4	5.1±1.1	3.9±0.2	3.6±0.6	4.8±0.5	4.6±0.5	5.5±0.6
	2	5.6±0.8	6.1±0.3	5.9±0.4	4.6±0.7	4.5±0.7	5.1±5.8	5.5±0.6	4.6±0.3
	3	2.5±0.6	10.1±1.1	3.7±0.6	3.8±1.2	4.0±0.8	4.4±0.3	5.3±0.7	4.4±0.5
	4	3.9±0.6	12.0±1.0	5.1±0.5	4.5±0.5	2.9±0.4	4.1±0.5	5.4±0.6	4.7±0.7
	5	3.9±1.6	10.7±1.8	5.2±0.7	5.8±0.8	1.7±0.3	3.7±0.5	5.5±0.6	5.1±0.5
Benzaldehyde	1	6.2±0.7	5.7±0.3	6.3±0.3	5.6±0.3	6.9±0.7	5.7±0.3	5.3±0.2	5.3±0.4
	2	6.4±0.5	6.0±0.2	6.3±0.3	5.2±0.3	7.1±0.4	5.8±0.4	5.0±0.2	5.4±0.2
	3	5.6±0.5	7.4±0.2	6.6±0.5	5.0±0.2	5.7±0.2	4.9±0.2	5.4±0.3	5.4±0.2
	4	5.4±0.4	7.4±0.4	6.5±0.3	5.1±0.3	5.6±0.6	5.1±0.2	5.7±0.4	5.4±0.5
	5	5.2±0.2	6.8±0.5	6.1±0.6	5.2±0.2	5.6±0.3	4.8±0.3	5.4±0.5	5.5±0.2
Phenylacetaldehyde	1	87.6±6.6	107.2±10.5	178.3±8.1	115.6±5.5	175.9±14.9	169.7±9.9	161.7±11.1	163.3±8.3
	2	97.1±7.2	116.1±9.0	168.5±7.9	109.6±10.5	176.4±8.7	169.5±11.0	155.3±6.0	162.1±6.7
	3	95.9±7.7	164.5±8.4	170.4±11.2	106.5±3.7	153.4±10.8	155.6±13.2	156.9±8.0	165.3±11.5
	4	96.1±6.6	165.6±7.9	161.3±7.7	103.5±5.8	149.2±6.8	153.4±11.0	159.2±7.5	171.2±9.3
	5	97.0±8.3	164.6±4.4	166.2±9.4	113.4±6.8	158.2±5.7	150.6±19.3	167.1±16.2	169.9±7.0
Lipid Oxidation aldehydes									
Hexanal	1	0.8±0.2	0.8±0.0	1.3±0.2	nq	1.0±0.1	1.2±0.4	1.0±0.2	0.9±0.1
	2	0.8±0.1	1.6±0.2	1.7±0.2	nq	1.4±0.2	1.9±0.2	2.1±0.2	1.9±0.3
	3	nd	2.2±0.4	1.4±0.1	nd	nd	nq	1.6±0.2	1.3±0.2
	4	nd	2.4±0.0	1.2±0.1	nd	nd	0.7±0.2	1.4±0.2	0.9±0.2
	5	nd	1.9±0.3	1.1±0.1	nd	nd	nq	1.6±0.1	1.1±0.1
Heptanal	1	nd	nd	nd	nd	nd	nd	nd	nd
	2	nd	nd	nd	nd	nd	nd	nd	nd
	3	nd	nd	nd	nd	nd	nd	nd	nd
	4	nd	nd	nd	nd	nd	nd	nd	nd
	5	nd	nd	nd	nd	nd	nd	nd	nd
Nonanal	1	4.0±0.5	2.6±0.1	1.9±0.4	4.3±0.6	1.7±0.4	2.5±0.2	1.7±0.2	2.2±0.3
	2	4.5±0.6	3.2±0.3	2.8±0.3	3.1±0.5	1.4±0.3	2.2±0.3	2.6±0.3	2.6±0.4
	3	2.2±0.2	nq	nq	2.0±0.3	1.3±0.3	2.7±0.3	2.1±0.2	nq
	4	2.1±0.2	nq	nq	2.1±0.3	1.4±0.3	4.8±0.4	2.0±0.2	nq
	5	1.8±0.5	nq	nq	2.2±0.3	2.2±0.2	2.1±0.1	1.9±0.2	1.8±0.2
Trans-2-nonenal	1	0.5±0.1	0.4±0.1	0.4±0.1	0.4±0.0	0.4±0.0	1.0±0.1	0.7±0.1	0.5±0.1
	2	0.7±0.1	0.7±0.1	0.4±0.1	0.6±0.3	0.4±0.0	1.4±0.1	0.9±0.2	0.7±0.1
	3	0.5±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.3±0.0	0.8±0.1	1.5±0.1	1.1±0.1
	4	0.7±0.1	0.5±0.1	0.5±0.0	0.6±0.1	nq	1.1±0.1	1.5±0.2	0.8±0.2
	5	0.5±0.1	0.7±0.1	0.4±0.0	0.8±0.1	0.4±0.1	0.8±0.1	1.4±0.3	1.0±0.2

nd: not detected; nq: not quantified