Universidade do Minho
Escola de Engenharia

Eduardo João Louro Coelho

Valorization of woods from wine ageing and development of methodologies for their reutilization

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Eduardo João Louro Coelho

Valorization of woods from wine ageing and development of methodologies for their reutilization

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Trabalho efetuado sob a orientação do
Professor José Maria Marques Oliveira
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Professora Maria Teresa de Jesus Simões Campos Tavares

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Abstract

Valorization of woods from wine ageing and development of methodologies for their reutilization

Ageing is a key step in the production of several premium products and has been for long used in the modification of sensory characteristics of alcoholic beverages. Scientific research has unraveled the complex phenomena occurring during ageing, focusing mainly on beverage transformations.

This thesis however, sets out on understanding the transformations occurring to cooperage wood when used in ageing beverages, and its impact when reused for ageing subsequent matrices.

Study of mass transfer showed that the porous structure of oak wood retains wine, along with its chemical composition. Uptake follows a saturation curve, with rate of uptake influenced by particle size and maximum uptake influenced by wood variety. Several wine volatile compounds are retained in wood, namely esters, alcohols and acids, while wood still maintains some content in its characteristic compounds, namely furans, lactones, phenolic compounds and aldehydes. Research on compound extraction showed that wine volatiles are adsorbed by hydrophobic interactions with wood, generating novel extractable composition. These compounds are subsequently extracted to other matrices when reusing cooperage wood, attaining a novel partition equilibrium. Ethanol concentration was shown to affect significantly extraction of compounds adsorbed by wood, whereas temperature showed no significant effect. Extraction of wood compounds is also affected by ethanol concentration as well as temperature for some compounds. Thus, control of extraction conditions can lead to targeted volatile composition. Impact of wood reuse was demonstrated by reapplying oak chips for the incorporation of volatile compounds in beer, wine and spirits, where beer stood out as a more favorable matrix. By using different wood concentrations and extraction temperatures, different volatile compositions were generated, leading to different perceived sensory characteristics. Transference of wine compounds was also shown in traditional barrel ageing of beer, where ageing methods further impacted volatile composition. A prominence of *D. bruxellensis* was identified, which motivated its isolation for the development of an accelerated process for beer ageing. *D. bruxellensis* was applied in co-culture with *S. cerevisiae* as multi-starter for beer ageing, which allowed the accelerated production of metabolites associated with barrel aged beer. Thus, the added value of used cooperage wood was shown along with methodologies for its reutilization and revalorization.

Keywords: extraction; oak wood; sensory properties; sorption; wood ageing;

Resumo

Valorização de madeiras de maturação de vinho e desenvolvimento de metodologias para a sua reutilização

A maturação é um passo chave na produção de vários produtos *premium*, e tem sido usada há muito na modificação de características sensoriais de bebidas. A investigação tem desvendado fenómenos complexos que ocorrem durante a maturação, focando principalmente nas transformações da bebida. Esta tese, no entanto, visa compreender as transformações que ocorrem na madeira de tanoaria quando usada na maturação de bebidas, e o seu impacto quando reutilizadas na maturação de matrizes subsequentes. O estudo da transferência de massa mostrou que a estrutura porosa da madeira de carvalho retém vinho, tal como a sua composição química. A absorção segue uma curva de saturação, sendo a taxa influenciada pelo tamanho da partícula e a absorção máxima influenciada pela variedade de madeira. Vários compostos voláteis do vinho são retidos na madeira, nomeadamente ésteres, álcoois e ácidos, enquanto a madeira mantém ainda algum teor em compostos característicos, nomeadamente furanos, lactonas, compostos fenólicos e aldeídos. A investigação da extração mostrou que os voláteis do vinho são adsorvidos por interações hidrofóbicas com a madeira, gerando uma nova composição extrativa. Estes compostos são subsequentemente extraídos para outras matrizes durante a reutilização, com um novo equilíbrio de partição. Demonstrou-se que a concentração de etanol afeta significativamente a extração de compostos adsorvidos, enquanto que a temperatura não mostrou efeitos significativos. A extração de compostos da madeira é também afetada pela concentração de etanol, tal como, para alguns deles, pela temperatura. Portanto, o controlo das condições de extração pode levar a uma composição volátil desejada. O impacto da reutilização foi demonstrado reaplicando aparas de carvalho para a incorporação de compostos voláteis em cerveja, vinho e destilados, onde a cerveja se destacou como matriz mais favorável. Diferentes concentrações de madeira e temperaturas de extração, permitiram obter diferentes composições voláteis, levando à perceção de diferentes características sensoriais. A transferência de compostos foi também demonstrada na maturação de cerveja em barrica, onde os métodos de maturação influenciaram adicionalmente a composição volátil. Foi identificada uma proeminência de *D. bruxellensis*, motivando o seu isolamento para desenvolvimento de um processo acelerado de maturação de cerveja. Aplicou-se *D. bruxellensis* em co-cultura com *S. cerevisiae* como inóculo *multi-starter* para a maturação de cerveja, permitindo a produção acelerada de metabolitos associados à cerveja maturada. O valor acrescentado da madeira de tanoaria usada foi demonstrado, tal como metodologias para a sua reutilização e revalorização.

Palavras-chave: adsorção; extração; madeira de carvalho; maturação; propriedades sensoriais;

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List of abbreviations, variables and acronyms

Abbreviations and acronyms

AOAC- association of official agricultural chemists

BET- Brunauer–Emmett–Teller

BJCP- Beer judge certification program

DMS – dimethyl sulfide

FTIR- fourier transform infrared

GC- gas chromatography

GC-MS - gas chromatography – mass spectrometry

HPLC- high performance liquid chromatography

HS- headspace

IBU- International bitterness unit

IT- ion-trap

LLE- liquid-liquid extraction

LLME- liquid-liquid micro-extraction

MS - mass spectrometry

PBS- phosphate buffered saline

PCA- principal component analysis

RI- refraction index

SDME- single drop micro-extraction

SEM- Scanning electron microscope

SPE- sorbent-phase extraction

SPME- solid-phase micro-extraction

UV- ultra-violet

Variables

a_1, b_1, c_1– linear coefficients

A_x, A_{is} – peak area of the compound x, peak area of the internal standard

b – slope of the regression curve; intercept of the regression curve

a_2, b_2, c_2– quadratic coefficients

C – concentration

C_{beer}, C_{spik} – concentration in beer, concentration spiked

C_{determ}, C_{expect}, C_{measur} – determined concentration, expected concentration, measured concentration

C_{EtOH}, C_{cell} – ethanol concentration, cellular concentration

C_x, C_{is} – concentration of the compound x, concentration of the internal standard

F – relative frequency

GM – geometric mean

H_{i} – initial moisture

I – relative intensity

K_U – time needed for reaching half of U_{max}

LOD – limit of detection

LOQ – limit of quantification

m/z – mass-to-charge ratio

n – number of replicates/essays

$^{\circ}P$ – plato concentration

P – purity

p – significance level

PT – perception threshold

R^2 – Pearson correlation coefficient

RE – relative error

Rec – compound recovery

R_{f} – response factor

RSD – relative standard deviation

s – standard deviation

SA – particle surface area

T – temperature

t – time

$U(t)$ – uptake expressed as mass gained

U_{max} – maximum uptake expressed as mass gained

$\overline{x}$ – average of the measured values

Latin expressions

e.g. – *exempli gratia* (for example)

et al. – *et alii* (and others)

i.e. – *id est* (that is)

Scientific outputs

Papers in peer reviewed journals:

Coelho, E., Lemos, M., Genisheva, Z., Domingues, L., Vilanova, M., & Oliveira, J. M. (2020). Validation of a LLME/GC-MS methodology for quantification of volatile compounds in fermented beverages. *Molecules*, 25(3), 1–10. https://doi.org/10.3390/molecules25030621

Coelho, E., Domingues, L., Teixeira, J. A., Oliveira, J. M., & Tavares, T. (2019). Understanding wine sorption by oak wood: Modeling of wine uptake and characterization of volatile compounds retention. *Food Research International*, 116, 249–257. https://doi.org/10.1016/j.foodres.2018.08.025

Coelho, E., Teixeira, J. A., Domingues, L., Tavares, T., & Oliveira, J. M. (2019). Factors affecting extraction of adsorbed wine volatile compounds and wood extractives from used oak wood. *Food Chemistry*, 295, 156–164. https://doi.org/10.1016/j.foodchem.2019.05.093

Coelho, E., Magalhães, J., Pereira, F. B., Macieira, F., Domingues, L., & Oliveira, J. M. (2019). Volatile fingerprinting differentiates diverse-aged craft beers. *LWT- Food Science and Technology*, 108, 129–136. https://doi.org/10.1016/j.lwt.2019.03.044

Coelho, E., Azevedo, M., Teixeira, J. A., Tavares, T., Oliveira, J. M., & Domingues, L. (2020). Evaluation of multi-starter *S. cerevisiae*/ *D. bruxellensis* cultures for mimicking and accelerating transformations occurring during barrel ageing of beer. *Food Chemistry*, 126826. https://doi.org/10.1016/j.foodchem.2020.126826

Oral communications by invitation:

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Coelho, E., Domingues, L., Teixeira, J.A., Tavares, T., Oliveira, J.M. The added value of used cooperage wood. *Winery2019 - 9th Specialized Conference on Sustainable Viticulture, Winery Wastes &Agroindustrial Wastewater Management*. Mons, Belgium, 2019

Poster communications:

Coelho, E.; Magalhães, J.G.; Oliveira, J.M.; Domingues, L. Exploring barrel aged beer heterogeneity using volatile fingerprinting. *Microbiotec'17 - Congress of Microbiology and Biotechnology 2017*. No. P-244, Porto, Portugal, Dec 7-9, 361, 2017

Motivation and Outline of the Thesis

Motivation

Production of alcoholic beverages is maybe one of the most ancient models of biotechnology, where tradition meets innovation in the constant search for better products and processes. Despite being carried out for so long, some processes still maintain their secrets and are yet being studied and reinvented. Such is the case of barrel ageing of alcoholic beverages, an ancient and complex process, which is widely acknowledged as a key step in the production of several well-known premium products.

In its lifecycle, cooperage wood, namely barrels and their alternatives, is produced, used and reused, during which it suffers transformations that affect the quality of the aged beverages. Throughout ageing, the transference of compounds between wood and the beverage is bilateral, and most of the existing work focuses on the transformations occurring to the beverage.

This thesis is set out to understand what particularly occurs to wood during ageing, which can impart subsequent ageing processes. With this purpose, a special focus is put on sorption of beverage compounds by wood, along with the mechanisms involved, in order to understand the transformations that can add value to used cooperage wood. Such compounds can be transferred to other beverages by reuse of wood, making it a vector for recombination of aromas between beverages.

Further on, this thesis also aimed at studying the conditions for the extraction of adsorbed beverage compounds from wood to new matrices, along with available wood extractives, envisaging the development of accelerated ageing methodologies for mimicking additive maturation stages. Lastly, this thesis also aimed at shedding a light on the impact of wood when reused in ageing beverages, with a particular interest on sensory active compounds.

Considering the high diversity of wood and beverages to be combined, this work focused on the transference of compounds from fortified wine to wood, and subsequently to other matrices. A special emphasis is also put on wood ageing of beer, taking into account the current growth in the craft beer market and the scarce scientific knowledge existent regarding this theme. In order to develop an accelerated ageing method, particularly for beer, this work also aims for the study and control of the microbiological related transformations occurring during ageing, in order to develop mixed culture fermentations to be combined with wood usage.

Therefore, the specific objectives were envisaged:

- Determination of the content of specific wine compounds (phenolics, volatile compounds and organic acids) in woods reused from wine ageing;
- Characterization of sorption and retention dynamics of wine compounds in wood;
- Development of extraction methodologies that allow the transference and incorporation of wood and wine compounds metabolites in the focused beverages by reusing wood;
- Isolation and characterization of specific barrel ageing microorganisms for the development of mixed culture fermentations to be applied for microbiological ageing;

With reaching these objectives this thesis intends to enrich the scientific knowledge about the theme, to develop methodologies for industrial application and to potentiate wood re-usage and valorization, in processes typically of empiric nature.

Outline

This thesis is organized in 7 different chapters, that compose a logical structure to understand the proposed research objectives:

Chapter 1 discloses the state of the art regarding cooperage wood characteristics and utilization in beverage ageing, setting the theoretical basis for the presented work.

Chapter 2 presents the validation of the LLME/GC-MS methodology widely used in the analyses performed throughout this thesis.

Chapter 3 presents a structured study of wood sorption of wine and its compounds. The uptake of wine by the porous structure of wood is demonstrated, along with mathematical models demonstrating mass transfer dynamics in different varieties of oak wood. This is complemented by the identification of volatile compounds in oak wood after contact with wine, which demonstrates the adsorbed wine compounds along with the availability of wood extractives.

Chapter 4 shows the impact of process variables in the breakage of the bond between beverage compounds and wood. With this, the chemical driver force of sorption is identified, which completes the work started in chapter 3. Also, the study of extraction demonstrates the impact and potential of wood reuse in subsequent ageing processes, providing also knowledge on the most desirable variables to be used in the development of an accelerated ageing methodology with the outline of extraction mathematical models for each compound.

Chapter 5 sets on the case study of wood reuse in the additive ageing of different beverages. Used wood is applied to beer, wine and spirit ageing, where the impact of transference of volatile compounds is demonstrated. Also, adsorption of further compounds is identified based on the different beverages studied, with impact in beverage and in wood composition. An additional focus is put on the case study of beer, where different formulations are studied along with their correlation with changes in sensory properties of beer.

Chapter 6 explores the transformations occurring to beer when aged in oak barrels previously used in wine ageing, using different approaches. In this work, the impact of wood reuse is shown in traditional barrel ageing of beer. Several compounds and transformations of interest are identified, as well as the impact of spontaneous barrel fermentation by endogenous barrel microbes.

Chapter 7 presents an additional effort in the development of a methodology for the particular case of beer ageing, by demonstrating the impact of co-cultures when applied for promoting transformations inherent to biological ageing of beer. Multi-starters of *S. cerevisiae* and *D. bruxellensis*, are demonstrated as a suitable strategy for controlling and accelerating the production of metabolites associated with aged beer aroma.

Chapter 8 sums up the general conclusions regarding the added value and reutilization of used cooperage wood and presents work perspectives on the theme.

Chapter 1. General Introduction

This chapter sums up the state of the art regarding cooperage wood lifecycle and its utilization in ageing of alcoholic beverages.

1. Cooperage production

1.1 Selection of wood

Cooperage wood and manufacture processes are highly dependent on the beverages to be aged and the intended product. Several different tree species can be used, for example chestnut (*Castanea sativa*), acacia (*Robinia pseudoacacia*), cherry (*Prunus* sp.), ash (*Fraxinus* sp.) and oak (*Quercus* sp.) (De Rosso, Cancian, Panighel, Dalla Vedova, & Flamini, 2009; De Simón, Esteruelas, Muñoz, Cadahía, & Sanz, 2009). Among them, oak is the most widely used wood in cooperage and beverage production, mainly due to its permeability, handling ease, flexibility and resistance (del Álamo-Sanza & Nevares, 2018; Martínez-Gil, del Álamo-Sanza, Sánchez-Gómez, & Nevares, 2018). Two main varieties can be highlighted among the species within the *Quercus* genus: American oak (*Quercus alba*) and French oak (*Quercus robur* or *Quercus petraea*). These varieties are designated after the major areas supplying such species, considering that forests from which oak trees are cut are mainly located in the United States of America and France, as depicted in Figure 1.1. Due to the high demand and price of American and French oak, several alternative varieties and provenances have been explored for cooperage production, namely Portuguese and Spanish species (*Quercus pyrenaica*) (Fernández de Simón, Cadahía, del Álamo-Sanza, & Nevares, 2010; Garcia, Soares, Dias, Freitas, & Cabrita, 2012), East European (Prida & Puech, 2006) and Colombian oak (*Quercus humboldtii*) (Martínez-Gil *et al.*, 2019).

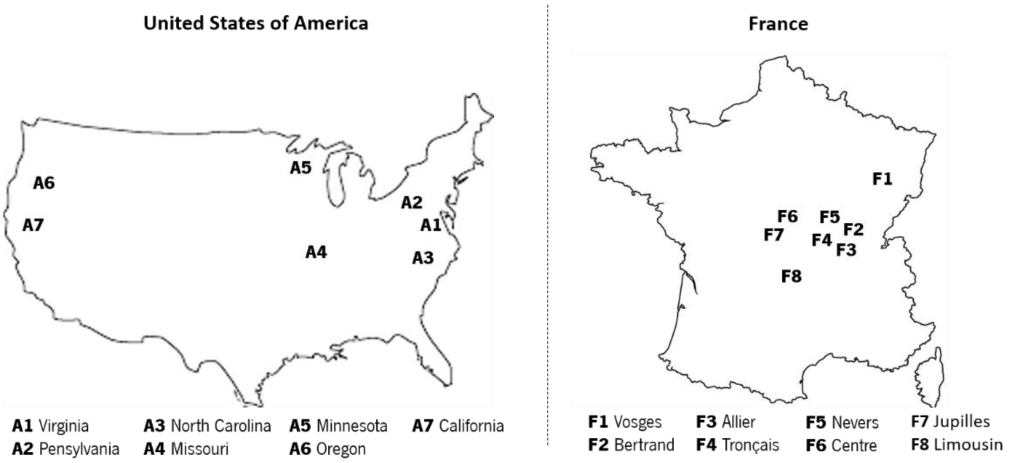


Figure 1.1: Main forests for cooperage oak production (adapted from Zamora, 2019).

Silviculture for oak production is ruled rigorously, as coopers exploit government controlled forests. Only trees above 100 years old can be exploited, being the best specimens found between 120 and 160 years old (Zamora, 2019). Apart from origin and species, a particular characteristic to take into account when selecting trees for cooperage production is grain. During growth, tree trunks expand radially and when examining a trunk cross section, a set of rings can be seen. Medullary rays cross the radial section of the tree trunk from the center to the edge, as better depicted in Figure 1.2, crossing the annual rings. Annual rings are comprised by two layers, one of spring wood and other of summer wood with different characteristics due to water availability, sap flow and growth rate. These rings compose wood grain, which is defined as ring dimension or number of rings per cm of wood. Oak wood can be classified as fine grain (> 5 rings per cm), medium grain (between 3 and 4 rings per cm) or coarse grain (< 2 rings per cm) (Vivas, 1995).

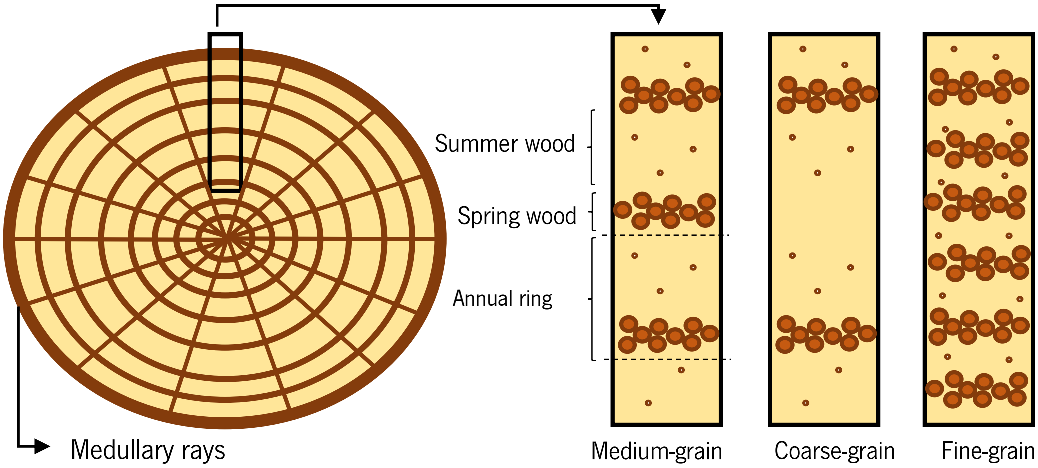


Figure 1.2: Tree trunk cross section representation (adapted from Zamora, 2019).

Grain is commonly accepted as a predictive measure of wood quality and characteristics, namely liquid/air permeability (del Álamo-Sanza & Nevares, 2018) and extractive composition, *e.g.* ellagitanin content (Jordão, Ricardo-da-Silva, & Laureano, 2007), being still the main parameter considered by coopers for wood selection. Composition and characteristics of oak wood are highly variable, depending on species, origin and edaphoclimatic conditions, which are further modulated and modified by the cooperage manufacturing steps.

1.2 Cooperage wood processing

For barrel manufacture, oak trees are selected, cut and prepared for cooperage processing, as depicted in Figure 1.3. Trunks are cut in the transverse direction, either using a wedge or a saw, for obtaining staves, which will compose the barrel. The type of oak wood used generally influences the cutting technique. American oak can be cut by saw because it has higher tylose content, making it more resistant and water tight when compared with the French variety, which generally must be split by wedge. To counteract the lower tylose content, wedge cutting of French oak generates staves with parallel medullary rays, which oppose liquid flow direction and consequently promote liquid permeability (Zamora, 2019). After cutting, staves are stacked and dried by air exposure or kiln-dried to harden the wood and enhance its durability. During this period, seasoning of wood occurs, hence its composition is modified, promoted by exposure to the elements (temperature, humidity, light) during a long period of time.

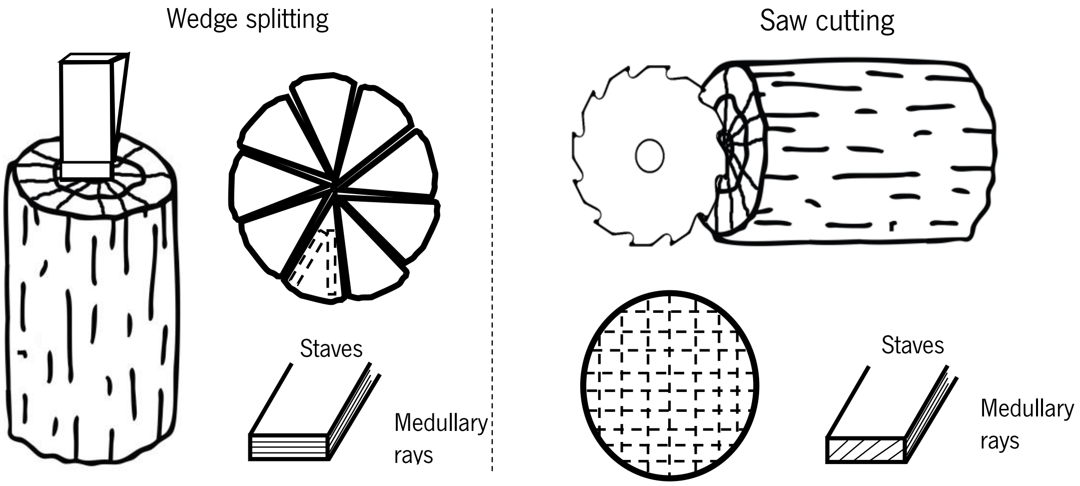


Figure 1.3: Stave cutting techniques (adapted from Zamora, 2019).

Several cumulative phenomena occur: exposure to light triggers degradation and oxidation phenomena, breaking wood macromolecules; rain promotes leaching of wood extractives and microorganisms and fungi grow on wood surface, releasing enzymes that further modify wood composition (Fernández de Simón *et al.*, 2010). With these phenomena, several chemical modifications occur in wood, decreasing content in undesired polyphenolic compounds (*e.g.* excess of ellagitanins or coumarins) and volatile compounds [*e.g.* 1-hexanal, 1-hexanol, 1-nonenal, (*E*)-2-octenal] (Martínez, Cadahía, de Simón, Ojeda & Rubio, 2008). Dried/seasoned staves are then bent with the aid of moisture or steam to assemble the barrel, followed by a toasting process, which technique also depends on the oak variety used (Figure 1.4). Generally, the American technique resorts to a heavy burn, applying flames directly onto wood surface and promoting the formation of char. The European technique applies a much lighter toast, with the flame placed in the center of the barrel, leading to a milder roasting of wood surface. Toasting affects profoundly wood composition, by promoting hydrothermolysis and pyrolysis reactions which degrade wood polymers. Pyrolysis of lignin produces volatile phenols and phenolic aldehydes, polysaccharides are degraded producing furanic compounds and lipid degradation produces lactones (Cadahía, Fernández de Simón, & Jalocha, 2003; Caldeira, Clímaco, Bruno de Sousa, & Belchior, 2006; Collins, Miles, Boulton, & Ebeler, 2015). Having such impact on wood extractive content, toasting is a fine tuned process, with each cooper having its own method. Toasting intensity depends on the binomial relation between heat intensity and contact time, allowing the discrimination of light, medium and heavy toasting levels (Chatonnet, Cutzach, Pons, & Dubourdieu, 1999). By modifying toasting intensity, the extractable pool available in wood is also modified.

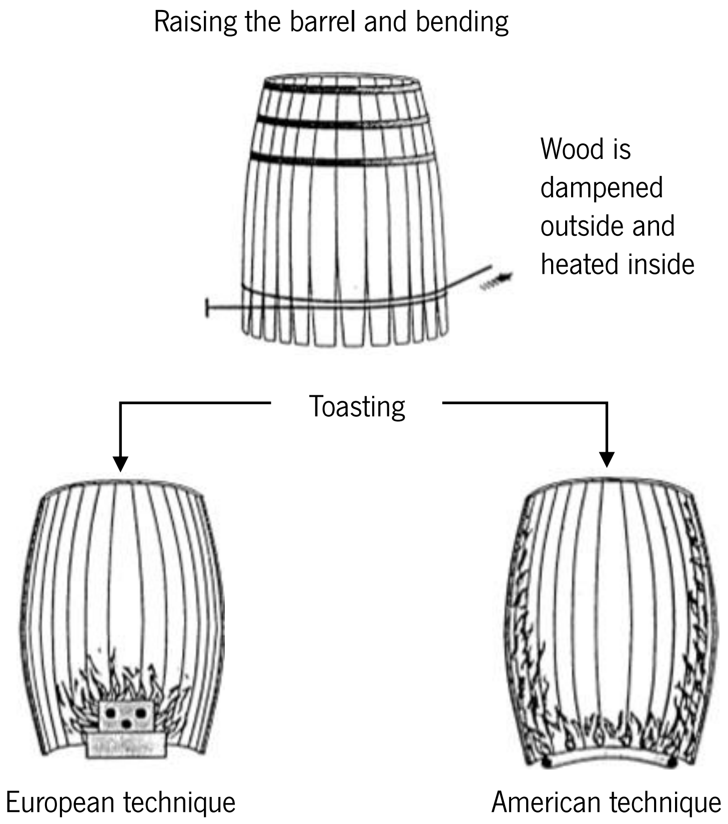


Figure 1.4: Techniques used for barrel burn (adapted from Mosedale & Puech, 1998).

Increasing toasting severity leads to the decrease of the extractable content of ellagitanins, due to their thermal degradation (Chira & Teissedre, 2014; Jordão *et al.*, 2007) and increase in extractable volatile compounds such as furan compounds, lactones and aldehydes, deriving from pyrolysis (Caldeira *et al.*, 2006; de Simón *et al.*, 2010).

1.3 Cooperage products

Apart from oak variety, seasoning and toasting, beverage producers can further direct and control ageing processes by selecting the most suitable cooperage product. Coopers supply the market with a wide variety of wood products, either following traditional or innovative approaches.

Traditionally, barrels are still the main traded product in the cooperage industry, as well as the main tool used for wine, spirits or beer ageing. Several variations can be found for container shape and volume, ranging from small barrels to large volume tuns, each with its specific intended purpose. Varying barrel volume and design alters not only the amount of liquid stored but also the amount of wood extractives incorporated in the beverage. A small vessel, will have a higher ratio of wood surface per liquid volume, leading to a higher concentration of wood compounds extracted to the beverage. Oppositely, large containers will represent a smaller wood surface exposed to the liquid and will incorporate lesser amounts of wood extractives in the aged beverage (Singleton, 1974).

In fact, specific ratio of wood per beverage volume, is one of the main parameters taken into account in creating oak alternatives and may be used for accelerating incorporation of wood extractives into aged beverages. These are innovative and technological approaches that envisage the enhancement of wood extraction, by increasing the amount of incorporated compounds and their transfer rates. Several barrel alternatives can be named, for example powder, chips, blocks, sticks, staves (Chatonnet, 2007) and even spirals (Karasch, Theisen, & Theisen, 2008). Barrel alternatives can be directly immersed in the beverage, providing additional extractive content. Multiple advantages can be associated with the use of barrel alternatives: seasoning and toasting of oak alternatives can be dissimilar from toasting of the barrel, generating distinct amounts of extractives (Fernández de Simón *et al.*, 2010), the amount to be applied to the beverage can easily be controlled, allowing a finer tuning of wood/beverage ratio (Dumitriu *et al.*, 2019), they can easily be combined with other accelerated ageing technologies (Canas, Caldeira, Anjos, & Belchior, 2019; Oberholster *et al.*, 2015). Furthermore, the production cost associated to ageing with oak alternatives is 5 to 100 folds lower when compared with the cost of traditional barrel ageing, which is of upmost interest for economic feasibility of ageing. Also, the sustainability of ageing using oak alternatives, measured as global warming impact, is significantly lower when compared with traditional barrel ageing (García-Alcaraz *et al.*, 2020).

1.4 Chemical composition of wood

Oak wood has in its composition typical lignocellulosic constituents as seen in Figure 1.5.

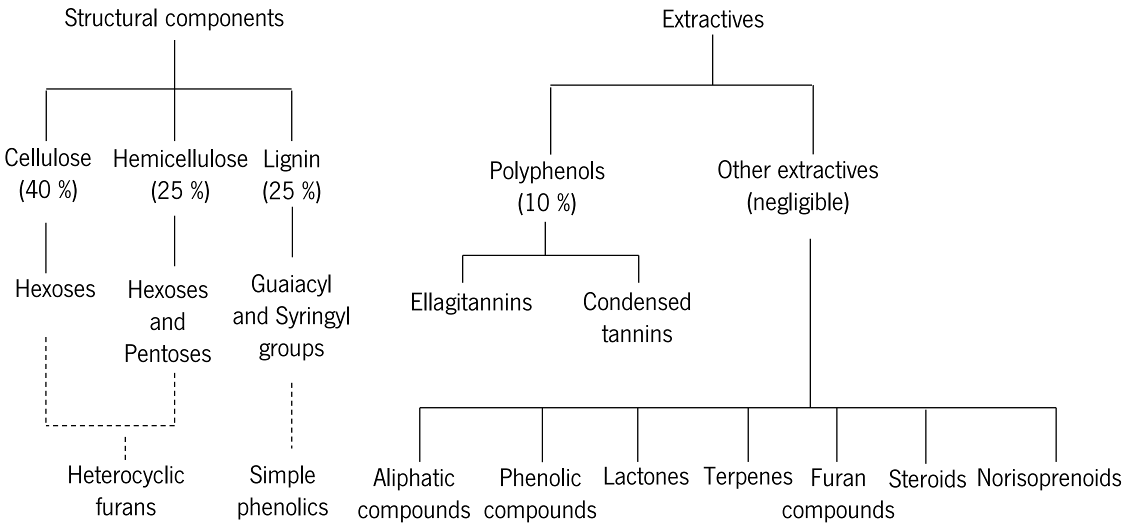


Figure 1.5: Main oak wood constituents (adapted from Mosedale & Puech, 2003).

When considering wood usage for ageing alcoholic beverages, extractives play a central role among its constituents. One major group to take into account are phenolic compounds, which have significant impact on the final product organoleptic characteristics and quality, participating in color, aroma and flavor of the aged beverage (Canas *et al.*, 2019; Castro-Vázquez *et al.*, Alañón, Ricardo-da-Silva, Pérez-Coello, & Laureano, 2013). According to their molecular structure, phenolic compounds occurring in plants can be split into different classes, presented in Table 1.1.

Table 1.1: Classes of phenolic compounds found in plants (adapted from Zhang, Cai, Duan, Reeves, & He, 2015)

Structure	Phenolic class	Structure	Phenolic class
C_6	Simple phenolics	C_6-C_3-C_6	Flavanonols
C_6-C_1	Phenolic acids and related compounds	C_6-C_3-C_6	Flavan-3-ols
C_6-C_2	Acetophenone and phenylacetic acid	C_6-C_3-C_6	Isoflavonoids
C_6-C_3	Cinnamic acids, cinnamyl aldehydes/alcohols	C_6-C_3-C_6	Anthocyanidins/Anthocyanins
C_6-C_3	Coumarins, isocoumarins, chromones	$(C_6$-C_3-$C_6)_2$	Biflavonoids
C_6-C_1- C_6	Benzophenones, xanthones	C_6,C_{10},C_{14}	Benzoquinones, naphthaquinones, anthraquinones
C_6-C_2- C_6	Stilbenes	C_{18}	Betacyanins
C_6-C_3- C_6	Chalcones, aurones, dihydrochalcones	Lignans, neolignans	Dimers or oligomers
C_6-C_3- C_6	Flavones	Lignin	Polymers
C_6-C_3- C_6	Flavonols	Phlobaphenes	Polymers
C_6-C_3- C_6	Flavanones		

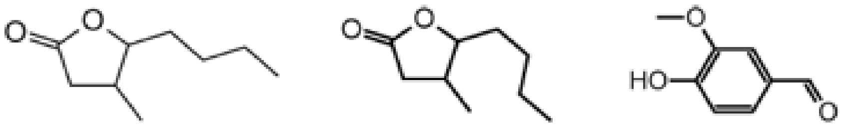
One class with particular interest are simple phenolics, which are listed in Table 1.2. These compounds participate actively in several organoleptic phenomena, namely pigmentation, aroma and flavor (Cano-López, López-Roca, Pardo-Minguez & Gómez Plaza, 2010; Zhang *et al.*, 2015). Also, they are involved in oxidation reactions or serve as substrates to microbial activity, to be further explored in sections 2.1.3 and 2.1.4 of this chapter. In a more familiar concept within the wine industry, tannins constitute a class of polyphenols with wider range of structures and intermediate to high molecular weights. These can be sorted in two main classes: condensed tannins (proanthocyanidins) with flavonoid cores as main basic unit, and hydrolysable tannins (gallotannins and ellagitannins), which are polymers of glucose esters of gallic and hexahydroxydiphenoic acids, respectively (Zhang *et al.*, 2015).

Table 1.2: Simple phenolic compounds available in oak wood (adapted from Zhang *et al.*, 2015)

Hydroxybenzoic	**Hydroxycinnamic**
Gallic acid	*p*-Coumaric acid
Gentisic acid	Caffeic acid
p-Hydroxybenzoic acid	Ferulic acid
Protocatechuic acid	Sinapic acid
Syringic acid	Sinapaldehyde
Vanillic acid	Coniferaldehyde
p-Hydroxybenzoic aldehyde	Eugenol
Protocatechuic aldehyde	
Syringaldehyde	
Vanillin	

Particularly, ellagitannins are of profound interest when focusing barrel use for winemaking (Jordão *et al.*, 2007). These polyphenols impart extremely important organoleptic qualities to the beverage, being also responsible for changes in pigmentation and, most of all, astringency (Zhang *et al.*, 2015) which imparts significantly the mouthfeel and body of aged beverage (Ma *et al.*, 2014). Their content in wood depends on species and processing methods. Generally, French oak is richer in ellagitanins when compared with the American variety (Chira & Teissedre, 2014). Also, ellagitanin content in wood can be further modified by cooperage manufacturing steps as stated previously in section 1.2.

Another fraction of wood composition, with high importance in beverage production, is the extractive volatile compounds (Figure 1.6). These comprise a negligible percentage of wood composition but strongly impart beverage characteristics. Even occurring at concentrations of few $\mu g\ L^{-1}$, some of these compounds have significant impact on beverage sensory properties due to their low perception thresholds. Oak lactones are a key marker of aged spirits, often associated with coconut and vanilla sensory descriptors, essential for whisky quality. Furan compounds, namely furfural and 5-methylfurfural contribute with caramel descriptors, and volatile phenols guaiacol and 4-methylguaiacol contribute with smoky character, whereas eugenol is commonly associated with clove aroma. Lastly, vanillin also contributes with desirable sweet vanilla descriptors (Russell & Stewart, 2014; Wilkinson, Li, Grbin, & Warren, 2013). Variation of these constituents can be observed among species and even among different trees from the same variety. For instance, American oak has lower content in polyphenols and higher in whisky lactone, when compared to the French variety. Even among French species variability can be observed. For instance, *Q. robur* has high level of ellagitanins and negligible amounts of whisky lactone, whereas *Q. petraea* is more similar to the American variety (Doussot, De Jéso, Quideau, & Pardon, 2002).



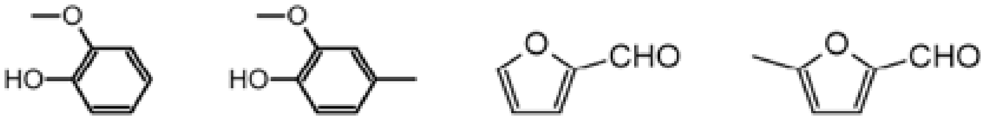
cis-oak lactone (coconut, vanilla)

trans-oak lactone (coconut, vanilla)

vanillin (Vanilla)

eugenol (clove)

isoeugenol (spicy)



guaiacol (smoky)

4-methylguaiacol (smoky)

furfural (caramel, vanilla)

5-methylfurfural (caramel)

Figure 1.6: Main extractible oak volatile compounds (adapted from Wilkinson *et al.*, 2013, and Fan, Xu, & Yu, 2006).

Such variability between oak from different provenance has brought up the need to control oak wood by a denomination of origin in order to make oak wood quality more predictable and controlled (Doussot *et al.*, 2002; Gougeon *et al.*, 2009; Martínez-Gil *et al.*, 2018).

2. Use of cooperage wood in ageing of beverages

2.1 Transformations occurring during wood ageing

2.1.1 Subtractive ageing

Throughout the ageing period, beverage penetrates barrel wood pores soaking it and mass transfer through wood structure is widely acknowledged. Water flow through wood has been extensively studied in material science, which provided further basis to comprehend mass transfer during barrel ageing. Wood is an anisotropic material, with different cellular structures and orientations (Zillig, Janssen, Carmeliet, & Derome, 2006). Water entry or evaporation causes wood fibers to swell or shrink, which furthermore influences barrel permeability (Noack, Schwab, & Bartz, 1973). Two different regimes have been identified for water transport. Above fiber saturation point, defined as moisture concentration where cell walls accommodate the maximum water content without losing structural integrity (Engelund, Thygesen, Svensson, & Hill, 2013), wood behaves as a non-hygroscopic material and liquid water flows by capillarity following Darcy's law. Below fiber saturation point, water most predominantly flows as vapor or bound water, following the principles of Fick's law (Kang & Chung, 2009). These two mechanisms take place during barrel ageing, as water will penetrate the stave by capillarity until fiber saturation point is reached, point from which water will evaporate due the contact with the dry air (del Álamo-Sanza & Nevares, 2018).

Another type of mass transfer occurring during barrel ageing is gas exchange. Gas exchange is an important feature of barrel ageing, being that oxygen is involved in several ageing phenomena, to be further discussed in section 2.1.3. Several entry points were identified in barrels as air can diffuse into the beverage either between stave joints, the bung hole and even through wood staves (del Álamo-Sanza & Nevares, 2018). Oxygen transfer rate through wood has been shown to be dependent on moisture content, being that a wet stave has lower oxygen transfer rates than a dry stave (Nevares, Crespo, Gonzalez, & del Álamo-Sanza, 2014). Further contributing to air diffusion, liquid evaporation generates a headspace in the top of the barrel. Wood in contact with this headspace will become dry and consequently become more permeable to gas exchange. Liquid volume losses also cause variations in the barrel internal pressure and wood reaction and adaptation to such pressure changes causes additional changes to the dissolved oxygen in the beverage (Nevares & del Álamo-Sanza, 2018). Oxygen transfer rate will decrease with successive reutilization of the barrel, as a consequence of the formation of precipitates in the porous structure of wood (Zamora, 2019). Gas and liquid transfer through the barrel overall contribute to a process often designated as "Angel's share", which refers to the loss of beverage components by evaporation (Canas *et al.*, 2002; Russell & Stewart, 2014). This will reduce beverage content in volatile components as well as increase concentration in non-volatile components due to water losses. As for most ageing parameters, permeability is influenced by wood characteristics, namely origin, grain and cooperage processing (Acuña, Gonzalez, De La Fuente, & Moya, 2014). Also, producers pay special attention to cellar atmospheric conditions such as temperature and humidity, which can amplify or reduce losses during ageing (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006; Ruiz De Adana, López, & Sala, 2005).

Apart from the losses caused by mass transfer through wood, compounds from the beverage can also be adsorbed by wood, which is another subtractive ageing mechanism. Sorption was studied by promoting wood-beverage contact and accompanying compound partition using synthetic matrixes mimicking wine. Ramirez and collaborators studied wood sorption for a wide range of compounds including terpenic alcohols, esters, aldehydes and norisoprenoids commonly found in wine, with particular interest on their disappearance and the underlying impact on wine quality. Sorption of wine volatile compounds, especially linalool and ethyl octanoate, was reported and described as a selective process, not related with their solubility and hydrophobicity. Instead, Ramirez suggested that acid-base and polar characteristics were the main causers of the sorption phenomenon (Ramirez-Ramirez *et al.*, 2001). This hypothesis was contradicted in posterior works of Ramirez and collaborators when studying the effect of wine constituents in wood sorption of a small pool of wine volatiles, they proposed that hydrophobicity could indeed be involved in the sorption of wine compounds (Ramirez-Ramirez, Chassagne, Feuillat, Voilley, & Charpentier, 2004). Barrera-Garcia and collaborators also studied sorption on the wood/wine interface, reporting sorption of monomeric volatile phenols following two distinct behaviors (Barrera-García, Gougeon, Voilley, & Chassagne, 2006). Moreover, Barrera-Garcia *et al.* (2007) studied the sorption of wine polyphenols, namely monomeric anthocyanins, (+)-catechin, (-)-epicatechin, gallic acid and *trans*-resveratrol, having found that except for gallic acid, all compounds disappeared in the presence of wood (Barrera-García *et al.*, 2007). Later on Barrera-Garcia and collaborators proposed that hydrophobic interactions were involved in sorption of volatile phenolic compounds. All wood macromolecules demonstrated ability of adsorbing the studied volatile phenols with lignin presenting the highest selectivity and sorption affinity (Barrera-García, Gougeon, Karbowiak, Voilley, & Chassagne, 2008). One particular feature regarding studies on wood sorption is that they targeted mainly subtractive modifications occurring in the beverage. Nonetheless, sorption also modifies the composition of wood, which must be accounted for regarding its lifecycle.

2.1.2 Additive ageing

With high impact on beverage quality, wood compounds are extracted during the contact with the hydroalcoholic matrix. Phenolic compounds (including volatile phenols, phenolic acids and tannins), furanic compounds and lactones are broken and extracted, affecting profoundly the aromatic characteristics of the beverage (Mosedale & Puech, 1998). The presence of ethanol in the alcoholic beverage modifies the polarity of the medium, leading to the extraction of compounds often with low water solubility. This will lead to the partition of the available compounds, which will be extracted from the solid phase (wood) to the liquid phase (beverage/spirit) until an equilibrium is reached (Jung, Park, & Yang, 2016). Extraction rates and the obtained equilibria are intrinsically related with each compound's nature, the aged beverage and the cooperage product applied.

Extraction kinetics of volatile compounds, namely phenolic compounds, furans and lactones, follows a hyperbolic profile, with an initial pronounced increase in concentration followed by a less pronounced linear increase, probably due to progressive breakdown of wood macromolecules by ethanolysis (Caldeira *et al.*, 2016; García-Estévez, Alcalde-Eon, Le Grottaglie, Rivas-Gonzalo, & Escribano-Bailón, 2015). Similar kinetics was also observed for extraction of wood compounds by beer (Sterckx, Saison, & Delvaux, 2012a), wine (Arapitsas, Antonopoulos, Stefanou, & Dourtoglou, 2004) and whisky (Jeffery & Berglund, 2016).

Considering this kinetic behavior, time is usually a parameter to be considered in the control of the extractive content to be incorporated in beverages (Cerdán & Ancín-Azpilicueta, 2006).

In traditional processes, the control of wood extractives transferred to the beverage highly depends on the selection of wood barrel and on the control of contact conditions. As referred previously, wood provenance and cooperage manufacturing methods highly influence the extractable content of the barrel, either regarding the amount of compounds as the type of compounds available. The extractable pool of compounds also depends on barrel usage history. Throughout ageing and during successive ageing cycles wood will lose richness in extractible compounds, becoming exhausted of its content (Cerdán, Mozaz, & Azpilicueta, 2002; Gomez-Plaza *et al.*, 2004; Jackson, 2014). Thus, barrel selection focuses not only on barrel type but also on its history in beverage ageing. Incorporation of wood extractives in the beverage can be further controlled by selecting different barrel volumes, which leads to different wood surface to beverage ratios and consequently higher or lower extraction of wood compounds (Jeffery & Berglund, 2016). Also, the use of oak alternatives allows a further enhancement of wood compounds extraction, by modifying wood/liquid ratios and mass transfer kinetics.

2.1.3 Chemical ageing

As previously referred in section 2.1.2, the extraction of wood compounds occurs during ageing, which modifies the beverage's chemical composition, but this is not the only modification occurring. Throughout time, the beverage will also undergo several transformations involving reactions between the compounds present in the matrix such as oxidation, condensation and nucleophilic reactions (Coetzee & Du Toit, 2015). Due to oxygen permeability of the barrel, micro-oxygenation can occur throughout ageing, which leads to the oxidation of several compounds. As described for wines and spirits, phenolics are the main substrates for oxidation (Oliveira, Ferreira, De Freitas, & Silva, 2011; Rodriguez Madrera, Garcia Hevia, & Suárez Valles, 2013). Oxidation affects specially polyphenols containing a catechol ring or a galloyl group such as (+)-catechin/(−)-epicatechin, gallocatechin, gallic acid and its esters, and caffeic acid. These substrates are oxidized to semiquinone radicals and benzoquinones, as represented in Figure 1.7, while oxygen is reduced to hydrogen peroxide, mediated by the redox cycle of Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+} given that oxygen does not react directly with phenolic compounds without the presence of transition metal ions (Oliveira *et al.*, 2011). Quinones and other primary products are unstable and can undergo other reaction with nucleophilic products (other phenols, thiols or amines) and the produced dimers or polymers can rearrange and be further oxidized (Coetzee & Du Toit, 2015; Oliveira *et al.*, 2011). Therefore, oxidation of phenolic compounds can trigger and accelerate the formation of polymers and other oxidation products as represented in Figure 1.7. Considering the importance of phenolic compounds in pigmentation, their oxidation and polymerization alter the color of the aged beverage, by stabilizing or intensifying content on pigmented complexes.

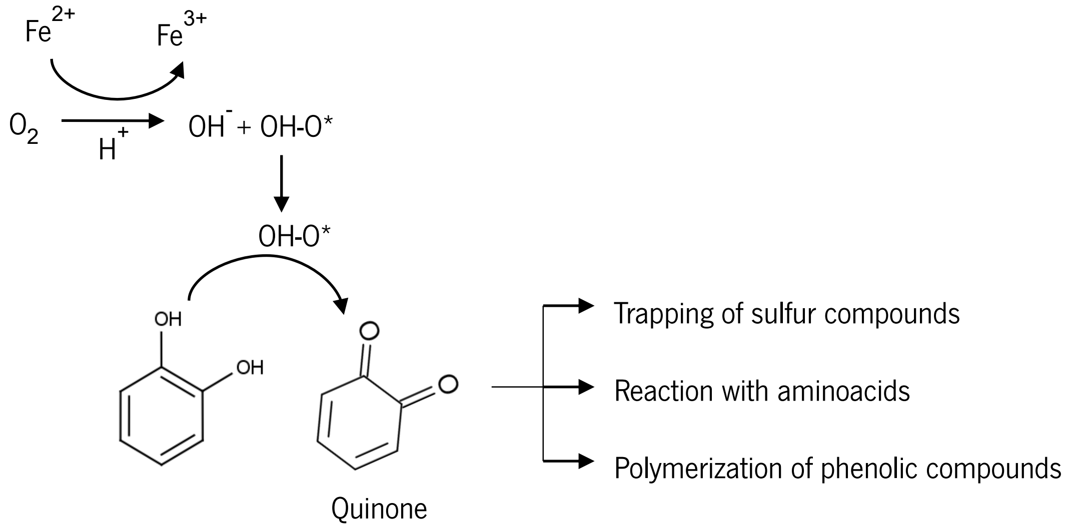


Figure 1.7: Oxidation of phenolic compounds mechanism (adapted from Coetzee & Du Toit, 2015).

In wine, for instance, oxidation and reactions involving phenolic compounds are described to be responsible for the appearance of yellow pigmentation throughout ageing (Cano-López *et al.*, 2010). Also, condensation and polymerization of phenolics affect the taste of the beverage, being that more polymerized chains are correlated with a reduction on bitterness and astringency (Gómez-Plaza & Cano-López, 2011). Oxidation mechanisms also have high importance on reduction of volatile thiols, contributing to the beverage quality by reducing the unpleasant descriptors associated with these sulfur compounds (Coetzee & Du Toit, 2015). In whiskies, dimethyl sulfide (DMS) is the main sulfur compound to take into account, which is oxidized to dimethyl sulfoxide by hydrogen peroxide formed during oxidation of phenolic compounds. Also, DMS can be adsorbed by wood or evaporate through the barrel due to its low boiling point (Macnamara & Wyk, 2001).

Other reactions occurring slowly during ageing affect compounds more related to the beverage. Wines and especially spirits have high content in acrolein (2-propenal), diacetyl (2,3-butanodione), 2-butanol, allyl alcohol (2-propen-1-ol) or acetic acid, compounds that confer pungent and unpleasant notes to the distillate. Acetaldehyde and acrolein are the main compounds responsible for unpleasant flavors in distilled spirits, which react with ethanol during ageing, to form the acetals 1,1-diethoxyethane and 1,1,3-triethoxypropane, respectively, leading to a reduction of the pungent character (Christoph & Bauer-Christoph, 2007). In wines, the mechanisms of reaction and corresponding products are more diverse, due to microbial conversion pathways (Han, Webb, & Waterhouse, 2019; Zea, Serratosa, Mérida, &

Moyano, 2015). Another type of reactions occurring during ageing is esterification, where carboxylic acids react with alcohols to form ethyl esters, which generally is favored by the presence of a basic catalyst. Ethyl esters can be formed from several carboxylic acids, being ethyl acetate the most predominant in wine and spirits, resulting from the esterification reaction involving acetic acid and ethanol (Christoph & Bauer-Christoph, 2007; Russell & Stewart, 2014). Esterification reactions however are rather slow and when focusing spirits such as whisky and rum, ester content is derived mainly from yeast during fermentation of wort, being esters derived from the ageing process considered residual (Nascimento, Cardoso, & Franco, 2008).

2.1.4 Biological ageing

Besides chemical ageing, beverages in the barrel can undergo biological ageing, especially for low alcohol products. Barrel wood can become naturally populated by wild microbes introduced by beverages matured in it or by exposure to the winery/cellar/brewery environment. For instance, wine is naturally populated by yeast (*e.g. Saccharomyces* sp. and *Brettanomyces* sp.) and bacteria (*e.g. Lactobacillus* sp., *Acetobacter* sp.), which will become a part of used barrel microflora (Snauwaert *et al.*, 2016; Spitaels *et al.*, 2014). *Brettanomyces* sp., which can also be referred as *Dekkera sp.* (the teleomorph form of the yeast), has been reported to penetrate up to 8 mm in wood enduring harsh cleaning procedures and remaining active in the barrel (Suárez, Suárez-Lepe, Morata, & Calderón, 2007). Several efforts are usually undertaken in wine production to avoid the growth and appearance of *Brettanomyces* sp., involving sanitation of winery equipment and use of sulfur dioxide (Barata *et al.*, 2008). The main feature of this yeast metabolism is the production of volatile phenols from the decarboxylation of phenolic acids as represented in Figure 1.8. These are considered off-odors in wine and therefore harmful for wine quality, mainly related to “phenolic”, “leader”, “varnish” and “horse sweat” descriptors (Suárez *et al.*, 2007). Moreover, *Brettanomyces* sp. has been described to produce esterases, enzymes involved in ester hydrolytic and/or synthetic activity, which will also influence ester content and volatile quality of the beverage (Spaepen & Verachtert, 1982). Despite being a disadvantageous contaminant for wine quality, *Brettanomyces* is considered responsible for certain desired traits in some styles of beer which are considered active aroma contributors, namely in the Lambic beer style and in sour ales (Spitaels *et al.*, 2014; Steensels *et al.*, 2015). For instance, as shown for gueuze beer, an initial complex and diverse microbial community converges throughout time to *Dekkera* sp. as the sole isolated microorganism, which is also observed for the produced metabolites (Spitaels *et al.*, 2015). Focusing other yeasts, several species can also appear in barrels such as *Pichia* sp., *Candida* sp., *Cryptococcus* sp. and *Debaryomyces* sp. These can impart the imbalance of typical fermentation associated metabolites such as higher alcohols, volatile fatty acids or esters, which are already produced by *Saccharomyces* sp. during fermentation (Snauwaert *et al.*, 2016).

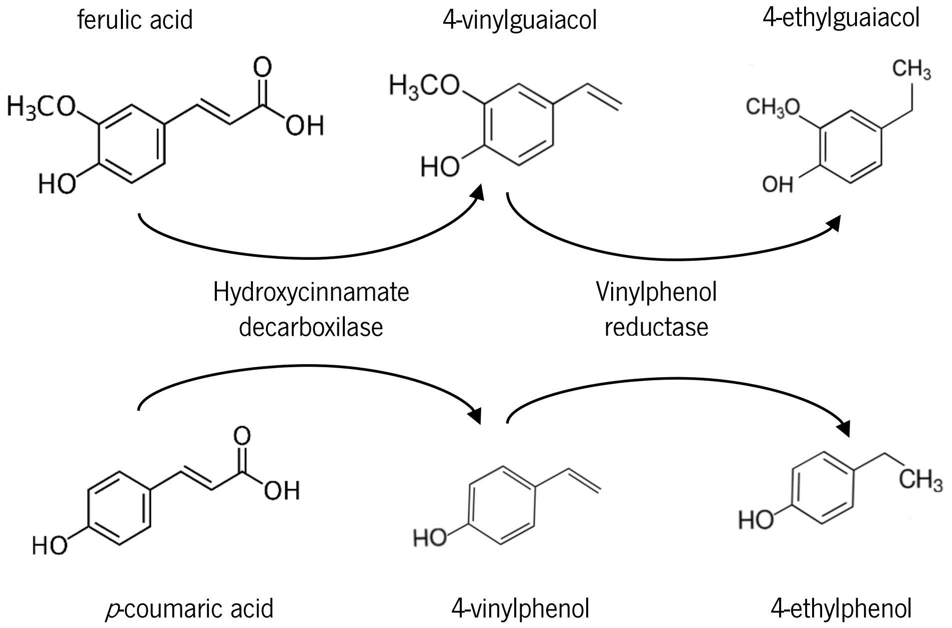


Figure 1.8: Production of volatile phenols by *Brettanomyces*/*Dekkera* sp. (adapted from Suárez *et al.*, 2007).

Lactic acid bacteria such as *Lactobacillus* sp., *Pediococcus* sp., *Oenococcus oeni* and *Leuconostoc* sp. can also appear and be of benefit to some products. In wine, they are responsible for carrying out malolactic fermentation, where malic acid (a tart harsh tasting acid) is converted to lactic acid (a softer tasting more pleasant organic acid). Besides modifying organic acid content, malolactic fermentations performed by these bacteria also modify volatile composition, being also responsible for diacetyl production, an undesired metabolite often associated with butter aroma descriptors (Fischer, 2007; Menz *et al.*, 2010). Another class of bacteria to take into account is the acetic acid bacteria. These microorganisms are of extreme importance when controlling wine quality. One of their main features is the production of acetic acid from ethanol oxidation, being also capable of oxidizing other compounds and modifying the organoleptic characteristics of the beverage. Besides appearing in wine, *Acetobacter* sp. have already been reported in beer samples, which derives from beer ageing in barrels previously used in wine (Fischer, 2007; Spitaels *et al.*, 2014). Considering barrel ageing processes, growth and metabolism of these microorganisms can occur sequentially or simultaneously, being some species predominant over time. As described by Spitaels and collaborators when studying spontaneous fermentation of beers in wood barrels, several microbial species predominate over time becoming more diverse for late stages of maturation. *Saccharomyces* sp. are predominant during the first stage of fermentation, being replaced by *Dekkera* and *Debaromyces* yeasts. For the late stages of fermentation other species arise as the diversity of barrel microflora increases (Spitaels *et al.*, 2014).

2.2 Beverages aged in wood

Ageing beverages in wood barrels goes back to ancient times, when the only options for storing food products were clay or wood containers. As containers from other materials were developed, barrels began to be exclusively used for storing beverages due to their impact on product properties and their characteristics as dynamic containers. Among the beverages that can be stored in the barrel, three major types can be highlighted, with very distinct properties and provenances: beer, wine and spirits.

2.2.1 Beer

Beer is one example of barrel aged beverages. Ancient fermenters were mostly wooden vats and beer was stored and transported in wood barrels (Kunze, 2004). It was only after the First World War that wood fermenters began to be replaced by alternative materials, firstly aluminum and posteriorly stainless steel, which is currently the preferred material for production of beer fermenters. Wood storage containers also followed the same trend, as wood barrels were replaced by stainless steel kegs, which are more durable and ensure maintenance of beer quality throughout shelf-life (Priest & Stewart, 2006). However, some specialty beers still maintain the use of wood barrels. Traditional English Ales still perform fermentation in barrel (Briggs, Boulton, Brookes, & Stevens, 2004), as well as several styles of Belgian Ales (Pavsler & Buiatti, 2008). The use of wood chips is also reported in beer production in processes other than ageing, where the sorbent properties of wood are used to filter colloids from hazy beer (Priest & Stewart, 2006). During contact with oak wood, beer extracts characteristic wood compounds, namely furan compounds and aldehydes (Sterckx, Saison, & Delvaux, 2012b; Sterckx *et al.*, 2012a; Wyler, Angeloni, Alcarde, & da Cruz, 2015). However, barrel aged beers are more deeply affected by biological ageing phenomena, which modify beer composition and consequently its sensory properties. As reported for barrel aged Belgian beers, the presence of endogenous barrel microbes transforms beer composition, with the induction of sour and acidic features, much appreciated in the *Lambic, Gueuze* and *Flanders-red* Belgian ales (Smith & Divol, 2016; Snauwaert *et al.*, 2016; Spitaels *et al.*, 2015, 2014).

2.2.2 Wine

Wine is another, and maybe one of the most acknowledged, barrel aged beverage. Originally, the Romans stored and transported wine in clay amphorae, but after reaching Gaul they learned barrel making techniques, which were believed to be brought from the Celts. Due to their high resistance and durability, barrels rapidly replaced amphorae and became a perennial tool in the winemaking process (McGovern, Katz, & Fleming, 2003). Several table wines resort to barrel storage as a tool to enhance their sensory properties, mainly applied in red winemaking, which benefits strongly with overall modification of astringency, color stabilization and aroma enhancement (Ribéreau-Gayon *et al.*, 2006).

Fortified wine production also resorts to wood ageing, in order to promote transformations and induce oak character. Sherry wine, Port wine and Madeira wine are among the main examples of barrel aged fortified wines. Sherry wine for instance has a vast tradition and a very well established ageing process. During Sherry production, wine is transferred from one barrel to another, in a system designated by *solera* and *criaderas* (Ángeles Pozo-Bayón & Victoria Moreno-Arribas, 2011). Port wine is also another well-established fortified wine that resorts to barrel ageing. This fortified wine is aged in either 600 L and 650 L casks or in large volume wood vats of 10 000 L to 100 000 L, also called *balseiros,* commonly found in Port cellars, which are a trademark image of the product (Moreira & Guedes de Pinho, 2011). Madeira wine is commonly stored in wood barrels, in cellars where temperatures are typically higher than the common cellar temperature, where it acquires its characteristic sensory properties (Perestrelo, Albuquerque, Rocha, & Câmara, 2011). Vermouth, which also derives from fortification of wine, also undergoes barrel ageing (Panesar, Joshi, Bali, & Panesar, 2017).

Wine is probably the beverage for which the benefits of barrel ageing are better known and described. Additive ageing imparts wine with several wood extractives that enhance its sensory properties, namely volatile compounds and especially tannins. Wine also benefits from chemical ageing due to oxidation processes, with color stabilization and astringency modification caused by oxygenation during storage as described in section 2.1.3. Biological ageing of wine also occurs during barrel storage, which is tightly controlled by winemakers. Oppositely to beer, wine resorts to the control of microbial growth in order to minimize *Brettanomyces* and other potential spoilage microorganisms, allowing only the growth of the desirable ones, such as lactic acid bacteria responsible for malolactic fermentation (Barata *et al.*, 2008).

2.2.3 Spirits

Lastly, spirits are also well-established products that resort to barrel ageing. Whisky (Bourbon and Scotch) and Brandy are widely produced and consumed spirits with several well acknowledged premium products. Bourbon ageing generally resorts to American oak barrels, toasted by the American technique, which contribute to its rich vanilla and coconut characteristics.

Scotch is also aged in oak barrels, usually previously used in ageing other beverages, mainly Bourbon or fortified wines – Sherry, Port or Madeira (Russell & Stewart, 2014). Brandy also resorts to an initial short contact with new barrels, after which is generally transferred to reused barrels (Caldeira *et al.*, 2006).

Several other examples of barrel aged spirits can be mentioned, as for example rum, “cachaça” and grape marc spirit (Bortoletto, Correa, & Alcarde, 2016; Rodríguez-Solana, Salgado, Domínguez, & Cortés-

Diéguez, 2014; Roullier-Gall *et al.*, 2018). Due to their high alcoholic content, biological ageing is not considered in spirits, which benefit mainly from subtractive, additive and chemical ageing.

2.3 Lifecycle of cooperage wood

As stated, cooperage wood is an important raw material in the production of aged beverages. Similarly to any raw material, wood for ageing beverages is produced, used, reused and discarded. Cooperage production specializes mainly in creating barrels for wine, bourbon and brandy production, which are the main products that use new barrels. After the first use, barrels can be reused for ageing the same beverage, which mostly depends on the extractable content available in wood, the winemaker/distiller evaluation and the intended product (Russell & Stewart, 2014; Zamora, 2019). When barrels are considered unsuitable or exhausted from extractives by the producer, they can either be discarded, reused or treated for regeneration.

Regeneration treatment involves disassembly of the barrel, scraping the surface for removal of the spent wood, re-assembly and toasting of the barrel which generates novel extractable content (Wilkinson *et al.*, 2013). However, re-treating wood involves further processing costs and labor, and it is not always an attractive alternative for coopers or winemakers (Russell & Stewart, 2014).

Another option is the addition of oak alternatives as oak chips, sticks or staves, in combination with spent oak barrels to replace the extractable content of oak wood. This allows reutilization of the barrel for chemical and biological ageing of wine, being the extractable content supplied by the added oak alternatives (García-Alcaraz *et al.*, 2020). Nonetheless, this also generates further used wood residues, as chips also become depleted of extractives. Used barrels can be reused for ageing other beverages. Ageing of whisky (scotch and irish) and beer resort mostly to the reuse of barrels previously used for ageing Bourbon, Brandy, Sherry, Port or Madeira wines (Quinn, 2014; Spitaels *et al.*, 2014). Barrel provenances were shown to differentiate whiskies aged in bourbon casks from whiskies aged in sherry casks as whiskies aged in sherry barrels showed significant content of wine-related chemical markers, for example quercetin-glucuronide and myricetin-glucoside, which have been transferred by the barrel (Roullier-Gall *et al.*, 2018). Apart from barrels, the increasing interest on alternative accelerated ageing technologies and application of barrel alternatives leads to an ever increasing amount of used wood residues, either chips, staves or sticks for which reutilization procedures are still to be established.

3. Technology for the accelerated ageing of beverages

Due to the high costs associated with barrel ageing, including costs of raw materials and maintenance of large stocks during long time periods, several methodologies have been developed to mimic and accelerate barrel ageing of beverages. Efforts were made to integrate and control variables involved in the physical-chemical transformations naturally occurring in the barrel, which were previously described. Process variables such as wood-beverage contact, oxygenation, pressure and other physical-chemical variables have been combined to design reactors to work as alternatives to barrel ageing (Pielech-Przybylska & Balcerek, 2019; Tao, García, & Sun, 2014).

The most popular approaches involve mixing the beverage and wood fragments in stainless steel tanks. These allow the intensification of extraction of wood components by enhancing the surface area of wood in contact with the beverage, along with control of process variables such as temperature, pressure, pH and mixing. Furthermore, ageing vessels can include strategies to further improve and accelerate ageing, integrating other physical treatments such as micro-oxygenation, high pressure, catalysts, ultrasounds, gamma irradiation and electric fields (Tao *et al.*, 2014). For instance, extensive research work has been performed to develop accelerated ageing methodologies with particular focus on wine production, with several works and achievements compiled in Table 1.3.

Most of the approaches involve mixing of wood fragments and/or yeast lees with wine to promote additive ageing, which can be further combined with micro-oxygenation to mimic the oxygenation occurring in barrel and the inherent oxidative chemical ageing phenomena. Accelerated ageing methodologies have also been developed focusing application to distilled spirits. These technologies often resort to the same approaches mentioned for wine ageing, mainly mixing wood fragments with the beverage which can be further combined with other physical chemical transformations. Several examples of accelerated ageing of spirits have been developed. Accelerated ageing of grape marc spirit by application of wood chips (Rodriguez-Solana, Rodriguez-Freigedo, Salgado, Domínguez, & Cortés-Diéguez, 2017), accelerated ageing of brandy in stainless steel tanks with wood tablets or staves without micro-oxygenation (Canas, Caldeira, & Belchior, 2013), with micro-oxygenation (Canas *et al.*, 2019), or by applying wood chips in combination with ultrasound (Schwarz, Rodríguez, Sánchez, Guillén, & Barroso, 2014).

Table 1.3: Technologies available for accelerating wine ageing, with corresponding advantages and disadvantages (adapted from Tao *et al.*, 2014)

Ageing technology		**Main chemical modification**	**Wine type**	**Advantages**	**Disadvantages**	**References**
Wood barrel system	Wood barrel ageing	a. Wood-related aromatic compound increase b. Polymerization, copigmentation, and oxidation of phenolic compounds promotion	Grape wine (*e.g.*, red wine, white wine)	a. The longest history b. Reliable in producing high-quality wines	b. Expensive c. Space-consuming d. Limited barrel life e. Potential microbiological contamination f. Storage loss due to evaporation	(Bozalongo, Carrillo, Torroba, & Tena, 2007) (Ortega-Heras, González-Huerta, Herrera, & González-Sanjosé, 2004) (Ruiz De Adana *et al.*, 2005)
	Wood barrel ageing + lees	a. Similar effects to wood barrel ageing b. Polysaccharides, aminoacids, peptides, fatty acids, and lipids enrichment c. Aromatic and phenolic composition modification d. Biogenic amines production		a. Same advantages as wood barrel aging b. Nutritional value enhancement c. Improvement of structure, roundness and body of wines d. Aromatic notes of wines more complex and persistent e. Color stability improvement	a. Same disadvantages as wood barrel aging b. Sulfur off-odors generation c. Biogenic amines production	(Del Barrio-Galán, Pérez-Magariño, & Ortega-Heras, 2011) (Escot, Feuillat, Dulau, & Charpentier, 2001) (Palacios, Vasserot, & Maujean, 1997)
Other vessels (usually stainless steel tanks)	Wood fragments	Wood-related aromatic compounds increase	Grape wine (*e.g.*, red wine, white wine)	a. Reducing aging time b. Money saving	a. Wine sensorial quality not as good as long-term barrel-aged wines b. Not benefiting color evolution	(Bautista-Ortín *et al.*, 2008) (Morales, Benitez, & Troncoso, 2004) (Miriam Ortega-Heras, Pérez-Magariño, Cano-Mozo, & González-San José, 2010)
	Wood fragments + micro-oxygenation	Similar effects to wood barrel aging		a. Same advantages as wood fragments b. Wood barrel aging simulation	a. Problems of potentially excessive oxidation	(Durán Guerrero *et al.*, 2011) (Cano-López *et al.*, 2010) (Gay *et al.*, 2010)
	Wood fragments + micro-oxygenation + lees	a. Color and red polymeric pigments increase b. Slight phenolic composition modification	Red wine	a. Same advantages as the combination of wood fragments and micro-oxygenation b. Wine color more stable during bottling	a. Same disadvantages as the combination of wood fragments and micro-oxygenation b. More studies are needed to understand this technology	(Sartini, Arfelli, Fabiani, & Piva, 2007)
Physical methods	Ultrasound (<100 kHz)	a. Alcohol content reduction b. Acetaldehyde content decrease c. Ethyl acetate content increase d. Polyol concentration reduction	Rice wine	a. Aging time reduced drastically b. Money saving c. Space saving d. Labor saving e. No storage loss due to evaporation f. Shelf life extended after ultrasonic treatment	a. More studies needed on different types of wine b. Critical wine regulations c. People's worry about the security of these technologies, especially gamma irradiation and nanogold photocatalysis	(Chang & Chen, 2002) (Leonhardt & Morabito, 2007)
		a. Accelerating chemical reactions	Grape wine (*e.g.*, red wine, white wine)			
	Gamma irradiation	a. Polyol concentration reduction b. Ethyl acetate content increase	Rice wine			(Chingzu Chang, 2003) (Chang, 2004)
		a. Acetaldehyde content increase b. Polyol concentration reduction	Maize wine			
	Electric field	a. Higher alcohols and aldehydes content decrease b. Esters and free amino acids content increase	Red wine			(Zeng, Yu, Zhang, & Chen, 2008)
	Nanogold photocatalysis	Acetic acid and ethyl acetate content increase	Sorghum sprit			(Lin *et al.*, 2008)
	High pressure	a. The ultraviolet-visible spectra (190 nm to 560 nm) change b. Boiling point, relative density, redox potential, electrical conductivity, and total acidity change	Red wine			Li *et al.* (2005a) Li *et al.* (2005b)

Several processes focusing a wide variety of spirits have been developed, which has led to the filing of several patents which are compiled in Table 1.4.

Table 1.4: Compilation of some technologic alternatives for the ageing of spirits

Process	**Principle**	**Reference**
Process for the accelerated maturing of distilled spirits	Activated carbon	(Brown, 1938)
Process for maturing strong beverages	Electromagnetic field	(Gubiev *et al.*, 1986)
Oak aged alcoholic beverage extract and accelerated whisky maturation method	Solvent extraction	(Zimlich & Effler, 2003)
Accelerated ageing of wine and spirits	Packed columns or vessels mixing ground wood and lees combined with micro-oxygenation	(Niazi, 2012)
Apparatus and method for accelerating ageing of alcoholic liquids such as wines, brandies, spirits and the like	Low-frequency polarized pulsating magnetic fields	(Ceccarani, 1999)
Method of treatment for fermented and distilled beverages and the like	Compressional waves of super-sonic frequency	(August & Roy, 1937) (Chambers & Smith, 1937)
Method for ageing whisky	Closed system for gas and liquid recirculation through wood	(Nickol, 1957)
Accelerated ageing of alcoholic beverages	Actinic light	(Weber, 1974)
Acoustic cavitation of distilled spirits and other beverages	Cavitation	(Mahamuni, 2013)
Whiskey making method	Pressure cycles	(Lix, 2013)

On the opposite hand, few accelerated ageing processes are described for mimicking barrel ageing of beer. Main knowledge on barrel ageing of beer mostly focuses on describing the microbial aspects of spontaneous fermentations (Snauwaert *et al.*, 2016), rather than controlling the ageing process. There are works that report the application of wood chips to induce oak character in beer (Sterckx *et al.*, 2012a, 2012b; Wyler *et al.*, 2015), but most brewers still resort to traditional approaches for the production of aged beer (Cantwell & Bouckaert, 2016). Apart from additive ageing, processes focusing microbiological, subtractive and chemical ageing still remain to be studied and developed in order to develop technology for the accelerated ageing of beer.

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Chapter 2. Validation of a LLME/GC-MS methodology for quantification of volatile compounds in fermented beverages

Abstract

Knowledge of composition of beverages volatile fraction is essential for understanding their sensory attributes. Analysis of volatile compounds predominantly resorts to gas chromatography coupled with mass spectrometry (GC-MS). Often a previous concentration step is required to quantify compounds found at low concentrations. This work presents a liquid-liquid micro-extraction method combined with GC-MS (LLME/GC-MS) for the analysis of compounds in fermented beverages and spirits. The method was validated for a set of compounds typically found in fermented beverages comprising alcohols, esters, volatile phenols, and monoterpenic alcohols. The key requirements for validity were observed, namely linearity, sensitivity in the studied range, accuracy, and precision within the required parameters. Robustness of the method was also evaluated with satisfactory results. Thus, the proposed LLME/GC-MS method may be a useful tool for the analysis of several fermented beverages, which is easily implementable in a laboratory equipped with a GC-MS.

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1. Introduction

The flavor, which is one of the most important sensory attributes of fermented, alcoholic, and distilled beverages (cider, wine, beer, vinegar, spirits, vodka, whiskey, among others), is determined by a vast and diverse number of volatile compounds, arising either from raw material (*e.g.*, grapes, barley, hops), yeast/bacteria fermentations, which are secondary metabolites (Briggs, Boulton, Brookes, & Stevens, 2004; Hirst & Richter, 2016; Robinson *et al.*, 2014a), or from ageing when applied (*e.g.*, in oak wood) (Le Floch, Jourdes, & Teissedre, 2015). These volatile compounds belong to diverse chemical families like alcohols, esters, aldehydes and carbonyls, volatile fatty acids, volatile phenols, sulfur compounds, terpenes, norisoprenoids, lactones, furans, and more (Coelho, Genisheva, Oliveira, Teixeira, & Domingues, 2017; Coelho *et al.*, 2015; Hirst & Richter, 2016; Ugliano & Henschke, 2009), which are often found in very low concentrations.

Since volatile compounds of fermented/alcoholic beverages are highly correlated with the sensory characteristics of the products, its identification and quantification acquires crucial significance for understanding beverages organoleptic properties and further develop product quality. In addition, the presence/absence or the amount of each individual component may be a marker of the used technology or the indication of a product defect. The analysis of individual volatile compounds must comprise a chromatographic separation, which is followed by a generic or a selective identification (*e.g.*, flame ionization detector, electron capture detector, flame photometric detector, mass spectrometric detector) (Robinson *et al.*, 2014b). Recently, some authors have correlated FTIR spectra with some specific compounds or groups of compounds (Dambergs, Gishen, & Cozzolino, 2015; Genisheva *et al.*, 2018).

Apart from the major volatile compounds present in amounts of mg L^{-1} (*e.g.*, 3-methyl-1-butanol), which may be analyzed by direct injection, those presented in lower amounts ranging from a few µg L^{-1} (*e.g.,* linalool) or even scarce ng L^{-1} (*e.g.,* 4-methyl-4-mercapto-2-pentanone) must be concentrated before the chromatographic separation. This step could be achieved by mixing a solvent with the sample, as in liquid-liquid extraction (LLE) (Andujar-Ortiz, Moreno-Arribas, Martín-Álvarez, & Pozo-Bayón, 2009; Perestrelo, Fernandes, Albuquerque, Marques, & Câmara, 2006) and liquid-liquid micro-extraction (LLME) (Ferreira, López, Escudero, & Cacho, 1998; Ortega, López, Cacho, & Ferreira, 2001). Several adaptations/modifications of LLE/LLME methods can be envisioned. The evaporation of solvent for increasing concentration, adsorption of volatiles in a solvent drop (single-drop microextraction – SDME) (Jain & Verma, 2011), or even adsorption/desorption of the compounds using a polymeric phase (sorbent-phase extraction – SPE) (Oliveira, Oliveira, Baumes, & Maia, 2008). Solvent-free techniques include solid-phase micro-extraction (SPME) (Souza-Silva, Gionfriddo, & Pawliszyn, 2015; Zhu, Zhu, Wang,

& Li, 2016), usually in the headspace of the sample (HS-SPME) (Cirlini, Caligiani, Palla, & Palla, 2011; García-Martín, Herrero, Peña, & Barciela, 2010), and stir-bar sorptive extraction (SBSE) (Castro & Ross, 2015; Hórak *et al.*, 2010). Some of these methods, developed to analyze volatiles in alcoholic/fermented beverages, are generic considering that they allow the identification and quantification of the majority of compounds, where their range of application depends on the solvents and/or the sorbents' polarity. Specific methodologies based on polymeric materials, sometimes applying derivatization procedures, were developed to quantify specific compounds or classes of compounds (Rodríguez-Bencomo, Schneider, Lepoutre, & Rigou, 2009).

For a method to be applied in the laboratory, it must be validated to ensure its reliability and the quality of the obtained results. Several points must be addressed for a method to be valid, namely its linearity, specificity, quantification range, limits of detection and quantification, sensitivity, precision, and accuracy. Optionally, robustness and reproducibility studies can be performed to reinforce the methods applicability and efficiency (Hartmann, Smeyers-Verbeke, Massart, & McDowall, 1998; Kruve *et al.*, 2015a, 2015b).

This work aims to validate a liquid-liquid micro-extraction method (LLME) first published by Oliveira and collaborators (Oliveira, Faria, Sá, Barros, & Araújo, 2006), which only reported its use for the analysis of three C6-alcohols (1-hexanol, *E*-3-hexenol and *Z*-3-hexenol), exclusively in wine. As the method provided satisfactory performance and results, its feasibility for the analysis of a broader range of compounds and a wider variety of matrices remained to be validated. The presented LLME method combined with GC-MS poses as an additional alternative to analyze volatile compounds in alcoholic/fermented beverages. This procedure can be applied in any laboratory equipped with a GC-MS by any technician, using only ordinary glassware and low amounts of sample and solvents. High throughput applications are envisioned as the procedure enables handling a substantial number of samples and screening a large number of volatiles in a short period of time.

2. Materials and Methods

2.1 LLME-GC/MS Method

2.1.1 Liquid-Liquid Micro-Extraction of Volatile Compounds

In a 10 mL culture tube (Pyrex, ref. 1636/26MP), 8 mL of sample, clarified by centrifugation if necessary, 2.46 µg of internal standard (4-nonanol, Merck ref. 818773), and a magnetic stir bar were added. Extraction was done by stirring samples with 400 µL of dichloromethane (Merck, ref. 106054), at room temperature for 15 min, using a magnetic stirrer. Tubes were placed vertically and agitation was regulated in order to maintain dispersion of solvent micro-drops without reaching the sample surface. After cooling at 0 °C for 10 min, the magnetic stir bar was removed and the organic phase was detached by centrifugation (5118 *g*, 5 min, 4 °C). Using a glass Pasteur pipette, the extract was recovered into a vial, dried with anhydrous sodium sulphate (Merck, ref. 1.06649), and transferred to a new vial for storage at -20 °C before analysis.

2.1.2 Chromatographic Analysis

Gas chromatographic analysis of volatile compounds was performed using a GC-MS Varian Saturn 2000 (Varian,Walnut Creek, CA, USA) equipped with a 1079 injector, an ion-trap mass spectrometer, and a Sapiens-Wax MS capillary column (30 m × 0.15 mm, 0.15 µm film thickness, Teknokroma, Barcelona, Spain). The temperature of the injector and the MS transfer line were both set to 250 °C. The oven temperature was held at 60 °C, for 2 min, programmed to rise from 60 °C to 234 °C, at 3 °C min^{-1}, and from 234 °C to 260 °C at 5 °C min^{-1}. Lastly, it was held for 10 min at 260 °C. The carrier gas was helium GHE4× (Praxair, Maia, Portugal), at a constant flow rate of 1.3 mL min^{-1}. A 1 µL injection was made in the split-less mode, for 30 s (split vent of 30 mL min^{-1}). The detector was set to an electronic impact mode (70 eV) with an acquisition range (*m/z*) from 35 to 300 at an acquisition rate of 610 ms.

2.1.3 Identification of Volatile Compounds

Identification of volatile compounds was preformed using the software Star – Chromatography Workstation version 6.9.3 (Varian), by comparing mass spectra and retention indices with those of pure standard compounds.

2.2 Method Validation

2.2.1 Base Standard Solution

To perform the method validation, a hydroalcoholic solution (7 %, by volume; ethanol Fisher, 99.8 %), using Milli-Q water, was initially prepared, which was the solvent used for compound dilution. First, a concentrated solution of the volatile compounds was prepared by adding each compound, by weighing using an analytical scale (Kern ABJ), to the hydroalcoholic solution, at a concentration of 1000× the highest concentration presented in Table 2.1. The base standard solution was then prepared by diluting the concentrated solution by a factor of 1000 with the hydroalcoholic solution to attain the highest concentrations specified in Table 2.1 (maximum value of the cited range). Compounds were chosen as being representative of the chemical groups with the higher impact in the volatile fraction and sensory properties of fermented beverages, such as wine, beer, and vinegar. These were purchased as pure standards with the purity and suppliers indicated in Table 2.1.

2.2.2 Linearity

Calibration curves were constructed by using six points, corresponding to different concentrations obtained by the dilution of the base standard solution in the hydroalcoholic solution. Each solution was analyzed in triplicate by the proposed method. The average area ratios (*i.e.*, peak area of the compound x, A_x, to the peak area of the internal standard, A_{IS}) were plotted against the concentration ratios (*i.e.*, concentration of the compound x, C_x, to the concentration of the internal standard, C_{IS}) to obtain the calibration curves in accordance with Equation 2.1.

$$\frac{A_x}{A_{\mathrm{IS}}} = b \times \frac{C_x}{C_{\mathrm{IS}}} \qquad \text{(Equation 2.1)}$$

From each curve, slope (*b*) and regression coefficient (R^2) were calculated, and linearity was evaluated by a lack-of-fit *F*-test. Response factors (R_{f}) were also calculated for each compound as the inverse of the slope $\left(R_{\mathrm{f}} = \frac{1}{b}\right)$. The limit of quantification (*LOQ*) was determined as the minimum concentration of the compound that could be trustily quantified.

2.2.3 Precision

Two different measures of precision were evaluated for validation of the LLME method such as repeatability and intermediate precision. Repeatability was evaluated by the analysis of five replicate samples in the same conditions of the proposed method. As a measure of repeatability, the relative standard deviation (*RSD*) was calculated according to Equation 2.2.

$$\frac{RSD}{\%} = \frac{s}{\overline{x}} \times 100 \qquad \text{(Equation 2.2)}$$

where s stands for standard deviation and $\overline{x}$ represents the average of the measured values. To evaluate intermediate precision, independent measurements of dissimilar samples were performed at different times by independent operators, where the *RSD* was also calculated as stated in Equation 2.2.

2.2.4 Accuracy

In the absence of reference materials, accuracy was investigated by spiking and recovery. A commercial beer was used for analysis by the proposed method in its original state and after the addition (spiking) of a known mass of the analyte to the sample. The relative error (*RE*) of the determined concentration was calculated based on Equation 2.3, *i.e.*, calculating the concentration of each compound in the spiked beer (C_{determ}) against its expected concentration (C_{expect}).

$$\frac{RE}{\%} = \frac{C_{\text{determ}} - C_{\text{expect}}}{C_{\text{expect}}} \times 100 \qquad \text{Equation 2.3}$$

2.2.5 Robustness

Other parameters were studied to evaluate the susceptibility of the method to changes that might occur during routine analysis (use a different matrix or an extended extraction time). The matrix effect was evaluated using two different matrices mimicking a wine and a vinegar, respectively, by adding the same volatile compounds under evaluation at an intermediate concentration. Apart from volatile compounds, the synthetic wine comprises ethanol (12 % by volume, Fisher, 99.8 %), tartaric acid (5 g/L, Sigma, 99.5 %), glycerol (7.5 g/L, Himedia, 99.5 %), and malic acid (2 g/L, Acros Organics, 99 %). Synthetic vinegar was prepared using 10 g/L of citric acid (Panreac, 99.5 %) and 50 g/L of acetic acid (Sigma). Three replicates were carried out for each matrix. The effect of the change of the duration of the extraction time was also evaluated. Accordingly, three replicates of the extraction procedure were done by stirring the sample for 30 min instead of 15 min of the proposed method. Recovery (*Rec*) of target compounds, expressed as a percentage, was evaluated by calculating the measured concentration (C_{measur}) *vs.* the expected concentration (C_{expect}), as stated in Equation 2.4.

$$\frac{Rec}{\%} = \frac{C_{\text{measur}}}{C_{\text{expect}}} \times 100 \qquad \text{Equation 2.4}$$

3. Results and Discussion

3.1 Linearity and Sensitivity

Linearity and sensitivity of the proposed LLME method were evaluated by outlining calibration curves for each analyte, using a solution of pure standards. Compounds selected for calibration of the method were chosen on the basis of their contribution for the volatile and aromatic fraction of fermented products, which are considered to be representative of the analytes generally found in beer, wine, spirits, and vinegar. Acids were left out of the validation study by considering the difficulties of maintaining them in a standard solution due to their reaction with some other components in the mixture.

Regressions were performed from the obtained data with the corresponding coefficients presented in Table 2.1. Good linear regressions were obtained for extraction and quantification using the LLME method, with values of $R^2 > 0.995$ for all of the studied analytes. The R^2 value is a useful indicator of the regression quality. However, according to Kruve and collaborators (Kruve *et al.*, 2015b) and Araujo (Araujo, 2009), it cannot be considered as a standalone measure to validate a method linearity, which must be further validated by a statistical lack-of-fit *F*-test. Lack–of-fit tests were performed for the regression curves obtained for each analyte, according to the recommendations of Araujo (Araujo, 2009), since all regressions were demonstrated to be linear, with the *F* obtained being lower than the tabulated one for the corresponding degrees of freedom. This linearity reflects not only the directly proportional response of the MS detector, but also the direct proportionality in the extraction of analytes by LLME.

Table 2.1: Reference, purity (*P*), and concentration range (*C*) for each analyte, and Pearson correlation coefficient (R^2), limit of quantification (*LOQ*), and response factor of the method (R_f), with respective confidence limits ($p = 0.05$), obtained from the calibration curves.

Compound	**Reference**	***P*/%**	**Range** ***C*/**(μg L^{-1})	R^2	***LOQ*/**(μg L^{-1})	R_f
4-methyl-2-pentanone	Fluka 02474	≥ 99.7	24.8 to 248	0.9991	6.9	1.32 ± 0.05
Ethyl butyrate	Aldrich E15701	99	5.76 to 576	0.9995	4.7	1.58 ± 0.04
Ethyl 2-methylbutyrate	Aldrich 306886	99	2.48 to 248	0.9997	1.8	0.87 ± 0.02
Ethyl 3-methylbutyrate	Aldrich 112283	98	3.12 to 312	0.9993	2.2	0.91 ± 0.03
3-methyl-1-butyl acetate	Aldrich 306967	≥ 99	21.32 to 2132	0.9990	3.9	2.00 ± 0.07
Ethyl hexanoate	Aldrich 148962	≥ 99	9.64 to 964	0.9978	2.2	1.32 ± 0.07
Hexyl acetate	Aldrich 108154	99	2.76 to 276	0.9983	2.9	1.57 ± 0.08
3-methyl-1-pentanol	Aldrich 111112	99	25.6 to 256	0.9968	14.2	4.63 ± 0.30
Ethyl lactate	Aldrich E34102	98	113.2 to 1132	0.9978	107.4	44.90 ±2.45
1-hexanol	Fluka 73117	> 99.9	14.72 to 1472	0.9976	6.7	3.63 ± 0.20
E-3-hexen-1-ol	Aldrich 224715	97	6.32 to 632	0.9971	5.1	5.11 ± 0.32
Z-3-hexen-1-ol	Fluka 53056	≥ 98	7.20 to 720	0.9968	5.9	5.23 ± 0.34
Linalool	Aldrich L2602	97	4.76 to 476	0.9998	3.2	1.71 ± 0.03
Diethyl succinate	Aldrich 112402	99	6.12 to 612	0.9977	2.4	1.25 ± 0.07
α-terpineol	Merck 8.21078	≥ 98	2.60 to 260	0.9979	2.6	1.37 ± 0.07
Citronellol	Aldrich C83201	95	2.72 to 272	0.9999	2.2	1.43 ± 0.02
Nerol	Aldrich 268909	97	3.04 to 304	0.9988	3.1	1.83 ± 0.07
2-phenylethyl acetate	Fluka 46030	> 99	10.32 to 1032	0.9995	2.6	1.39 ± 0.03
Geraniol	Aldrich 163333	98	3.08 to 308	0.9994	2.4	1.26 ± 0.04
Guaiacol	Aldrich G10903	98	2.92 to 292	0.9984	5.1	2.65 ± 0.12
4-ethylphenol	Aldrich E44205	99	4.88 to 488	0.9983	4.2	2.03 ± 0.10

Extraction selectivity was maintained throughout the tested concentrations, which enabled proper quantification of the analytes in the mixture. All regressions presented intercept values not significantly different from zero ($p > 0.05$) and, therefore, equations are only based on the slope, similarly to the previously reported works for other LLE methods (Andujar-Ortiz *et al.*, 2009). Moreover, the baseline value is subtracted for the integration of peaks in the chromatogram using background correction in the software, which also justifies the absence of the intercept value.

Sensitivity is defined as the change in the method response, which corresponds to a change in the measured quantity and is intrinsically related to the slope of the calibration curve (Magnusson & Örnemark, 2014). In this case, R_f is the inverse of the slope. This factor is, therefore, a measure of the method's sensitivity in terms of the relative response of each compound in relation to the response of the internal standard. A higher response factor means a higher variation of the compound's concentration for a given variation of the signal, which, therefore, accounts for a lower sensitivity. Overall, response factors obtained for esters ranged between 1 and 2, with the exception of ethyl lactate for which the response factor was highly superior while attaining the value of 44.9 and accounting for a lower sensitivity of the method toward this compound. Similar to esters, monoterpenic alcohols as well as 4-methyl-2-pentanone presented response factors between 1.2 and 1.8, which was followed by volatile phenols that presented slightly higher R_{f} values of about 2. With higher response factors, and, therefore, lower sensitivity alcohols, presented R_{f} values between 3.6 and 5.1. This variation in the response factor is a combination of different extraction selectivity by the LLME method and differences in ionization and detection in the MS. Response factors seem to be similar within groups of compounds, which, despite not excluding the need for determining a specific analyte response for a proper quantification, can aid in the prediction of the response for compounds within the same group. With response factors between 1 and 5 for the majority of compounds, it is believed that the method has good sensitivity. Therefore, the method complies with the first base requirements for validation, being that the LLME method in the study presents proper sensitivity and linearity.

3.2 Limits of Detection and Quantification

Limits of detection (*LOD*) and quantification (*LOQ*) deal with the minimum amount of compound possible to detect and quantify, respectively. As stated by Brettell and Lester (Brettell & Lester, 2004), two strategies can be used for determining *LOD*: a statistical approach, which is more likely to generate artificially low *LOD* values, and an experimental approach, which is attained by decreasing the analyte concentration until the identification criterion is no longer met. This generates higher and closer values to reality *LOD* values (Brettell & Lester, 2004). Since the mass spectrum of a given compound/peak can be compared with spectra of private or commercial spectrum libraries, the occurrence of a match at a given retention time ensures reliability of compound detection and identification. Additionally, considering the GC-MS method used, the *LOD* value will be related with the *LOQ* value as, if a given compound identification is reliable, its quantification from the chromatogram is possible. Hence, the statistical calculation of the *LOD* value has no practical application, and the experimental approach was performed for determining the *LOQ* value, which is of greater use. Several recommendations can be found for determining the *LOQ* value but considering the focus of the method. A conservative approach was chosen for its establishment by following the recommendations of Kruve and collaborators (Kruve *et al.*, 2015a). Therefore, the minimum amount of analyte detected and quantified was taken into account for determining *LOQ*, with the obtained values presented in Table 2.1. As demonstrated, *LOQ* values ranged from 2 µg L^{-1} to 7 µg L^{-1} for most compounds, with the exception of 3-methyl-1-pentanol (14.2 µg L^{-1}) for which the minimum tested concentration was higher, and ethyl lactate (107.4 µg L^{-1}) due to the lower sensitivity obtained. The obtained values are, in their majority, about 2 to 10 folds lower when compared with the values reported by Ferreira and collaborators (Ferreira *et al.*, 1998), which worked with similar compounds and concentrations. As a cross validation for acceptance of this value, the measurements are within the 20 % of the relative standard deviation (*RSD*), as stipulated by Brettel and Lester (Brettell & Lester, 2004).

3.3 Precision

As reported by Kruve and collaborators (Kruve *et al.*, 2015b), precision can be quantified as the relative standard deviation/coefficient of variation of replicate analysis. In this work, we evaluated two types of precision, the repeatability (a single operator in the same run conditions), and the intermediate precision (different operators, different run conditions but the same laboratory). The *RSD* values were calculated for the two scenarios which were presented in Table 2.2. For evaluating the intermediate precision, analyses using the proposed LLME method were performed by two operators with one experienced in its execution and one with reduced experience in the laboratory and with the method. Five replicates were measured by each operator using an independent equipment and apparatus, where the GC-MS is the only equipment in common for the analysis of extracts.

As visible in Table 2.2, *RSD* observed for evaluation of repeatability was considerably low, ranging from 3.3 % to 9.0 %. When analyzing the *RSD* values obtained for intermediate precision (involving two different operators), a higher variation can be observed ranging from about 6.0 % to 19.7 %. This higher dispersion of the measurements can be justified by the differences in the experience of the operators, where deviations in the addition of an internal standard or differences in the interpretation and integration of chromatograms can lead to a higher dispersion of results. Establishment of critical *RSD* values for a method to be precise depends strongly on the application intended. Several limits have been proposed, which are the most common considered *RSD* < 15 % of the nominal value (González *et al.*, 2014; Kruve *et al.*, 2015b) or as high as 20 % for environmental or food samples (Huber, 1998). As seen in the results, values of *RSD* regarding repeatability were all below the minimum level accepted. In addition, despite the higher *RSD* values obtained for intermediate precision, the majority of compounds were still below the acceptable limit of 15 % with the exception of 3-methyl-1-pentanol, ethyl lactate, *E*-3-hexen-1-ol, and *Z*-3-hexen-1-ol, which still fall below the limit of 20 % proposed by Huber (Huber, 1998). Thus, the method is considered precise and can be performed with satisfactory outputs.

Table 2.2: Values obtained for evaluation of precision, measured as relative standard deviation (*RSD*), accuracy, expressed as relative error (*RE*), and robustness, quantified by compound recovery (*Rec*)

Compound	**Repeatability**	**Intermediate Precision**	**Accuracy**	**Robustness**		
	RSD/%	***RSD***/%	***RE***/%	***Rec***/% (t = 30 min)	***Rec***/% **(Synthetic Wine)**	***Rec***/% **(Synthetic Vinegar)**
4-methyl-2-pentanone	6.5	9.3	11.2	103.2	100.6	103.1
Ethyl butyrate	5.3	7.4	10.8	99.9	91.0	95.2
Ethyl 2-methylbutyrate	9.0	9.3	13.2	96.6	86.0	83.0
Ethyl 3-methylbutyrate	5.3	6.7	20.5	98.0	88.8	91.8
3-methyl-1-butyl acetate	4.5	5.7	1.6	104.7	93.9	96.2
Ethyl hexanoate	3.3	6.0	2.9	100.6	101.9	100.3
Hexyl acetate	3.8	11.2	15.5	98.0	92.3	97.8
3-methyl-1-pentanol	6.3	18.0	2.3	96.1	148.6	113.6
Ethyl lactate	8.4	18.9	2.8	88.5	161.6	106.7
1-hexanol	5.0	12.5	15.8	91.3	115.3	95.9
E-3-hexen-1-ol	7.0	18.0	14.2	86.0	116.3	96.2
Z-3-hexen-1-ol	6.8	19.7	15.9	85.8	116.4	90.5
Linalool	4.0	10.8	9.5	103.8	98.6	93.4
Diethyl succinate	3.3	10.3	13.3	113.0	117.7	113.4
α-terpineol	4.9	10.2	10.8	109.7	112.7	105.6
Citronellol	4.4	12.5	6.6	87.8	90.6	86.1
Nerol	5.9	13.8	13.7	108.6	100.0	93.2
2-phenylethyl acetate	3.4	7.9	0.7	108.6	109.6	109.9
Geraniol	2.6	9.6	16.7	107.2	97.3	93.9
Guaiacol	6.3	14.0	1.6	97.3	120.5	105.5
4-ethylphenol	4.5	9.9	5.0	72.9	112.5	97.1

3.4 Accuracy

Accuracy was determined by the addition of a known amount of the analytes in the study to a real sample (spiking) and quantification of the analytes in the spiked sample. For this purpose, a commercial beer was analyzed using the proposed LLME method both in its original state and after spiking, as recommended by the guidelines for method validation (Kruve *et al.*, 2015b). To better assess accuracy, the theoretical expected concentration ($C_{\mathrm{expect}} = C_{\mathrm{beer}} + C_{\mathrm{spik}}$) was compared with the concentration measured using the LLME method (C_{determ}). According to multiple t-tests for comparisons ($p \leq 0.05$), no differences were found between the expected and the measured concentrations. As a more appropriate measure of accuracy, the deviation of the measured concentrations regarding the expected values was calculated, and expressed as a relative error (*RE*) (González *et al.*, 2014). In agreement with the results reported by González and collaborators (González *et al.*, 2014), this value cannot exceed 15 % for the method to be accurate (except for determinations at the *LOQ* where 20 % is accepted). As shown in Table 2.2, *RE* values were within the 15 % limit established for the studied compounds. Thus, the method is considered to be accurate when complying with another key requirement for validity.

3.5 Robustness

As stated, robustness can be defined as the ability of the method to endure slight variations and maintain its result (Kruve *et al.*, 2015a). To assess the robustness of the method, two criteria were evaluated including variation of contact time and the matrix effect, which were identified as the main variables affecting the LLME method. The effect of an increased stirring and extraction time was tested to evaluate the possible occurrence of differences in compound extraction. Again, as performed for accuracy, possible differences in compound recovery and quantification were statistically determined by the *t*-test, comparing the measured concentration with the expected concentration of the compounds, and evaluating the recovery of target analytes by taking into account the known dilution and concentration of the solution of standards.

Regarding the increase of stirring time, no statistically significant differences were observed for the measurements performed with 30 min of stirring ($p > 0.05$). Extraction of the compounds using 15 min stipulated in the method is shown to be sufficient for the total recuperation of analytes, which is maintained independently of the longer contact time. For a better assessment of robustness, recovery was calculated for each compound in accordance with Kruve and collaborators (Kruve *et al.*, 2015b), with the values presented in Table 2.2. Similarly, when observed for accuracy, critical recovery values can be established for the acceptance of recovery in determining robustness. In this sense, values of recovery between 70 % and 110 % for measurements ranging from 10 μg L^{-1} to 100 μg L^{-1}, or 80 % to 110 % for measurements above 100 μg L^{-1}, are considered acceptable (AOAC International, 2013). Therefore, the recovery values obtained with increased stirring time were within the acceptable range.

Regarding the matrix effect, the main focus was to evaluate if the recovery and quantification of the analyses would be affected by other components in the mixture. For control purposes, two synthetic matrixes were tested including a solution mimicking wine composition and another mimicking vinegar composition. For the majority of compounds, recovery values were also within the acceptable ranges previously referred, with the exception of those obtained for 3-methyl-1-pentanol and ethyl lactate in the synthetic wine matrix. The higher recovery observed for these compounds can be caused by a higher efficiency and selectivity in their extraction and, therefore, an accuracy test or validation in wine would be advised for the specific quantification of these compounds. Nevertheless, only two compounds in one matrix showed recovery values outside the acceptable range. The remaining compounds were properly quantified in the synthetic wine as well as all compounds in the synthetic vinegar matrix. Considering the overall results obtained under multiple conditions, global robustness of the method can be considered satisfactory.

4. Conclusions

The LLME method presented in this work is a reliable alternative for the analysis of compounds participating in the volatile fraction of fermented beverages. The method is linear for the studied ranges and has good sensitivity, which varies depending on the chemical group of compounds. The method is precise and has shown good repeatability and intermediate precision. Variations were performed for the analytical matrix and for protocol execution. The LLME method was also demonstrated to be robust. Lastly, the method is accurate and adequate for application in real samples. Having complied with all the parameters needed, the LLME method presented in this work is, therefore, valid for application in the analysis of fermented beverages and, certainly, to distilled beverages/spirits, after a convenient dilution with water to reach an alcoholic strength, by volume, of about 15 %.

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Chapter 3. Understanding wine sorption by oak wood: modeling of wine uptake and characterization of volatile compounds retention

Abstract

Cooperage wood is a porous material and beverages exchange compounds with it by penetrating into its pores. This work demonstrates the enrichment of wood with wine during ageing. Three oak varieties were cut into different sized chips and immersed in fortified wine and water. Wine and water uptake were measured along time and sorption was described based on a saturation empirical model. Maximum uptake varied among wood types and was independent of particle size, which affected only equilibrium time. Sorption of wine volatiles such as alcohols, esters and acids in wood was shown, which was also dependent on wood type and independent of particle size. Multivariate analysis demonstrated differences and similarities in depletion of wood extractives and sorption of wine volatiles depending on wood variety. Sorption shown in this work demonstrates wood as a vector for aroma recombination, when reused for ageing between different beverages.

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1. Introduction

Ageing is a key factor in the production of alcoholic beverages and spirits, essential for the final quality of the product. Traditionally, ageing is performed by storing the beverage in wood barrels. Oak is the preferred cooperage wood, where the extraction of its compounds (additive ageing) and reaction and evaporation of beverage compounds (subtractive ageing) enhance the sensory character of the final product (Mosedale & Puech, 1998). Accelerated ageing processes are also used, which resort to soaking wood in the beverage in vats, mimicking and accelerating phenomena occurring in the barrel (Canas, Caldeira, & Belchior, 2013; del Álamo-Sanza, Nevares, Gallego, Martin, & Merino, 2008). Traditionally, oak wood can be reused and goes from the ageing of one beverage to another. For instance, scotch whisky production resorts to woods previously used in other beverages ageing such as Bourbon or Sherry, being also reported the reuse of Port or Madeira wine casks for the finishing steps (Mosedale, 1995; Russell & Stewart, 2014). Beer ageing also resorts to the reuse of woods from other beverages, as the example of Lambic beers which reuse casks previously used in wine ageing (Spitaels *et al.*, 2014).

During ageing, several wood compounds are extracted by the hydroalcoholic matrix with well documented impact on the beverage properties (De Rosso, Cancian, Panighel, Dalla Vedova, & Flamini, 2009; Garde-Cerdán & Ancín-Azpilicueta, 2006; Le Floch, Jourdes, & Teissedre, 2015). Wood can be reused a finite number of times during which it becomes depleted of extractives (Mosedale & Puech, 1998; Wilkinson, Li, Grbin, & Warren, 2013). Besides becoming depleted of extractives, several other additive transformations occur to wood during ageing. For instance, wine has a complex endogenous microflora which during contact adheres and grows on the wood surface in the form of biofilms (Bastard *et al.*, 2016). This has been shown to impart the ageing of other beverages when reusing the cask (Spitaels *et al.*, 2014). Also, tartaric acid present in wine precipitates mainly in the form of potassium hydrogen tartrate crystals at wood surface (Ortega-Heras, González-Sanjosé, & González-Huerta, 2007). With more interest to the present work, wood sorption of beverage compounds, namely volatile compounds which influence sensory properties, also occurs. Ramirez and collaborators demonstrated that terpene alcohols, esters, aldehydes and norisoprenoids concentrations in wine decreased in the presence of wood, especially for linalool and ethyl octanoate. Such decrease was described as selective and did not depend on solubility of the compounds but instead on acid, base and polar characteristics (Ramirez-Ramirez *et al.*, 2001). Barrera-García and collaborators demonstrated the sorption of monomeric volatile phenols in the wood-wine interface (Barrera-García, Gougeon, Voilley, & Chassagne, 2006) and also the sorption of polyphenols in wood, with the disappearance of monomeric anthocyanins, (+)-catechin, (-)-epicatechin, and *trans*-resveratrol in the presence of wood (Barrera-García *et al.*, 2007). Later, Barrera-García and collaborators also demonstrated that cellulose, hemicellulose and lignin were involved in sorption selectivity of phenolic compounds by wood (Barrera-García, Gougeon, Karbowiak, Voilley, & Chassagne, 2008). To our knowledge, these works cover most of the scientific research on wood sorption of chemical compounds during ageing of alcoholic beverages, and focus mainly on the subtractive changes that occur to the beverage. Therefore, considering that wood reuse is common, detailed knowledge of beverage sorption is of upmost importance to understand its impact during the subsequent reutilizations. If we consider that wood becomes enriched with the beverage, it can be a vector for transferring sensory properties from one product to another.

This work focuses on transformations occurring to wood by sorption of wine, taking into account mass transfer and retention of wine volatiles in wood. For the first time, sorption was studied using a real fortified wine in three different types of wood, covering mass transfer into wood with the establishment of empirical mathematical models for wine uptake. Retention of volatiles was not only demonstrated but was also shown to differ among the studied woods.

2. Materials and Methods

2.1 Chemicals and materials

The following chemicals were used as standards for the GC-MS analysis: 3-methyl-1-butanol (≥ 99.8 %), 2-methyl-1-propanol (≥ 99.9 %), 1-hexanol (≥ 99.9 %), *Z*-3-hexenol (≥ 90 %), 2-phenylethanol (≥ 99 %), hexanoic acid (≥ 98 %), furfural (99 %), vanillin (≥ 98 %), from Fluka; benzyl alcohol (≥ 99 %), etyl butyrate (≥ 99 %), isoamyl acetate (≥ 99 %), ethyl hexanoate (≥ 99 %), ethyl lactate (98 %), ethyl octanoate (≥ 99 %), ethyl-3-hydroxybutyrate (99 %), diethyl succinate (99 %), diethyl malate (≥ 97 %), ethyl hexadecanoate (≥ 99 %), monoethyl succinate (90 %), octanoic acid (≥ 99.5 %), 5-methylfurfural (99 %), 4-methylguaiacol (≥ 98 %), 4-propylguaiacol (≥ 99 %), guaiacol (98 %), 2,6-dimethoxyphenol (99 %), eugenol (99 %), *cis*/*trans*-oak lactone (≥ 98 %), 4-ethylbenzaldehyde (98 %), acetovanillone (98 %), syringaldehyde (98 %) from Aldrich; 5-hydroxymethylfurfural (98 %) from Acros Organics and 4-ethylguaiacol (98 %) from Alfa Aesar. The remaining compounds were identified on the basis of the NIST spectrum collection.

Fortified wine used for the sorption assays was kindly provided by Quinta do Portal S.A., and is used for Port wine production in the Douro demarcated region. Wine had an ethanol content, by volume, of 20.9 % ± 0.3 %, and a volumetric mass density of 0.9861 g cm^{-3} ± 0.0007 g cm^{-3}. Toasted French oak (M+ toast) 950×50×18 mm^3, toasted American oak (M+ toast) 950×50×6 mm^3 and untoasted American oak 950×50×6 mm^3 staves, from the Oenostave® series (kindly provided by Seguin Moreau), were used in this work. Toasted staves were used with the M+ toasting level, which in cooperage generally corresponds to a 68 min toast at 62 °C ± 3 °C without water addition (Chira & Teissedre, 2014).

2.2 Structural analysis of oak woods

Structural properties of woods were characterized at Instituto Pedro Nunes (Coimbra, Portugal), using whole wood pieces without grinding. Mercury porosimetry, which analyses pores between 5.5 nm and 360 µm, was performed using a Micromeritics AutoPore IV 9500 following the ISO 15901-1:2016 procedures. BET porosimetry, which analyses pores between 1 nm and 0.3 µm, was measured using a Micromeritics ASAP 2000 surface area analyzer following the procedures of ISO 9277:2010. A desktop Scanning Electron Microscope (SEM) (Phenom ProX, Netherlands) was used for microscopy. Samples were added to aluminum pin stubs with electrically conductive carbon adhesive tape (PELCO Tabs™), which were placed inside a Phenom Standard Sample Holder. The analysis was conducted at 5 kV with intensity image. All results were acquired using the ProSuite software.

2.3 Sorption conditions

The three different oak woods used for the uptake and sorption studies were cut into 3 mm × 3 mm × 6 mm (small- S), 7 mm × 7 mm × 6 mm (medium- M) and 10 mm × 10 mm × 6 mm (large- L) chips. Wood staves were cut with a vertical saw in the transversal direction and then individually cut using a blade in the longitudinal direction of wood. All chips were verified for their dimensions with a pachymeter. Woods were put in contact with fortified wine, and distilled water as control, at a concentration of 50 g L^{-1} in Pyrex tubes fitted with Teflon caps. Isotherm conditions were maintained at 20 °C with 150 min^{-1} orbital agitation. Uptake was accompanied by determination of mass variation in wood throughout contact time, until equilibrium was attained. For such, wood chips were recovered periodically, excess wine at wood surface was eliminated with an absorbent and wood chips were weighed in a Mettler AE 200 analytical scale. Uptake was calculated as percentage of mass variation referred to the initial mass, taking also into account the initial moisture of wood, determined with a Radwag MAC/50/1/NH moisture analyzer. All woods remained in contact with wine until equilibrium was observed for all the tested conditions. The state of equilibrium was assumed when no mass variation was observed for three consecutive measurements. Wood-wine contact was performed in independent triplicates for each condition.

2.4 Modeling of uptake

Modeling of uptake by wood was performed by non-linear regression. For that a regression was performed to verify the adjustment of the model proposed in Equation 3.1 to the experimental data:

$$U(t) = \frac{t \times U_{\max}}{K_U + t} + H_{\mathrm{i}} \qquad \text{Equation 3.1}$$

where $U(t)$ is the uptake expressed as percentage of mass gained, t is time, $U_{\max}$ is the maximum uptake expressed as percentage of mass gained, K_U is the time needed for reaching half of $U_{\max}$ and H_{i} is the initial moisture in mass percentage. For the regression, the sum of squared errors between the predicted and experimental data was minimized using the Excel solver, by the modification of $U_{\max}$ and K_U values. Errors associated to the regression coefficients were calculated for a 95 % confidence interval.

2.5 Extraction of volatiles from wood

In order to determine the presence of volatile compounds in woods, an extraction step was performed on the basis of previous works (Caldeira, Clímaco, Bruno de Sousa, & Belchior, 2006). Woods were ground to particles of diameter <1 mm, and mixed at a concentration of 50 g L^{-1}, using a 55 % hydroalcoholic solution, pH 4.6. Contact between wood and the hydroalcoholic solution was performed in 10 mL Pyrex tubes with Teflon caps, at 20 °C with rotary agitation during 24 h to ensure total extraction of wood content. Afterwards, the hydroalcoholic solution was separated from woods by centrifugation at 2700 g during 10 min followed by decantation of the clear extracts.

2.6 Analysis of volatile compounds

Volatiles in the hydroalcoholic extracts were analyzed by gas chromatography coupled with mass spectrometry (GC–MS). Extraction was performed in 8 mL of diluted wood extract samples, by a factor of 4, with 400 µL of dichloromethane (SupraSolv for gas chromatography, Merck), after adding 4-nonanol as internal standard (2.4 µg). Extracts were analyzed in a gas chromatograph Varian 3800 equipped with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000. Each 1 µL injection was made in splitless mode (30 s) in a Sapiens-Wax MS column (30 m × 0.15 mm; 0.15 µm film thickness, Teknokroma). Carrier gas was helium 49 (Praxair) at a constant flow of 1.3 mL min^{-1}. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range (m/z) from 35 to 260 and 610 ms acquisition interval. The oven temperature was initially set to 60 °C for 2 min and then raised to 234 °C at a rate of 3 °C min^{-1}, raised again to 260 °C at 5 °C min^{-1} and finally maintained at 260 °C for 10 min. Injector temperature was set to 250 °C with a 30 mL min^{-1} split flow and transfer line was maintained at 250 °C. Compounds were identified using MS Workstation version 6.9 (Varian) software, by comparing mass spectra and retention indices with those of pure standards and quantified as 4-nonanol equivalents. PCA analysis was performed using Statsoft Statistica 7 software.

3. Results and discussion

3.1 Structural analysis of oak wood

In order to investigate oak wood capacity as a sorbent, its porosity was assessed by mercury porosimetry and BET porosimetry. The results are presented in Table 3.1.

Table 3.1: Structural characterization of French and American oak woods by mercury and BET porosimetry

	Toasted French oak	**Toasted American Oak**
Mercury porosimetry		
Intrusion Specific Volume (cm^3 g^{-1})	$74.8\times10^{-2} \pm 2.1\times10^{-2}$	$75.2\times10^{-2} \pm 2.1\times10^{-2}$
Total Pore Specific Area (m^2 g^{-1})	49.3 ± 1.4	41.3 ± 1.1
Average Pore radius (nm)	30.4 ± 0.1	36.4 ± 0.1
Porosity (%)	50.9 ± 1.4	51.9 ± 1.4
BET		
Specific Surface area (m^2 g^{-1})	$27.2\times10^{-2} \pm 1.3\times10^{-2}$	$32.8\times10^{-2} \pm 1.3\times10^{-2}$
Total Pore Specific Volume (cm^3 g^{-1})	$4.6\times10^{-4} \pm 0.2\times10^{-4}$	$5.1\times10^{-4} \pm 0.2\times10^{-4}$
Average Pore diameter (nm)	6.8 ± 0.1	6.2 ± 0.1

Wood is acknowledged as a porous material due to its structure, where hollow lumens exist within the individual fibers, interlinked with lignin (Mackay & Gschwend, 2000; Zillig, Janssen, Carmeliet, & Derome, 2006). This can be observed in Figure 3.1, where the pores are clearly visible on the surface of cut French wood. Porosities were of 50.9 % for French and 51.9 % for American oak, with similar intrusion volumes of 74.8×10^{-2} cm^3 g^{-1} and 75.2×10^{-2} cm^3 g^{-1}, respectively. Such values are coherent with the ones previously reported for oak woods (Ding, Koubaa, Chaala, Belem, & Krause, 2008; Plötze & Niemz, 2011). As a consequence of its porosity, oak wood demonstrated a large internal surface area associated to pores, of 49.3 m^2 g^{-1} for French oak and 41.3 m^2 g^{-1} for American oak. The difference in surface area among the analyzed oak types can be directly correlated with the differences observed in average pore diameters. French oak presented a lower average pore diameter than American oak, which were of 30.4 nm and 36.4 nm, respectively. Porosity in oak wood was characterized mainly in the form of mesopores, within the 2 nm and 50 nm interval (Leofanti, Padovan, Tozzola, & Venturelli, 1998). When comparing the results of mercury porosimetry with those of BET porosimetry, most of surface area was associated with pores averaging a 30 nm diameter, whereas smaller pores had low expression in the overall void volume.

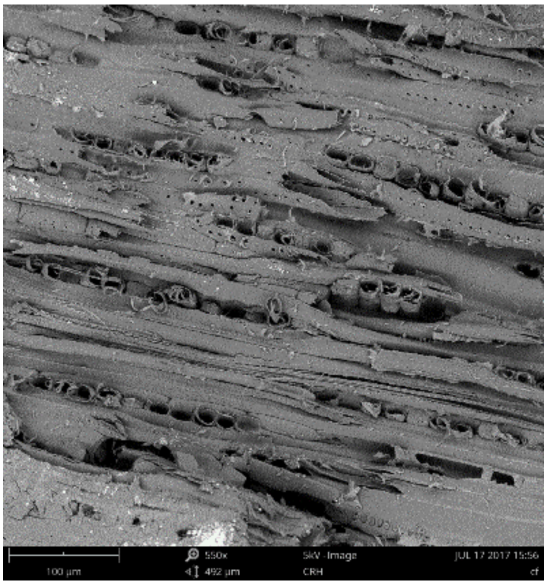

Figure 3.1: Scanning Electron Microscopy (SEM) image of toasted French oak chip surface.

For instance in French oak, the volume associated with pores averaging 6.8 nm radius was only of 4.6×10^{-4} cm^3 g^{-1}, largely inferior to the one previously discussed for mercury porosimetry. The same behavior was observed for American oak where the volume associated with pores was slightly higher than the French variety but still only accounted for 5.1×10^{-4} cm^3 g^{-1} for an average pore diameter of about 6.2 nm. Contrary to the observed for micropores, average diameter of pores in American oak was lower than the observed for French oak, which justifies the higher BET surface area observed for American oak.

3.2 Modeling of wine uptake by wood

Taking into account the porous nature of wood and on the basis of a submerged wood-wine contact system, chips of different sizes of toasted French, toasted American and untoasted American oak were put in contact with wine and water, which served as control. Uptake of liquid was monitored on a mass percentage basis, with the sorption kinetics presented in Figure 3.2. It can be seen that both wine and water uptake showed saturation profiles, with most of the uptake occurring in the first two days for French oak and in the first five days for American and untoasted American oak. Uptake profiles are coherent with the previously described for water sorption in isotherm conditions (Engelund, Thygesen, Svensson, & Hill, 2013). For the overall particle sizes, French oak stabilized earlier than American oak, needing only 14 days for full stabilization whereas American oak stabilized later, at around 35 days to 40 days for toasted and 30 days to 35 days for the untoasted variety. For a better understanding of the differences in uptake kinetics, non-linear regressions were performed, which allowed the comparison between the different conditions studied regarding two main variables: maximum uptake of wood ($U_{\max}$) and time needed to attain half of the observed maximum uptake (K_U).

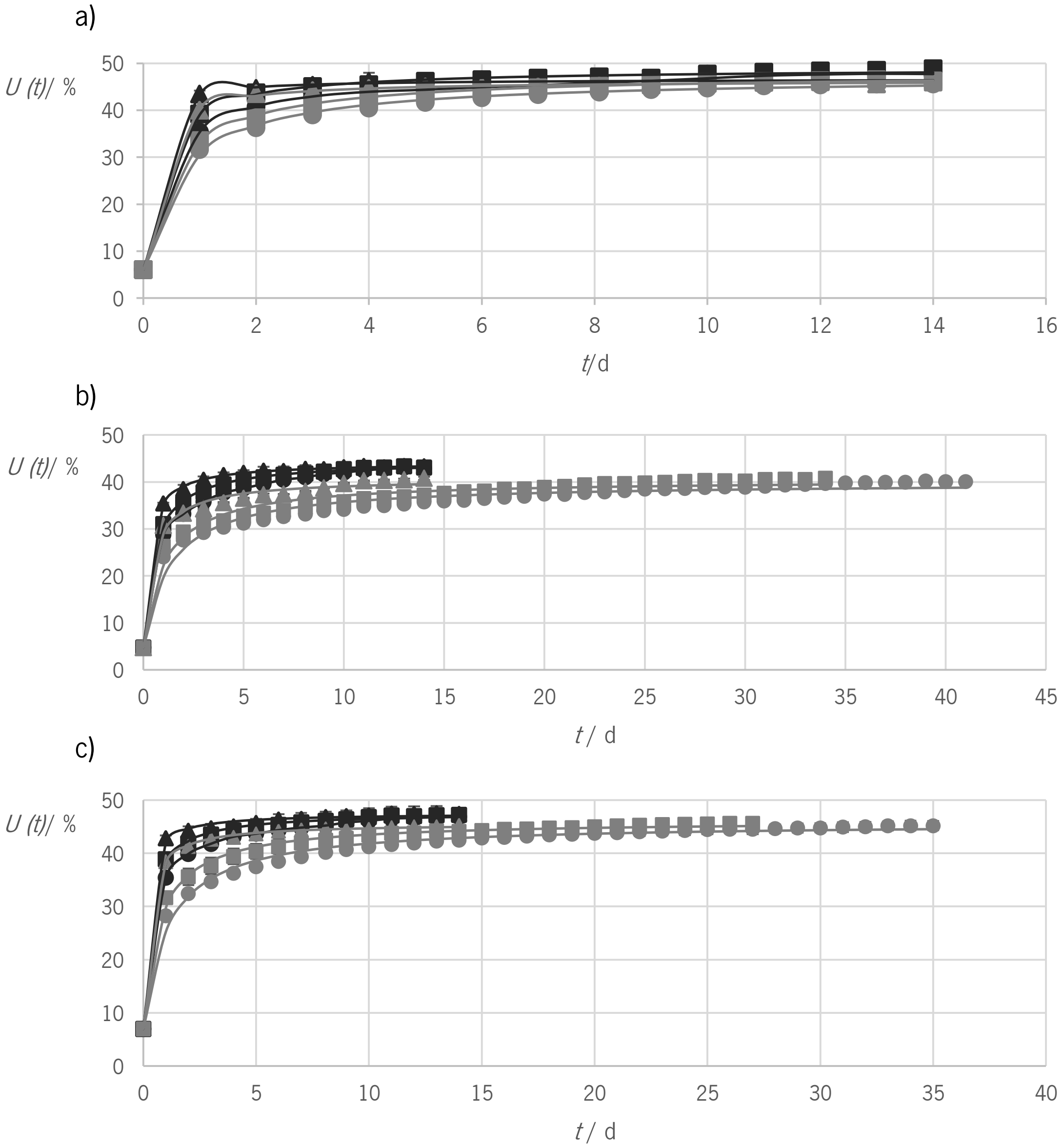

Figure 3.2: Uptake [*U(t)*] throughout time (t) of small (triangles), medium (squares) and large (circles) chips for a) toasted French oak, b) toasted American oak and c) untoasted American oak in contact with fortified wine (grey) and water (black), along with the corresponding kinetic models obtained by non-linear regression (lines).

Initial moisture (H_i) in wood was also taken into account considering that it would influence maximum uptake capacity. U_max and K_U obtained for each condition are listed in Table 3.2. A good correlation was found between wood uptake kinetics and the proposed empirical model, as visible by the R^2 obtained from the non-linear regressions. In the majority of conditions tested, regression coefficients were higher than 0.99, only with the exception of uptake of wine by toasted American oak which presented lower R^2 values but still higher than 0.95. Therefore, the proposed empirical model properly represents the sorption kinetics under study. Maximum uptake observed (U_max), ranged between 38 % and 43 % for all the conditions tested. French oak showed slight differences in water and wine uptake but these were not statistically significant for a 95 % confidence interval. However, statistically significant differences in water and wine uptake were found between toasted and untoasted American oak. For all particle sizes, maximum uptake of water was lower than maximum uptake of wine, with the highest difference observed in toasted American oak, when compared with the untoasted variety. Such differences in uptake can be explained by the different densities of each matrix. Fortified wine used in this work presented a density of 0.9861 g cm^{-3} and therefore lower than water, which can be attributed to its high ethanol content (21 %, by volume) (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Therefore differences in $U_{\max}$ can be expected considering that it was determined on a mass basis. Regarding comparisons between oak varieties, no statistically significant differences were found in water uptake for toasted and untoasted American oak in the different chip sizes tested. However, wine uptake was significantly higher for untoasted American oak when compared with the toasted variety. For French oak, water uptake was higher when compared with American oak, either toasted or untoasted, with only the small chips being significantly different. Wine uptake was also higher in French oak when compared with both American varieties for all the particle sizes. Considering the similar porosities observed for each wood, differences in maximum uptake are not justified by differences in volume associated to pores. Therefore, further wood characteristics may be involved in the maximum uptake capacity, which remain to be further investigated. Focusing K_U values, a higher variation was observed either among the chips of different size or water and wine contact. Water uptake was faster than wine uptake for all the chip sizes and oak varieties, as demonstrated by the lower time needed to attain half of $U_{\max}$, being also visible in the uptake profiles as wood in contact with water reached equilibrium earlier. Uptake is not only dependent on the sorbent material, but also on the absorbed matrix. Wood is a hygroscopic porous material, where liquid uptake occurs mainly through diffusion, following the established by Darcy's Law (Kang & Chung, 2009). As described, wine viscosity is higher than water, due to its content in sugar, ethanol and glycerol (Nurgel & Pickering, 2005; Pickering, Heatherbell, Vanhanen, & Barnes, 1998). Therefore, taking into account Darcy's Law, as discussed by Morris Muskat (Muskat, 1937), the higher viscosity of wine when compared to water can be correlated with the slower uptake observed, hence reinforcing the validity of the models. K_U values also demonstrate the importance of particle size on uptake kinetics. It can be clearly seen in the results that K_U increased with increase of particle size, which is expected taking into account the described for liquid mass transfer into wood. Again, this observation can be explained by Darcy's Law, which postulates that flow varies in inverse proportion with the length of the section (Muskat, 1937). Therefore, the increase observed in the modeled K_U values is again coherent with the postulated for liquid transport by diffusion.

Table 3.2: U_{max} and K_U kinetic coefficients obtained by non-linear regression for fortified wine (FW) or water (H_2O) uptake by toasted French oak (TFO), toasted American oak (TAO) and untoasted American oak (UAO) in the form of small (S), medium (M) and large (L) chips. Errors represent standard deviation for independent triplicates

		K_U/d			$U_{\mathrm{max}}/\%$			R^2		
Wood	Matrix	S	M	L	S	M	L	S	M	L
TFO (H_i = 6 %)	H_2O	0.09 ± 0.03	0.30 ± 0.04	0.47 ± 0.05	40.54 ± 0.31	42.87 ± 0.42	42.51 ± 0.46	0.9988	0.9983	0.9982
	FW	0.18 ± 0.02	0.56 ± 0.03	0.68 ± 0.07	40.27 ± 0.20	41.84 ± 0.28	41.08 ± 0.58	0.9995	0.9994	0.9977
TAO (H_i = 4 %)	H_2O	0.30 ± 0.02	0.56 ± 0.04	0.76 ± 0.10	39.40 ± 0.20	39.82 ± 0.36	39.86 ± 0.71	0.9996	0.9989	0.9961
	FW	0.49 ± 0.14	1.06 ± 0.20	1.36 ± 0.23	35.99 ± 1.09	35.77 ± 0.72	35.17 ± 0.66	0.9866	0.9633	0.9590
UAO (H_i = 7 %)	H_2O	0.16 ± 0.04	0.31 ± 0.04	0.47 ± 0.05	40.50 ± 0.45	40.72 ± 0.40	40.76 ± 0.44	0.9976	0.9983	0.9983
	FW	0.24 ± 0.02	0.72 ± 0.07	1.12 ± 0.12	38.59 ± 0.18	39.16 ± 0.36	38.73 ± 0.43	0.9996	0.9938	0.9864

In further detail, for chips with a rectangular prism geometry, when plotting K_U values *versus* particle surface area (*SA*), a linear correlation between particle size and K_U was hinted. For proper assessment of this hypothesis, further particle sizes were tested using French oak submerged in fortified wine, in order to check if the direct proportion and linear behavior would be maintained, being the results presented in Figure 3.3. As seen by the R^2 of 0.9685, a good correlation between particle size and K_U was obtained, demonstrating the correlation between uptake time and particle size in isotherm conditions. Therefore, the empirical model proposed goes in good agreement with the reported for liquid transport in wood, being considered a good predictive tool for the process in matter.

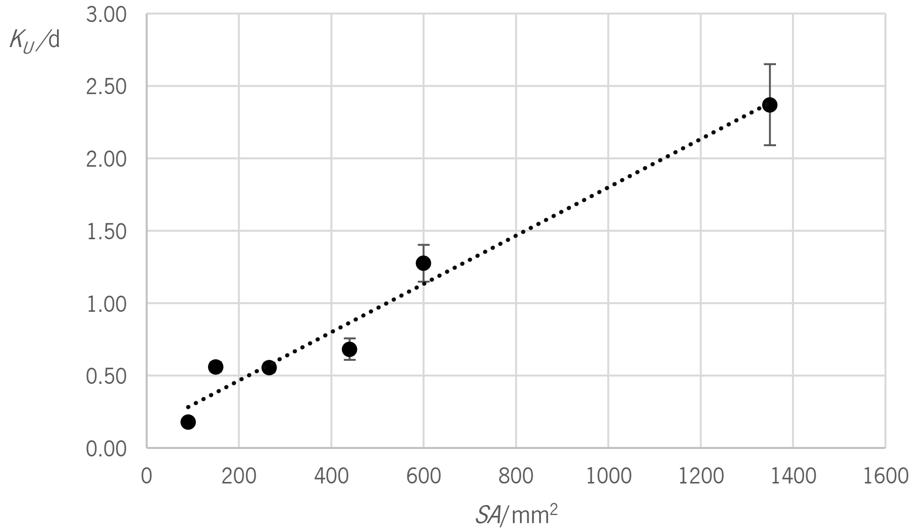

Figure 3.3: Correlation between particle surface area (*SA*) and the time needed to attain half of the maximum uptake (K_U) for toasted French Oak in contact with Fortified Wine $\frac{K_U}{\mathrm{d}} = 0.0017 \times \frac{SA}{\mathrm{mm}^2} + 0.1332$

As demonstrated, oak wood is a porous material and wine enters wood pores by diffusion during contact. Mass transfer of wine into wood was modeled and demonstrated as being of upmost importance in the enrichment observed, with uptake speed depending on particle size, and maximum uptake depending on wood type. At equilibrium, maximum retention capacity of wood was observed, with differences depending on wood type.

3.3 Sorption of wine volatiles by oak wood

After modeling uptake kinetics, characterization of wine volatiles in wood was performed at equilibrium, in order to assess its retention. Volatiles quantified in wood are presented in Table 3.3, which represent the compounds identified Control wood samples (N) showed typical wood extractives, such as volatile phenols, aldehydes, ketones and lactones, coherent with the reported for oak wood (De Rosso *et al.*, 2009; Wilkinson *et al.*, 2013). Toasted American oak presented higher levels of oak lactone and volatile phenols than toasted French oak which on its hand presented higher concentrations of furan compounds. The observed differences in oak composition are in good agreement with the previously reported for these wood varieties (Cadahía, Fernández de Simón, & Jalocha, 2003; Chira & Teissedre, 2014). Furan compounds, aldehydes and some volatile phenols were absent or found in lower concentrations in untoasted American oak when compared with the toasted variety. Concentration of these compounds in wood is strongly dependent on the burning step during cooperage (Caldeira, Mateus, & Belchior, 2006; Canas, 2017; Fernández de Simón, Cadahía, del Álamo-Sanza, & Nevares, 2010), which justifies the lower concentrations found in untoasted American oak.

After contact with wine, overall concentration of wood extractives was lower when compared to the control, as a result of extraction of wood volatiles, which are highly soluble in ethanol-water solutions (Mosedale & Puech, 1998). No statistically significant differences were found in wood extractives concentration between the 3 particle sizes for toasted American and French oak and untoasted American oak. Therefore, extraction of wood volatiles occurred at the same level for the different particle sizes within each wood variety. Regarding sorption, several wine volatiles were found in wood after contacting with wine, namely 3-methyl-1-butanol, 2-phenylethanol, ethyl lactate, monoethyl and diethyl succinate, diethyl malate, ethyl hexadecanoate, 4-ethylbenzaldehyde and octanoic acid.

Most of these compounds were initially present in the wine matrix, as seen in Table 3.3, being that the ones appearing in wood correspond to those found in higher concentration in wine. Taking this and the previously discussed for wine uptake into account, absorption of wine can be the main contributor for the enrichment of wood with such compounds. On the other hand, Ramirez and his collaborators have previously demonstrated wood sorption of ethyl esters (ethyl hexanoate and ethyl octanoate), 2-phenylethanol and benzaldehyde from a synthetic hydroalcoholic matrix (Ramirez-Ramirez *et al.*, 2001). Baiano and his collaborators have also demonstrated the disappearance of 2-phenylethanol, isoamyl acetate and 3-methyl-1-butanol along several other compounds in wines treated with oak chips, which implied that compounds were retained by wood (Baiano *et al.*, 2016).

In the present work, using a real wine matrix, ethyl esters, alcohols and aldehydes were also found in wood. Moreover, the concentration of volatiles retained in wood was not proportional to the concentrations observed in wine, indicating that selectivity took place, which has also been reported in Ramirez's work. Finally, 2-phenylethanol concentrations found in this work are within the same values reported for wood sorption of this compound (Ramirez-Ramirez *et al.*, 2001), which confirms the previous findings and validates the results obtained.

Table 3.3: Characterization of volatiles, expressed as concentration (*C*) of 4-nonanol equivalents, in Fortified Wine, unused oak controls (N) and oak chips with the dimensions of small (S), medium (M) and large (L) after contact with Fortified Wine for toasted French and American oak and untoasted American oak. Errors represent standard error of the mean from independent duplicates

			toasted French oak				**toasted American oak**				**untoasted American oak**			
N.º	**Compound**	**Wine** C/(µg L^{-1})	**N** C/(µg g^{-1})	**S** C/(µg g^{-1})	**M** C/(µg g^{-1})	**L** C/(µg g^{-1})	**N** C/(µg g^{-1})	**S** C/(µg g^{-1})	**M** C/(µg g^{-1})	**L** C/(µg g^{-1})	**N** C/(µg g^{-1})	**S** C/(µg g^{-1})	**M** C/(µg g^{-1})	**L** C/(µg g^{-1})
	Alcohols													
1	3-methyl-1-butanol	85076.1±2269.0	—	20.9±1.2	30.1±3.3	30.1±2.7	—	33.9±0.0	41.2±6.4	49.0±19.1	—	22.8±3.1	26.8±10.6	25.0±1.9
2	2-methyl-1-propanol	3051.4±414.4	—	—	—	—	—	—	—	—	—	—	—	—
3	1-hexanol	1364.8±26.1	—	—	—	—	—	—	—	—	—	—	—	—
4	*Z*-3-hexenol	118.6±7.0	—	—	—	—	—	—	—	—	—	—	—	—
5	benzyl alcohol	128.8±3.2	—	—	—	—	—	—	—	—	—	—	—	—
6	2-phenylethanol	25456.4±359.8	—	69.4±6.5	81.3±15.7	71.2±10.8	—	75.3±0.9	92.9±26.8	114.4±40.2	—	60.3±2.7	63.0±19.1	64.6±7.7
	Esters													
7	ethyl butyrate	81.8±18.8	—	—	—	—	—	—	—	—	—	—	—	—
8	isoamyl acetate	625.5±32.8	—	—	—	—	—	—	—	—	—	—	—	—
9	ethyl hexanoate	744.2±33.5	—	—	—	—	—	—	—	—	—	—	—	—
10	ethyl lactate	6918.2±596.6	—	2.0±0.3	2.6±0.9	3.7±1.4	—	3.8±0.1	5.1±1.6	5.2±1.7	—	3.8±0.5	4.2±1.5	4.1±0.6
11	ethyl octanoate	787.5±122.6	—	—	—	—	—	—	—	—	—	—	—	—
12	ethyl-3-hydroxybutyrate	135.6±18.6	—	—	—	—	—	—	—	—	—	—	—	—
13	diethyl succinate	19907.8±1451.9	—	26.5±1.2	27.5±1.3	26.2±1.8	—	31.2±7.2	45.6±8.7	43.1±14.5	—	24.0±2.0	21.7±4.5	25.5±2.7
14	diethyl malate	2651.0±53.6	—	2.6±0.2	3.3±0.2	2.6±0.5	—	2.7±0.0	3.6±0.9	3.6±0.4	—	2.7±0.6	2.4±0.6	15.8±13.5
15	ethyl hexadecanoate	—	—	—	—	—	—	1.4±0.3	1.1±0.2	0.9±0.3	—	1.1±0.1	0.5±0.1	0.9±0.4
16	monoethyl succinate	10415.3±1520.3	—	1.2±0.1	1.3±0..3	1.5±0.3	—	1.1±0.1	1.4±0.6	1.3±0.6	—	—	—	—
	Acids													
17	hexanoic acid	692.6±22.9	—	—	—	—	—	—	—	—	—	—	—	—
18	octanoic acid	2042.4±196.9	—	9.0±1.8	9.7±0.0	7.2±0.2	—	11.3±0.7	13.8±1.7	9.7±1.8	—	4.7±1.7	7.2±1.1	7.1±0.2

— not detected

Table 3.3 (continuation): Characterization of volatiles, expressed as concentration (*C*) of 4-nonanol equivalents, in Fortified Wine, unused oak controls (N) and oak chips with the dimensions of small (S), medium (M) and large (L) after contact with Fortified Wine for toasted French and American oak and untoasted American oak. Errors represent standard error of the mean from independent duplicates

			toasted French oak				**toasted American oak**				**untoasted American oak**			
N.º	**Compound**	**Wine**	**N**	**S**	**M**	**L**	**N**	**S**	**M**	**L**	**N**	**S**	**M**	**L**
		C/(µg L^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})	*C*/(µg g^{-1})
	Furan Compounds													
19	furfural	—	135.6±14.4	13.9±0.2	15.1±1.0	10.0±1.5	66.9±8.5	8.5±1.1	11.1±2.5	12.7±3.3	14.5±2.9	—	—	—
20	5-methylfurfural	—	30.8±2.8	3.3±0.2	3.2±0.8	2.3±0.0	18.2±4.0	5.4±0.9	6.4±0.4	5.0±0.1	—	—	—	—
21	5-acetoxymethyl-2-furaldehyde	—	6.9±0.3	—	—	2.4±0.5	12.5±4.1	3.6±0.4	4.9±1.4	5.0±2.3	—	—	—	—
22	5-hydroxymethylfurfural	—	1.65±0.3	—	—	—	—	—	—	—	—	—	—	—
	Volatile phenols													
23	guaiacol	—	—	—	—	—	3.5±0.8	1.1±0.1	1.5±0.5	2.4±0.7	—	—	—	—
24	4-methylguaiacol	—	3.0±0.3	—	—	—	2.3±0.7	0.9±0.0	1.4±0.4	1.5±0.3	—	—	—	—
25	4-ethylguaiacol	—	—	—	—	—	0.5±0.2	—	—	—	—	—	—	—
26	4-propylguaiacol	—	5.7±0.5	—	—	—	2.8±0.3	—	—	—	—	—	—	—
27	2,6-dimethoxyphenol	—	2.3±0.3	—	—	—	23.9±6.1	7.3±1.4	11.9±4.6	12.5±4.1	1.3±1.1	—	—	—
28	eugenol	—	0.9±0.2	—	—	—	2.3±0.7	—	3.5±0.8	3.4±1.5	4.7±0.1	2.4±0.1	3.0±0.6	2.7±0.0
	Lactones													
29	*cis*-oak lactone	—	—	—	—	—	69.4±14.7	35.5±4.9	49.3±13.6	57.6±21.2	7.7±0.4	—	—	—
30	*trans*-oak lactone	—	—	—	—	—	26.5±7.1	12.5±2.5	17.7±3.4	20.1±7.4	24.1±1.5	4.6±0.3	6.1±1.1	1.9±1.9
	Aldehydes													
31	4-ethylbenzaldehyde	—	—	2.0±0.5	—	12.9±6.7	-	7.8±2.0	35.4±25.5	5.6±2.9	—	3.6±0.3	8.7±2.1	50.6±7.9
32	vanillin	—	41.5±0.2	10.9±0.8	14.1±1.1	10.7±2.1	66.3±14.2	25.0±2.3	36.3±13.4	36.3±11.0	4.0±0.2	—	1.1±0.2	0.9±0.1
33	syringaldehyde	—	121.5±7.1	36.0±3.0	57.5±17.0	33.2±0.9	371.4±82.5	163.2±20.8	243.7±77.5	318.4±113.5	15.9±3.9	—	—	—
34	sinapaldehyde	—	679.2±95.7	375.2±40.1	383.1±20.1	330.4±48.7	888.4±240.1	514.8±106.9	656.7±120.6	743.4±361.5	18.9±6.0	—	—	—
	Ketones													
35	acetovanillone	—	1.1±0.0	—	—	—	5.3±0.9	2.5±0.5	3.3±1.1	4.2±0.4	—	—	—	—
36	propiovanillone	—	3.3±0.2	—	—	—	4.1±1.3	1.9±0.7	1.7±0.2	2.3±0.8	—	—	—	—

— not detected

Therefore, the occurrence of wood sorption of wine volatiles, previously proposed by Ramirez, can be also involved in the wood enrichment described.

Focusing differences in volatile retention, no statistically significant differences (p>0.05) were found in sorption of wine volatiles between the different particle sizes within each wood variety. Therefore, at equilibrium, retention of wine volatile compounds was independent of particle size or surface area to volume ratio. Such behavior is coherent with the discussed for wine uptake and again demonstrates, at compound level, that particle size did not affect maximum sorption capacity.

Considering that no statistically significant differences were found between the chips of different size, the average concentration of retained wine volatiles was calculated for each wood, allowing an overall comparison between the different oak varieties. In this sense, American oak presented higher overall concentrations of 3-methyl-1-butanol, diethyl succinate and octanoic acid than other woods. Ethyl hexadecanoate was not found in French oak, but found in both American oaks at similar concentrations. Monoethyl succinate was only found in the toasted oak varieties, being absent in untoasted American oak. No statistically significant differences were found between the different woods for the remaining volatiles deriving from wine sorption. Summing up, during contact with wine, besides being subjected to extraction, wood also becomes enriched with wine compounds, either by absorption or sorption. Volatile extraction and retention at equilibrium was independent of particle size, coherent with the observed for wine uptake. Some differences in volatiles retention were observed between the analyzed woods, suggesting that sorbent characteristics can be involved in sorption selectivity.

3.4 Multivariate analysis of volatiles in wood

For a better knowledge of the correlations between wood samples and their composition in volatiles, a Principal Component Analysis (PCA) was performed. Eleven components were extracted, which explained 96.47 % of sum of squares, with the first three components explaining 63.90 % of the overall correlations. With the obtained principal components, and the corresponding loadings regarding each variable, a cluster analysis was performed with the scatterplots presented in Figure 3.4. Most aspects previously discussed for volatile characterization were confirmed with the cluster analysis. As it can be seen, control wood samples were placed in opposite quadrants in the scatterplot, demonstrating the differences observed in volatile composition between each wood. On the other hand, woods contacted with wine were placed in similar clusters in the PCA, and different from the Control woods. Moreover, chips of different size appeared in most cases almost overlapped in the scatterplot, confirming the absence of statistically significant differences previously discussed between the different particles sizes within each wood. Toasted French oak and untoasted American oak showed similar loadings regarding the first and second component extracted, and minor differences regarding the third component. Overall, similar loadings regarding the third component were found between all woods after contact with wine and retained wine volatiles, namely 3-methyl-1-butanol, diethyl succinate, 2-phenylethanol, octanoic acid, ethyl lactate and ethyl hexadecanoate.

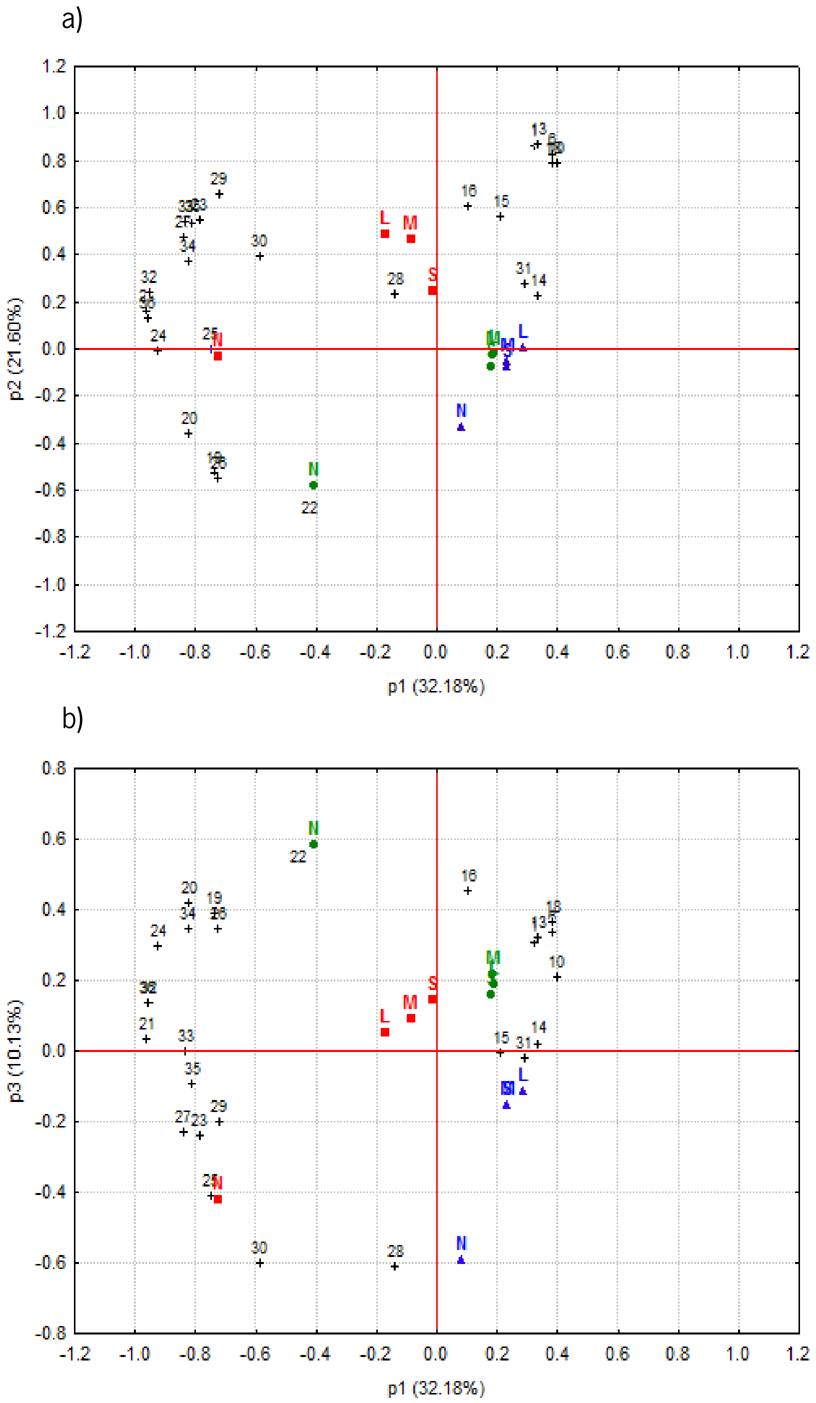

Figure 3.4: Loadings scatterplot correlating toasted French oak (●), toasted American oak (🟥) and untoasted American oak (▲) for unused oak controls (N) and after contact with fortified wine for small (S), medium (M) and large (L) oak chips, with the analyzed volatiles (represented by the numbers presented in Table 3.3) regarding a) the first and second components and b) the first and third components.

4. Conclusions

During ageing processes wine penetrates oak wood, entering its porous structure which is mainly driven by diffusion. Maximum sorption capacity depends principally on wood type and not on chip size. Nevertheless, particle size influences the time needed for attaining equilibrium. As a result of sorption, wine volatiles are retained in wood, which also depends on wood characteristics and is independent of particle size. Sorption and absorption phenomena are involved in the enrichment of wood with wine, the knowledge gained on these phenomena paves the way to make wood a controlled vector for transferring aroma compounds when applied for ageing different beverages.

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Chapter 4. Factors affecting extraction of adsorbed wine volatile compounds and wood extractives from used oak wood

Abstract

During ageing, wood adsorbs volatile compounds from beverages. However, chemical interactions involved in sorption still remain unclear, as well as wood capacity to transfer such compounds to subsequent matrices when reused. Therefore, extractions were conducted from used wood manipulating variables such as ethanol concentration, contact temperature and pH, in order to determine their effect in the interaction and consequent recovery of wine volatiles from wood. Mathematical models were outlined, which demonstrated an exclusive effect of ethanol concentration on the extraction of wine volatiles adsorbed in wood, more prominent for compounds of higher hydrophobicity. Thus adsorption of wine volatiles was shown to be based on hydrophobic interactions. Recovery of wood extractives was also modeled, confirming the known positive effect of ethanol and temperature on the overall extraction of characteristic wood compounds. When reused, wood transferred wine compounds to hydroalcoholic matrices, demonstrating its impact and potential as a vector for aroma transference.

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1. Introduction

Generally, spirits and wines are aged by contact with oak wood. During contact, wood compounds are extracted into the beverage and several other reactions and transformations occur which enhance the beverage sensory properties. Several works have identified the main wood extractives, namely phenolic compounds (Zhang, Cai, Duan, Reeves, & He, 2015), furanic compounds (Garcia, Soares, Dias, Freitas, & Cabrita, 2012), lactones and aldehydes (De Rosso, Cancian, Panighel, Dalla Vedova, & Flamini, 2009; Mosedale & Puech, 1998) which determine the beverage sensory quality. Depending on the beverage to be aged, the casks employed can be new or reused from ageing other beverages. Bourbon is aged in new unused cooperage wood (Gonzalez, 2014; Lyons, 2014), as well as brandy which undergoes a short first contact with unused wood and a second one in used barrels for a longer ageing period (Caldeira, Mateus, & Belchior, 2006). Whisky and beer are generally aged in previously used casks from the production of other beverages, generally from bourbon or fortified wine (Quinn, 2014; Roullier-Gall *et al.*, 2018; Spitaels *et al.*, 2014). Wine can be aged either in new or used casks (Cerdán & Ancín-Azpilicueta, 2006). The content of extractives in wood depends highly on the species used (De Rosso *et al.*, 2009), geographic origin, coarseness of wood grain (Jordão, Ricardo-da-Silva, & Laureano, 2007) drying/seasoning methods, and the toasting methods used in cooperage manufacture (Chira & Teissedre, 2014; de Simón, Cadahía, del Álamo-Sanza, & Nevares, 2010; González-Centeno, Chira, & Teissedre, 2016). During successive reutilizations, wood will lose richness in extractible compounds and become depleted, because their content in wood is finite (Gómez-Plaza *et al.*, 2004; Wilkinson, Li, Grbin, & Warren, 2013). Besides becoming depleted of extractives, wood also becomes enriched with the beverage composition. Several different families of compounds were found to adsorb onto wood during contact, namely monomeric volatile phenols (Barrera-García, Gougeon, Voilley, & Chassagne, 2006) polyphenols, namely monomeric anthocyanins, (+)-catechin, (-)-epicatechin, gallic acid, and *trans*-resveratrol (Barrera-García *et al.*, 2007) and wine volatile compounds, namely terpenes, alcohols, esters, aldehydes, norisoprenoids and acids (Coelho, Domingues, Teixeira, Oliveira, & Tavares, 2019; Ramirez-Ramirez *et al.*, 2001). Several mechanisms were proposed for interaction of wine compounds with wood. Ramirez and collaborators proposed that sorption of wine volatiles relied on polar and acid base characteristics of wood, stating that solubility and hydrophobicity of compounds was not related with the phenomenon (Ramirez-Ramirez *et al.*, 2001). Later on, in a study focusing interaction of wine constituents on wood sorption of wine aromas, Ramirez and collaborators hypothesized that hydrophobicity could indeed be involved in wood sorption, for a small pool of wine volatile compounds in a synthetic matrix (Ramirez-Ramirez, Chassagne, Feuillat, Voilley, & Charpentier, 2004). This hypothesis was later confirmed in the study of sorption monomeric volatile phenols by wood, where Barrera-García and collaborators demonstrated that the main wood macromolecules, namely cellulose, hemicellulose and lignin were involved in the interactions established (Barrera-García, Gougeon, Karbowiak, Voilley, & Chassagne, 2008). Sorption of these compounds was found to be dependent both on wood macromolecules and on sorbate chemical structure, with lignin demonstrating selective adsorption, involving mainly hydrophobic interactions (Barrera-García *et al.*, 2008). In recent works, focusing also wood sorption of wine compounds, the importance of mass transference was demonstrated in the uptake of wine by wood, accompanied by the identification of the wine volatiles adsorbed (Coelho *et al.*, 2019). However, the adsorption mechanism of wine volatile compounds to wood remained to be confirmed, on the basis of the previously mentioned works. Thus the first hypothesis proposed in this work is that wine volatiles interact with oak wood due to hydrophobic interactions. Furthermore, despite the knowledge available on the sorption of wine volatiles and depletion of typical wood extractives, little is known about extraction of these compounds from used wood when applying it in successive ageing processes. Hence, the second proposed hypothesis is that wood is a vector for transferring volatile compounds between beverages, such as wine, beer or spirits, when reused consecutively.

To test both proposed hypotheses, this work explores the extraction of wine volatile compounds from used wood by studying the effect of variables capable of disrupting binding between sorbent and sorbate.

2. Materials and methods

2.1 Chemicals and materials

Determination of GC-MS spectra and retention indexes was performed with the following compounds with the corresponding purity. From Fluka, 3-methyl-1-butanol (≥ 99.8 %), 2-phenylethanol (≥ 99 %), furfural (99 %) and vanillin (≥ 98 %). From Aldrich, isoamyl acetate (≥ 99 %), ethyl hexanoate (≥ 99 %), ethyl lactate (98 %), ethyl octanoate (≥ 99 %), diethyl succinate (99 %), diethyl malate (≥ 97 %), octanoic acid (≥ 99.5 %), 5-methylfurfural (99 %), 4-methylguaiacol (≥ 98 %), guaiacol (98 %), 2,6-dimethoxyphenol (99 %), eugenol (99 %), *cis*/*trans*-oak lactone (≥ 98 %), acetovanillone (98 %) and syringaldehyde (98 %). From Acros Organics, 5-hydroxymethylfurfural (98 %) and, from Alfa Aesar, 4-ethylguaiacol (98 %). Only sinapaldehyde was identified using the retention index and the NIST08 mass spectral library.

Fortified wine was chosen for the prior contact with wood considering that is the type of barrel reused by most industries and that it poses a lower risk for spreading undesirable microbial spoilage traits due to its higher ethanol content. Unaged fortified wine utilized for the preparation of the used oak woods was kindly provided by Quinta do Portal S.A., with an ethanol content, by volume, of 20.9 % ± 0.3 %. Before contact with wood, unaged fortified wine didn't have any content in wood extractives, being its composition reported in Coelho *et al.* (2019). Toasted French oak (*Q.robur*) 950×50×18 mm^3 and toasted American oak (*Q. alba*) 950×50×6 mm^3 staves, from the Oenostave® series (kindly provided by Seguin Moreau, France), were used in this work. Staves of both varieties were supplied with the M+ toasting level, that in cooperage corresponds to a 68 min toast at 62 °C ± 3 °C without water addition (Chira & Teissedre, 2014). Toasted American and French oak without any prior usage did not have any content in wine volatile compounds, which where adsorbed during contact with fortified wine as reported in Coelho *et al.* (2019), where compositions of unused and used woods are presented.

2.2 Preparation of used wood samples

The designation of used wood in this work refers to cooperage wood after contact with fortified wine, characterized by presence of adsorbed wine volatile compounds and a lower content in wood extractives, in opposition with the characteristics of unused cooperage wood as demonstrated in Coelho *et al.*, (2019). For reproducibility and control purposes, used wood samples were prepared in the laboratory in accordance with the procedures described in Coelho *et al.* (2019). Wood staves were cut into cubes of 3 mm side with a vertical saw in the transversal direction and with a blade in the longitudinal direction, using a pachymeter for confirmation of individual chip dimensions. Chips were then immersed in the fortified wine, at a ratio (wood/wine) of 50 g L^{-1}, and maintained under isothermal conditions of 20 °C ± 1 °C until uptake equilibrium was attained, confirmed by determination of mass variation. Prior to utilization, wood chips were collected and excess wine was removed with absorbent paper.

2.3 Extraction methodology

Extractions of compounds from wood were conducted in Pyrex tubes fitted with Teflon caps, with a wood/hydroalcoholic solution ratio of 20 g L^{-1}, varying ethanol concentration and pH of the hydroalcoholic solutions as presented in section 2.4. Contact was promoted in an incubator maintaining isothermal conditions and orbital agitation of 150 min^{-1} for 48 h, determined in preliminary assays to be the sufficient time for extraction to stabilize for the least favorable conditions. At the end of the contact period, wood was separated from the hydroalcoholic solutions which were analyzed for total phenolic content and volatile composition.

2.4 Experimental planning

For a better understanding of variables effect on the extraction of chemical compounds from used wood, a 1 block – 3 level Box-Behnken factorial design was outlined, with triplicates on the central point. Extractions were performed using hydroalcoholic solutions varying ethanol concentration, by volume, from 5 % to 35 %, temperatures ranging from 30 °C to 50 °C and pH values ranging from 3 to 5, covering the commonly found in spirits, wines and beer. Detailed information regarding outline and runs of the Box-Behnken experimental planning can is presented in Table 4.1.

Table 4.1: Ethanol concentration (*C_{EtOH}*), pH and temperature (*T*) in the runs of the Box-Behnken design experimental planning.

Run	C_{EtOH}/%	pH	*T*/°C
1	5	3	40
2	35	3	40
3	5	5	40
4	35	5	40
5	5	4	30
6	35	4	30
7	5	4	50
8	35	4	50
9	20	3	30
10	20	5	30
11	20	3	50
12	20	5	50
13	20	4	40
14	20	4	40
15	20	4	40

Factorial designs allowed the mathematical modeling of the extraction of each compound, correlating extraction of each compound with variable values according Equation 4.1:

$$C_x = b + a_1 \times \frac{C_{\mathrm{EtOH}}}{\%} + a_2 \times \left(\frac{C_{\mathrm{EtOH}}}{\%}\right)^2 + b_1 \times \mathrm{pH} + b_2 \times \mathrm{pH}^2 + c_1 \times \left(\frac{T}{°\mathrm{C}}\right) + c_2 \times \left(\frac{T}{°\mathrm{C}}\right)^2$$

(Equation 4.1)

Where C_x is the concentration of each compound, b is the y-intercept, a_1, b_1 and c_1 are the linear and a_2, b_2, c_2 the quadratic coefficients for ethanol, pH and temperature respectively. C_{EtOH} is the concentration of ethanol in the hydroalcoholic solution, as percentage by volume, pH is the cologarithm of material concentration of H^+ ions in the hydroalcoholic solution and *T* is the temperature of contact. After screening of the variables with impact on extraction of used wood chemical compounds, a replicate assay mimicking ethanol concentrations typically found in aged beverages (15 % – wine, 25 % – fortified wine and liquors, 45 % – spirits) was performed at 30 °C and 50 °C using four replicates (*n* = 4) for proper statistical analysis. For a better comprehension of the work rationale, schematic representation of experimental work can be found in Figure 4.1.

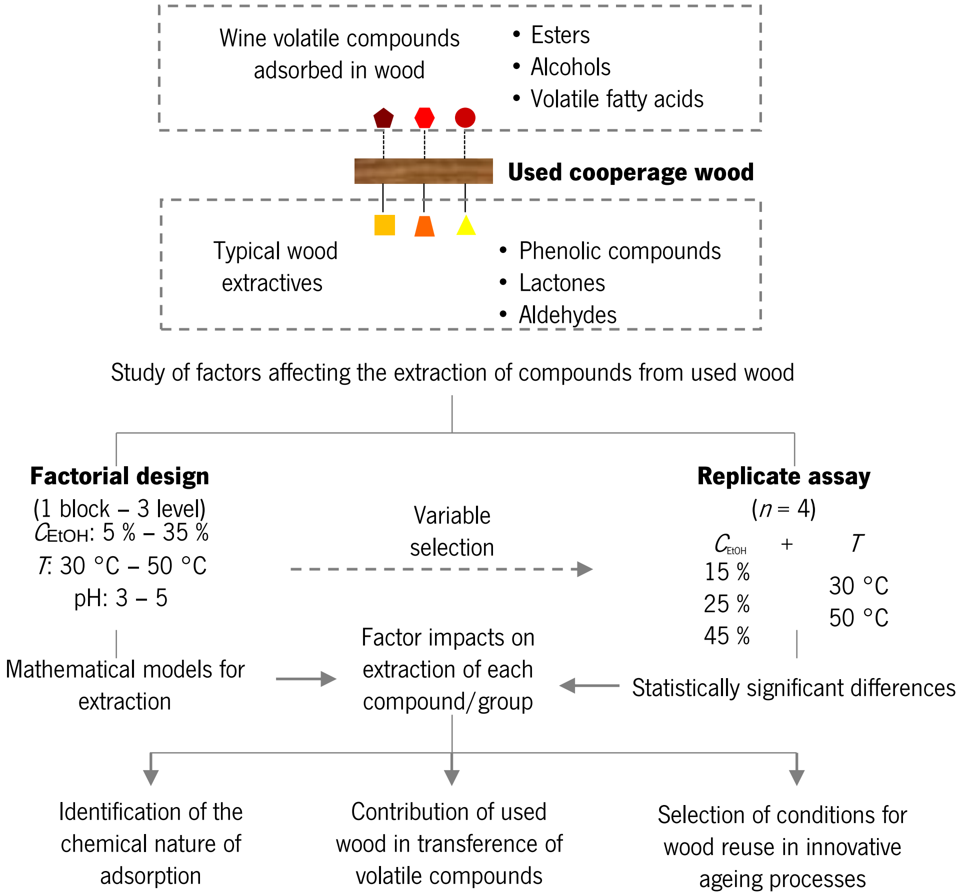

Figure 4.1: Schematic representation of overall work rationale for studying extraction of volatile compounds from wood.

2.5 Analysis of total phenolic content

Total phenolic content in the hydroalcoholic extracts was assessed by spectrophotometry using an adaptation of the method for tannin quantification (Mercurio, Dambergs, Herderich, & Smith, 2007). A saturated ammonium sulfate solution was added to the hydroalcoholic extracts for protein precipitation and the supernatants absorbance was read at 280 nm using a Greiner UV-Star® 96 well microplate to determine total phenolic content. Total phenolics were expressed as equivalents of (-)-epicatechin using a calibration curve prepared with pure (-)-epicatechin standards.

2.6 Analysis of volatile compounds

Volatile compounds in the hydroalcoholic extracts were analyzed by gas chromatography coupled with mass spectrometry (GC–MS), following the procedure proposed by Oliveira and collaborators (Oliveira, Faria, Sá, Barros, & Araújo, 2006). Wood extract samples were diluted, when necessary, to achieve ethanol concentration below 15 % for a final volume of 8 mL, extracted with 400 μL of dichloromethane (SupraSolv for gas chromatography, Merck), after adding 4-nonanol as internal standard (3.2 μg). Extractions were performed in Pyrex tubes fitted with Teflon caps, with agitation promoted by a stir bar during 15 min. Extracts were recovered with Pasteur pipette, dehydrated with anhydrous sodium sulfate and analyzed in a gas chromatograph Varian 3800 equipped with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000. Each 1 μL injection was made in splitless mode (30 s) in a Sapiens-Wax MS column (30 m × 0.15 mm; 0.15 μm film thickness, Teknokroma). Carrier gas was helium 49 (Praxair) at a constant flow of 1.3 mL min^{-1}. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range (m/z) from 35 to 260 and 610 ms acquisition interval. The oven temperature was initially set to 60 °C for 2 min and then raised to 234 °C at a rate of 3 °C min^{-1}, raised again to 260 °C at 5 °C min^{-1} and finally maintained at 260 °C for 10 min. Injector temperature was set to 250 °C with a 30 mL min^{-1} split flow and transfer line was maintained at 250 °C. Compounds were identified using MS Workstation version 6.9 (Varian) software, by comparing mass spectra and retention indices with those of pure standards and quantified as 4-nonanol equivalents.

2.7 Statistical analysis

Factorial designs and corresponding regressions for each response factor were performed in Statistica 7 software (Statsoft). Significant effects on the validation assay were evaluated by pairwise comparisons for each compound in the different studied conditions using a Kruskall-Wallis analysis, without Dunn correction, performed in XLSTAT software (Addinsoft).

3. Results and discussion

Extraction of compounds from used woods was studied with the aim of understanding adsorption of wine volatile compounds by wood during the previous contact, and to demonstrate its potential and impact when reused for ageing other beverages. In a first step a factorial design was performed for a preliminary screening of the effect of variables in the extraction of wood and wine compounds. The extraction of each volatile compound was modeled individually according to Equation 4.1, with the modeled coefficients presented in Table 4.2.

As seen, good regression coefficients were obtained for the extraction of compounds from used American oak, with R^2 values ranging mostly from 0.8 up to 0.98 with the exception of 3-methyl-1-butanol, guaiacol and octanoic acid which presented R^2 between 0.6 and 0.8. Extraction of compounds from French oak presented lower regression coefficients, mostly in the range of 0.7 to 0.9. Despite the lower regression coefficients, models obtained for extraction of used French oak compounds are still satisfactory for describing most of the interactions and allow an evaluation of effects, as long as properly supported by the replicates assay also presented. Analyzing the models for compound extraction, it can be seen that esters (isoamyl acetate, ethyl hexanoate, ethyl lactate, diethyl succinate and diethyl malate) alcohols (2-phenylethanol and 3-methyl-1-butanol) and acids (octanoic acid) were extracted from both used American and French oak. These compounds are not usually found in oak wood and their presence and extraction is a consequence of wood enrichment with fortified wine composition during the preceding contact as previously demonstrated (Coelho *et al.*, 2019). Therefore, the previously established hypothesis that wood can be a vector for transferring and recombining volatile compounds from one matrix to another is confirmed.

Analyzing the models and the observed effects, it can be seen that ethanol concentration in the hydroalcoholic solution had a significant effect on the overall extraction of volatile compounds from either American or French oak, being the most influent variable among the ones studied. Ester extraction seemed to be favored by ethanol concentration as seen by a_1 and a_2 coefficients obtained for the models from used American oak and used French oak. Correlating the data obtained by the models with the observed in the assay mimicking ethanol concentration typically found in beverages presented in Figure 4.4 and Figure 4.5, a positive relation between ethanol concentration and extraction is visible for isoamyl acetate, diethyl succinate and ethyl hexanoate, being only statistically significant in the case of ethyl hexanoate. Ethyl hexanoate was the only ester whose extraction was affected by temperature, in the specific case of American oak, as seen by the c_1 coefficients obtained in the model. Nevertheless, such effect of temperature was not observed in the models for used French oak, neither it is statistically significant in the replicates assay for both oak woods. For ethyl lactate and diethyl malate, no trends were verified in the replicate assay, whereas for isoamyl acetate and diethyl succinate trends were noticeable but without statistical significance. These trends are justified by the response surfaces obtained from the models for each compound presented in Figure 4.2. In fact, for ethyl lactate and diethyl malate, a significant effect of ethanol is found when increasing its content up to about 15 %, point from where the concentration of these compounds stabilizes, whereas for isoamyl acetate and diethyl succinate, this stabilization was not observed. Therefore, a weaker adsorption with wood can be indirectly observed for ethyl lactate and diethyl malate, which are easily extracted with lower ethanol concentrations, whereas isoamyl acetate and diethyl succinate extraction is improved with increasing ethanol concentration. Presenting a more efficient extraction with increasing ethanol concentration, ethyl hexanoate and ethyl octanoate demonstrate a stronger interaction with wood. Extraction of ethyl octanoate was clearly influenced by ethanol concentration, as demonstrated in the replicates assay, which is in good agreement with the behavior obtained for the remaining esters. Moreover, higher partition coefficients have been also reported for ethyl hexanoate and ethyl octanoate in wood sorption of volatiles from a model wine (Ramirez-Ramirez *et al.*, 2001). Therefore, the stronger interactions and affinities observed and discussed in this work are in good agreement with the previously described by Ramirez and collaborators for these compounds, reinforcing the validity of the obtained results.

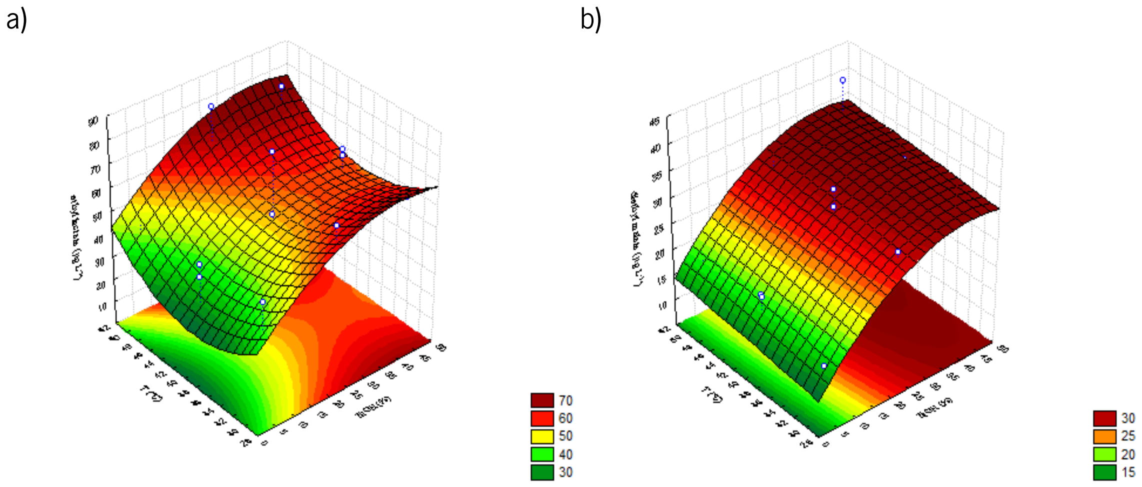

Figure 4.2: Response surfaces correspondent to ester extraction models from used American oak for a) ethyl lactate and b) diethyl malate.

Thus, the hypothesis that interaction of esters with oak wood is of hydrophobic nature gains strength, since increasing ethanol concentration breaks the bond of esters with wood more efficiently. Ethanol is a low polar solvent compared to water which is strongly polar. Increasing ethanol concentration in ethanol-water solutions modifies the polarity of the solvent and allows the extraction of compounds of more hydrophobic nature by a "like dissolves like" principle (Zhang *et al.*, 2007). Taking into account the values of XLogP3, a computational prediction of the log *P* value (Cheng *et al.*, 2007) which indicates a molecule hydrophobicity (Barrera-García *et al.*, 2008), presented in Table 4.3, a clear relation between XLogP3 values reported (National Center for Biotechnology Information, 2018) and the extraction profiles can be established.

Diethyl malate and ethyl lactate, which were easily extracted in all the conditions of the validation assays, have low XLogP3 values. Diethyl succinate and isoamyl acetate, which demonstrate intermediate XlogP3 values, showed the effect of ethanol concentration on compound extraction but this was not statistically significant on the validation assay. Lastly, ethyl hexanoate and ethyl octanoate with the higher XLogP3 values showed significant effect of ethanol concentration on extraction either both in the models and the validation assay. The behaviors of 3-methyl-1-butanol and 2-phenylethanol were similar to the ones previously discussed for esters.

Table 4.2: Model coefficients of Equation 4.1 for the extraction of each compound from used American and French oak obtained from the Box-Behnken experimental planning.

		Used American Oak								Used French Oak							
	Compound	R^2	b	a_1	a_2	b_1	b_2	c_1	c_2	R^2	b	a_1	a_2	b_1	b_2	c_1	c_2
Wine volatile compounds adsorbed in wood	**Esters**																
	isoamyl acetate	0.90400	14.80019*	3.65908*	-1.48734*	0.04154	0.17047	0.17548	0.73242	0.63733	7.927436*	1.191950	1.036599*	0.557106	0.102958	-0.988346	-0.006156
	ethyl hexanoate	0.91975	25.98559*	0.92703	-3.56334*	-0.87767	-0.36912	1.60980*	-0.00774	0.91293	21.51498*	-2.30109*	4.08575*	-0.32937	-0.01146	0.85656	0.13502
	ethyl lactate	0.79359	56.01251*	12.66402*	2.65064	-4.84432	-1.39300	0.51088	1.18300	0.76749	57.06880*	10.16696*	6.80726*	-7.39785	-1.53277	-0.68594	-2.91732
	diethyl succinate	0.96502	475.8765*	102.3494*	-11.8061	-1.9410	2.6861	2.7183	6.3067	0.84131	309.9605*	47.9950*	-0.6333	-8.5789	4.6263	-1.1582	-3.2482
	diethyl malate	0.81252	24.62538*	6.85824*	1.67529	-1.68279	0.82950	-1.13260	1.21548	0.69495	24.83909*	4.35763*	2.09375	-1.71796	-0.16600	-0.95744	-1.19341
	Alcohols																
	3-methyl-1-butanol	0.70047	577.3549*	98.9753*	37.2161	21.4584	2.9590	2.0677	15.3149	0.70358	500.6570*	66.9925*	17.7958	-80.8727*	-17.3520	-6.2772	-24.5621
	2-phenylethanol	0.95365	781.8344*	194.2933*	27.3028*	18.0457	6.9237	5.1024	18.8565	0.75928	610.2244*	106.1317*	18.8215	-56.4714	4.4043	24.7938	-29.3953
	Volatile fatty acids																
	octanoic acid	0.73017	66.41817*	17.47776*	1.63379	1.17351	2.82730	9.45560	3.56468	0.79595	53.08467*	12.62963*	1.65728	-0.72422	-2.13678	3.94711	-0.11369
Characteristic wood extractives	**Furan compounds**																
	furfural	0.96023	159.3218*	24.3818*	2.0132	-3.4999	-3.4465	27.9146*	4.0677	0.54962	177.8887*	25.1715	-0.0759	-18.0572	-6.2652	12.7842	-6.2681
	5-methylfurfural	0.95379	57.16781*	12.37206*	-0.39395	-0.86926	0.09452	4.06594*	1.17242	0.77124	44.00520*	8.20830*	0.32661	-1.85320	-0.13164	-0.03994	0.08520
	Volatile phenols																
	guaiacol	0.60088	11.13915*	1.72992*	-0.16793	-0.15618	0.18191	-0.17716	-0.14162	-	-	-	-	-	-	-	-
	4-methylguaiacol	0.87708	16.55411*	4.64245*	-1.33493*	-0.41989	0.82299	-0.37842	1.21219	-	-	-	-	-	-	-	-
	eugenol	0.95881	13.91413*	6.70476*	-0.19004	-0.31868	0.23372	0.43275	0.39088	-	-	-	-	-	-	-	-
	2,6-dimethoxyphenol	0.82498	65.18232*	19.30949*	-1.44754	2.50086	1.45696	-0.59677	3.05793	0.35998	9.24464	3.71584	7.38221	1.54876	7.12737	-4.51129	4.93132
	Lactones																
	cis-oak lactone	0.95942	276.9982*	107.0425*	-3.9728	2.8264	6.9129	16.3243	3.8999	-	-	-	-	-	-	-	-
	trans-oak lactone	0.95376	92.26293*	37.00651*	-2.21205	1.44930	2.91637	3.61239	2.47773	-	-	-	-	-	-	-	-
	Aldehydes																
	vanillin	0.9242	323.3094*	90.8262*	-7.5153	15.2043	7.3883	40.6386*	9.3851	0.73326	118.4160*	16.8592*	2.8481	-1.1431	2.0543	12.9984*	-5.9832
	syringaldehyde	0.91926	1727.474*	512.558*	-72.130	127.696	57.321	283.756*	67.135	0.7238	327.9433*	81.7169*	-33.6997	-18.4218	22.1911	67.5063	-48.0495
	sinapaldehyde	0.96866	3485.483*	1705.197*	-319.500*	361.397*	57.134	727.596*	203.335	0.89876	910.723*	548.305*	-250.762*	2.641	110.364	-11.193	-102.299
	Ketones																
	acetovanillone	0.89315	64.64908*	28.39521*	-3.08806	-0.13696	3.35425	3.58694	2.42456	-	-	-	-	-	-	-	-
	Total phenolics	0.98395	352.2807*	144.7368*	1.5789	1.0526	11.5789	75.2632*	2.6316	0.9696	657.8947*	258.4211*	24.4737	43.6842	23.4211	173.6842*	13.4211

*statistically significant ($p<0.05$); - not detected

Their extraction from used wood was mainly affected by ethanol concentration, with 2-phenylethanol and 3-methyl-1-butanol presenting stabilization of extraction around 20 %, by volume, according to the obtained models. Comparing this value with the replicates assay, the tendency predicted by the models are hinted but the deviations obtained hinder statistically significant differences, in similarity to the observed for isoamyl acetate and diethyl succinate.

Table 4.3: Values of XlogP3 reported for wine volatiles adsorbed by wood (National Center for Biotechnology Information, 2018).

Compound	XLogP3	Compound	XLogP3	Compound	XLogP3
diethyl malate	0.1	isoamyl acetate	2.0	3-methyl-1-butanol	1.2
ethyl lactate	0.2	ethyl hexanoate	2.4	2-phenylethanol	1.4
diethyl succinate	1.2	ethyl octanoate	3.5	octanoic acid	3.0

Lastly, octanoic acid extraction was also affected by increasing ethanol concentration. A slight effect of temperature was also observed but it was not considered significant neither by the models nor the replicates assay. A clear relation can be seen between the compounds XLogP3 values and their extraction behavior, demonstrating that interaction with wood is influenced by hydrophobicity of compounds. Previous works have suggested that acid-base and polar characteristics of wood were involved in the sorption mechanism (Ramirez-Ramirez *et al.*, 2001). Overall pH effect was not considered significant in the extraction of wine volatiles from used oak wood, nor the extraction of typical wood extractives, with the exception of sinapaldehyde. Results demonstrate that pH changes in the range typically found in alcoholic beverages (from 3 to 5) did not affect binding of compounds to wood, putting again more emphasis on polarity of sorbent and sorbate. This finding is in good agreement with the demonstrated by the work of Barrera-García *et al.* (2008), which reported two types of interactions for the sorption of volatile phenols onto wood. Wood macromolecules were reported to be involved in sorption, with cellulose and hemicellulose retaining sorbate by non-specific hydrophilic interactions and lignin adsorbed volatile phenols by selective interactions of hydrophobic nature. Based on our results, sorption of wine volatiles is also of hydrophobic/hydrophilic nature, confirming Barrera-Garcia's observations (Barrera-García *et al.*, 2008) and clarifying the hypothesis established by Ramirez's work (Ramirez-Ramirez *et al.*, 2001). Focusing on typical wood extractives, several groups of compounds were extracted from used wood, namely volatile and non-volatile phenolic compounds, lactones, furan compounds and aldehydes, as visible in Table 4.2 and Figure 4.3, Figure 4.4 and Figure 4.5. These are in good agreement with the reported for wood (Wilkinson *et al.*, 2013) and the previously reported for used woods produced in the same conditions (Coelho *et al.*, 2019). These compounds were described as the main contributors to wood aroma in wines and spirits, and are easily extracted from wood by ethanol-water solutions taking into account their solubility and ethanolysis of lignin (Mosedale & Puech, 1998). Models for extraction of phenolic compounds were outlined, with the corresponding coefficients presented in Table 4.2 and response surfaces in Figure 4.3. Models obtained for both used American and French oak clearly demonstrate a positive effect of temperature and ethanol concentration on the overall extraction of phenolic compounds, as demonstrated by the response surfaces as well as by the coefficients presented in Table 4.2.

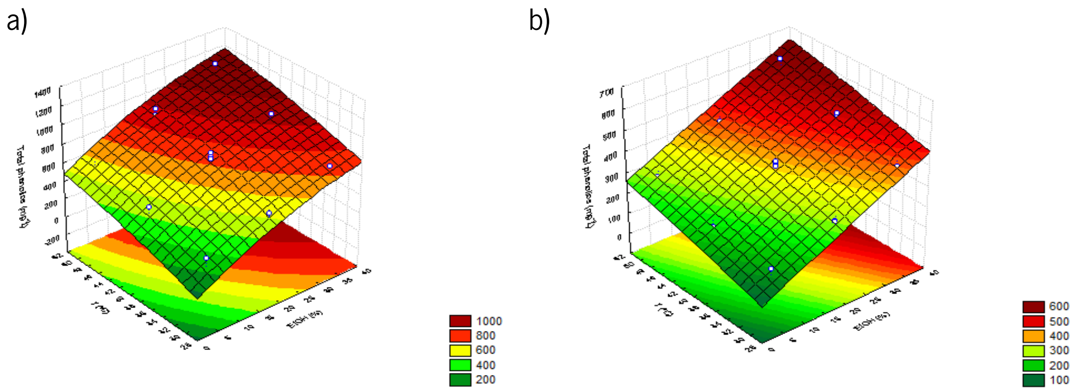

Figure 4.3: Response surfaces for extraction of phenolic compounds from a) used French oak and b) used American oak.

Several works focusing on extraction of phenolic compounds from wood have also identified ethanol concentration and temperature as the main contributors for the recovery of these compounds (Ghitescu *et al.*, 2015; Jung, Park, & Yang, 2016), as well as the absence of influence of pH on the extraction of such compounds, as seen for ellagic tanins and ellagic acid (Jordão, Ricardo-da-Silva, & Laureano, 2005). Thus, the obtained models are in good agreement with the described in the literature. Overall extraction of phenolic compounds was higher in used French oak when compared with used American oak. This was expected, taking into account the higher content of French oak in ellagitannins (Navarro *et al.*, 2016) and phenolic acids, especially ellagic acid, in relation to the American variety (Garcia *et al.*, 2012). Regarding the extraction of typical wood volatiles, several groups were extracted either from used French and American oak, but models for guaiacol, 4-methylguaiacol and eugenol are absent for used French oak due to difficulties in determining their concentrations in some runs of the factorial designs, with co-eluting peaks in the chromatograms. Nevertheless, such models were determined for used American oak, which demonstrate that extraction of those compounds was mainly affected by ethanol concentration, being temperature and pH not considered significant factors. These results were confirmed by the replicates assay, where increased extraction with increasing ethanol concentration can be seen, with prominent significance for eugenol.

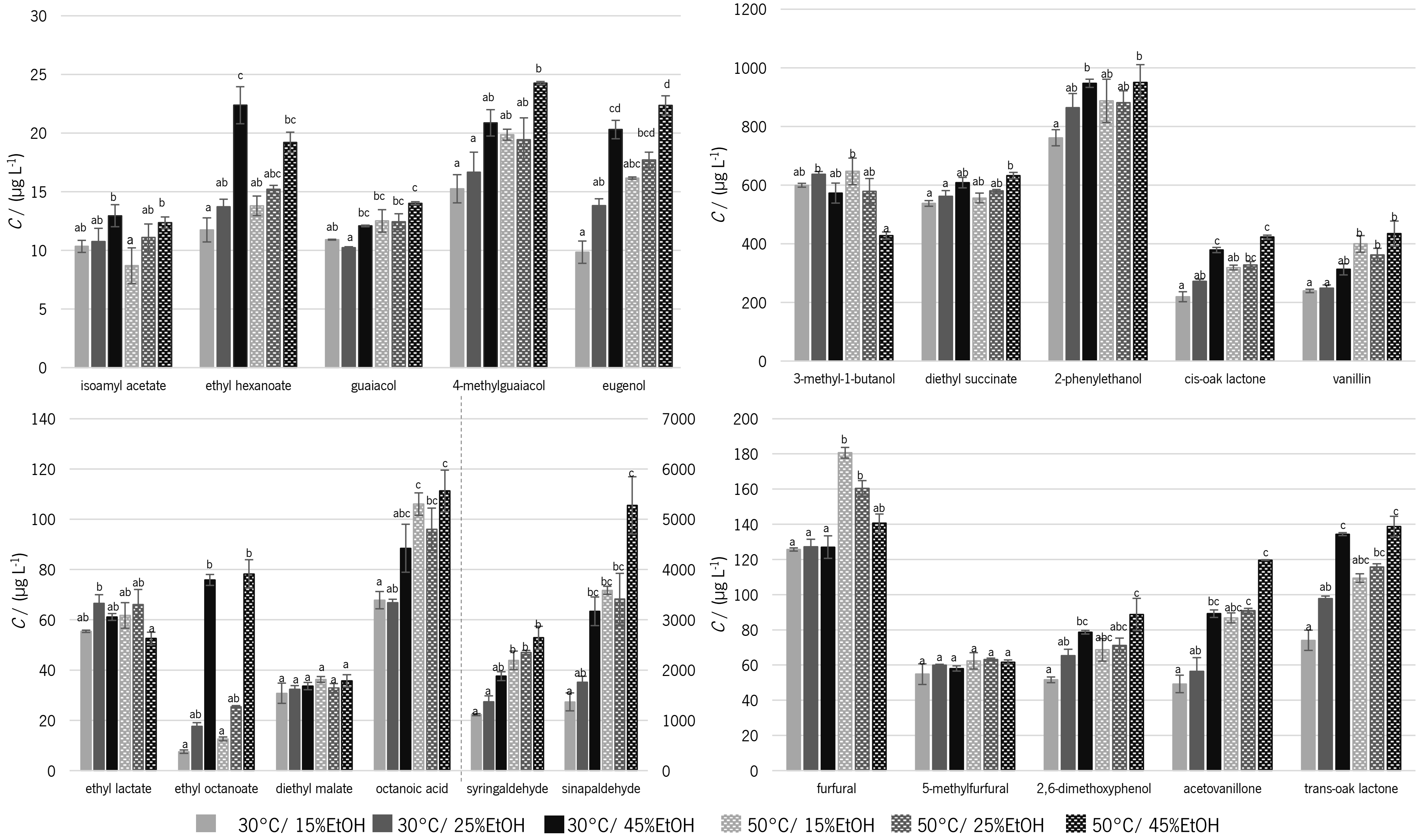

Figure 4.4: Volatile compounds concentrations (*C*) obtained for replicates extraction at different conditions from Used American Oak. Syringaldehyde and sinapaldehyde are found plotted on the secondary axis.

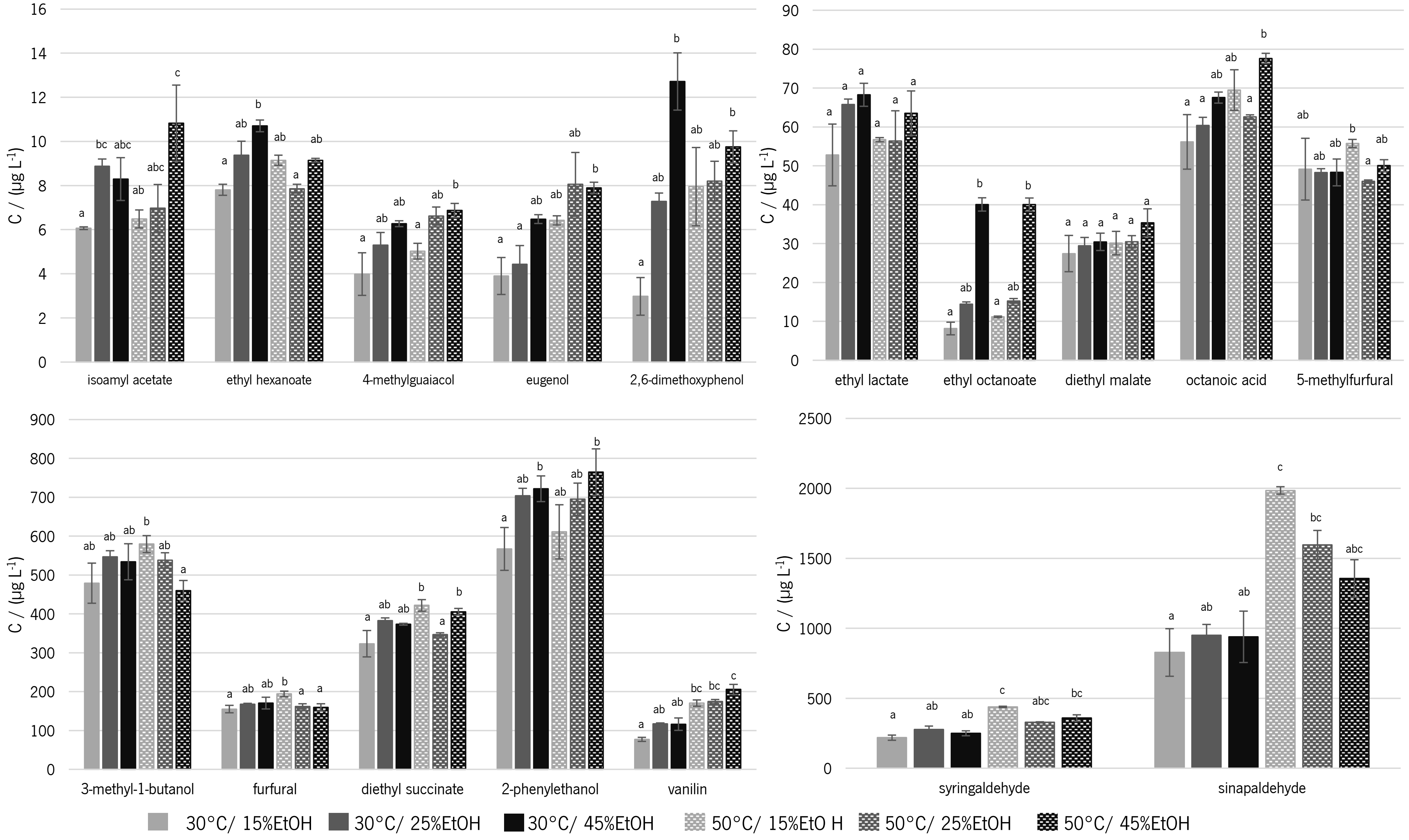

Figure 4.5: Volatile compounds concentrations (*C*) obtained for replicates extraction at different conditions from Used French Oak.

Validation data obtained for used French oak is coherent with the observed for used American oak. Extraction of furan compounds demonstrated characteristic behavior when comparing used American oak with used French oak. Models for extraction of furfural and 5-methylfurfural showed a positive effect of ethanol concentration and temperature. This is also verified in the validation assay where a significant positive effect of temperature is observed, mainly for extractions conducted with lower ethanol percentages. A negative effect of ethanol is visible mainly in the extractions conducted at 50 °C. However, in the case of French oak, effects were hindered by the poor quality of the model obtained for furfural as no significant effects could be observed. These were also not clear for 5-methylfurfural in the validation assay, with no statistically significant tendencies observed. Lactones were only extracted from used American oak, while the staves of French oak had no detectable amounts of these compounds as demonstrated in previous works (Coelho *et al.*, 2019). The obtained models demonstrate that only ethanol concentration affected significantly the extraction of those molecules, which was also verified in the validation assay. Lastly, another important group of compounds is aldehydes, with special focus on vanillin due to its characteristic aromatic properties. The effect of pH was only significant for sinapaldehyde extraction, as the extraction of aldehydes was mainly affected by ethanol and temperature, which is also clear in the validation assay.

On a broader analysis of the results, the different effects observed demonstrate that different volatile compositions can be attained reusing the same wood in subsequent beverages. As demonstrated, extraction of wine compounds adsorbed in wood (esters and alcohols) was only affected by ethanol concentration, and independent of temperature, whereas recovery of wood extractives (aldehydes and phenolic compounds) was generally affected both by ethanol concentration and by contact temperature. If a technological approach for application of wood in accelerated ageing processes is envisaged, different chemical and sensory profiles can be obtained manipulating temperature, ethanol concentration in the beverage or wood concentration and provenance. For instance, using low temperatures can favor the extraction of adsorbed wine volatiles, imparting the beverage mainly with esters. On the other hand, if higher temperatures are used, typical sensory characteristics associated to wood will be favored, as a consequence of a higher extraction of phenolic compounds and wood volatile compounds. Summing up, used wood can impact ageing beverages by transferring both compounds from the previous beverage and from wood itself. Esters and alcohols migrating through wood can confer a fruity and floral flavor to aged beverages, along with characteristic wood aromas such as vanilla, coconut, and nuts. Profound knowledge of sorption and extraction processes occurring in wood converts it into an even more useful tool to be used as a transference vector in beverage ageing. Recombination of volatile compounds can be performed by controlling wood/beverage combinations and ageing conditions, on the basis of the obtained knowledge.

4. Conclusions

Ethanol concentration of a solution/beverage is the main variable affecting extraction of wine volatiles adsorbed in wood and characteristic wood extractives. Hence, the hypothesis that sorption of wine volatile compounds by wood is mainly due to polar or hydrophobic interactions is confirmed, being refuted or diminished the importance of acid base characteristics on this sorption phenomenon. Considering that hydrophobic characteristics are involved in sorption of wine volatiles by wood, the interaction established depends on both wood composition and volatile compounds intrinsic structure. Extraction of phenolic compounds is not only affected by ethanol concentration in the beverage but also by contact temperature, which is also verified for main wood extractives with the exception of volatile phenols. Also the second hypothesis is confirmed, when reused between ageing different beverages, wood transports sensory active compounds from one beverage to another, being the extraction of these compounds strongly dependent on beverage characteristics and contact conditions used.

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Chapter 5. Reuse of oak chips for modification of the volatile fraction of alcoholic beverages

Abstract

New or used barrels can be applied in ageing of alcoholic beverages. Compounds adsorbed in wood migrate between beverages along with wood extractives. As barrel ageing is costly and time-consuming, processes using wood fragments have been gaining interest. These generate wood residues for which the reuse is still not well established. This work aims at the reuse of oak fragments for the additive ageing of alcoholic beverages. Oak chips, previously immersed in fortified wine, were applied to beer, wine and grape marc spirit. Wood compounds and adsorbed wine volatiles were extracted, with more impact and satisfactory yields on beer composition. Also, wood adsorbed beverages compounds in a subtractive ageing phenomena. Beer formulations using different binomial wood concentration/temperature combinations were generated and presented to trained tasters. Higher temperatures and wood concentrations led to prominence of wood descriptors and lower perception of fruity and floral aromas, reflecting the changes in chemical composition.

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1. Introduction

Wood ageing is commonly used as a strategy to modify and enhance the composition of alcoholic beverages. Quite a few alcoholic beverages traditionally resort to ageing in contact with wood, either beer, wine or spirits. During contact with wood, several ageing phenomena can occur namely additive ageing – the extraction of wood constituents to the beverage – (Canas, Caldeira, & Belchior, 2013; García-Estévez, Alcalde-Eon, Le Grottaglie, Rivas-Gonzalo, & Escribano-Bailón, 2015), subtractive ageing – the loss of compounds due to evaporation or wood sorption – (Barrera-García *et al.*, 2007; Coelho, Domingues, Teixeira, Oliveira, & Tavares, 2019), chemical ageing – reaction between beverage and wood components – (Coetzee & Du Toit, 2015) and biological ageing – conversion of constituents in the matrix due to the action of microbes – (Coelho *et al.*, 2020; Snauwaert *et al.*, 2016). Traditionally, ageing is performed by storing the beverage in wood casks or barrels during periods of time dependent on the beverage to be aged and the intended product. Oak is the most preferred wood for barrel production and beverage ageing due to its mechanical properties and its capacity to act as a dynamic container (del Álamo-Sanza & Nevares, 2018). Also it imparts beverage composition with several compounds that positively contribute to the sensory characteristics of aged beverages such as furan compounds, volatile phenols, tannins, lactones among several other (Mosedale & Puech, 2003). During its lifecycle, cooperage wood can be used, reused, regenerated and eventually discarded. For barrels, there is a well-established reuse flow depending on the aged beverages. Wine production usually resorts to new barrels, which may be reused depending on their extractive content (Zamora, 2019). Bourbon production also resorts to new barrels, whereas Scotch and Irish Whiskey production usually resort to previously used ones, either from Bourbon and Brandy production, or from fortified wine production, mainly Sherry but also Port and Madeira wines (Quinn, 2014). Beer ageing also resorts to reused casks, either from Bourbon or Sherry wine production (Spitaels *et al.*, 2014). Previous works have demonstrated that oak wood adsorbs compounds from the beverages during ageing (Coelho *et al.*, 2019), which are transported to other matrices in subsequent contacts (Coelho, Teixeira, Domingues, Tavares, & Oliveira, 2019). Transference of characteristic wine compounds such as quercetin-glucuronide and myricetin-glucoside, has also been identified in whiskies aged in Sherry casks, which were differentiated in their chemical composition from whiskies aged in Bourbon casks (Roullier-Gall *et al.*, 2018).

As an alternative to barrel ageing, several processes have been developed envisaging intensification of ageing phenomena, by immersing oak fragments in the beverage in combination with other physical-chemical treatments. Several efforts have been made for acceleration of wine ageing (Tao, García, & Sun, 2014), as well as ageing of several spirits, namely grape marc distillate (Rodríguez-Solana, Rodríguez-

Freigedo, Salgado, Domínguez, & Cortés-Diéguez, 2017), brandy (Canas, Caldeira, Anjos, & Belchior, 2019; Schwarz, Rodríguez, Sánchez, Guillén, & Barroso, 2014) and whisky (Pielech-Przybylska & Balcerek, 2019). A few works have also envisaged extraction of oak compounds to beer, by applying oak chips (Sterckx, Saison, & Delvaux, 2012a, 2012b; Wyler, Angeloni, Alcarde, & da Cruz, 2015) and further on combining wood with microorganisms for biological ageing (Coelho *et al.*, 2020). Accelerated ageing technologies often make use of oak alternatives such as chips, staves, cubes or sticks (Chatonnet, 2007), which are a cheaper, eco-friendlier and a more sustainable alternative for ageing (García-Alcaraz *et al.*, 2020). With the dissemination of these technologies, the use and demand of barrel alternative cooperage products is expected to increase, as well as the generation of their used residues, for which a reuse circuit is still not well established.

Moreover, research on beverage ageing mainly focuses the transference of oak compounds to the beverage along with the occurring chemical and biological transformations, whereas the contribution of wood reuse is often overlooked. This work aims to investigate the contribution of used wood to the volatile fraction of beverages, when reused for induction of aroma compounds in accelerated ageing processes. The main issue under study is the transference of compounds from one beverage to another through wood reutilization, which modifies not only the chemical composition but also the sensory properties of the beverage. Thus, revalorization of used wood fragments is proposed and demonstrated in order to promote their reuse in accelerated ageing processes.

2. Materials and methods

2.1 Materials and chemicals

The following compounds with the corresponding purities were used as standards for the GC-MS analyses. From Fluka: isobutyl acetate (99 %), ethyl pentanoate (≥99.7 %), ethyl phenylacetate (≥99 %), 2-phenylethyl acetate (≥99 %), ethyl dodecanoate (≥98 %), 2-methyl-1-propanol (≥99.9 %), 2-methyl-1-butanol (≥98 %), 3-methyl-1-butanol (≥99.8 %), 1-hexanol (≥99.9 %), *Z*-3-hexenol (≥98 %), 1-octanol (≥99.5 %), 2-phenylethanol (≥99 %), propanoic acid (≥99.5 %), butanoic acid (≥99.5 %), hexanoic acid (≥98 %), decanoic acid (≥98 %), dodecanoic acid (≥99 %), furfural (99 %), linalool furanic oxide (≥97 %), benzaldehyde (≥99 %), vanillin (≥98 %) and acetoin (97 %); from Aldrich: ethyl butyrate (99 %), ethyl-2-methylbutyrate (99 %), ethyl-3-methylbutyrate (98 %), isoamyl acetate (≥99 %), ethyl hexanoate (≥99 %), hexyl acetate (99 %), ethyl heptanoate (99 %), ethyl lactate (99 %), ethyl octanoate (≥99 %), ethyl-3-hydroxybutyrate (99 %), ethyl decanoate (≥99 %), diethyl succinate (99 %), methyl salycilate (≥99 %), diethyl malate (≥97 %), ethyl cinnamate (99 %), *E*-3-hexenol (96 %), benzyl alcohol (≥99.9 %), linalool (97 %), β-citronellol (95 %), isovaleric acid (99 %), octanoic acid (≥99.5 %), 5-methylfurfural (99 %), *cis*/*trans*-oak lactone (≥98 %), γ-nonalactone (≥98 %), γ-ethoxycarbonyl-γ-butyrolactone (95 %), syringaldehyde (98 %), octanal (99 %), nonanal (95 %), 4-methylguaiacol (≥98 %), eugenol (99 %), 4-ethylphenol (99 %), 4-vinylguaiacol (98 %), 2,6-dimethoxyphenol (99 %), 4-vinylphenol (10 %), acetovanillone (98 %), tyrosol (98 %), methionol (98 %) and β-damascenone (1.3 %); α-terpineol and acetic acid (≥99.8 %) from Merck, *cis*-nerolidol (98 %) and 4-ethylguaiacol (98 %) from Alfa Aesar and 5-hydroxymethylfurfural (98 %) from Acros Organics.

For generating the used oak wood, American oak was used (M+ toast) (kindly provided by Seguin Moreau), which was submerged in fortified wine used in Port wine production (kindly provided by Quinta do Portal S.A.) with contact conditions and wood and wine composition as described in Coelho *et al.* (2019). Prior to its application, wood was separated from the fortified wine and excess liquid was removed with sorbent paper. For the extraction assay with different beverages a commercial red wine (12.5 % ethanol), a commercial lager beer (4.7 % ethanol) and a craft grape marc spirit (41 % ethanol) were used. For the formulation and sensory evaluation assays a pilsner craft beer (5 % ethanol) was used (kindly provided by Cerveja Letra).

2.2 Wood contact with beverages

Extractions were performed to assess impact of reused wood on the volatile fraction of different beverages. Used wood chips were immersed in beer, wine and grape marc spirit at a proportion of 20 g L^{-1}. Contacts were conducted in Pyrex tubes (16 mm × 100 mm, wall thickness 1.8 mm) fitted with caps with Teflon membranes to prevent losses by evaporation. Tubes were placed horizontally in an incubator at 40 °C, with orbital agitation (150 min^{-1}) during 48 h. Also, for control purposes, beverages were placed in the same conditions without application of wood, in order to discriminate transformations caused only by temperature. After contact, beverages were separated from wood by decantation and further filtered with a 0.22 µm cellulose acetate filter for analysis of volatile compounds. Beer (B), wine (W) and grape marc spirit (S) were characterized in their initial state, after residence at 40 °C without wood (B40, W40, S40) and with wood (BW40, WW40, SW40).

2.3 Beer formulations

For sensory evaluation, various formulations were prepared combining different wood concentrations and contact temperatures. Three different wood concentrations were used, 10 g L^{-1}, 20 g L^{-1} and 30 g L^{-1} as well as three different contact temperatures, 30 °C, 40 °C and 50 °C. Extractions were conducted in 100 mL airtight flasks fitted with rubber stoppers and aluminum seals, placed during 48 h in an incubator with orbital agitation (150 min^{-1}). Beers were separated from wood by decantation followed by centrifugation at 2700 *g* during 15 min for removal of suspended particles. Samples were identified by a *CxTy* key, where *Cx* refers to wood concentration at *x* g L^{-1} and *Ty* refers to contact temperature performed at *y* °C. Controls of beer incubated at the same temperatures without addition of wood were also performed, identified as *BTy*, where *y* refers to contact temperature in °C.

2.4 Sensory evaluation of beer

Sensory evaluation of the different beer formulations was performed by five trained panelists. The panel was constituted by one certified Beer Judge Certification Program (BJCP) judge, one certified beer Sommelier and 3 experienced wine and beer tasters. Blinded samples were presented in tasting glasses simultaneously, to allow comparison between the different samples. Scoring was performed using a tasting sheet with identification of visual, aroma and taste descriptors, using a quantitative scale from 1 to 10, where 1 corresponds to very low and 10 to very high intensity. Results were processed in agreement with the standard ISO 11035 (International Organization for Standardization, 1994). Outliers were removed and geometric mean (*GM*) was calculated for each descriptor in each sample according to Equation 5.1.

$$GM/\,\% = \sqrt{I \times F} \times 100 \qquad \text{Equation 5.1}$$

Where I refers to relative intensity, which is the sum of the intensities given by the panelists for a given descriptor, divided by the maximum possible intensity, and F refers to the relative frequency, which is the number of times that the descriptor was mentioned divided by the maximum number of times that it could be mentioned.

2.5 Analysis of volatile compounds

Volatile compounds were analyzed by gas chromatography coupled with mass spectrometry (GC–MS), following the previously reported procedure (Coelho *et al.*, 2020). Each 8 mL sample was extracted with 400 µL of dichloromethane (SupraSolv for gas chromatography, Merck), after adding 4-nonanol as internal standard (3.2 µg). Extractions were performed in Pyrex tubes fitted with Teflon caps, with stir bar agitation during 15 min. Extracts were then recovered with a glass Pasteur pipette, dehydrated with anhydrous sodium sulfate and analyzed in a Varian 3800 gas chromatograph equipped with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000. Each 1 µL injection was made in splitless mode (30 s) in a Sapiens-Wax MS column (30 m × 0.15 mm; 0.15 µm film thickness, Teknokroma). Helium 49 (Praxair) at a constant flow of 1.3 mL min^{-1} was used as a carrier gas. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range (*m/z*) from 35 to 260 and a 610 ms acquisition interval. The oven temperature was initially set to 60 °C for 2 min and then raised to 234 °C at a rate of 3 °C min^{-1}, raised again to 260 °C at 5 °C min^{-1} and finally maintained at 260 °C for 10 min. Injector temperature was set to 250 °C with a 30 mL min^{-1} split flow and transfer line was maintained at 250 °C. Compounds were identified using MS Workstation version 6.9 (Varian) software, by comparing mass spectra and retention indices with those of pure standards and quantified as 4-nonanol equivalents.

2.6 Statistical analysis

Statistically significant differences in concentrations of volatile compounds in the assay with different beverages were evaluated by a pairwise multiple *t*-test using the Prism 6 software (GraphPad software Inc.). For the assay with different beer formulations, statistically significant differences were determined by a non-parametric Kruskall-Wallis analysis, with Conover-Iman multiple pairwise comparisons, using the XLStat software (Addinsoft). For assessment of correlations between sensory and chemical characterization a multivariate Principal Component Analysis (PCA) was performed using Statistica software (version 7, StatSoft Inc.)

3. Results and discussion

3.1 Impact of used oak chips in the aromatic fraction of different beverages

With the aim of determining their contribution in additive ageing, a preliminary assay was performed promoting contact of used oak chips with different alcoholic beverages, namely beer, red wine and grape marc spirit. Changes in the volatile composition of beverages was monitored by GC-MS with the results presented in Table 5.1. Statistically significant differences were evaluated by pairwise multiple *t*-test, without correction for multiple comparisons. An important initial observation is that low impact was observed for the residence of the studied beverages at 40 °C without wood, regarding their volatile composition. Statistically significant differences when comparing initial composition with composition after residence at 40 °C without wood were only found for 5-methylfurfural and benzaldehyde in beer, which were found in concentrations near the limit of detection and quantification; ethyl octanoate and ethyl decanoate in wine, which could arise from residual esterification of the corresponding acids with ethanol; and hexyl acetate, 2-phenylethanol, 4-ethylguaiacol and 4-ethylphenol in grape marc spirit, for which no significant trends or mechanisms could be identified. When applying used wood to beverages, several changes were observed. Despite having been previously in contact with fortified wine, used woods still had significant amount of wood compounds to be extracted, coherent with the previously reported (Coelho *et al.*, 2019).

Table 5.1: Concentration (*C*) of volatile compounds in beer (B), wine (W) and grape marc spirit (S) in their initial state, after 48 h at 40 °C without wood (B40, W40, S40), and after 48 h at 40 °C with reused wood (BW40, WW40, SW40). Errors represent standard deviation of independent duplicates

	B	B40	BW40	W	W40	WW40	S	S40	SW 40
Compound	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})
Esters									
isobutyl acetate	18.7±3.6	17.8±2.5	19.0±4.9	-	-	-	813.7±102.6	844.7±4.7*	604.8±71.8*
ethyl butyrate	41.1±0.1	40.7±4.7	46.8±9.5	106.6±2.0	100.0±2.8	111.2±5.5	1605.2±106.6	1677.0±43.9	1566.0±171.0
ethyl 2-methylbutyrate	-	-	-	-	-	-	771.1±87.5	823.0±13.0	740.0±91.3
ethyl 3-methylbutyrate	-	-	-	-	-	-	2989.6±181.7	3263.4±408.6	3175.2±362.3
isoamyl acetate	988.0±9.3	982.8±89.1	1029.7±166.0	580.1±35.9	604.3±6.4	556.3±65.2	4703.7±48.1	5001.5±212.6	4690.9±490.7
ethyl pentanoate	-	-	-	-	-	-	167.3±16.3	217.1±5.5	173.5±25.6
ethyl 2-butenoate	-	-	-	-	-	-	570.8±18.7	580.5±21.8	550.9±55.0
ethyl hexanoate	137.9±0.2	140.7±13.0	126.6±22.5	257.5±11.8	288.3±10.0	235.6±22.0	6393.0±309.9	6867.9±422.3	5964.1±632.6
hexyl acetate	-	-	-	-	-	-	247.8±6.8	268.7±0.4*	208.8±19.8*
ethyl heptanoate	-	-	-	-	-	-	569.2±20.2	550.8±8.8	474.6±36.5
ethyl lactate	-	-	59.0±16.3*	3454.7±937.4	3576.2±161.5	3907.3±925.5	1297.2±98.4	1411.6±2.1	1660.5±284.1
ethyl 2-hexenoate	-	-	-	-	-	-	365.5±10.6	358.3±13.3	361.7±41.1
ethyl octanoate	129.0±4.4	133.4±6.6*	68.0±13.4*	237.5±13.7	316.3±12.2*	152.8±12.2*	24531.9±534.8	26831.5±1570.6*	20902.4±1731.3*
ethyl-3-hydroxybutyrate	-	-	-	74.2±17.0	78.4±4.6	93.1±17.2	-	-	-
ethyl decanoate	18.6±0.8	20.7±2.4*	11.1±3.1*	30.0±0.9	33.7±0.3*	11.7±0.3*	25557.4±285.6	32014.4±6534.6*	11350.1±796.2*
diethyl succinate	-	-	666.1±123.7*	7562.9±913.9	7296.9±29.3	8087.6±642.2	8252.1±106.3	8317.3±206.9	8551.1±694.2
methyl salycilate	-	-	-	-	-	-	1167.4±27.4	1144.5±23.4	1058.7±62.3
ethyl phenylacetate	-	-	-	-	-	-	624.2±27.4	601.0±0.8	577.3±29.2
2-phenylethyl acetate	788.9±15.2	809.4±68.0	735.0±141.0	128.4±16.0	119.6±10.0	106.1±7.7	904.2±19.0	913.4±15.5*	752.3±34.3*
ethyl dodecanoate	-	-	-	-	-	-	8144.5±45.8	13516.4±4377.1*	1857.1±90.8*
diethyl malate	-	-	35.3±8.2*	151.4±34.6	150.2±5.3*	200.6±20.2*	-	-	54.8±11.4*
ethyl cinnamate	-	-	-	-	-	-	282.8±11.2	260.4±5.3	238.7±13.9

- not detected

Table 5.1 (continuation): Concentration (*C*) of volatile compounds in beer (B), wine (W) and grape marc spirit (S) in their initial state, after 48 h at 40 °C without wood (B40, W40, S40), and after 48 h at 40 °C with reused wood (BW40, WW40, SW40). Errors represent standard deviation of independent duplicates

	B	B40	BW40	W	W40	WW40	S	S40	SW 40
Compound	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})	C/(µg L^{-1})
Alcohols									
2-methyl-1-propanol	140.7±14.5	139.5±50.4	198.5±58.1	749.1±89.8	869.3±73.0	821.5±150.1	5820.2±205.8	5929.5±923.9	5984.5±675.3
2-methyl-1-butanol+ 3-methyl-1-butanol	5781.5±280.5	5787.7±1594.4	7796.4±2045.1	20560.5±2889.4	23359.9±57.9	23400.2±5011.6	103415.4±8988.9	116358.1±1318.9	128263.2±19837.8
1-hexanol	-	-	-	533.3±67.1	539.8±20.3	591.1±101.8	13181.2±1015.3	14152.3±196.8	14653.3±1890.5
Z-3-hexenol	-	-	-	15.0±2.4	14.5±0.5	16.6±2.7	330.9±25.1	367.4±11.3	384.7±55.8
E-3-hexenol	-	-	-	39.0±7.1	39.6±0.2	45.1±9.5	580.8±49.0	620.6±18.1	639.9±80.8
1-octanol	6.9±0.1	7.4±0.9	8.6±1.3	18.4±1.9	17.2±0.9	16.9±2.5	1481.4±4.4	1478.1±85.5	1414.6±129.3
benzyl alcohol	11.1±1.2	13.1±4.5	17.8±4.7	154.0±91.1	104.2±4.8	124.5±36.1	228.1±17.9	231.1±19.5	281.1±26.1
2-phenylethanol	6027.0±315.6	6477.9±1480.8	7893.9±1868.2	9337.1±1869.4	9286.7±99.0	9969.5±1515.8	9763.2±43.8	10462.5±211.8	11003.2±1480.6
Terpenic alcohols									
linalool	8.9±0.3	9.9±0.5	9.5±1.8	11.7±2.0	10.4±0.4*	8.8±0.5*	391.6±10.0	391.3±8.6	368.3±28.5
α-terpineol	5.6±0.1	7.4±1.0	7.6±1.4	34.9±5.6	33.1±0.4	30.0±1.9	861.6±17.7	889.9±13.3	864.0±44.8
β-citronellol	3.0±0.1	3.4±0.5	3.7±0.8	-	-	-	558.7±35.8	540.2±16.8	503.0±39.3
cis-nerolidol	-	-	-	-	-	-	377.3±7.8	355.9±42.0	315.3±1.4
Acids									
acetic acid	40.0±9.0	83.4±77.3	46.6±13.5	303.4±118.5	298.6±48.4	305.0±89.2	-	-	-
propanoic acid	12.0±0.1	15.0±3.7	7.6±2.5	47.3±33.6	46.3±14.9	25.6±10.6	-	-	-
butanoic acid	3.3±1.2	5.8±2.4	4.0±1.5	29.4±14.1	23.6±0.6	16.8±5.5	-	-	-
isovaleric acid	17.8±2.1	20.6±9.0	21.5±5.9	91.9±25.5	93.9±1.3	92.8±25.3	1272.2±73.2	1345.5±136.1	1505.3±207.7
hexanoic acid	295.1±20.7	314.4±94.4	327.0±83.1	742.8±191.2	660.2±44.8	742.6±127.9	3721.9±2867.8	1833.9±79.2	1976.4±318.7
octanoic acid	1896.3±74.6	2040.3±244.4	2182.2±408.4	1606.8±198.0	1555.1±0.3	1574.4±131.8	13013.4±197.0	14085.0±1057.2	12475.1±1241.8
decanoic acid	444.8±121.2	462.8±68.5*	257.7±59.0*	466.8±42.0	460.1±42.3*	292.2±13.6*	17977.0±118.4	16888.1±711.9	17033.0±1611.5
dodecanoic acid	-	-	-	-	-	-	5008.1±71.3	5176.7±869.5	4039.6±320.8

- not detected

Table 5.1 (continuation): Concentration (*C*) of volatile compounds in beer (B), wine (W) and grape marc spirit (S) in their initial state, after 48 h at 40 °C without wood (B40, W40, S40), and after 48 h at 40 °C with reused wood (BW40, WW40, SW40). Errors represent standard deviation of independent duplicates

	B	B40	BW40	W	W40	WW40	S	S40	SW 40
Compound	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})
Lactones									
cis-oak lactone	-	-	364.2±66.3*	-	-	359.4±22.7*	-	-	548.6±53.1*
trans-oak lactone	-	-	138.2±24.6*	-	-	144.1±6.4*	-	-	163.3±11.4*
γ-nonalactone	38.3±1.2	42.3±4.0	39.2±7.2	11.5±2.8	10.6±0.0	10.6±0.3	233.8±10.6	231.6±8.8	210.3±6.5
γ-ethoxycarbonyl-γ-butyrolactone	-	-	-	404.1±55.6	451.1±20.1	513.6±31.2	-	-	-
Aldehydes									
benzaldehyde	-	5.1±0.6*	16.3±2.9*	-	-	-	2337.5±16.8	2427.9±55.0	2295.7±212.0
vanillin	15.6±0.1	14.7±4.7*	459.0±119.8*	-	-	480.8±22.6*	-	-	542.1±93.7*
syringaldehyde	-	-	2513.2±707.3*	147.3±57.8	317.0±58.4*	2573.9±169.5*	-	-	2812.3±92.0*
sinapaldehyde	-	-	2377.4±761.7*	-	-	2258.2±636.4*	-	-	4012.7±569.8*
octanal	-	-	-	-	-	-	178.1±4.5	188.4±0.7*	155.7±11.7*
nonanal	-	-	-	-	-	-	1428.6±2.0	1544.6±40.0	1425.1±86.6
Volatile phenols									
4-methylguaiacol	-	-	18.7±3.4*	-	-	-	-	-	68.6±6.4*
4-ethylguaiacol	0.8±0.0	0.6±0.9*	4.4±0.9*	-	-	-	229.2±2.6	263.2±10.0	263.4±11.4
eugenol	-	-	17.6±3.3*	1.3±0.5	5.6±5.8	14.1±1.2	456.2±0.9	423.6±11.5	422.1±16.6
4-ethylphenol	-	-	-	-	-	-	281.8±4.8	236.4±0.0	249.4±27.1
4-vinylguaiacol	94.8±63.6	104.3±24.7*	38.2±8.0*	-	-	-	-	-	-
2,6-dimethoxyphenol	-	-	71.4±16.8*	31.5±9.5	32.5±3.3*	112.3±1.2*	-	-	-
4-vinylphenol	11.2±0.2	16.2±4.1*	5.5±1.7*	-	-	-	-	-	-
acetovanillone	8.3±0.0	11.3±3.5*	90.9±22.9*	-	-	-	-	-	-
tyrosol	22.1±5.2	47.1±31.7	43.4±17.5	-	-	-	-	-	-

Table 5.1 (continuation): Concentration (*C*) of volatile compounds in beer (B), wine (W) and grape marc spirit (S) in their initial state, after 48 h at 40 °C without wood (B40, W40, S40), and after 48 h at 40 °C with reused wood (BW40, WW40, SW40). Errors represent standard deviation of independent duplicates

	B	B40	BW40	W	W40	WW40	S	S40	SW 40
Compound	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})	*C*/ (μg L^{-1})
Furan compounds									
furfural	84.7±4.5	102.9±45.5*	342.3±77.8*	76.0±15.0	96.8±17.8*	320.6±56.6*	4207.7±64.7	4518.0±106.8	4894.0±682.3
linalool furanic oxide	-	-	-	-	-	-	313.1±3.9	316.9±14.5	319.0±35.1
5-methylfurfural	-	1.5±0.5*	109.7±21.9*	-	-	133.6±17.5*	678.8±49.8	719.4±16.5	783.5±90.1
5-hydroxymethylfurfural	13.1±2.5	16.7±8.6	24.6±9.4	-	-	-	-	-	-
Sulphur compounds									
methionol	-	24.4±9.4	17.6±5.0	131.9±36.0	124.9±11.7	96.8±20.6	-	-	-
C13-norisoprenoids									
β-damascenone	-	1.9±1.3	4.0±1.4	-	-	-	-	-	-
3-hydroxy-β-damascone	15.0±1.2	18.9±3.3	15.3±4.6	-	-	-	-	-	-
3-hydroxy-β-ionone	5.6±0.1	8.3±1.5	13.0±2.6	-	-	-	-	-	-
Other									
acetoin	-	-	-	143.1±48.0	153.9±5.3	141.2±28.9	-	-	-

- not detected

Characteristic wood extractives, namely volatile phenols and aldehydes, were transferred to the beverages by contact with wood, with statistically significant increases observed either in beer, wine and grape marc spirit. Increase in concentrations of furan compounds was only statistically significant for beer and wine, with variations in grape marc distillate masked by the initial high furan compounds concentrations. Along with characteristic wood extractives, fortified wine compounds were also transferred to the beverages with the reuse of wood. As reported previously, characteristic wine esters (ethyl lactate, diethyl succinate and diethyl malate) were adsorbed by wood during the previous contact with fortified wine (Coelho *et al.*, 2019) and the reuse of wood in a subsequent ageing step led to their transference to the studied matrices. However, transference of these aroma compounds was more significant in the application of used wood to beer, where ethyl lactate, diethyl succinate and diethyl malate were absent. Also, for the specific case of diethyl malate, statistically significant increases were observed for application of wood to all the studied beverages. Extraction of additional wine volatiles adsorbed in wood, namely alcohols 3-methyl-1-butanol and 2-phenylethanol, monoethyl succinate and octanoic acid was not evident, probably hindered by the deviations in GC-MS analysis and the already high content of these compounds in the beverages. Apart from extraction of volatile compounds, compound losses inherent to subtractive ageing were also observed, as a direct result of application of wood. Concentrations of ethyl octanoate and ethyl decanoate decreased significantly in all beverages put in contact with wood. Moreover, in grape marc spirit several esters were adsorbed, with decrease in concentrations of hexyl acetate, 2-phenylethyl acetate and ethyl dodecanoate. As stated previously, wood adsorbs several compounds from wine, mainly due to hydrophobic interactions (Coelho *et al.*, 2019) and these observations are corroborated by the results obtained in this work. Esters whose concentrations decreased due to wood contact, have high xlogP3 values, as presented in Table 5.2, again reinforcing that their hydrophobic nature is the main driver of wood sorption. Significant decreases were also observed for decanoic acid concentration in beer and wine and nonanal in grape marc spirit, which have comparable XLogP3 values. In a similar process, Sterckx and collaborators applied American oak wood chips for beer ageing, leaving wood in contact with beer during 60 d at 20 °C, following variation in concentration of volatile monophenols (Sterckx *et al.*, 2012a, 2012b). These authors reported syringaldehyde concentrations of about 600 µg L^{-1}, reached after 30 d of contact with medium toast unused American oak chips (Sterckx *et al.*, 2012a), which corresponds roughly to an extraction of syringaldehyde, per mass of dry wood, of 120 µg L^{-1} g^{-1}. Results presented in this work show a final syringaldehyde concentration of about 2500 µg L^{-1}, attained after application of 20 g L^{-1} of hydrated used oak wood, which accounts for an extraction of syringaldehyde, per mass of dry wood, of about 210 µg L^{-1} g^{-1}.

Table 5.2: XLogP3-AA values reported for the volatile compounds adsorbed in wood (National Center for Biotechnology Information, 2020)

Compound	XLogP3-AA	Compound	XLogP3-AA
2-phenylethyl acetate	2.3	decanoic acid	4.1
hexyl acetate	2.4	ethyl decanoate	4.6
nonanal	3.3	ethyl dodecanoate	5.6

Sterckx also reported concentrations of vanillin of about 90 µg L^{-1} (Sterckx *et al.*, 2012b) accounting roughly for the extraction, per mass of dry wood, of 15 µg L^{-1} g^{-1}, whereas in the presented work, the attained value was 38 µg L^{-1} g^{-1}. These differences in extraction yields can derive from differences in oak wood composition, considering the reported variability within oak origin (Doussot, De Jéso, Quideau, & Pardon, 2002). On the other hand, higher syringaldehyde and vanillin extraction concentrations, even from a previously used wood, can be a result of the higher contact temperature used, which was previously shown to influence significantly extraction of several characteristic wood compounds (Coelho *et al.*, 2019). Sterckx also reported an increase in 20 µg L^{-1} in eugenol concentration, accounting for about 4 µg L^{-1} g^{-1}, whereas in the presented results an extraction of about 2 µg L^{-1} g^{-1} was obtained. As also demonstrated by Coelho et. al. (2019), eugenol extraction is more influenced by ethanol concentration in the extractive matrix, which in our work was of 4.7 %, by volume, and in Sterckx and collaborators' work of 8.3 %, by volume, which in combination with depletion of eugenol in the preceding contact with wine justifies the observed differences. Lastly, Sterckx *et al.* (2012) also reported reduction in 4-vinylguaiacol concentration, similarly to the observed in this work.

In similar works focusing wine, Bautista-Ortín and collaborators have studied the application of oak alternatives for the ageing of wine, submerging American oak shavings in red wine in stainless steel tanks using a 2.6 g L^{-1} proportion (Bautista-Ortín *et al.*, 2008). The authors reported maximum concentrations of furfural of 326 µg L^{-1} accounting for an extraction, per mass of wood, of 125 µg L^{-1} g^{-1}, and of 5-methylfurfural of 37 µg L^{-1} accounting for extraction of 14 µg L^{-1} g^{-1} (Bautista-Ortín *et al.*, 2008). In the presented results similar concentrations of furfural (about 320 µg L^{-1}) and higher concentrations of 5-methylfurfural (133 µg L^{-1}) were attained with previously used oak chips. Lower extraction yield was observed for furfural, namely 27 µg L^{-1} g^{-1}, and similar extraction yield was observed for 5-methylfurfural, accounting for 11.2 µg L^{-1} g^{-1}. Focusing oak-lactones, Bautista-Ortín and collaborators reported maximum concentration of 300 µg L^{-1} of *cis*-oak lactone and 130 µg L^{-1} of *trans*-oak lactone, accounting for extractions yields of 115 µg L^{-1} g^{-1} and 50 µg L^{-1} g^{-1}, respectively. In the present work, concentrations of *cis*- and *trans*-oak lactone were 360 µg L^{-1} and 144 µg L^{-1}, accounting for lower extraction yields of

30 μg L^{-1} g^{-1} and 12 μg L^{-1} g^{-1}, respectively, attained with previously used wood. Bautista-Ortín and collaborators also reported maximum vanillin concentration of 443 μg L^{-1} corresponding to a yield of 170 μg L^{-1} g^{-1}, whereas in the present work a similar concentration of vanillin of 480 μg L^{-1} was attained with used wood, but corresponding to a lower yield of 40 μg L^{-1} g^{-1}. Lastly, Bautista-Ortín and collaborators reported concentrations of 4-ethylphenol and 4-ethylguaiacol of 900 μg L^{-1} and 60 μg L^{-1} respectively, which are often considered spoilage in wine, whereas in the presented work such compounds were absent. More recently, Dumitriu and collaborators also applied American wood chips for wine ageing, in stainless steel tanks, at 3 g L^{-1} and 5 g L^{-1}, for a maximum of 3 months. For 3 g L^{-1} of wood chips, the authors reported higher concentrations of furfural (900 μg L^{-1}) and 5-methylfurfural (about 400 μg L^{-1}), and similar concentrations of *cis*-oak lactone (400 μg L^{-1}) and *trans*-oak lactone (130 μg L^{-1}) (Dumitriu *et al.*, 2019).

Lastly, focusing on grape marc spirits, Rodríguez-Solana and collaborators reported significantly higher concentrations of vanillin and oak lactone in different grape marks aged in American oak barrels (Rodríguez-Solana *et al.*, 2017), using an optimized process. On a global analysis, beer was the beverage in which the additive effect of used wood was more pronounced, regarding extraction of characteristic wood volatiles as well as transference of wine compounds previously adsorbed by wood. Moreover, beer presented the most satisfactory extraction yields when compared with those reported in the literature. Therefore, it was considered as the more advantageous matrix for the application of reused wood and chosen for the development of formulation and preference studies.

3.2 Impact of used oak wood on the sensory profile of beer

Several formulations using different binomial combinations of wood concentration and temperature were prepared and presented to a panel of trained tasters, resorting to the same wood previously put in contact with fortified wine. Samples were analyzed by GC-MS, in order to search for correlations between volatile composition and aroma preference. Characterization of volatile composition of the different beer samples is presented in Table 5.3 and the sensory evaluation of the correspondent samples is presented in Figure 5.1. Again, the main adsorbed wine volatiles extracted to beer were ethyl lactate, diethyl succinate and diethyl malate, which were originally absent from beer, along with characteristic wood extractives, vanillin, *cis*- and *trans*-oak lactone, syringaldehyde, sinapaldehyde, furfural and 2,6-dimethoxyphenol. Concentration of wood affected significantly the incorporation of volatile compounds in beer, either regarding adsorbed wine volatiles or characteristic wood extractives, due to the increasing extractable amount supplied by higher wood to beer ratios.

Table 5.3: Composition of beer formulations using different wood concentration and temperature combinations. Samples are identified by a *CxTy* key, where *Cx* refers to wood concentration at *x* g L^{-1} and *Ty* refers to contact temperature performed at *y* °C. Controls of beer incubated at the same temperatures without addition of wood were also performed, identified as BT*y*, where *y* refers to contact temperature in °C. Statistical significant similarities/differences are marked by a to h letters. Errors represent standard deviation of independent duplicates

		*BT*30	*C*10*T*30	*C*20*T*30	*C*30*T*30	*BT*40	*C*10*T*40	*C*20*T*40	*C*30*T*40	*BT*50	*C*10*T*50	*C*20*T*50	*C*30*T*50
	Compound	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})	*C*/(μg L^{-1})
	Esters												
1	isoamyl acetate	842.7±9.8^{bc}	753.2±11.3^{abc}	723.4±43.0^{abc}	682.1±36.3^{a}	947.8±119.6^{bc}	795.2±47.2^{abc}	730.1±0.3^{abc}	826.7±182.8^{abc}	896.9±38.7^{c}	746.9±25.2^{abc}	744.2±48.6^{abc}	723.7±7.4^{ab}
2	ethyl hexanoate	89.0±3.2^{bc}	77.2±1.8^{ab}	83.1±4.2^{abc}	74.4±5.1^{a}	105.3±14.7^{c}	85.3±4.8^{abc}	83.4±0.1^{abc}	95.4±19.0^{abc}	90.3±6.4^{bc}	75.1±4.5^{ab}	81.7±3.1^{abc}	74.9±1.3^{a}
3	ethyl lactate	-	160.1±24.5^{ab}	278.2±23.7^{d}	314.1±20.6^{de}	-	142.9±0.2^{ab}	209.6±7.0^{bc}	494.9±142.6^{e}	-	117.7±3.6^{a}	245.7±27.5^{cd}	367.8±51.8^{e}
4	ethyl octanoate	462.9±9.7^{ef}	396.9±8.0^{cde}	410.6±20.1^{def}	354.2±31.9^{bcd}	571.6±110.6^{f}	467.8±23.6^{ef}	398.8±27.9^{cde}	436.3±127.6^{def}	172.8±4.0^{abc}	145.0±8.8^{ab}	145.1±4.2^{ab}	122.2±3.8^{a}
5	ethyl decanoate	149.5±0.3^{d}	105.6±5.2^{c}	96.0±9.2^{c}	70.4±5.3^{b}	234.4±40.5^{d}	157.4±11.4^{d}	111.8±6.5^{c}	108.3±31.1^{c}	71.3±0.0^{b}	50.2±3.5^{ab}	44.4±1.8^{a}	32.5±2.3^{a}
6	diethyl succinate	-	190.8±4.9^{a}	415.1±23.2^{cd}	558.2±18.8^{de}	-	199.4±8.2^{ab}	392.5±2.2^{bc}	689.4±148.7^{e}	-	180.3±1.9^{a}	395.9±19.77^{bc}	573.5±32.9^{e}
7	2-phenylethyl acetate	371.5±1.1^{d}	296.0±1.0^{bc}	269.2±13.5^{ab}	214.4±12.8^{a}	409.8±61.8^{d}	318.5±15.0^{cd}	267.4±2.9^{ab}	272.5±58.8^{abc}	370.9±6.2^{d}	296.2±1.6^{bc}	278.4±12.3^{abc}	245.7±9.4^{a}
8	diethyl malate	-	9.4±0.4^{a}	17.9±1.7^{bc}	24.5±0.3^{cd}	-	9.2±0.1^{a}	15.4±0.4^{b}	33.4±7.7^{d}	-	9.2±1.6^{a}	16.7±2.0^{b}	25.4±2.5^{cd}
	Alcohols												
9	2-methyl-1-propanol	1182.5±361.3^{bcd}	1209.7±353.3^{bcd}	1070.5±10.9^{bcd}	802.7±16.8^{ab}	1473.5±123.8^{d}	1092.0±19.7^{cd}	710.8±15.2^{a}	1289.7±414.5^{d}	1118.9±121.9^{bcd}	796.8±33.6^{abc}	901.9±69.0^{abcd}	992.9±101.3^{bcd}
10	2-methyl-1-butanol +3-methyl-1-butanol	18238.1±3147.1^{ab}	18197.0±2776.9^{ab}	17793.9±1257.0^{ab}	14573.2±124.8^{a}	22631.7±626.2^{b}	18113.6±109.2^{ab}	14755.3±319.4^{a}	23086.8±6907.8^{b}	20141.1±480.0^{b}	16260.9±461.6^{ab}	17323.4±1686.8^{ab}	17852.8±1828.8^{ab}
11	furfuryl alcohol	44.2±12.2^{abcd}	48.3±10.3^{bcd}	45.4±0.5^{bcd}	37.4±2.6^{abc}	56.1±1.9^{cd}	44.3±1.9^{bcd}	29.9±0.2^{a}	46.9±13.5^{abcd}	45.0±4.8^{bcd}	32.9±1.5^{ab}	39.0±3.0^{abc}	97.0±7.6^{d}
12	2-phenylethanol	23056.5±1501.2^{bc}	20874.6±341.4^{abc}	20867.5±2103.7^{abc}	17231.3±353.0^{a}	27280.9±3401.7^{c}	21707.8±676.7^{abc}	18805.0±380.2^{ab}	24556.2±6547.2^{abc}	23564.3±150.5^{bc}	19709.9±790.3^{abc}	20445.4±2005.2^{abc}	19428.1±1824.2^{ab}
	Monoterpenic alcohols												
13	Linalool	89.7±5.6^{c}	70.8±2.0^{abc}	69.8±6.0^{abc}	60.7±1.2^{a}	95.3±14.3^{c}	77.0±3.5^{bc}	68.7±0.5^{abc}	77.9±18.6^{abc}	86.7±3.7^{c}	71.4±0.2^{abc}	69.8±3.5^{abc}	63.2±3.8^{ab}
	Acids												
14	acetic acid	202.7±54.1^{cd}	199.9±93.1^{bcd}	154.3±7.9^{bcd}	111.7±8.7^{abc}	225.8±64.1^{d}	179.3±25.7^{cd}	85.7±0.1^{a}	189.5±7.1^{d}	99.2±17.6^{ab}	81.3±22.2^{a}	118.0±21.1^{abc}	136.9±13.4^{abcd}
15	isovaleric acid	95.5±25.9^{ab}	94.6±19.6^{ab}	93.2±3.4^{ab}	72.5±2.4^{a}	123.2±11.7^{b}	99.0±1.4^{ab}	76.7±1.3^{ab}	124.8±35.4^{ab}	89.4±12.1^{ab}	75.9±1.3^{a}	100.0±5.2^{ab}	97.0±7.6^{ab}
16	hexanoic acid	349.4±40.0^{ab}	387.8±26.4^{ab}	359.7±2.5^{ab}	305.5±13.6^{a}	462.6±46.1^{b}	369.4±15.5^{ab}	290.9±9.1^{a}	352.3±0.0^{ab}	364.8±22.0^{ab}	326.9±28.0^{a}	365.2±0.0^{ab}	336.3±61.2^{ab}
17	octanoic acid	2304.3±52.2^{c}	2012.7±94.9^{abc}	1857.2±67.2^{ab}	1505.8±93.5^{a}	2699.1±435.3^{c}	2192.1±117.3^{abc}	2027.0±49.9^{abc}	2187.8±467.4^{abc}	2336.5±105.9^{c}	2178.9±83.3^{abc}	2217.8±98.4^{bc}	2079.6±81.3^{abc}
18	decanoic acid	328.8±8.6^{fg}	245.5±3.6^{cd}	183.0±4.6^{ab}	134.0±11.1^{a}	380.7±72.1^{fg}	259.4±15.2^{de}	211.9±16.8^{bc}	194.1±30.8^{ab}	354.8±13.2^{g}	292.6±8.5^{ef}	257.4±13.5^{de}	213.1±4.9^{b}

\- not detected

Table 5.3 (continuation): Composition of beer formulations using different wood concentration and temperature combinations. Samples are identified by a *CxTy* key, where *Cx* refers to wood concentration at *x* g L^{-1} and *Ty* refers to contact temperature performed at *y* °C. Controls of beer incubated at the same temperatures without addition of wood were also performed, identified as BT*y*, where *y* refers to contact temperature in °C. Statistical significant similarities/differences are marked by a to h letters. Errors represent standard deviation of independent duplicates

		*BT*30	*C*10*T*30	*C*20*T*30	*C*30*T*30	*BT*40	*C*10*T*40	*C*20*T*40	*C*30*T*40	*BT*50	*C*10*T*50	*C*20*T*50	*C*30*T*50
	Compound	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})	*C*/(µg L^{-1})
	Furan compounds												
19	furfural	-	74.9±2.7^{a}	153.1±27.0^{cd}	188.9±6.3^{ef}	-	100.6±3.2^{ab}	183.1±1.1^{de}	397.6±96.2^{g}	-	113.2±5.3^{bc}	224.5±32.9^{f}	360.7±31.8^{g}
	Lactones												
20	*cis*-oak lactone	-	102.3±2.7^{a}	205.1±6.2^{cd}	274.3±12.7^{e}	-	131.4±4.3^{bc}	260.7±1.5^{de}	429.8±90.9^{f}	-	124.6±2.4^{ab}	271.8±9.6^{e}	377.9±15.4^{f}
21	*trans*-oak lactone	-	44.7±0.9^{a}	84.9±6.4^{cd}	109.6±2.9^{e}	-	58.4±2.6^{bc}	103.6±0.8^{d}	169.1±39.2^{f}	-	52.9±0.7^{ab}	109.5±4.6^{e}	147.1±11.4^{f}
	Phenolic compounds												
22	2,6-dimethoxyphenol	-	32.1±2.3^{a}	41.7±3.1^{bc}	48.2±0.9^{cd}	-	30.8±1.1^{a}	43.5±0.4^{bc}	76.3±19.0^{d}	-	29.4±0.9^{a}	41.1±2.5^{b}	57.9±3.2^{d}
	Aldehydes												
23	vanillin	-	68.2±0.9^{a}	125.4±16.1^{cd}	165.8±6.9^{de}	-	103.7±5.6^{bc}	183.0±2.3^{ef}	344.8±92.7^{g}	-	95.8±5.0^{ab}	206.7±20.2^{f}	300.8±24.2^{g}
24	syringaldehyde	-	312.3±3.9^{a}	578.9±49.6^{cd}	745.8±42.0^{e}	-	409.3±20.8^{bc}	714.2±6.0^{de}	1327.7±260.8^{f}	-	406.4±21.0^{ab}	854.2±66.5^{e}	1277.6±73.3^{f}
25	sinapaldehyde	-	338.6±9.9^{a}	561.8±61.2^{bc}	820.0±62.8^{de}	-	450.1±32.4^{ab}	1049.3±2.1^{ef}	1855.9±353.7^{gh}	-	749.1±62.8^{cd}	1565.1±99.9^{fg}	2303.2±191.4^{h}

- not detected

Mathematical models established in previous work have demonstrated that temperature mainly increases extraction of characteristic wood compounds, having low to null impact on the extraction of adsorbed wine volatiles (Coelho *et al.*, 2019). In fact, very low variation was observed for ethyl lactate, diethyl succinate and diethyl malate comparing the different temperatures for the same wood concentration. On the other hand, noticeable variations were observed for the extraction of characteristic wood extractives, when comparing application of similar wood concentrations at different temperatures. Statistically significant differences were found when applying the same wood concentration at different temperatures for furfural, oak lactones, vanillin, syringaldehyde and sinapaldehyde, with higher compound concentrations at upper temperature. Thus, results obtained are also in good agreement with the established in a previous work (Coelho *et al.*, 2019). Wood sorption was again observed, with decreases in beer volatile compound concentrations by application of oak wood. Esters, either acetates (isoamyl and 2-phenylethyl) or ethyl esters (hexanoate, octanoate and decanoate), acids (octanoic and decanoic) and linalool were found to adsorb in wood. Statistical significant differences were more prominent when comparing beer controls without wood with beer in contact with the highest concentration of wood (30 g L^{-1}). Ramirez-Ramirez and collaborators also reported wood sorption of linalool, ethyl octanoate and isoamyl acetate from synthetic wine matrices, again with increased sorption for higher wood concentrations, being the obtained results in good agreement with the previously reported (Ramirez-Ramirez *et al.*, 2001). Regarding the effect of temperature, statistical significant differences were only observed for ethyl octanoate and decanoate concentrations at 50 °C when compared with the remaining temperatures, which was observed for beers with and without wood. Thus, temperature possibly affected compounds stability, rather than sorption by wood. On the other hand, decrease in octanoic and decanoic acid concentrations was more pronounced for beer in contact with wood at 30 °C, suggesting an impact of temperature on their sorption.

Focusing on the sensory analyses of beer formulations, several different features can be highlighted. Descriptors were grouped in visual, aroma and taste attributes, according to the evaluated sensory properties. For a better interpretation of the results, a multivariate PCA analysis was performed in order to distinguish sensory features of the tasted samples, presented in Figure 5.2 a), and in a more detailed analysis, correlation between aroma descriptors and volatile composition of samples is presented in Figure 5.2 b). Regarding visual descriptors, a yellow color was described for beers put in contact with wood at 30 °C and golden for samples put in contact with wood at 40 °C and 50 °C. Haze was noticeable in all the evaluated samples, which was slightly higher in beers put in contact with wood at higher temperatures of 40 °C and 50 °C. Despite small variations, overall visual quality was similar for all the tasted samples.

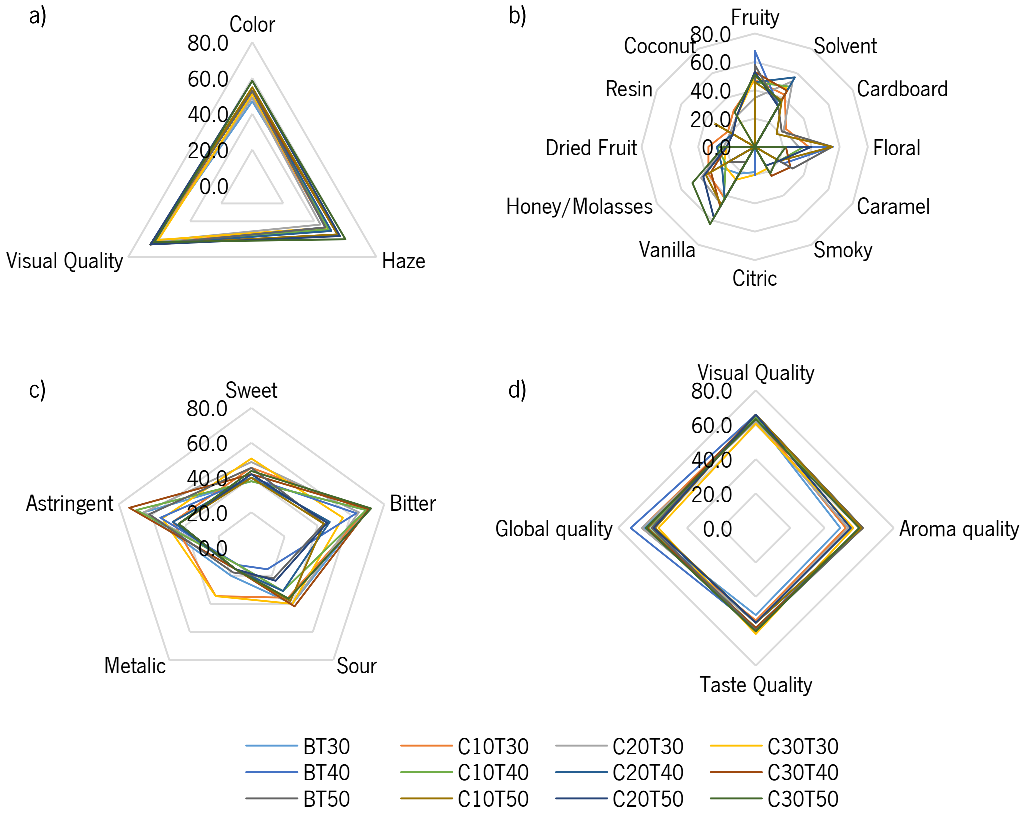

Figure 5.1: Sensory evaluation of the different beer formulations regarding a) visual descriptors, b) aroma descriptors, c) taste descriptors and d) overall quality.

Focusing aroma characteristics, fruity, solvent and floral were the descriptors with the higher overall ratings by the panelists for beer samples, as well as vanilla for the beers put in contact with wood. Higher fruity scores were attributed to beers without application of wood, especially for 40 °C and 50 °C, as visible in the scatterplot presented in Figure 5.2 a). The lower fruity scores of beers put in contact with wood can be correlated with adsorption of several esters, such as isoamyl acetate (perception threshold, *PT*, of 30 μg L^{-1}(Christoph & Bauer-Christoph, 2007)), ethyl octanoate (*PT* of 260 μg L^{-1} (Christoph & Bauer-Christoph, 2007)) and ethyl decanoate (*PT* of 50 μg L^{-1} (Christoph & Bauer-Christoph, 2007)) as previously discussed. In fact, Figure 5.2 b) demonstrates correlation of these esters with perception of fruity aroma by tasters, which were more prominent in control beers and less in beers put in contact with wood. Additional esters were extracted from wood, namely ethyl lactate (*PT* of 100 mg L^{-1} (Christoph & Bauer-Christoph, 2007)), diethyl succinate (*PT* of 100 mg L^{-1} (Christoph & Bauer-Christoph, 2007)) and diethyl malate, but their concentrations were low considering the perception threshold for each compound. Similarly to fruity aromas, scores of floral descriptors were also lower in beers put in contact with wood. Several compounds associated with floral descriptors, namely 2-phenylethyl acetate (*PT* of 250 µg L^{-1} (Christoph & Bauer-Christoph, 2007)), 2-phenylethanol (*PT* of 10 mg L^{-1} (Christoph & Bauer-Christoph, 2007)) and linalool (*PT* of 0.14 µg L^{-1} (Christoph & Bauer-Christoph, 2007)), were found in beer samples. In fact, linalool and 2-phenylethyl acetate concentrations decreased significantly with application of wood, again due to wood sorption, leading to a lower perception of floral aroma.

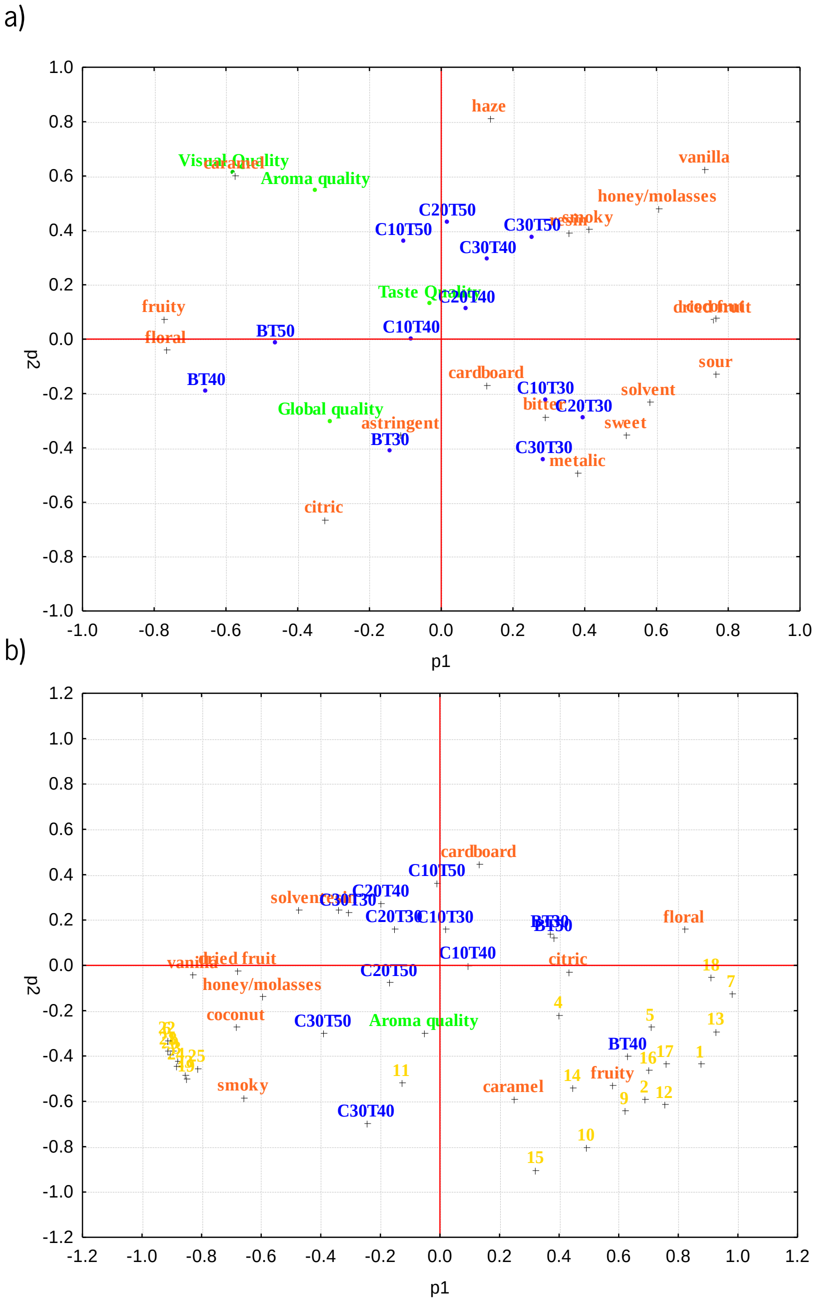

Figure 5.2: Scatterplots obtained of the Principal Component Analysis demonstrating a) differentiation of the different beer formulations according to their sensory features and b) correlation between the perceived aroma descriptors and volatile composition of the different beer formulations. Numbers in the scatterplot refer to the volatile compounds presented in Table 5.3.

Several descriptors commonly associated with aged wood products were also found. Vanilla, coconut and smoky descriptors were correlated with beers put in contact with wood, and more prominent in the contacts performed at higher temperatures of 40 °C and 50 °C. This is coherent with the higher extraction of characteristic wood volatiles such as vanillin, *cis-* and *trans-*oak lactone, 2,6-dimethoxyphenol, syringaldehyde, sinapaldehyde and furfural, often associated with barrel ageing descriptors. The contribution of temperature is furthermore highlighted by the perception of different binomial formulations by the panelists. Formulations using lower wood concentrations and higher temperatures had similar perception of wood descriptors of formulations using higher wood amounts and lower contact temperatures. Considering that extraction of adsorbed wine volatiles migrating to beer through wood is not affected by temperature, the use of different wood concentration/temperature binomials allows further fine tuning of the intended aromatic bouquet, as previously hypothesized (Coelho *et al.*, 2019). Despite the presence of volatile fatty acids, no faulty aromas were perceived by the trained panelists. Focusing on taste quality, beers were marked mainly by perception of bitterness and astringency. Astringency was overall higher in control beers and beers placed with wood at low to moderate temperatures of 30 °C and 40 °C, especially for sample *C*30*T*40. This was also observed for bitterness, with the exception of sample *C*30*T*50. Sweetness was higher for samples put at 30 °C but overall variation of sweetness perception was rather low. Also sour descriptors were more associated with beers placed at 30 °C but no significant trends could be observed. Perception of metallic taste was low for the overall beers, being higher only for *C*10*T*30 and *C*30*T*30 samples. Focusing on quality perception by the panelists, visual quality was similar for all the tasted samples, being only slightly lower for beers placed with wood at 30 °C. Higher aroma quality was attributed to beers with greater perception of wood descriptors, when compared with beer controls without wood. Moderate variations were also observed for taste quality, which was considered a little higher for beers placed with wood at temperatures of 40 °C and 50 °C. Panelists attributed slightly higher global quality scores to control beers, probably considering them a more balanced or recognizable product, with more pronounced fruity and floral aromas, taking into account what would be expected from the corresponding beer type.

4. Conclusions

Due to the reuse, wood transfers compounds from one beverage to another, along with a significant amount of wood extractives. The impact of wood reuse is strongly dependent on the beverages to be aged and their chemical composition. Transference of compounds from the previous beverage is perceived in the sensory characteristics of beer aged with reused wood. Wood also adsorbs compounds from the beverages during contact, which further modifies the perceived sensory features. Different binomial combinations of wood concentration and contact temperature lead to differentiated volatile compositions and consequently different organoleptic characteristics. Higher wood concentrations and contact temperatures lead to a sensory profile more marked by vanilla and coconut descriptors, whereas lower wood concentrations lead to more dominant fruity and floral characteristics. Reuse of oak chips is a feasible alternative for additive and subtractive ageing of alcoholic beverages, to be incorporated in accelerated ageing processes.

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Chapter 6. Volatile fingerprinting differentiates diverse-aged craft beers

Abstract

Beer ageing on wood is a complex and difficult to control process involving several reactions and compounds. Difficulties in understanding the underlying phenomena often lead to empirical and unpredictable processes and heterogeneous products. This work resorts to volatile fingerprinting along with multivariate analysis as tools to differentiate and highlight differences in beers derived from diverse production processes. Volatile composition of beers originating from barrel ageing processes and unaged beer were analyzed by GC-MS. The collected data was processed by principal component analysis, which allowed the evaluation of relations between samples and volatile compounds. Beers were distinguished by clusters comprising different groups of volatiles. Beer with the longest period in barrel was in the cluster with the most volatiles. Beer produced by resident barrel microbiota fermentation was characterized by presence of *Brettanomyces sp.* metabolites. Beer aged in barrel by a shorter time period showed characteristic content of ethyl esters and oak extractives. Beer produced in inox vat and beer fermented in barrel with pitching of *S. cerevisiae* appeared in the same cluster, relating with fermentative esters. Volatile fingerprinting was a viable approach to characterize and distinguish the analyzed beers, providing relevant information regarding the impact of production methodologies in volatile composition.

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1. Introduction

Ageing of beverages in oak barrels is a common practice and is widely recognized for improving stability and organoleptic properties of the final product. During contact, several phenomena occur which impact beverage composition, namely the extraction of wood compounds, evaporation of beverage volatile compounds, oxidation of compounds in the beverage, and reaction between wood and beverage components (Mosedale & Puech, 1998). Extraction of volatiles from oak barrels depends mainly on wood extractible content, contact time, cellar conditions and beverage composition (Chira & Teissedre, 2014). Barrel usage is a crucial factor for ageing. The pool of extractable oak compounds is finite and the rate and amounts of compounds extracted diminish as the barrel is used in successive years (Wilkinson, Li, Grbin, & Warren, 2013). Furthermore, casks also retain compounds from the beverages aged in them, due to sorption of beverages components. As shown, oak wood retains compounds from the aged beverages, which can be transferred from one beverage to another through the reuse of the cask (Coelho, Domingues, Teixeira, Oliveira, & Tavares, 2019). Presence of lees in the cask can also take part on enhancing sensorial characteristics of alcoholic beverages, leading to higher body and aroma complexity as acknowledged in wine production (Del Barrio-Galán, Pérez-Magariño, & Ortega-Heras, 2011). Therefore, volatile composition of the aged beverage is influenced not only by the previously referred reactions occurring in the barrel but also by transference of compounds from one matrix to another, with the barrel posing as a vector for carrying such compounds and aromatic properties. Additionally, when envisaging beverages such as wine or beer, biological transformations occur during barrel ageing which furthermore modify chemical and sensory properties. Casks are often populated with microorganisms that alter significantly the beverage composition. Bacteria, such as *Lactobacillus sp.* and *Enterobacter sp.* and wild yeasts such as *Brettanomyces sp., Debaryomyces sp.* and *Candida sp.* can be found in wood and wine barrels with impact on beverage composition (Spitaels *et al.*, 2014). Whilst mainly considered as spoilage organisms responsible for off-flavor production in wine, an increasing number of authors report that these endogenous microbes can add beneficial (or at least interesting) aromas that increase the flavor complexity of fermented beverages. On this field, *Brettanomyces sp.* strains are rising in importance, due to the ability to produce volatile compounds that enhance sensory properties of beer (Colomer, Funch, & Forster, 2019). Several beer styles traditionally produced by fermentation in barrel rely on typical *Brettanomyces sp.* flavors such as seen for Lambic beers (Steensels *et al.*, 2015) and Belgian red-brown acidic ales (Snauwaert *et al.*, 2016).

With an increasing market which strives for innovative specialty products, mainly in craft beer, barrel ageing has been applied at great extent. Variables such as wood type and origin, presence of lees, resident barrel microbiota and ageing conditions lead to a large number of possible combinations to be performed. With such extent of variables to be manipulated and taking into account the multitude of compounds involved in beer sensory characteristics, ageing results are often unpredictable lead to heterogeneous products in regard to their chemical composition and sensory characteristics. In the case of wine, which has also complex composition, fingerprinting methodologies have been applied as *omics* tools for the discrimination, identification and quality control of dissimilar samples (Laaks, Letzel, Schmidt, & Jochmann, 2012) and for the investigation of the importance and effect of wine volatiles (González Álvarez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2011).

The aim of this work was the application and evaluation of volatile fingerprinting along with multivariate analysis as a tool to discriminate and characterize differences in barrel diverse-aged beers.

2. Materials and methods

2.1 Samples

Beer samples were kindly provided by "Fermentum – Engenharia das Fermentações Lda.", originating from "Letra" craft brewery. All samples originated from the same Belgian Dark Strong Ale wort with a specific gravity of 21 in the Plato scale (°*P*), leading to a beer with bitterness (*IBU*) of 27 and ethanol concentration, by volume, of 9 %. Wort was fermented with *Saccharomyces cerevisiae* 1762 Belgian Abbey II (Wyeast Laboratories Inc.), pitched according with the instructions of the supplier, with the exception of ab2 beer which was fermented by resident barrel microbiota. American oak barrels previously used once in Port wine ageing were kindly provided by Quinta do Portal S.A. Barrels were directly used for beer ageing without any kind of cleaning or processing. A minimum of two independent samples of each treatment were collected from the brewery, namely two for ab1, ab2, and ub and three for ab3 and ab4. For a better interpretation, information on samples, production process and ageing parameters are presented in Figure 6.1.

2.1 Chemical characterization

2.1.1 HPLC analysis

Ethanol was quantified by high performance liquid chromatography (HPLC), using a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI) and an 87H Chrompack column (300 mm × 7.8 mm) at 60 °C. A 5 mmol L^{-1} H_2SO_4 aqueous solution was used as mobile phase at a constant flow of 0.7 mL min^{-1}.

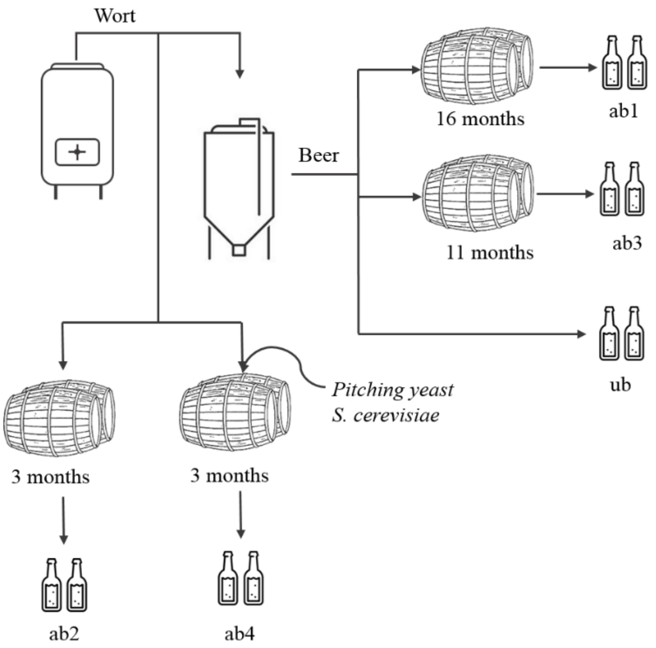

Figure 6.1: schematic representation of the strategies used for the production of the analyzed samples.

2.1.2 Analysis of volatile compounds

In a 10 mL culture tube (Pyrex, ref. 1636/26MP), 8 mL of sample, 3.08 µg of internal standard (4-nonanol), and a magnetic stir bar (22.2 mm × 4.8 mm) were added. Extraction was done by stirring the sample with 400 µL of dichloromethane according to Oliveira *et al.* (2006) (Oliveira, Faria, Sá, Barros, & Araújo, 2006). After cooling at 0 °C during 15 min, the magnetic stir bar was removed and the organic phase was separated by centrifugation (4000 min^{-1}, 7 min, 4 °C) and the extract recovered into a vial, using a Pasteur pipette. Then, the aromatic extract was dehydrated with anhydrous sodium sulfate and placed into a new vial. Analysis of volatile compounds was performed using a gas chromatograph-mass spectrometer (GC-MS) constituted by a Varian Saturn 2000 chromatograph with a 1079 injector and an ion-trap mass spectrometer (IT-MS). Samples of 1 µL were injected in splitless mode (30 s) in a Sapiens-Wax MS Teknocroma column (30 m × 0.15 mm; 0.15 µm film thickness). The temperature of the injector was held at 250 °C. The temperature of the oven was held at 60 °C, for 2 min, then programmed to rise to 234 °C at 3 °C min^{-1}, raised from 234 °C to 260 °C at 5 °C min^{-1} and finally held 5 min at 260 °C. The carrier gas was helium GHE4× (Praxair) at a constant flow rate of 1.3 mL min^{-1}. The detector was set to electronic impact mode (70 eV) with an acquisition range (*m/z*) from 35 to 300 (delay of 2.18 min). The identification of volatile compounds was performed using the software Star – Chromatography Workstation version 6.9.3 (Varian), by comparing retention index with those of pure standard compounds. Volatile compounds were determined as 4-nonanol equivalents.

2.2 Statistical analysis

Tukey's test was conducted to determine statistically significant differences in ethanol concentration between samples, for a 95 % confidence interval. Statistically significant differences in volatile compounds concentrations were assessed using a non-parametric Kruskall-Wallis analysis for multiple comparisons and a 95 % confidence interval, using XLSTAT software (Addinsoft). In order to establish the volatile fingerprint of the analyzed samples, a PCA analysis was performed using StatSoft, Inc. (2004) STATISTICA (data analysis software), version 7.0 (www.statsoft.com). Considering the parameters of the obtained PCA, three first components were selected for the profiling of the volatile fingerprints.

3. Results and Discussion

Analysis and characterization of beers was conducted in order to highlight differences between ageing strategies and their corresponding impact on chemical profile. As seen in Figure 6.2, ethanol concentration in barrel aged beers was significantly higher when compared with the unaged beer. Firstly, contact with Port wine lees (previously aged in the same barrels) can provide additional ethanol, considering that fortified wines contain alcoholic strength by volume between 19 % and 21 % (Tredoux & Silva Ferreira, 2012). As stated previously, barrels were supplied with lees and used without any prior treatment. Considering that composition and amount of lees is very difficult to measure without compromising the barrel and its application, the link between lee content and ethanol concentration cannot be directly established, but it cannot also be ruled out on the basis of the studied process. Secondly, alcoholic fermentation occurs further in the barrel, either directly from the beer wort or from residual sugars in beer. The presence of endogenous yeast in the barrel, adapted to higher ethanol concentrations, can lead to a more efficient sugar to ethanol conversion. More specifically, when comparing ab2 with ab4 samples, which were both fermented in barrel, a higher ethanol content for the ab2 sample is noticeable despite not being considered statistically significant. As referred, ab2 beer samples were obtained from barrel fermentation of wort without addition of inoculum, whereas ab4 beer samples derived from the same wort also fermented in barrel but pitched with a commercial *S. cerevisiae* yeast. Thus, fermentation in ab2 samples is exclusively attributed to resident microbiota in the barrel, whereas in ab4 the predominance of *S. cerevisiae* was imposed by pitching. In fact, sample ab2 showed higher content in *Brettanomyces sp.* strains, by detection with selective media, which are reported to be better adapted to high ethanol and stresses (Steensels *et al.*, 2015). Focusing ab1 and ab3 samples, secondary fermentation could also contribute to the higher ethanol content considering that the endogenous microflora could further consume residual sugar in beer.

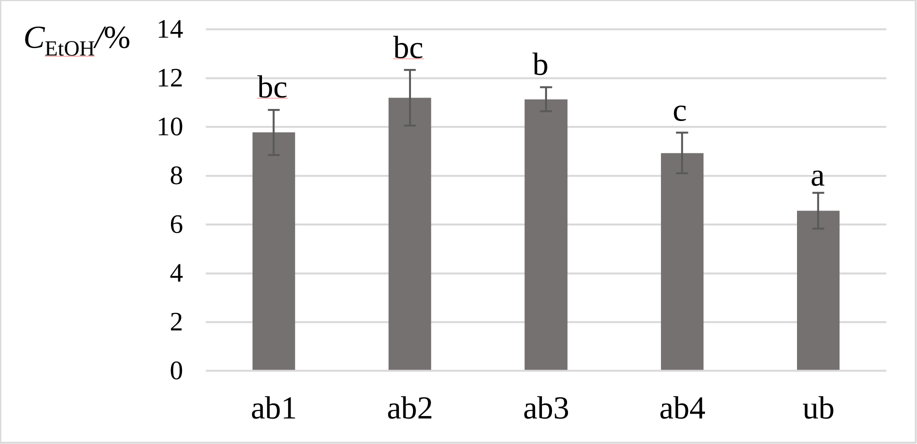

Figure 6.2: Ethanol concentration, by volume (*C*), in barrel aged beer and control samples. Results are shown with standard deviations of independent replicates (*n*=2 for ab1, ab2, and ub and *n*=3 for ab3 and ab4).

3.1 Volatile compounds

Besides increasing ethanol content, barrel ageing has a great impact on volatile composition of beers, being responsible for specific sensory traits of barrel aged beer. Volatiles were analyzed in the provided beer samples and are presented in Table 6.1. GC-MS analysis allowed the identification of a total of 56 volatile compounds, comprising 9 groups according to their classification. The higher alcohols 3-methyl-1-butanol, 2-methyl-1-butanol and 2-phenylethanol were the main volatile alcohols found, followed by 2-methyl-1-propanol at lower proportion. These alcohols derive from sugar and amino acid metabolisms in alcoholic fermentation and can be found either in wine (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006) or in beer (Briggs, Boulton, Brookes, & Stevens, 2004). Overall proportions of alcohols found in barrel aged beer were higher than the ones found in control beer. This can be linked either to a higher secondary alcohol production due to the more efficient ethanol conversion in barrel aged beers or due to the extraction of these compounds from wood and lees. Other alcohols, 3-ethoxy-1-propanol, *Z*-3-hexenol, 1-pentanol and 1-butanol, for instance, were only found in barrel aged beer samples, being absent in the unaged beer. Such compounds can be present either in the barrel, by wood sorption (Coelho *et al.*, 2019), or in lees (Liberatore, Pati, Nobile, & Notte, 2010) and can be transferred to beer in the subsequent reutilization of the barrel. The C_6-alcohol *Z*-3-hexenol for instance is directly related to wine considering that derives from polyunsaturated fatty acids found in grapes (Oliveira *et al.*, 2006; Ribéreau-Gayon *et al.*, 2006). Its exclusive presence on barrel aged samples demonstrates that characteristic wine traits were transferred to beer. Monoterpenic alcohols, namely linalool, α-terpineol, and β-citronellol were found in all beer samples. These monoterpenes can originate from hops used in brewing (Briggs *et al.*, 2004) and were found in all beer samples.

Table 6.1: Identification of volatile compounds in beer samples, expressed as mean concentration of analysis duplicate (*C*), including standard deviations, along with the corresponding linear retention indexes (*LRI*) perception thresholds (*PT*) and reported descriptors. Statistically significant similarities are represented grouped by a, b and c letters. Deviations represent standard error of independent replicates (*n*=2 for ab1, ab2, and ub and *n*=3 for ab3 and ab4)

No.	Compound	*LRI*	C_{ab1}/(μg L^{-1})	C_{ab2}/(μg L^{-1})	C_{ab3}/(μg L^{-1})	C_{ab4}/(μg L^{-1})	C_{ub}/(μg L^{-1})	*PT*/(μg L^{-1})	Descriptors
	C$_6$-Alcohols								
1	1-hexanol	1344	246.5±5.1 ^{c}	98.4±4.7 ^{ab}	160.6±29.1 ^{bc}	127.1±25.6 ^{abc}	18.5±2.4 ^{a}	8000 [1, 2] **	Coconut, green [3]
2	*Z*3-hexenol	1375	19.5±5.2 ^{c}	8.5±0.7 ^{ab}	13.6±3.3 ^{bc}	12.9±3.0 ^{abc}	nd	400 [1] **	Lettuce [4]
	Alcohols								
3	2-methyl-1-propanol	1077	980.8±63.7 ^{b}	504.4±33.0 ^{a}	580.3±194.2 ^{ab}	767.8±239.5 ^{ab}	875.7±252.3 ^{ab}	550 [4] ***	Malty [4]
4	3-methyl-1-butanol + 2-methyl-1-butanol	1199	15946.2±3779.0 ^{b}	9659.4±1838.7 ^{a}	12488.8±3503.1 ^{ab}	12176.7±3011.6 ^{ab}	11191.7±2578.5 ^{ab}	220 [4] ***	Malty [4]
5	1-pentanol	1239	13.3±5.4 ^{b}	6.5±1.2 ^{ab}	6.5±0.8 ^{ab}	10.2±3.5 ^{b}	nd		
6	*Z*2-penten-1-ol	1312	7.1±3.2 ^{a}	7.3±4.4 ^{a}	11.9±3.2 ^{a}	10.7±2.5 ^{a}	9.3±3.7 ^{a}		
7	4-methyl-1-pentanol	1307	19.8±2.7 ^{a}	11.1±1.3 ^{a}	10.9±3.1 ^{a}	11.8±2.3 ^{a}	11.3±3.8 ^{a}	50000 [2] **	
8	3-ethoxy-1-propanol	1366	22.8±5.4 ^{b}	10.5±2.4 ^{ab}	12.3±3.8 ^{ab}	11.0±2.5 ^{ab}	nd	50000 [2] **	
9	2-ethyl-1-hexanol	1483	5.9±1.5 ^{ab}	25.6±0.2 ^{b}	2.3±0.6 ^{a}	13.8±8.9 ^{ab}	1.4±2.0 ^{a}		
10	1-octanol	1551	34.4±0.2 ^{c}	28.2±3.1 ^{bc}	21.8±3.7 ^{ab}	23.5±3.5 ^{abc}	16.4±2.6 ^{a}	10000 [1] **	Coconut, nuts, oily [3]
11	1-butanol	1131	205.9±16.4 ^{b}	67.9±3.6 ^{ab}	115.5±31.3 ^{b}	29.2±9.1 ^{a}	nd	590 [4] ***	Malty, solvent [4]
12	3-methyl-1-pentanol	1318	105.0±7.3 ^{c}	74.1±0.4 ^{bc}	76.4±12.1 ^{bc}	61.6±5.9 ^{ab}	46.5±7.8 ^{a}		
13	Furfuryl alcohol	1653	365.2±15.6 ^{b}	220.5±17.4 ^{ab}	150.9±44.2 ^{a}	174.2±40.4 ^{ab}	162.6±34.8 ^{a}	15000 [5] *	Hay, Moldy [5]
14	Benzyl alcohol	1862	60.3±6.5 ^{b}	38.7±13.6 ^{ab}	46.3±19.7 ^{ab}	52.2±13.3 ^{b}	10.7±0.6 ^{a}	200000 [6] *	Almonds, Bitter [3]
15	2-phenylethanol	1896	14310.5±938.8 ^{b}	6169.9±145.4 ^{ab}	8300.3±2067.2 ^{ab}	3559.1±616.0 ^{a}	3363.6±1353.9 ^{a}	140 [4] ***	Flowery, Honey [4]
	Monoterpenic alcohols								
16	Linalool	1542	168.9±2.8 ^{ab}	122.8±14.1 ^{a}	198.2±28.6 ^{b}	126.0±21.3 ^{a}	128.1±22.3 ^{a}	25.2 [7] *	Aniseed, terpene [3] lemon [8]
17	α-terpineol	1684	100.7±3.4 ^{b}	53.4±13.3 ^{ab}	50.9±14.0 ^{ab}	43.7±4.0 ^{a}	16.9±1.4 ^{a}	250 [7] *	Pine, terpene [3]
18	β-citronellol	1759	31.0±1.8 ^{c}	26.4±4.4 ^{bc}	24.1±4.1 ^{bc}	7.6±1.1 ^{ab}	6.5±1.2 ^{a}	100 [1] **	Citronella [14]

nd – not detected

Table 6.1 (continuation): Identification of volatile compounds in beer samples, expressed as mean concentration of analysis duplicate (*C*), including standard deviations, along with the corresponding linear retention indexes (*LRI*) perception thresholds (*PT*) and reported descriptors. Statistically significant similarities are represented grouped by a, b and c letters. Deviations represent standard error of independent replicates (*n*=2 for ab1, ab2, and ub and *n*=3 for ab3 and ab4)

No.	Compound	*LRI*	$C_{\mathrm{ab1}}/(\mu g\ L^{-1})$	$C_{\mathrm{ab2}}/(\mu g\ L^{-1})$	$C_{\mathrm{ab3}}/(\mu g\ L^{-1})$	$C_{\mathrm{ab4}}/(\mu g\ L^{-1})$	$C_{\mathrm{ub}}/(\mu g\ L^{-1})$	$PT/(\mu g\ L^{-1})$	Descriptors
	Esters								
19	Ethyl butyrate	1033	99.2±19.0 ^{a}	122.3±36.5 ^{ab}	216.0±12.2 ^{b}	103.7±19.2 ^{a}	65. 9±3.9 ^{a}	20 [1, 2] **	Fruity [4] Papaya, Sweet, Butter, Apple [3]
20	Ethyl 2-methylbutyrate	1052	27.3±4.6 ^{ab}	79.3±15.9 ^{b}	11.5±3.5 ^{a}	30.1±23.5 ^{ab}	nd	18 [7] *	Fruity [8]
21	Ethyl 3-methylbutyrate	1070	30.0±6.3 ^{abc}	38.6±3.8 ^{c}	29.7±10.9 ^{bc}	7.9±6.8 ^{ab}	nd	6 [7] *	Fruity [8]
22	Isoamyl acetate	1119	132.5±14.2 ^{ab}	142.1±14.3 ^{ab}	489.2±272.8 ^{b}	78.8±9.9 ^{a}	1011.4±184.6 ^{b}	30 [1, 2] **	Banana, apple, solvent [3]
23	Ethyl pentanoate	1131	22.5±7.8 ^{b}	19.8±13.3 ^{b}	7.3±2.2 ^{ab}	nd	2.4±3.4 ^{ab}		
24	Ethyl hexanoate	1229	301.8±15.9 ^{ab}	411.2±247.4 ^{b}	344.3±86.5 ^{b}	235.9±111.4 ^{ab}	81.3±16.3 ^{a}	14 [7] *	Apple, Fruity, Aniseed, Sweet [3]
25	Ethyl lactate	1335	2539.0±297.3 ^{b}	999.7±127.4 ^{ab}	923.3±169.9 ^{ab}	902.4±122.4 ^{ab}	35.1±18.3 ^{a}	157; 810	Strawberry, raspberry, perfumed [9] [3]
26	Ethyl octanoate	1429	418.9±196.6 ^{ab}	433.0±151.6 ^{ab}	779.4±441.8 ^{b}	594.6±193.5 ^{b}	174.4±61.3 ^{a}	5 [7] *	Apple, Sweet, Fruity [3]
27	Ethyl 3-hydroxybutyrate	1509	29.0±0.0 ^{b}	13.5±0.3 ^{ab}	25.6±9.7 ^{b}	5.7±1.1 ^{a}	nd	20000 [6] *	
28	Ethyl decanoate	1632	131.0±45.1 ^{b}	52.0±13.2 ^{a}	89.2±42.1 ^{ab}	104.9±14.6^{ab}	72.2±10.4 ^{ab}	200 [7] *	Fatty acid, fruity, apple, solvent [3]
29	Diethyl succinate	1668	1338.3±177.1 ^{c}	685.9±167.4 ^{abc}	1023.4±141.4 ^{ab}	591.5±176.0 ^{ab}	7.3±1.8 ^{a}	100000 [2] **	
30	Ethyl phenylacetate	1774	66.1±0.9 ^{bc}	104.3±26.5 ^{c}	25.8±4.2 ^{abc}	19.7±7.5 ^{ab}	2.1±2.9 ^{a}		
31	2-phenylethyl acetate	1801	34.1±10.9 ^{a}	55.1±14.5 ^{ab}	306.7±151.8 ^{b}	40.6±13.3 ^{a}	156.4±23.6 ^{ab}	250 [1] **	Roses, honey, apple, sweet [3]
32	Diethyl malate	2032	367.8±16.3 ^{abc}	74.6±10.0 ^{ab}	518.7±94.9 ^{c}	468.2±200.9 ^{bc}	nd	760000 [2] **	
33	Monoethyl succinate	2448	550.5±3.2 ^{c}	206.1±75.0 ^{abc}	406.3±124.5 ^{bc}	120.2±33.7 ^{ab}	nd	1000000 [2] **	Sweat, sour, fruity [3]

nd – not detected

Table 6.1 (continuation): Identification of volatile compounds in beer samples, expressed as mean concentration of analysis duplicate (*C*), including standard deviations, along with the corresponding linear retention indexes (*LRI*) perception thresholds (*PT*) and reported descriptors. Statistically significant similarities are represented grouped by a, b and c letters. Deviations represent standard error of independent replicates (*n*=2 for ab1, ab2, and ub and *n*=3 for ab3 and ab4)

No.	Compound	*LRI*	C_{ab1}/(µg L^{-1})	C_{ab2}/(µg L^{-1})	C_{ab3}/(µg L^{-1})	C_{ab4}/(µg L^{-1})	C_{ub}/(µg L^{-1})	*PT*/(µg L^{-1})	Descriptors
	Volatile fatty acids								
34	Propanoic acid	1545	21.4±10.4 ^{b}	7.6±1.1 ^{ab}	10.8±4.7 ^{ab}	8.7±3.1 ^{ab}	5.3±0.9 ^{a}		
35	2-methylpropanoic acid	1574	66.8±12.3 ^{b}	60.3±10.5 ^{ab}	67.3±19.5 ^{b}	60.8±5.1 ^{ab}	28.6±6.5 ^{a}	2 300 [7] *	Sweaty, bitter, vinegar [3]
36	Butanoic acid	1634	28.2±7.3 ^{ab}	25.1±4.7 ^{ab}	41.9±12.9 ^{b}	23.8±11.2 ^{ab}	5.7±1.2 ^{a}	173 [7] *	Butter, cheesy, sweat [3]
37	Isovaleric acid	1675	204.4±23.2 ^{c}	169.2±5.7 ^{bc}	135.6±36.2 ^{abc}	127.3±17.2 ^{ab}	84.5±19.4 ^{a}	33.4 [7] *	Cheesy, sweaty, old hops [3]
38	Hexanoic acid	1851	799.0±51.9 ^{bc}	395.4±23.5 ^{abc}	774.8±163.9 ^{c}	392.3±114.8 ^{ab}	269.2±22.0 ^{a}	420 [7] *	Fatty acid, oily, sweaty [3]
39	2-methyl-2-Pentenoic acid	1984	118.1±42.7 ^{b}	102.6±27.8 ^{b}	70.3±12.7 ^{ab}	54.4±15.6 ^{a}	64.6±4.2 ^{ab}		
40	Octanoic acid	2065	2423.5±221.1 ^{b}	941.6±76.3 ^{a}	2122.5±281.5 ^{b}	1569.7±680.1 ^{ab}	2045.0±432.8 ^{ab}	500 [7] *	Fatty acid, oily, sweaty [3]
41	Decanoic acid	2279	1144.9±23.1 ^{b}	105.7±105.4 ^{a}	339.6±143.8 ^{ab}	379.4±211.4 ^{ab}	1148.2±335.4 ^{b}	10000 [7] *	Wax, tallow, rancid, soap [3]
42	Benzoic acid	2400	82.0±49.4 ^{b}	32.9±24.8 ^{ab}	29.2±27.4 ^{ab}	13.3±9.1 ^{ab}	nd		
43	Benzeneacetic acid	2657	243.5±45.1 ^{a}	135.4±66.4 ^{bc}	118.9±54.5 ^{bc}	55.5±26.6 ^{ab}	nd		
	Pyrazines								
44	2-methylpyrazine	1255	19.9±2.3 ^{b}	14.2±5.0 ^{b}	1.2±1.3 ^{a}	9.9±5.5 ^{ab}	4.6±1.0 ^{ab}		
45	2,3,5,6-tetramethylpyrazine	1460	4.8±1.2 ^{ab}	4.1±0.7 ^{ab}	9.8±1.5 ^{b}	2.5±0.5 ^{a}	3.6±0.8 ^{ab}		
	Lactones								
46	*cis*-oak lactone	1870	14.5±3.6 ^{c}	9.0±0.0 ^{abc}	10.9±1.8 ^{bc}	5.1±1.1 ^{ab}	nd		
47	*trans*-oak lactone	1938	8.4±3.1 ^{a}	12.8±0.7 ^{ab}	50.9±23.1 ^{b}	13.6±0.8 ^{ab}	nd		
48	γ-nonalactone	2009	190.0±22.3 ^{c}	70.0±13.3 ^{ab}	120.3±9.5 ^{bc}	82.1±10.7 ^{ab}	50.0±14.1 ^{a}	30 [10] *	
49	4-Ethoxycarbonyl-γ-butanolactone	2241	29.0±0.9 ^{b}	31.5±6.5 ^{b}	19.0±4.4 ^{ab}	9.2±1.7 ^{a}	1.7±1.9 ^{a}		
50	Furaneol	2020	218.1±88.1 ^{b}	293.2±14.5 ^{b}	72.8±24.1 ^{a}	108.7±13.4 ^{ab}	116.1±20.6 ^{ab}	37 [11] *	Caramel [4]

nd – not detected

Table 6.1 (continuation): Identification of volatile compounds in beer samples, expressed as mean concentration of analysis duplicate (*C*), including standard deviations, along with the corresponding linear retention indexes (*LRI*) perception thresholds (*PT*) and reported descriptors. Statistically significant similarities are represented grouped by a, b and c letters. Deviations represent standard error of independent replicates (*n*=2 for ab1, ab2, and ub and *n*=3 for ab3 and ab4)

No.	Compound	*LRI*	C_{ab1}/(μg L^{-1})	C_{ab2}/(μg L^{-1})	C_{ab3}/(μg L^{-1})	C_{ab4}/(μg L^{-1})	C_{ub}/(μg L^{-1})	*PT*/(μg L^{-1})	Descriptors
	Volatile phenols								
51	4-ethylguaiacol	2017	77.8±15.1 ^{ab}	1103.6±59.9 ^{b}	69.5±8.6 ^{ab}	108.9±82.1 ^{ab}	nd	47 [5] *	Smoky, Spicy [5]
52	4-ethylphenol	2165	93.2±13.1 ^{bc}	397.3±13.9 ^{c}	67.9±13.8 ^{abc}	48.4±32.0 ^{ab}	nd	440 [5] *	Phenolic, astringent [3] horse [5]
53	2,6-dimethoxyphenol	2249	16.8±0.8 ^{b}	8.0±4.8 ^{ab}	4.4±1.4 ^{a}	4.7±0.8 ^{a}	5.2±0.1 ^{ab}		
54	tyrosol	2989	108.4±4.6 ^{b}	28.0±12.9 ^{ab}	24.4±8.1 ^{ab}	11.2±4.8 ^{a}	9.6±0.9 ^{a}		
	Sulfur compounds								
55	methionol	1705	100.1±4.3 ^{a}	41.7±11.8 ^{a}	86.1±28.5 ^{a}	92.5±26.4 ^{a}	60.1±21.5 ^{a}		

[1] (Guth, 1997)
[2] (Moreno, Zea, Moyano, & Medina, 2005)
[3] (Meilgaard, 1975)
[4] (Czerny *et al.*, 2008)
[5] (Boidron, Chatonnet, & Pons, 1998)
[6] (Gómez-Míguez, Cacho, Ferreira, Vicario, & Heredia, 2007)
[7] (Ferreira, López, & Cacho, 2000)
[8] (Escudero *et al.*, 2004)
[9] (Siebert *et al.*, 2005)
[10] (Nakamura, Crowell, Ough, & Totsuka, 1988)
[11] (Kotseridis & Baumes, 2000)

* – threshold in model solution
** – threshold in hydroalcoholic solution
*** – threshold in water
nd – not detected

α-terpineol concentration was higher in ab1 sample, when compared with the remaining, which was the beer that had a longer residence time in the barrel. β-citronellol concentration was also higher in aged beer samples, when compared to the unaged beer, with the exception of ab4 sample. These monoterpenes can derive from nerol, linalool or geraniol by different reaction mechanisms (Briggs *et al.*, 2004; Takoi *et al.*, 2012) which can alter their concentration during residence in barrel.

Isoamyl acetate was the main ester found in unaged beer, which is a characteristic volatile compound produced by yeast during sugar metabolism (Priest & Stewart, 2006). In barrel aged beers a higher predominance of ethyl lactate and diethyl succinate was observed. The predominance of these esters is generally observed in wine. Ethyl esters can derive from reaction of organic acids with ethanol (Lin, Zeng, Yu, & Sun, 2012), or in some cases are products of non *S. cerevisiae* strains metabolism, as for example ethyl lactate which derives from malolactic fermentation in wine (Ribéreau-Gayon *et al.*, 2006). Moreover, these esters are reported as the main ones found adsorbed in wood, along with several other wine compounds (Coelho *et al.*, 2019). The appearance and higher concentration of these wine characteristic esters in the aged beer is believed to be linked with the extraction of compounds previously adsorbed in wood to beer due to barrel reuse. Monoethyl succinate and diethyl malate are other example of this mechanism. These esters were also reported adsorbed in wood (Coelho *et al.*, 2019) and were exclusively found in barrel aged beers along with ethyl 2-methylbutyrate, ethyl 3-methylbutyrate and ethyl 3-hydroxybutyrate. Other esters typically produced by yeast during alcoholic fermentation were also found such as ethyl hexanoate and phenylethyl acetate. Besides being extracted from wood, esters found in barrel aged beers can also derive from lees, which were also found to increase the concentration of these volatiles in wine during ageing (Liberatore *et al.*, 2010; Pérez-Serradilla & de Castro, 2008).

Regarding volatile acids, octanoic and decanoic acids were the main found in beer samples. C4, C6, C8 and C10 acids derive from fatty acids yeast metabolism during alcoholic fermentation and can be found either in wine or in beer (Briggs *et al.*, 2004). Only in barrel aged beers bezeneacetic and benzoic acids were found, being absent in control beer. Benzoic acid occurs in wines and is a precursor of phenolic compounds (Ribéreau-Gayon *et al.*, 2006) and its detection in barrel aged beer reinforces the enrichment with wine traits. Overall concentration of the remaining volatile fatty acids was higher in the barrel aged beers, when compared to unaged beer. As reported for wine, the contact with lees can lead to higher volatile fatty acids concentrations (Pérez-Serradilla & de Castro, 2008), justifying the differences observed. Another important group of compounds in regard to ageing in oak wood is lactones. Oak lactone was found in all barrel aged beers and null in the control beer, which demonstrates extraction of characteristic wood compounds from reused barrels. The ab3 samples presented the higher oak lactone content whereas the remaining barrel aged beers showed similar concentrations. This can be dependent either on residence time in barrel or more importantly from the barrel content in these extractives which are depleted during subsequent utilizations (Wilkinson *et al.*, 2013), leading to the differences observed. Volatile phenols, were also found in higher proportions in aged beer samples, when compared to the control beer. Phenolic compounds can be extracted from wood during ageing (Le Floch, Jourdes, & Teissedre, 2015) or derive from fermentation by the microflora in the barrel. For instance, 4-ethylguaiacol and 4-ethylphenol can be associated with the presence and growth of *Brettanomyces/ Dekkera sp.* strains. These are typically associated to wine spoilage and can endure in the barrel, being found penetrating up to 8 mm in the wood (Suárez, Suárez-Lepe, Morata, & Calderón, 2007). Lambic and sour beers are marked by the predominance of *Brettanomyces/ Dekkera sp.*, which are responsible for their characteristic sensory properties (Spitaels *et al.*, 2014). The concentration of these phenolic compounds was higher in ab2, where wort was fermented by the resident barrel microbiota. High substrate availability and low stress environment favored the growth of endogenous strains present in the barrel leading to higher *Brettanomyces/Dekkera* growth, detected by analysis using differential media, and consequently higher 4-ethylguaiacol and 4-ethylphenol concentrations. These volatile can also derive from *Saccharomyces sp.* metabolism (Ribéreau-Gayon *et al.*, 2006) or be extracted from cooperage wood (Fernández de Simón, Cadahía, del Álamo-Sanza, & Nevares, 2010), which can justify their appearance in the remaining samples at lower concentrations.

Tyrosol and 2,6-dimethoxyphenol were found at higher concentrations in sample ab1. Tyrosol is the fermentative product of tyrosine degradation via Ehrlich pathway (Briggs *et al.*, 2004), and its higher concentration in ab1 when compared with the other samples was not expected. Nevertheless, considering heterogeneity of the used barrels, lees in ab1 could contribute to this difference. 2,6-dimethoxyphenol is extracted from oak wood and the higher concentration of this compound can be related to the higher ageing period of sample ab1.

3.2 Volatile fingerprinting

Considering the high number of volatiles identified, the heterogeneity in their concentrations among the samples and the overall complexity of the determinations, a principal component analysis was performed in order to assess the distinguishing traits of the different beer samples.

PCA allowed the extraction of 5 principal components, explaining 84 % of the sum of squares. The loadings obtained in the PCA allowed the establishment of clusters relating samples and volatile compounds, highlighting the impact of specific volatiles on overall beer composition. Considering that approximately 72 % of the sum of squares in the PCA were explained by three of the five components extracted, these were focused for the cluster analysis. For a better interpretation of PCA data, scatterplots were outlined from the three principal components extracted, represented in Figure 6.3. Figure 6.4 shows a closer look on the volatiles standing closely in the cluster with each sample, taking into account the 3 extracted components.

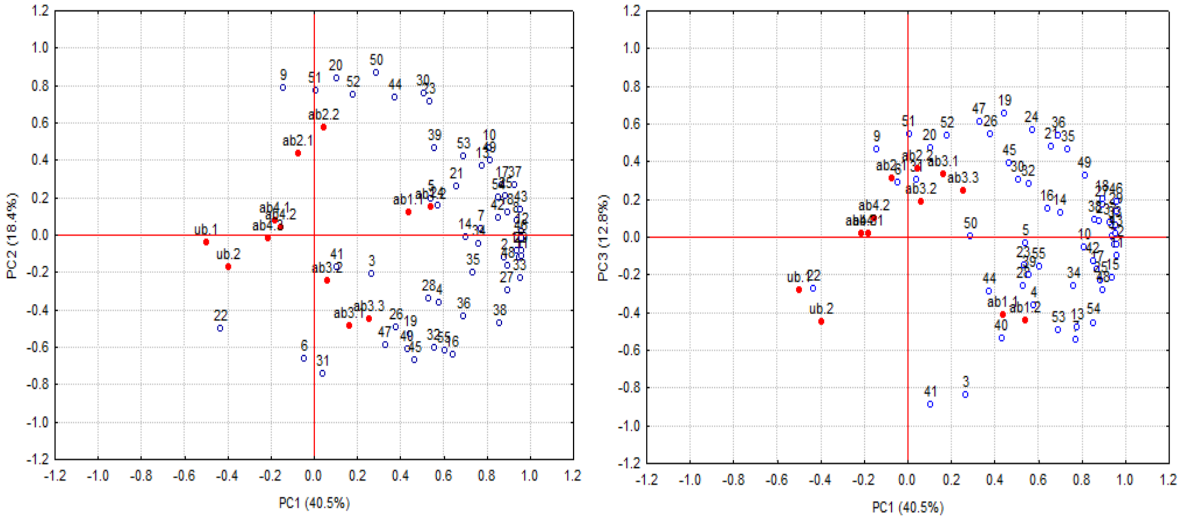

Figure 6.3: Scatterplots obtained from the three extracted components of the PCA analysis representing the loadings obtained for the samples and volatile compounds (represented by numbers in Table 6.1).

Beer samples were located in opposite quadrants of the scatterplots, demonstrating the existence of distinctive chemical compositions. Sample ab1 appeared closely with the most volatiles, mainly in the first component. This demonstrates the richer and more diverse volatile composition of ab1 beer and goes accordingly with the previously discussed for the volatile compounds. This higher richness can be a result of its longer residence time in the barrel, during which slow occurring extraction, oxidation or condensation reactions previously discussed occurred. Figure 6.4 a) demonstrates the closest volatiles to ab1 in the PCA, relatively to the 3 components. 4-methyl-1-pentanol, furfuryl alcohol, ethyl lactate, propanoic acid and tyrosol were found in the cluster of ab1 sample, and are characteristic traits of ab1 beer in comparison with the remaining. Represented in Figure 6.4 b), ab2 sample was located in another cluster of the scatterplot along with several volatiles, namely ethyl-2-methylbutyrate, 2-ethyl-1-hexanol, 4-ethylguaiacol and 4-ethylphenol. Among these, proximity of ab2 samples with 4-ethylphenol and 4-ethylguaiacol clearly illustrates the impact of barrel fermentation by resident barrel microbiota in beer composition, going accordingly with the previously discussed and reinforcing the validity of the volatile fingerprint outlined. Finally, as shown in Figure 6.4 c), ab3 samples were also in a separate cluster, comprising ethyl butyrate, ethyl octanoate, 2,3,5,6-tetramethylpyrazine, 2-phenylethyl acetate and oak lactone. Esters were the main volatiles associated with these samples, which contribute to a distinguished sweet and fruity profile of these samples. Also, oak lactone was more characteristic of ab3 samples, imparting typical oak related aroma to these beers.

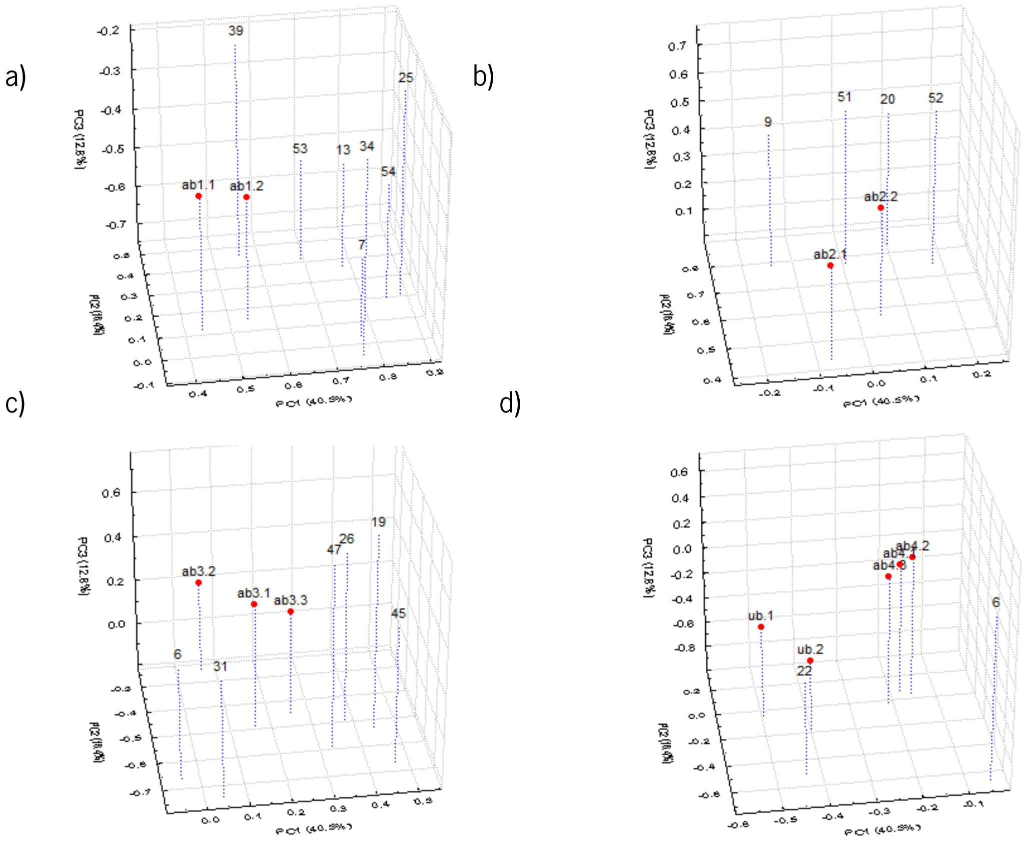

Figure 6.4: Detailed three-dimensional scatterplots focusing a) ab1, b) ab2, c) ab2 and d) ab4 and ub samples and the volatile compounds (represented by numbers in Table 6.1) in each cluster regarding the three extracted components.

On the opposite side, as seen on Figure 6.3 and Figure 6.4 d), ab4 and ub samples appeared in an isolate cluster in the scatterplot. These samples appeared on a cluster comprising the lowest number of volatiles, being mostly associated with isoamyl acetate and *Z*-2-penten-1-ol. Both samples were obtained from the same wort fermented with pitched *S. cerevisiae* differing only in the vessel on which they were fermented. The appearance of these samples in the same cluster goes accordingly with their similar production process and illustrates the similarity between both. Overall volatile fingerprinting allowed the identification of tendencies from a large complex dataset, which were coherent with the differences between the studied samples. Therefore, this multivariate analysis tool can aid brewers in controlling and understanding brewing processes, shedding a light on processes which are often empirical and difficult to analyze.

4. Conclusions

Characterization of the barrel aged beers allowed the determination of the chemical differences between the beer samples as well as the impact of barrel reuse on beer ageing. Such was seen for the absence or pitching of *S. cerevisiae*, where wort fermented in barrel by endogenous microflora related strongly with 4-ethylphenol and 4-ethylguaiacol in the volatile fingerprint (typical *Brettanomyces/Dekkera* metabolites), which was less noticeable for wort also fermented in cask but with yeast inoculation. Beer aged in barrel during the longer time period appeared in the cluster comprising the higher number of volatiles in the outlined fingerprint. Beers aged in cask during a lower time period were characterized by oak lactone, a typical wood extractible. Specific wine traits were found in the barrel aged beers and absent in the control beer, demonstrating for the first time the impact of barrel reutilization in aged beer production. Volatile fingerprinting manifestly demonstrated to be appropriate for the analysis and detection of differences in beer composition, which clearly reflected the alterations in the production processes, posing as a promising tool for application in brewing and ageing processes' monitoring.

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Chapter 7. Evaluation of multi-starter *S. cerevisiae/ D. bruxellensis* cultures for mimicking and accelerating transformations occurring during barrel ageing of beer

Abstract

During beer ageing, endogenous barrel microbes grow spontaneously and transform wort/beer composition, being *Dekkera bruxellensis* and *Saccharomyces cerevisiae* among the main contributors to the chemical and sensory profile of aged beer. This work aims at the application of multi-starter cultures to mimic and accelerate biological modifications occurring during barrel ageing of beer, in controlled fermentation processes. Co-cultures of *D. bruxellensis/S. cerevisiae* were conducted under conditions commonly found in barrel aged beer production: different pitching rates, high glucose concentration and presence of ethanol and wood extracts. Selective pressures and competition between yeasts influenced microbial growth and metabolite production, namely ethanol, acetic acid and target volatile compounds (esters, alcohols, terpenols, volatile acids and volatile phenols). Metabolic profiles of co-cultures combined traits of both species, and differed from those of pure cultures. Lastly, multi-starters were successfully applied in combination with wood in a controlled and accelerated fermentation process for mimicking barrel ageing transformations.

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1. Introduction

With the increasing innovation and interest on craft and specialty beers, there is the constant search for alternative processes, microorganisms and raw materials, leading to an ever increasing multitude of alternative products. One example of specialty products are barrel aged beers, produced either by fermentation of wort in wood barrels or by storage of previously fermented beer in wood casks. During contact with wood several phenomena occur: wood compounds are extracted by the hydroalcoholic matrix (additive ageing), beverage compounds are adsorbed by wood (subtractive ageing), oxidative and polymerization reactions (chemical ageing) and, more importantly, a secondary fermentation takes part (biological ageing), transforming chemical and sensorial characteristics of the product (Coelho *et al.*, 2019). In most barrel aged beers, this secondary fermentation is the result of the spontaneous growth of microbial population, mostly endogenous to the barrel or populating the brewery atmosphere (Spitaels *et al.*, 2014). Previous works have identified the more prominent microorganisms in the biological ageing of some beers, highlighting bacteria such as Enterobacteriaceae sp.*, Lactobacillus* sp. and *Pediococcus* sp. as well as yeasts such as *Saccharomyces* sp.*, Debaryomyces* sp. and *Dekkera* sp. (Snauwaert *et al.*, 2016; Spitaels *et al.*, 2014). Among these, *Dekkera* sp. is considered the main contributor to aroma characteristics of barrel aged products (Colomer, Funch, & Forster, 2019). Despite being commonly regarded as a spoilage microorganism in wine, this yeast is gaining increasing importance in the brewing and bioethanol industries due to its unique metabolic features, such as the bioconversion of hydroxycinnamic acids to volatile phenols, the Custer effect, and characteristic esterase and β-glucosidase activity (Kuo *et al.*, 2018; Schifferdecker, Dashko, Ishchuk, & Piškur, 2014; Smith & Divol, 2016; Steensels *et al.*, 2015).

Traditional barrel ageing has some disadvantages. It is usually a costly process, consuming time and space, and commonly poorly controlled, often with unpredictable end products. Several recent works have been performed to develop fermentative processes aiming to replicate biological transformations associated with barrel ageing, in a more controlled and efficient manner. Most of them have explored the application of alternative non-*Saccharomyces* species for the souring and flavoring of beer by sequential fermentation, being the end products profoundly affected by yeast metabolomics (Holt, Mukherjee, Lievens, Verstrepen, & Thevelein, 2018; Osburn *et al.*, 2018). Another alternative, acknowledged in wine production, is the simultaneous culture of one or more species within the same matrix, by the addition of a multi-starter culture. This can be advantageous, as the interaction between both species can lead to distinct metabolomic profiles, contrasting with those of pure cultures (Ciani & Comitini, 2015; Comitini *et al.*, 2011). However, despite having been hypothesized and proposed in several recent works (Holt *et* *al.*, 2018; Steensels *et al.*, 2015), the possibility of using multi-starter cultures, involving *Dekkera* sp. and *Saccharomyces* sp., for biological ageing of beer still remains to be tested. This hypothesis is studied in this work by evaluating the growth and metabolomics of *S. cerevisiae* and *D. bruxellensis* co-cultures under selective pressures commonly found in barrel aged beer production. The application of *D. bruxellensis*/*S. cerevisiae* multi-starter cultures for the fermentation of beer in combination with the application of wood, is proposed in this work as an accelerated and controlled approach for mimicking and furthermore controlling the transformations typically occurring in barrel ageing.

2. Materials and methods

2.1 Chemicals, materials and strains

Calibration of GC-MS was performed resorting to the following pure standards. From Aldrich: ethyl butyrate (99 %), isoamyl acetate (≥99 %), ethyl hexanoate (≥99 %), ethyl lactate (98 %), ethyl octanoate (≥99 %), ethyl decanoate (≥99 %), diethyl succinate (99 %), ethyl hydrocinnamate (99 %), diethyl malate (≥97 %), linalool (97 %), β-citronellol (95 %), geraniol (98 %), isovaleric acid (99 %), octanoic acid (≥99.5 %), 5-methylfurfural (99 %), *cis*-oak lactone (≥98.0 %), *trans*-oak lactone (≥98.0 %), 4-methylguaiacol (≥98.0 %), eugenol (99 %), 2,6-dimetoxyphenol (99 %), 4-ethylphenol (99 %), syringaldehyde (98 %), tyrosol (98 %) and methionol (98 %). From Fluka: isobutyl acetate (99 %), ethyl phenylacetate (≥99 %), 2-phenylethyl acetate (≥99 %), 2-methyl-1-propanol (≥99.9 %), 2-methyl-1-butanol (≥98 %), 3-methyl-1-butanol (≥99.8 %), furfuryl alcohol (98 %), 2-phenylethanol (≥99 %), propanoic acid (≥99.5 %), isobutyric acid (≥99.5 %), butanoic acid (≥99.5 %), hexanoic acid (≥98.0 %), decanoic acid (≥98.0 %), phenylacetic acid (99 %), furfural (99 %), vanillin (≥98.0 %) and acetoin (97 %). From Alfa Aesar: 4-ethylguaiacol (98 %). Oak wood used in this work (kindly provided by Seguin Moreau) was submerged in fortified wine used in Port wine production (kindly provided by Quinta do Portal S.A.), until total sorption was reached, with sorption conditions and wood and wine composition as described previously (Coelho, Domingues, Teixeira, Oliveira, & Tavares, 2019). For the pure and co-culture fermentations *Saccharomyces cerevisiae* Belgian Abbey II (strain 1762, Wyeast Laboratories Inc.) was used. *Dekkera bruxellensis* used in this work was directly isolated from barrel fermentation of beer wort, using a modified DBDM medium (6.2 g L^{-1} yeast extract, 48 g L^{-1} ethanol, 0.1 g L^{-1} *p*-coumaric acid, 0.01 g L^{-1} cycloheximide, 0.022 g L^{-1} bromocresol green, 0.1 g L^{-1} chloramphenicol, 0.1 g L^{-1} tetracycline, 20 g L^{-1} agar, pH 5.4), based on the previously described (Rodrigues, Gonçalves, Pereira-da-Silva, Malfeito-Ferreira, & Loureiro, 2001). Beer samples were aseptically collected from barrels in a local craft beer producer (Fermentum Lda.) and plated in the modified DBDM medium. Colonies of *D. bruxellensis* were preliminarily detected and identified by a green halo, isolated and confirmed by sequencing of the 18S ITS rDNA region, presenting 97 % homology to *Dekkera bruxellensis*. The yeast was stored at -80 °C in a cryovial with glycerol for permanent stock and at -20 °C for working stocks to be used in the fermentation assays.

2.2 Inoculum preparation

For the fermentation assays, inocula of *S. cerevisiae* and *D. bruxellensis* were grown from stocks of each species stored in glycerol at -20 °C. For this purpose, shake flasks with MYPG medium (3 g L^{-1} malt extract, 3 g L^{-1} yeast extract, 10 g L^{-1} glucose and 2 g L^{-1} peptone) were pitched separately with each yeast and incubated at 28 °C with orbital agitation, at a rotational speed of 150 min^{-1}, until reaching stationary phase, as determined by optical density measurement at 600 nm.

2.3 Fermentation assays

Different conditions were tested in the study of the co-culture growth and metabolomics by using different culture media. All fermentations were carried out in Erlenmeyer shake flasks fitted with cotton stoppers, incubated at 28 °C with orbital agitation, at a rotational speed of 150 min^{-1}. Fermentations were started by pitching the studied media with the desired cell concentration, attained by previous cell count in the inoculum using a Neubauer chamber and pitching at the desired dilution. Periodic sampling in aseptic conditions assessed the profiling of cell growth and metabolite production. For an easier interpretation of the inoculum concentration used, each condition was labeled as *x*S*y*D, where *x*S stands for *S. cerevisiae* cellular concentration of $x \times 10^6$ mL^{-1} and *y*D stands for *D. bruxellensis* cellular concentration of $y \times 10^6$ mL^{-1} in the starter inoculum.

2.3.1 Impact of pitching rate

Co-culture fermentations were conducted in MYPG medium using different initial cell concentrations of *D. bruxellensis* and *S. cerevisiae*. Pitching rates were tested for both yeasts in co-culture, by increasing initial cell concentrations (2×10^6 mL^{-1}, 5×10^6 mL^{-1} and 10×10^6 mL^{-1}) of one yeast while maintaining constant the initial cell concentration of the other yeast (1×10^6 mL^{-1}). Cell growth was accompanied for each yeast in co-culture by flow cytometry and production of primary metabolites accompanied by High Performance Liquid Chromatography (HPLC).

2.3.2 Increased sugar concentration

A synthetic medium was used for mimicking wort fermentations with increased concentrations of MYPG components (10 g L^{-1} yeast extract, 10 g L^{-1} malt extract, 8 g L^{-1} peptone and 140 g L^{-1} glucose).

2.3.3 *Impact of ethanol and wood extract concentration*

For the evaluation of the impact of ethanol and wood extracts, MYPG medium was supplemented with a hydroalcoholic oak wood extract. Hydroalcoholic wood extract was obtained by extraction of used oak wood content by contact of grounded used wood with a hydroalcoholic solution of 55 % ethanol, by volume, at 20 °C, during 48 h. The liquid fraction of the extract was sterilized by filtration at 0.22 µm and diluted in MYPG medium to final ethanol concentrations, by volume, of 6 % (WE6%), 8 % (WE8%) and 10 % (WE10%).

2.3.4 *Application to beer ageing*

Lastly, co-culture fermentations were carried out using real beer matrices. For mimicking barrel aged beer, a commercial lager beer was put in contact with the used oak wood at different temperatures, namely 30 °C (WB30) and 50 °C (WB50) during 48 h following the reported in previous works (Coelho, Teixeira, Domingues, Tavares, & Oliveira, 2019). Aged beer was sterilized by filtration with 0.22 µm sterile filters, and pitched with each species at the desired rate.

2.4 Determination of culture growth

Growth of each species in the co-culture was evaluated by flow cytometry using an EC800 flow cytometer (Sony Biotechnology). Gates defining *S. cerevisiae* and *D. bruxellensis* events were previously determined by analyzing pure cultures of each yeast, combining the FS, SS and FL1 parameters, indicators of size, morphology and autofluorescence of each species. Sterile PBS was used as carrier liquid with a 40 µL min^{-1} flow. Analysis and quantification were performed using the equipment software.

2.5 Analysis of primary metabolites

Primary metabolites (glucose, ethanol and acetic acid) were analyzed by a high performance liquid chromatography (HPLC) system composed by a Jasco PU-1580 pump, a Jasco AS-2057 autosampler, a Jasco RI-2031 Plus RI detector and a Knauer K-2501 UV detector at the wavelength of 210 nm. Each sample of 20 µL was injected in an Aminex HPX-87H (BioRad) column, maintained at 60 °C, using 5 mmol L^{-1} H_2SO_4 as mobile phase. Chromatograms were analyzed using the Star-Chromatography Workstation software (version 6.9.3, Varian), and compounds were quantified against calibration curves prepared from pure standards.

2.6 Analysis of secondary metabolites

Volatile compounds in the hydroalcoholic extracts were analyzed by gas chromatography coupled with mass spectrometry (GC–MS), following the procedure proposed by Oliveira and collaborators (Coelho *et al.*, 2020). Each 8 mL sample was extracted with 400 µL of dichloromethane (SupraSolv for gas chromatography, Merck), after adding 4-nonanol as internal standard (3.2 µg). Extractions were performed in Pyrex tubes fitted with Teflon caps, with agitation promoted by a stir bar during 15 min. Extracts were then recovered with a glass Pasteur pipette, dehydrated with anhydrous sodium sulfate and analyzed in a Varian 3800 gas chromatograph equipped with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000. Each 1 µL injection was made in splitless mode (30 s) in a Sapiens-Wax MS column (30 m × 0.15 mm; 0.15 µm film thickness, Teknokroma). Carrier gas was helium 49 (Praxair) at a constant flow of 1.3 mL min^{-1}. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range (*m/z*) from 35 to 260 and a 610 ms acquisition interval. The oven temperature was initially set to 60 °C for 2 min and then raised to 234 °C at a rate of 3 °C min^{-1}, raised again to 260 °C at 5 °C min^{-1} and finally maintained at 260 °C for 10 min. Injector temperature was set to 250 °C with a 30 mL min^{-1} split flow and transfer line was maintained at 250 °C. Compounds were identified using MS Workstation version 6.9 (Varian) software, by comparing mass spectra and retention indices with those of pure standards and quantified as 4-nonanol equivalents.

2.7 Statistical analysis

Statistically significant differences in concentrations of volatile compounds were evaluated by a non-parametric Kruskall-Wallis analysis, with Conover-Iman multiple pairwise comparisons, using the XLStat software (Addinsoft). For evaluation of correlations between samples and volatile compounds a multivariate approach was performed by Principal Component Analysis (PCA) using Statistica software (version 7, StatSoft Inc.)

3. Results and discussion

3.1 Effect of pitching rate

A preliminary assay evaluating the impact of pitching concentration on the growth of each yeast in co-culture was performed. Synthetic MYPG medium was pitched with different concentrations of *D. bruxellensis* and *S. cerevisiae* for the evaluation of growth and metabolite production, with results presented in Figure 7.1. As seen, the favoring of each species by means of higher inoculum led to its more prominent growth in the co-culture. A difference in cellular concentration of only 1×10^{6} mL^{-1} higher in the pitching rate was sufficient for one yeast to be dominant over the other. This is noticeable in Figure 7.1a), where *D. bruxellensis* growth is clearly dominant over *S. cerevisiae* for all of the favoring inoculum concentrations of *D. bruxellensis.* Despite contributing for a dominant growth, increasing cellular pitching concentration above 2×10^{6} mL^{-1}, did not contribute to a significant increase of *D. bruxellensis* growth rate or maximum growth.

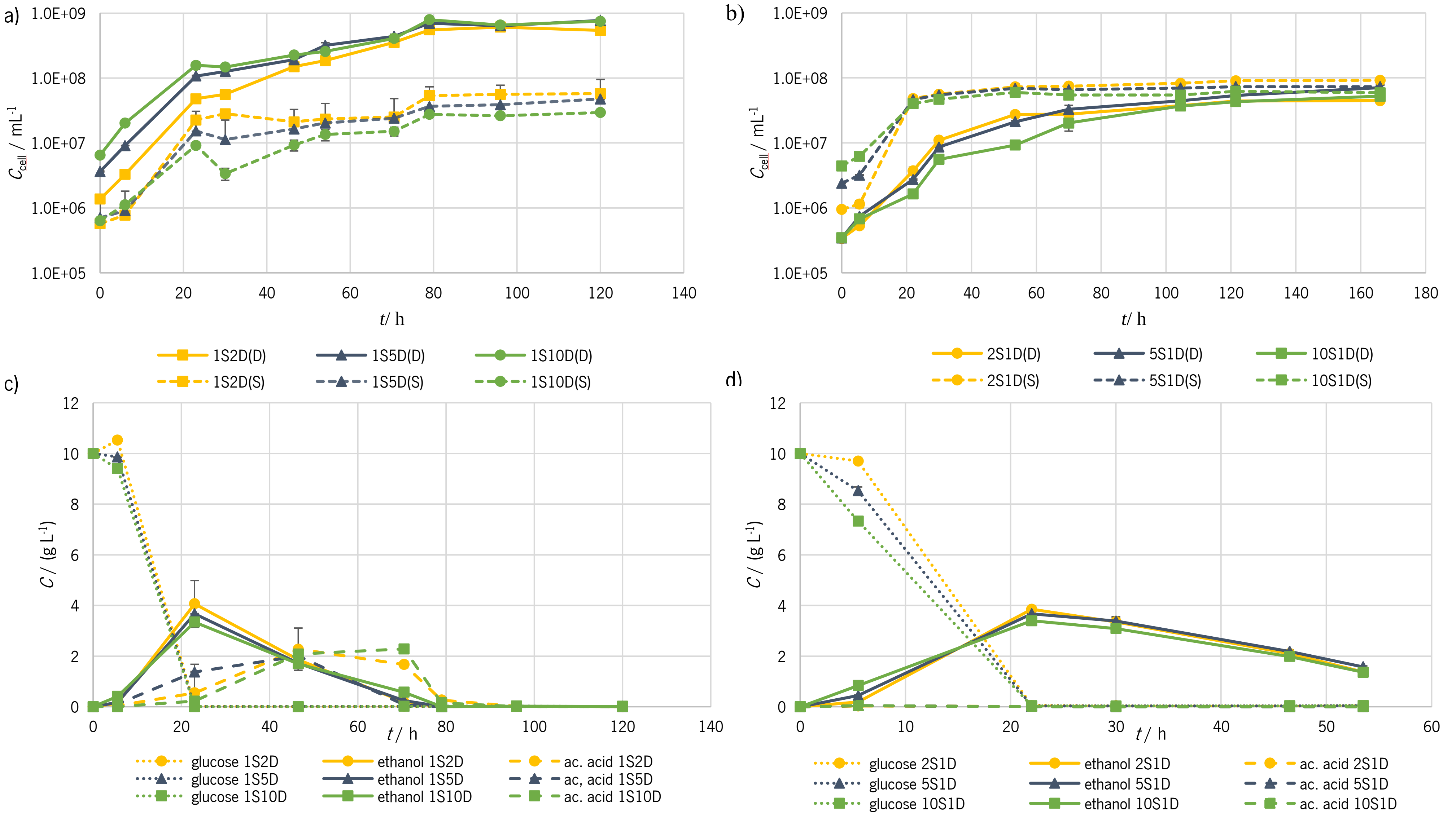

Figure 7.1: Impact of initial inoculum concentration on the growth of *D. bruxellensis* (D) and *S. cerevisiae* (S) in co-cultures varying pitching rates. Variation of cellular concentration (C_{cell}) and primary metabolite concentrations (C) throughout time (t) in fermentations with inoculum favoring *D. bruxellensis* are presented in a) and c) respectively, whereas fermentations with inoculum favoring *S. cerevisiae* are presented in b) and d) respectively. xSyD stands for *S. cerevisiae* cellular concentration of $x\times10^6$ mL^{-1} and yD stands for *D. bruxellensis* cellular concentration of $y\times10^6$ mL^{-1} in the starter inoculum. Error bars depict standard error of independent duplicates.

However, the increasing concentrations of *D. bruxellensis* in the inoculum led to a proportionally lesser growth of *S. cerevisiae* in the co-culture. On the other hand, this behavior may also be seen when favoring *S. cerevisiae* in the inoculum, as visible in Figure 7.1b). In this case, a higher pitching rate of *S. cerevisiae* also led to a dominance in its growth and an inhibition of *D. bruxellensis*. Increasing *S. cerevisiae* initial cellular concentration in the inoculum over 2×10^6 mL^{-1} also did not further favor *S. cerevisiae* growth, but on this hand no proportionality in *D. bruxellensis* growth inhibition could be observed. This phenomenon of growth dominance of one species over the other also impacts the production of primary metabolites in the co-culture. As visible in Figure 7.1c) the predominance of *D. bruxellensis* led to a higher production of its typical metabolites, particularly acetic acid. In fact, acetic acid production was only observed in the co-cultures favoring *D. bruxellensis* growth, being absent in the co-cultures promoting *S. cerevisiae,* as visible in Figure 7.1d). Maximum ethanol concentration was similar in both scenarios, reaching values of about 4 g L^{-1} either favoring *D. bruxellensis* or *S. cerevisiae* in the inoculum. In both conditions, when sugar depletion was complete, ethanol concentration began to decrease significantly, reaching residual values even in the *D. bruxellensis* favoring condition. Both yeast can metabolize ethanol as carbon source as part of a make-accumulate-consume mechanism, natural for both species (Rozpedowska *et al.*, 2011). However, cell growth accompanying the depletion of ethanol was more prominent in the case of *D. bruxellensis*, which is better adapted to grow under this condition (Smith & Divol, 2016). In contrast, on the *S. cerevisiae* favoring condition, no acetic acid production was observed, despite the occurrence of *D. bruxellensis* growth and the decrease in ethanol concentration. This absence of acetic acid production can be firstly due to the rapid depletion of glucose by the outcompeting *S. cerevisiae*, leaving no substrate available to *D. bruxellensis* other than ethanol. On the other hand, there is the rapid decrease of available oxygen in the fermentation medium and an anaerobic condition being imposed by the CO_2 produced by *S. cerevisiae*. As described, anaerobic conditions hinder acetic acid production by *D. bruxellensis* (Smith & Divol, 2018). This absence in the production of acetic acid is of particular interest when envisaging beverage production, which can be targeted or not depending on the intended product. Therefore, the presence or absence of *S. cerevisiae* in the starter culture can be a tool to manage acetic acid concentrations in the end product.

3.2 Fermentation with high glucose concentration

In order to evaluate the production of secondary metabolites and co-culture performance with alternative conditions, a fermentation with high glucose concentration (140 g L^{-1}) was performed. Based on the previous results, two different inoculum concentrations were chosen, one favoring *D. bruxellensis* and another favoring *S. cerevisiae*, accompanied by controls of each species grown in pure cultures. Cell growth and primary metabolite production profiles are presented in Figure 7.2.

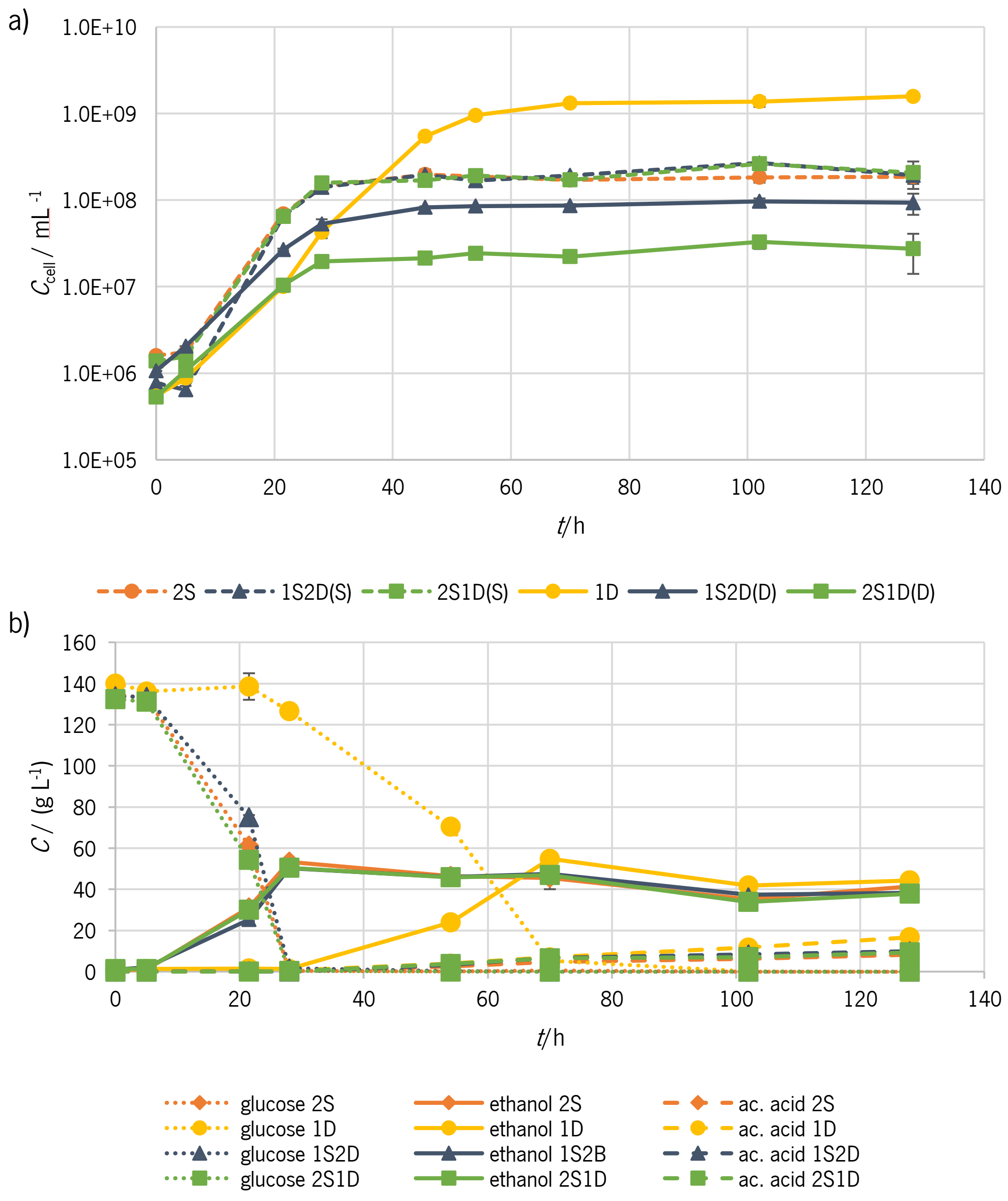

Figure 7.2: Fermentation profiles depicting a) Cellular concentration (C_{cell}) of *S. cerevisiae* (S) and *D. bruxellensis* (D) and b) primary metabolite concentrations (C) throughout time (t), in fermentations using pure cultures (2S and 2D) and co-cultures favoring *S. cerevisiae* (2S1D) or *D. bruxellensis* (1S2D) in the multi-starter. *x*S*y*D stands for *S. cerevisiae* cellular concentration of $x \times 10^6$ mL^{-1} and *y*D stands for *D. bruxellensis* cellular concentration of $y \times 10^6$ mL^{-1} in the starter inoculum. Error bars depict standard error of independent duplicates.

In contrast with the previous results in synthetic medium with low sugar concentration (10 g L^{-1}), a clear dominance of *S. cerevisiae* has been observed for all the conditions in the co-cultures, as visible in Figure 7.2a). The well acknowledged adaptation of *S. cerevisiae* to high sugar concentrations and its higher growth rates make it a dominant species over *D. bruxellensis* when such condition is imposed (Abbott, Hynes, & Ingledew, 2005; Blomqvist, 2011). Aguilar-Uscanga and collaborators have previously demonstrated the inhibitory effect of this selective pressure on *D. bruxellensis* performance, reporting decreases in growth rate, substrate consumption and metabolite production with increasing glucose concentrations, being 165 g L^{-1} the borderline from which these losses become more significant (Aguilar-Uscanga *et al.*, 2011). Therefore, even with inoculum favoring *D. bruxellensis*, *S. cerevisiae* was dominant in both co-cultures contrasting with the previous results. In fact, *D. bruxellensis* is only able to outcompete *S. cerevisiae* when glucose concentrations in the medium are low, due to different efficiencies of glucose transporters with dissimilar affinities (Blomqvist, 2011), justifying the distinct behaviors observed for low and high sugar concentrations. Despite the superiority of *S. cerevisiae*, *D. bruxellensis* was still able to grow in both co-cultures, but at lower levels when compared to the pure culture, due to the rapid depletion of substrate. Even with the dominance of *S. cerevisiae*, *D. bruxellensis* still produced considerable amounts of acetic acid, as visible in Figure 7.2b), being at lower concentrations in the co-cultures when compared with the pure culture. Again, maximum ethanol production was similar for *S. cerevisiae* and *D. bruxellensis* pure cultures, but it was reached at different times due to the differences in the specific growth and production rates of the two species.

Digging deeper into species metabolomics, in either pure or co-culture conditions, secondary metabolite production was characterized, and is presented in Table 7.1. Also, to better describe the correlations in production of secondary metabolites, a PCA was performed and a scatterplot was outlined, which is presented in Figure 7.3. Production of metabolites was found to be highly species dependent, either in terms of the compounds formed or their levels of production. For instance, isobutyl acetate was only found in cultures with *D. bruxellensis*. Production of this ester was even higher in co-cultures of *D. bruxellensis* and *S. cerevisiae*, when compared with pure culture of *D. bruxellensis*, contrasting with the lower growth and primary metabolite production observed. Moreover, increasing *D. bruxellensis* concentration in the co-culture inoculum led to an increase in isobutyl acetate production when compared with the co-culture using lower concentrations of *D. bruxellensis*. This may indicate a synergy between the two yeasts in the production of this ester. Tashiro and collaborators have proposed a two-way pathway for the production of isobutyl acetate involving glucose with 2-methyl-1-propanol and/or acetate as intermediaries (Tashiro, Desai, & Atsumi, 2015). Taking into account that *S. cerevisiae* produced high concentrations of 2-methyl-1-propanol, which *D. bruxellensis* produces at lower levels, and that *D. bruxellensis* produces high concentrations of acetate, the synergy between these two yeasts can be the driver of the higher isobutyl acetate production observed, with one species producing a substrate used by the other. Ethyl hexanoate and ethyl octanoate also showed higher concentrations in the co-culture than in pure cultures of each yeast. These esters can be formed by reaction of an alcohol and a carboxylic acid group, either driven by enzymatic activity or from chemical reaction at low pH (Sumby, Grbin, & Jiranek, 2010). One of the features of *D. bruxellensis*, which highly imparts beverage composition, is its characteristic esterase activity that can lead to both hydrolysis and synthesis of esters. Therefore, *D. bruxellensis* can be responsible for esterification of middle and long chain fatty acids with high specificity towards the formation of ethyl esters (Colomer *et al.*, 2019; Spaepen & Verachtert, 1982). On the other hand, these esters can derive from chemical reaction of the corresponding fatty acids with ethanol. In fact, concentration of several volatile fatty acids was higher in *D. bruxellensis* pure cultures when compared with *S. cerevisiae.* Synthesis of fatty acids is acknowledged as another typical *D. bruxellensis* trait (Steensels *et al.*, 2015). These compounds are generally a product of sugar and aminoacid catabolism, via the Ehrlich pathway (Hazelwood, Daran, Van Maris, Pronk, & Dickinson, 2008). In contrast with *S. cerevisiae*, *D. bruxellensis* typically diverts aminoacid conversion towards production of fusel acids instead of producing fusel alcohols, due its increased alcohol and aldehyde dehydrogenase activity (Piškur *et al.*, 2012). As expected, *D. bruxellensis* fermentation in pure culture produced high concentrations of volatile fatty acids. However, production of fatty acids in co-culture fermentations occurred at levels close to those seen in the *S. cerevisiae* pure cultures and significantly lower to those observed for *D. bruxellensis* pure cultures. This can be a direct consequence of the competitive advantage of *S. cerevisiae* for sugar and aminoacid metabolism. As *S. cerevisiae* has an advantage, conversion of sugars and aminoacids via the Ehrlich pathway will be more directed towards the production of fusel alcohols instead of fusel acids (Hazelwood *et al.*, 2008; Yu, Pratomo Juwono, Foo, Leong, & Chang, 2016). In agreement, concentrations of alcohols were higher in *S. cerevisiae* pure culture, when compared with *D. bruxellensis*, attaining similar concentration values in the co-culture fermentations. This is a clear advantage of co-fermentation of *D. bruxellensis* with *S. cerevisiae*, considering that fatty acids impart beverages with unwanted sensory features and are commonly regarded as spoilage compounds. Typical volatile phenols, 4-ethylphenol and 4-ethylguaiacol were exclusively found associated with *D. bruxellensis* growth, widely acknowledged for this yeast (Suárez, Suárez-Lepe, Morata, & Calderón, 2007). Co-culture of *D. bruxellensis* with *S. cerevisiae* led to a decrease in ethylphenol production, again due to the hindered *D. bruxellensis* growth by competition with *S. cerevisiae* for the substrate. Despite being considered spoilage compounds in wine, volatile phenols produced by *D. bruxellensis* are a desirable trait in barrel aged beers being characteristic in the aroma of such products. Also advantageous to beer aroma, monoterpenic alcohols were produced in higher concentrations by *D. bruxellensis* when compared with *S. cerevisiae.*

Table 7.1: Characterization of secondary metabolites in the fermentations with *D. bruxellensis*/*S. cerevisiae* multi starters in: synthetic medium with high glucose concentration (140 g L^{-1}), synthetic medium with addition of hydroalcoholic wood extract (WE) for final ethanol concentrations by volume of 6 %, 8 % and 10 %, and accelerated aged beer previously put in contact with oak wood at 30 °C (WB30) and 50 °C (WB50). *x*S*y*D stands for *S. cerevisiae* cellular concentration of $x\times10^6$ mL^{-1} and *y*D stands for *D. bruxellensis* cellular concentration of $y\times10^6$ mL^{-1} in the starter inoculum. Statistically significant similarities are represented by a to k letters. Deviations represent standard error of independent duplicates.

		High glucose concentration				**Addition of alcoholic wood extract**			**Application to beer aged in wood**							
	Compound	**2S**	**1D**	**2S1D**	**1S2D**	**WE6% 1S2D**	**WE8% 1S2D**	**WE10% 1S2D**	**WB30**	**WB30 1S**	**WB30 2D**	**WB30 1S2D**	**WB50**	**WB50 1S**	**WB50 2D**	**WB50 1S2D**
No.	**Esters**	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})	(μg L^{-1})
1	isobutyl acetate	-	44.1 ± 8.4^{de}	77.4 ± 30.5^{de}	103.3 ± 2.5^{e}	19.4 ± 6.2^{cd}	9.3 ± 3.5^{bc}	-	-	-	5.2 ± 1.0^{ab}	8.7 ± 0.6^{bc}	-	-	3.9 ± 0.7^{a}	9.5 ± 1.2^{bc}
2	ethyl butyrate	21.8 ± 4.4^{a}	-	20.9 ± 1.2^{a}	17.7 ± 3.2^{a}	-	-	-	-	-	-	-	-	-	-	-
3	isoamyl acetate	14.3 ± 1.6^{bc}	425.3 ± 49.8^{f}	160.0 ± 73.3^{ef}	242.1 ± 52.5^{ef}	64.2 ± 25.7^{de}	23.5 ± 11.9^{c}	-	-	-	14.8 ± 2.5^{bc}	17.1 ± 1.4^{cd}	-	-	10.0 ± 0.6^{ab}	9.3 ± 0.2^{a}
4	ethyl hexanoate	14.2 ± 2.7^{de}	4.4 ± 0.3^{ab}	29.6 ± 4.9^{e}	27.6 ± 2.3^{e}	4.7 ± 0.7^{ab}	6.8 ± 0.2^{cd}	-	-	-	9.0 ± 0.5^{de}	5.3 ± 1.0^{bc}	-	-	3.6 ± 0.4^{a}	4.4 ± 1.1^{ab}
5	ethyl lactate	6.0 ± 1.0^{a}	7.6 ± 1.9^{ab}	8.5 ± 0.5^{abc}	7.2 ± 1.2^{a}	5.5 ± 1.0^{a}	8.9 ± 4.3^{a}	17.2 ± 2.4^{cde}	22.2 ± 0.6^{de}	16.8 ± 1.7^{abcd}	22.1 ± 3.8^{de}	16.6 ± 1.0^{bcd}	20.9 ± 2.3^{de}	22.5 ± 0.7^{e}	18.7 ± 2.6^{de}	17.5 ± 2.9^{cde}
6	ethyl octanoate	24.3 ± 3.3^{a}	242.4 ± 36.2^{bc}	313.2 ± 59.4^{c}	313.4 ± 48.1^{c}	226.5 ± 37.7^{abc}	182.3 ± 2.7^{ab}	-	-	-	-	-	-	-	-	-
7	ehtyl decanoate	2.8 ± 0.3^{a}	38.4 ± 4.3^{de}	12.6 ± 2.2^{c}	17.1 ± 3.9^{cd}	103.4 ± 9.0^{e}	66.5 ± 32.0^{e}	-	-	-	8.4 ± 0.7^{ab}	13.6 ± 0.1^{cd}	-	-	8.9 ± 1.3^{ab}	13.2 ± 0.4^{bc}
8	diethyl succinate	-	-	-	-	75.3 ± 13.4^{de}	73.7 ± 33.9^{ef}	152.0 ± 19.9^{f}	50.2 ± 1.0^{de}	51.5 ± 1.0^{def}	45.6 ± 2.4^{cd}	43.1 ± 1.0^{bcd}	17.2 ± 0.3^{a}	16.4 ± 0.3^{a}	18.8 ± 1.4^{ab}	18.6 ± 0.5^{abc}
9	ethyl phenylacetate	7.1 ± 1.3^{abc}	7.8 ± 1.6^{c}	9.3 ± 0.1^{cd}	7.2 ± 0.6^{bc}	4.4 ± 1.5^{ab}	2.8 ± 0.9^{a}	-	-	22.2 ± 4.2^{d}	-	-	-	21.5 ± 1.0^{d}	-	-
10	2-phenylethyl acetate	419.1 ± 95.8^{g}	332.7 ± 30.2^{g}	275.0 ± 12.5^{fg}	279.6 ± 7.6^{fg}	142.1 ± 46.2^{de}	20.4 ± 9.0^{bc}	2.6 ± 0.4^{a}	-	12.5 ± 1.1^{bc}	107.5 ± 1.7^{de}	121.9 ± 3.2^{ef}	-	8.1 ± 0.3^{ab}	92.7 ± 13.5^{d}	94.1 ± 4.6^{cd}
11	ethyl hydrocinnamate	14.5 ± 2.1^{a}	-	15.9 ± 0.9^{a}	20.4 ± 3.1^{a}	-	-	-	-	-	-	-	-	-	-	-
12	diethyl malate	-	-	-	-	5.2 ± 1.8^{a}	4.8 ± 2.3^{a}	10.0 ± 1.3^{bc}	11.2 ± 0.1^{cd}	8.2 ± 0.1^{cd}	11.2 ± 1.3^{bc}	9.5 ± 1.0^{bc}	12.6 ± 1.0^{d}	9.1 ± 0.2^{abc}	11.2 ± 1.2^{cd}	9.5 ± 0.3^{bc}
	Sum of esters	524.0 ± 112.5^{i}	1102.8 ± 132.8^{k}	906.5 ± 185.1^{jk}	1022.1 ± 4.0^{k}	650.7 ± 121.8^{j}	398.9 ± 100.8^{hi}	181.8 ± 24.1^{ef}	83.6 ± 0.5^{bc}	111.3 ± 0.3^{cd}	223.7 ± 0.3^{fg}	235.8 ± 4.6^{gh}	50.8 ± 3.0^{a}	77.6 ± 0.1^{ab}	167.8 ± 19.3^{de}	176.0 ± 1.3^{e}

- not detected

Table 7.1 (continuation): Characterization of secondary metabolites in the fermentations with *D. bruxellensis*/*S. cerevisiae* multi starters in: synthetic medium with high glucose concentration (140 g L^{-1}), synthetic medium with addition of hydroalcoholic wood extract (WE) for final ethanol concentrations by volume of 6 %, 8 % and 10 %, and accelerated aged beer previously put in contact with oak wood at 30 °C (WB30) and 50 °C (WB50). *x*S*y*D stands for *S. cerevisiae* cellular concentration of $x\times10^6$ mL^{-1} and *y*D stands for *D. bruxellensis* cellular concentration of $y\times10^6$ mL^{-1} in the starter inoculum. Statistically significant similarities are represented by a to k letters. Deviations represent standard error of independent duplicates.

		High glucose concentration				**Addition of alcoholic wood extract**			**Application to beer aged in wood**							
	Compound	**2S**	**1D**	**2S1D**	**1S2D**	**WE6% 1S2D**	**WE8% 1S2D**	**WE10% 1S2D**	**WB30**	**WB30 1S**	**WB30 2D**	**WB30 1S2D**	**WB50**	**WB50 1S**	**WB50 2D**	**WB50 1S2D**
No.	**Alcohols**															
13	2-methyl-1-propanol	1254.0±168.5^{h}	70.0±8.8^{def}	1127.7±61.1^{gh}	1023.8±213.2^{gh}	19.5±0.2^{bc}	31.9±17.8^{bc}	-	111.0±14.2^{fgh}	59.1±6.5^{de}	14.3±2.0^{ab}	20.6±2.4^{bc}	81.4±2.0^{efg}	54.3±5.6^{cd}	9.9±0.6^{a}	14.1±5.1^{ab}
14	2-methyl-1-butanol+ 3-methyl-1-butanol	14413.6±1867.4^{i}	5198.4±1296.6^{ghi}	14335.9±771.9^{i}	11672.5±1843.8^{hi}	1311.1±12.1^{efg}	725.2±354.8^{cd}	156.5±35.0^{a}	1839.4±68.3^{fgh}	985.6±55.2^{def}	652.9±4.0^{cd}	600.0±40.8^{bc}	718.7±58.5^{cde}	665.0± 22.8^{cd}	332.2±36.7^{ab}	333.6±65.1^{ab}
15	furfuryl alcohol	12.4±9.2^{abc}	23.2±6.2^{bcd}	16.0±0.7^{abc}	18.4±4.5^{abc}	27.5±0.9^{cd}	30.7±15.5^{bcd}	43.7±8.7^{d}	15.1±1.0^{abc}	12.6±0.4^{a}	26.6±8.0^{bcd}	16.6±1.6^{abc}	14.5±0.5^{ab}	52.4±1.0^{d}	53.8±9.4^{d}	50.1±12.4^{d}
16	2-phenylethanol	20308.3±2936.6^{g}	9214.7±1945.7^{efg}	20898.1±431.1^{g}	18363.7±1398.8^{fg}	3468.1±184.3^{b}	1649.5±578.6^{a}	2.7±0.5^{a}	3468.5±10.8^{b}	5296.6±129.2^{def}	4780.6±219.8^{de}	4159.4±132.8^{cd}	2583.8±124.8^{a}	3424.6±68.5^{b}	3824.1±431.2^{bc}	3470.1±229.8^{b}
	Sum of alcohols	35988.3±4963.3^{j}	14506.3±3257.4^{hi}	36377.7±1264.7^{j}	31078.5±3460.4^{ij}	4826.1±197.0^{de}	2437.3±966.7^{ab}	202.9±44.2^{a}	5434.1±94.2^{fg}	6354.0±68.0^{gh}	5474.3±234.2^{fg}	4796.7±91.3^{ef}	3398.5±185.8^{ab}	4196.3±98.0^{cd}	4219.8±477.9^{cde}	3867.9±312.3^{bc}
	Monoterpenic alcohols															
17	linalool	3.3±0.6^{cde}	15.9±1.6^{fgh}	3.0±0.5^{bc}	2.8±0.1^{bc}	8.8±2.3^{def}	4.9±2.5^{cd}	-	-	1.5±0.4^{a}	26.8±0.8^{h}	16.9±0.3^{gh}	-	1.7±0.4^{ab}	17.7±1.9^{h}	9.8±1.0^{efg}
18	β-citronellol	24.3±5.4^{cd}	15.1±1.0^{bc}	28.4±2.3^{d}	22.9±1.0^{cd}	5.3±2.0^{ab}	2.3±1.0^{a}	-	-	25.2±3.9^{cd}	2.0±0.2^{a}	2.3±0.4^{a}	-	15.2±0.5^{bc}	-	2.4±0.2^{a}
19	geraniol	5.6±1.3^{ab}	108.4±9.2^{c}	25.0±0.6^{ab}	22.1±3.7^{a}	20.1±8.3^{a}	-	-	-	18.3±0.9^{a}	75.4±10.3^{c}	75.1±9.0^{c}	-	24.3±1.3^{ab}	78.3±16.6^{c}	62.9±8.9^{bc}
	Sum of monoterpenic alcohols	53.7±13.3^{bcd}	204.3±13.9^{f}	56.4±2.1^{cd}	47.7±2.6^{bc}	34.3±12.6^{ab}	7.2±3.5^{a}	-	-	45.0±5.2^{abc}	104.2±7.5^{ef}	94.3±9.1^{ef}	-	41.2±1.3^{ab}	96.0±18.4^{ef}	75.0±8.0^{de}

- not detected

Table 7.1 (continuation): Characterization of secondary metabolites in the fermentations with *D. bruxellensis*/*S. cerevisiae* multi starters in: synthetic medium with high glucose concentration (140 g L^{-1}), synthetic medium with addition of hydroalcoholic wood extract (WE) for final ethanol concentrations by volume of 6 %, 8 % and 10 %, and accelerated aged beer previously put in contact with oak wood at 30 °C (WB30) and 50 °C (WB50). xSyD stands for *S. cerevisiae* cellular concentration of x×10^{6} mL^{-1} and yD stands for *D. bruxellensis* cellular concentration of y×10^{6} mL^{-1} in the starter inoculum. Statistically significant similarities are represented by a to k letters. Deviations represent standard error of independent duplicates.

		High glucose concentration				**Addition of alcoholic wood extract**			**Application to beer aged in wood**							
	Compound	**2S**	**1D**	**2S1D**	**1S2D**	**WE6% 1S2D**	**WE8% 1S2D**	**WE10% 1S2D**	**WB30**	**WB30 1S**	**WB30 2D**	**WB30 1S2D**	**WB50**	**WB50 1S**	**WB50 2D**	**WB50 1S2D**
No.	**Fatty acids**															
20	propanoic acid	36.0±9.9^{f}	33.4±9.0^{ef}	37.4±1.0^{f}	34.2±10.1^{ef}	16.5±5.2^{bcd}	8.6±0.4^{abc}	3.0±1.0^{ab}	-	2.2±0.4^{a}	20.9±5.0^{cde}	25.3±1.1^{def}	-	6.2±2.9^{ab}	17.1±6.5^{bcd}	26.9±0.2^{ef}
21	isobutyric acid	209.7±40.1^{ef}	503.2± 156.8^{f}	215.9±3.6^{ef}	225.8±68.1^{ef}	97.8±23.1^{de}	31.7±19.8^{bc}	-	-	11.4±0.2^{a}	17.2±5.2^{abc}	30.9±3.6^{bcd}	-	21.0±0.2^{abc}	17.5±2.4^{ab}	39.7±8.6^{cd}
22	butanoic acid	88.4± 21.2^{gh}	106.6±29.8^{gh}	115.0±24.9^{h}	74.0±25.8^{fgh}	25.5±4.6^{cde}	19.5±8.7^{bcd}	4.2±2.1^{ab}	-	4.1±0.5^{abc}	63.6±22.4^{fg}	48.4±5.9^{fg}	2.4±0.2^{a}	9.7±1.6^{abc}	39.4±5.5^{def}	42.3±7.8^{ef}
23	isovaleric acid	325.9± 81.3^{ghi}	2957.9± 933.3^{i}	448.9±21.0^{hi}	394.5± 89.2^{chi}	185.8± 38.4^{fgh}	55.8±34.9^{bcd}	8.3±4.3^{a}	20.3±1.4^{ab}	35.8±2.5^{bc}	75.9± 22.4^{def}	94.3±6.6^{efg}	22.5±1.0^{abc}	51.2±0.8^{cde}	62.4±13.8a^{cde}	99.4± 13.8^{efg}
24	hexanoic acid	106.6± 36.5^{cd}	575.9± 133.9^{fg}	212.2± 43.6^{de}	201.7± 39.9^{de}	311.7± 2.1^{ef}	368.1± 168.3^{e}	14.8±5.5^{a}	53.6±3.5^{bc}	4.1±0.8^{a}	889.6±3.9^{h}	921.2±31.7^{h}	52.9±5.8^{bc}	22.8±0.8^{ab}	672.6± 12.8^{fg}	743.4±47.3^{gh}
25	octanoic acid	350.0± 104.5^{de}	2719.7± 287.5^{i}	491.9±72.9^{ef}	500.1±26.5^{ef}	845.0±48.7^{f}	805.4± 360.5^{ef}	66.9±18.9^{bc}	111.2±5.4^{cd}	6.1±1.6^{a}	1685.6± 91.3^{g}	1936.8±45.2^{hi}	30.7±3.9^{ab}	30.8±6.2^{ab}	1625.6±3.7^{g}	1754.3± 132.4^{gh}
26	decanoic acid	10.3±3.5^{a}	707.6±52.0^{f}	19.8±8.1^{ab}	16.2±1.5^{ab}	562.8±138.3^{ef}	230.2± 133.0^{d}	12.5±1.9^{a}	-	-	97.6±11.9^{cd}	144.7±25.2^{d}	-	-	66.7±21.0^{bc}	168.8±17.4^{de}
27	phenylacetic acid	14.0±4.2^{bcd}	100.7±21.5^{d}	18.5±5.4^{cd}	4.0±0.2^{a}	6.6±3.0^{ab}	-	-	-	-	-	9.8±1.5^{abc}	-	12.1±4.0^{abc}	4.4±1.4^{a}	13.8±2.7^{bcd}
	Sum of fatty acids	1140.9±301.1^{de}	7705.1±1623.8^{j}	1559.5±178.5^{ef}	1450.4±261.3^{e}	2051.8±259.1^{g}	1519.4±724.9^{ef}	109.7±33.6^{ab}	185.2±10.3^{cd}	63.6±3.8^{a}	2850.4±44.4^{hi}	3211.3±118.5^{ij}	108.6±10.9^{ab}	153.8±5.3^{bc}	2505.6±25.1^{gh}	2888.6±74.9^{hi}
	Furan compounds															
28	furfural	-	-	-	-	2.8±1.9^{bc}	0.5±0.3^{ab}	148.2±13.2^{de}	59.9±2.0^{cd}	0.3±0.1^{a}	-	-	457.2±33.3^{e}	2.9±0.2^{bc}	-	-
29	5-methylfurfural	-	-	-	-	-	-	94.5±10.9^{ab}	22.4±0.7^{a}	-	-	-	121.8±5.7^{b}	-	-	-
30	5-hydroxymethylfurfural	3.3±0.6^{bc}	2.2±0.6^{ab}	2.3±0.3^{ab}	1.4±0.1^{a}	-	-	35.5±5.5^{e}	5.5±0.4^{cd}	3.3±0.5^{bc}	4.0±1.3^{bc}	2.7±0.4^{ab}	11.2±1.4^{e}	8.0±0.2^{de}	8.7±1.7^{de}	8.0±1.4^{de}
	Sum of furan compounds	3.3±0.6^{cd}	2.2±0.6^{abc}	2.3±0.3^{abc}	1.4±0.1^{ab}	2.8±1.9^{abc}	0.5±0.3^{a}	278.3±29.6^{fg}	87.8±2.4^{fg}	3.6±0.4^{cde}	4.0±1.3^{cde}	2.7±0.4^{bc}	590.2±40.4^{f}	10.9±0.4^{fg}	8.7±1.7^{ef}	8.0±1.4^{def}

- not detected

Table 7.1 (continuation): Characterization of secondary metabolites in the fermentations with *D. bruxellensis*/*S. cerevisiae* multi starters in: synthetic medium with high glucose concentration (140 g L^{-1}), synthetic medium with addition of hydroalcoholic wood extract (WE) for final ethanol concentrations by volume of 6 %, 8 % and 10 %, and accelerated aged beer previously put in contact with oak wood at 30 °C (WB30) and 50 °C (WB50). *x*S*y*D stands for *S. cerevisiae* cellular concentration of $x\times10^{6}$ mL^{-1} and *y*D stands for *D. bruxellensis* cellular concentration of $y\times10^{6}$ mL^{-1} in the starter inoculum. Statistically significant similarities are represented by a to k letters. Deviations represent standard error of independent duplicates.

		High glucose concentration				**Addition of alcoholic wood extract**			**Application to beer aged in wood**							
	Compound	**2S**	**1D**	**2S1D**	**1S2D**	**WE6% 1S2D**	**WE8% 1S2D**	**WE10% 1S2D**	**WB30**	**WB30 1S**	**WB30 2D**	**WB30 1S2D**	**WB50**	**WB50 1S**	**WB50 2D**	**WB50 1S2D**
No.	**Lactones**															
31	*cis*-oak lactone	-	-	-	-	-	-	-	16.5±1.5^{e}	13.3±1.1^{cd}	15.7±0.4^{e}	15.1±0.1^{de}	10.4±0.0^{bc}	6.0±0.4^{a}	8.0±0.7^{ab}	8.7±0.2^{ab}
32	*trans*-oak lactone	-	-	-	-	-	-	-	7.9±0.9^{abc}	7.5±0.7^{abc}	11.5±1.8^{c}	8.5±1.0^{bc}	7.7±0.6^{abc}	6.5±0.8^{ab}	6.5±0.4^{ab}	6.3±0.5^{a}
	Sum of lactones	-	-	-	-	-	-	-	24.5±2.4^{de}	20.9±1.9^{cd}	27.1±1.4^{e}	23.6±1.1^{de}	18.2±0.6^{bc}	12.6±0.4^{a}	14.5±0.3^{ab}	15.1±0.7^{ab}
	Volatile phenols															
33	4-methylguaiacol	-	-	-	-	2.5±0.4^{a}	1.6±0.8^{a}	2.7±0.3^{a}	-	-	-	-	-	-	-	-
34	4-ethylguaiacol	-	36.4±3.8^{c}	13.7±6.8^{b}	17.1±3.0^{b}	172.6± 31.0^{d}	185.8± 78.2^{d}	5.1±1.6^{a}	-	-	1080.0± 27.2^{ef}	1041.9± 18.8^{e}	-	-	1111.1±3.2^{fg}	1126.3±6.8^{g}
35	eugenol	-	-	-	-	11.1±3.9^{c}	6.5±2.9^{c}	8.2±0.2^{c}	-	0.7±0.2^{a}	-	1.1±0.0^{ab}	-	0.9±0.2^{ab}	-	2.4±0.1^{bc}
36	2,6-dimethoxyphenol	-	1.6±0.4^{a}	-	-	2.9±0.0^{ab}	5.0±0.3^{abc}	9.7±0.1^{bcd}	23.1±1.9^{ef}	20.6±3.8^{de}	25.9±0.7^{fg}	19.0±28.0^{cde}	23.2±1.7^{ef}	28.0±1.5^{fg}	26.0±4.7^{fg}	33.5±3.1^{g}
37	4-ethylphenol	-	12.3±2.4^{b}	2.0±1.0^{a}	1.8±0.6^{a}	15.5±2.6^{b}	12.8±6.0^{b}	0.9±0.0^{a}	-	-	283.2±2.0^{de}	242.4±3.2^{c}	-	-	294.9±2.0^{e}	259.2±12.6^{cd}
38	vanillin	-	-	-	-	-	1.0±0.0^{a}	115.7±17.9^{ab}	102.2±3.2^{ab}	-	-	-	152.7±10.5^{b}	-	-	-
39	syringaldehyde	-	-	-	-	21.8±8.0^{a}	20.5±1.8^{a}	475.9±42.4^{b}	573.3±52.5^{bc}	-	-	-	760.3±30.8^{c}	-	-	-
40	tyrosol	65.0±13.5^{e}	8.2± 1.4^{a}	57.4±2.5^{e}	67.0±18.2^{e}	1.6±0.3^{a}	-	-	16.0±1.4^{ab}	22.1±0.5^{cd}	47.2±17.2^{e}	20.4±3.8^{bcd}	20.4±0.6^{bc}	25.6±1.0^{de}	19.5±1.9^{bc}	-
	Sum of volatile phenols	65.0±13.5^{b}	58.5±8.0^{ab}	73.1±10.4^{b}	85.9±21.9^{bc}	228.0±46.2^{cd}	233.1±86.4^{cd}	618.2±62.3^{de}	714.5±59.1^{def}	43.4±3.1^{a}	1436.3±8.6^{h}i	1324.8±25.8^{fg} h	956.7±43.6^{efg}	54.5±2.3^{ab}	1451.5±2.5^{i}	1421.4±16.2^{g} hi
	Other															
41	acetoin	330.9± 105.8^{ef}	-	169.5±47.5^{def}	84.2±7.5^{cd}	7.3±0.6^{a}	-	-	-	95.9±3.5^{de}	28.9±9.2^{b}	20.9±0.4^{ab}	-	457.8±15.4^{f}	24.8±4.3^{b}	27.0±5.4^{bc}
42	methionol	200.7± 28.2^{f}	-	165.6±17.5^{ef}	165.6±38.4^{ef}	14.8±1.4^{de}	16.1±8.4^{cd}	2.6±0.4^{a}	16.8±1.2^{def}	9.0±0.6^{abc}	16.7±3.5^{de}	12.6±2.5^{bcd}	12.6±0.6^{bcd}	6.6±0.9^{ab}	9.0±1.8^{abc}	13.2±1.9^{cd}

- not detected

De novo production of terpenoids is an acknowledged trait of yeast, already documented for *Saccharomyces* sp. (Carrau *et al.*, 2005) and multiple other non-*Saccharomyces* sp. yeasts (Rossouw & Bauer, 2016) with the exception of *D. bruxellensis* for which it is still not well documented.

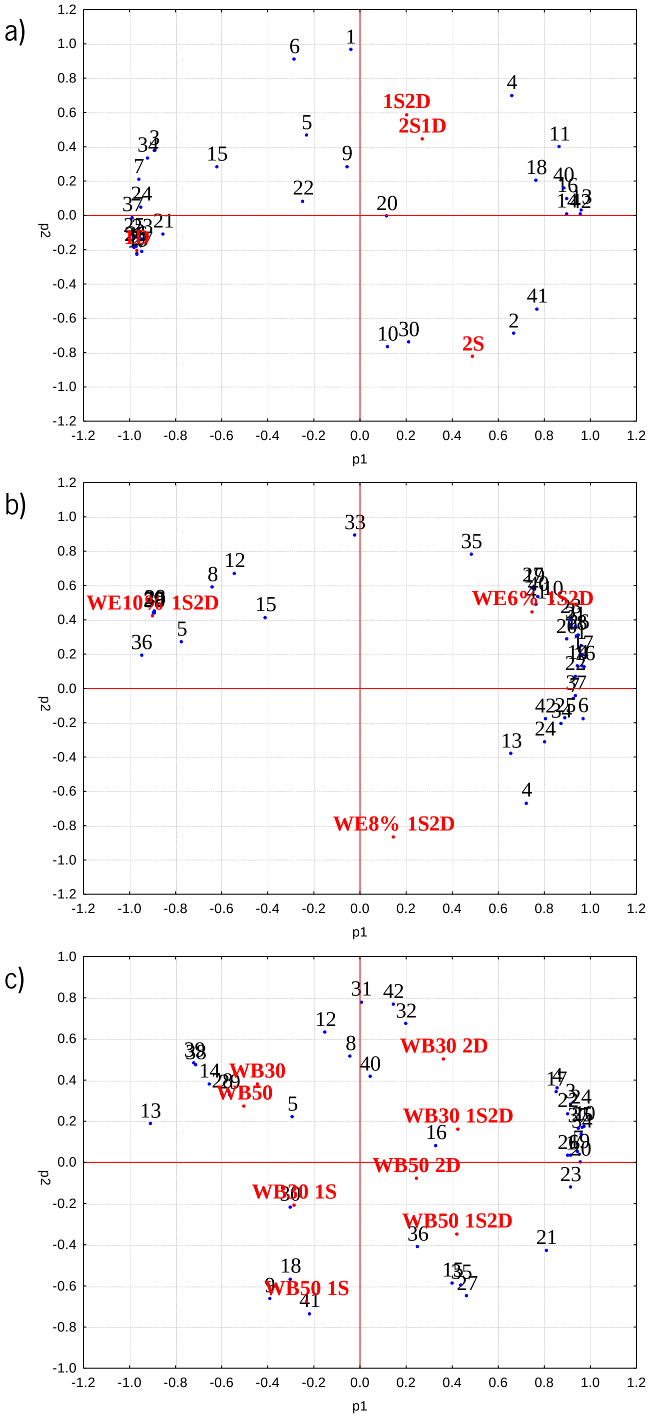

Figure 7.3: Scatterplots correlating metabolites and pure and/or co-cultures of *S. cerevisiae* and *D. bruxellensis* for fermentation of a) synthetic medium with high glucose concentrations, b) synthetic medium with addition of oak extract at ethanol concentrations by volume of 6 % (WE6%), 8 % (WE8%) and 10 % (WE10%) and c) biological ageing of beer after contact with reused oak wood at 30 °C (WB30) and 50 °C (WB50). Numbers in the scatterplot refer to the volatile compounds presented in Table 7.1. *x*S*y*D stands for *S. cerevisiae* initial cellular concentration of *x*×10^6 mL^{-1} and *y*D stands for *D. bruxellensis* initial cellular concentration of *y*×10^6 mL^{-1} in the starter inoculum.

Previous works have provided clues about the capacity of *Dekkera* sp. to produce terpenes, hinting production of terpenoids possibly linked to β-farnesene (Joseph, Gorton, Ebeler, & Bisson, 2013; Joseph, Albino, Ebeler, & Bisson, 2015). Nevertheless, terpenes produced by *D. bruxellensis* in this work were more similar to the ones produced by *S. cerevisiae*, however at significantly higher titers. *De novo* production of linalool and geraniol by *D. bruxellensis* is a novel and significant feature reported for this yeast. These monoterpenes impart beer with desirable floral aroma, being generally correlated with hops descriptors (Denby *et al.*, 2018).

Similarly to other metabolites previously discussed, geraniol and linalool concentrations where higher in pure culture of *D. bruxellensis*, being their concentration lower in co-cultures due to *S. cerevisiae* competition. Analyzing correlations among the produced metabolites presented in the scatterplot in Figure 7.3a), three clusters can be distinguished from the two extracted components highlighting the compounds that better correlate with each species. As previously referred, several metabolites were closely related to *D. bruxellensis* pure culture, namely the volatile phenols 4-ethylphenol and 4-ethylguaiacol, the monoterpenes linalool and geraniol and the volatile acids isovaleric and phenylacetic. On the *S. cerevisiae* cluster, the esters ethyl butyrate and 2-phenylethyl acetate and acetoin arise with closer relation to this species. More importantly, co-cultures of *S. cerevisiae* and *D. bruxellensis* clearly show the combination of metabolite features of both yeasts, correlating with typical and common metabolites of each species either by the first and/or the second component.

3.3 Fermentation with addition of wood extract

In order to mimic fermentation of barrel aged beer, a preliminary assay was conducted using synthetic MYPG medium with addition of oak wood extract. To better assess the effect of ethanol in combination with wood composition, generally extracted from oak barrels, co-cultures were conducted by addition of oak extracts at different ethanol percentages. Growth of each species in the co-culture is presented in Figure 7.4a). As visible, yeast growth was affected by increasing concentrations of alcoholic oak extract in the medium, possibly due to a synergistic inhibition effect of both ethanol and compounds extracted from oak. Inhibitory effect of ethanol on yeast growth is well known both for *S. cerevisiae* and for *D. bruxellensis.* As described by Bassi and collaborators, an increase of ethanol concentration from 9 % to 10 % causes a significant decrease both in *S. cerevisiae* and *D. bruxellensis* growth, being this inhibition complete when increasing ethanol percentage up to 11 % (Bassi, Silva, Reis, & Ceccato-Antonini, 2013). Besides ethanol, increasing oak extract concentration also increases content of typical wood inhibitors in the fermentation medium. Oak extracts have significant amounts of phenolic compounds such as furans and phenolic acids (Coelho, Teixeira, *et al.*, 2019).

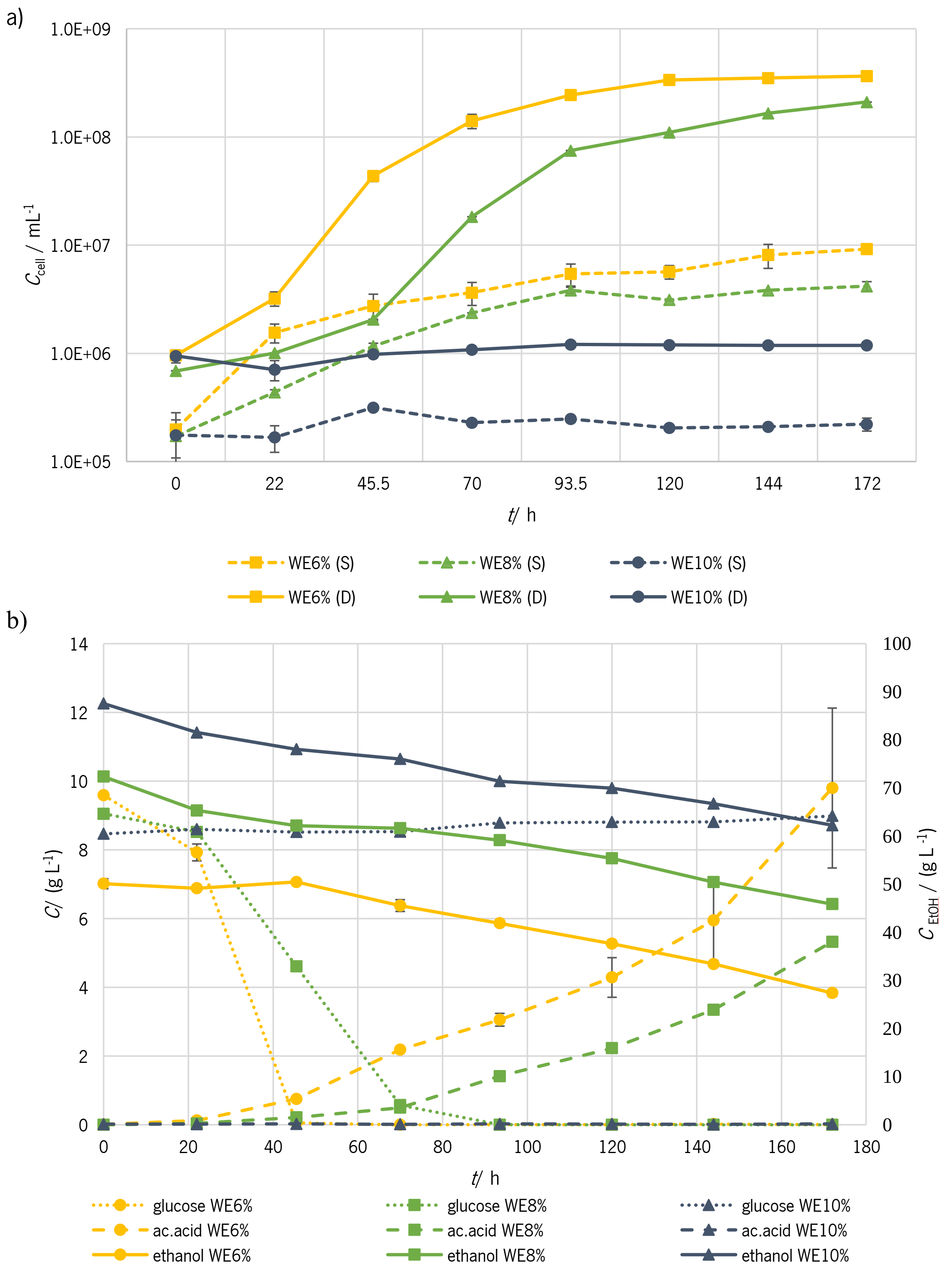

Figure 7.4: Fermentation profiles of synthetic medium with addition of hydroalcoholic wood extracts depicting a) Cellular concentration (C_{cell}) of *S. cerevisiae* (S) and *D. bruxellensis* (D) and b) primary metabolite concentration (C) throughout time (t), in fermentations pitched with cellular concentration of 1×10^6 mL^{-1} of *S. cerevisiae* and 2×10^6 mL^{-1} *D. bruxellensis* in the starter inoculum. Ethanol concentration (C_{EtOH}) is plotted in the secondary axis, whereas the remaining are plotted in the main axis. Error bars depict standard error of independent duplicates.

This is visible in Table 7.1 for the dilution corresponding to 10 % ethanol, where microbial growth was absent. Thus syringaldehyde, vanillin, furfural, 5-methylfurfural and 5-hydroxymethylfurfural were initially present in the medium, all coming from the oak wood extract. Impact of these compounds on *S. cerevisiae* and *D. bruxellensis* metabolism is well known in the field of bioethanol production; for instance, furan compounds are strong inhibitors of *S. cerevisiae* and *D. bruxellensis* growth in pretreated lignocellulosic medium (Blomqvist *et al.*, 2011; Kelbert *et al.*, 2016). It is important to highlight that final concentration of the abovementioned inhibitors was practically residual in the cultures where *S. cerevisiae* and *D. bruxellensis* were able to grow. As reported, *S. cerevisiae* can detoxify the medium by converting furans to least harmful compounds, enduring considerable concentrations of these inhibitors (Cunha, Romaní, Costa, Sá-Correia, & Domingues, 2019). On its hand, *D. bruxellensis* also endures low to moderate concentrations of these compounds but with its fermentative performance strongly affected (Galafassi *et al.*, 2011).

In contrast with the previous fermentation using high sugar concentration, *D. bruxellensis* showed a competitive advantage over *S. cerevisiae* in the co-culture fermentations with oak extract. Ethanol and glucose concentrations initially available in the medium were key aspects for this competitive advantage. As previously mentioned, *D. bruxellensis* is able to outcompete *S. cerevisiae* only when initial glucose concentrations in the medium are low (Blomqvist, 2011). Another aspect to take into account in its competitive advantage is *D. bruxellensis* ability to grow and metabolize alternative carbon sources efficiently, for example ethanol, pentoses and even cellobiose (Blomqvist, Eberhard, Schnürer, & Passoth, 2010). As presented in Figure 7.4b), a total depletion of glucose is observed in the co-cultures for the dilutions corresponding to 6 % and 8 % ethanol. For the condition of 10 %, glucose, concentration did not vary due to absence of microbial growth. A slower sugar conversion and acetic acid production rates were observed in the co-cultures for the dilution of oak extract to 8 % ethanol, when compared with the 6 % condition, naturally linked to the increased selective pressure on the co-culture. This selective pressure affected both species equally as seen for microbial growth, which is reflected in all of the primary metabolites studied.

Focusing on secondary metabolites, several compounds, previously identified in the high sugar concentration fermentations, were produced in the fermentations with addition of oak extract. Several esters were found, with particular emphasis on ethyl esters previously associated to *D. bruxellensis*, namely ethyl octanoate, ethyl decanoate and 2-phenylethyl acetate. Isobutyl acetate, previously identified as deriving from a potential synergy between *S. cerevisiae* and *D. bruxellensis*, also appeared in fermentations where microbial growth occurred. Considerable amounts of fatty acids were also found in fermentations with oak extract, along with rather low concentrations of alcohols, reflecting the more pronounced growth of *D. bruxellensis* in the fermentations with oak extract. As visible in Table 7.1, concentration of furan compounds was almost null in the fermentations where microbial growth was observed, an indicator of their detoxification. Also, a more pronounced formation of ethylphenols was observed, a direct result of a higher concentration of hydroxycinnamic acids in the medium, which typically derives from the wood extract. Several other compounds were found, which derived from the oak wood extract and are not metabolites of neither of the species. Such is the case of diethyl succinate, ethyl lactate and diethyl malate, which were adsorbed by wood in its prior contact with wine and posteriorly extracted from wood, coherent with the reported in previous works (Coelho, Domingues, *et al.*, 2019). Among these, diethyl succinate and diethyl malate concentration decreased significantly in the cultures where microbial growth was observed, potentially due to *D. bruxellensis* esterase activity. Scatterplot presented in Figure 7.3b), demonstrates the correlation of samples corresponding to 6 % and 8 % dilutions of oak extract with the compounds derived from yeast metabolism. Higher correlations were found for co-culture fermentations of diluted oak extract to 6 % due to the increased microbial growth and metabolism. Nevertheless, fermentations of oak extract corresponding to 8 % ethanol appeared closely in the first component. On the other hand, sample corresponding to 10 % ethanol appeared in the cluster correlating closer to wood compounds from oak extract, demonstrating the absence of microbial activity.

3.4 Application to beer ageing

Lastly, to further validate the results, single species and co-culture fermentations were conducted in real beer matrixes. For this purpose, beer was previously put in contact with used oak wood from wine ageing to induce oak wood characteristics by an accelerated approach. Two different temperatures were used for contact between oak wood and beer, in order to obtain different beer compositions, following previous findings (Coelho, Teixeira, *et al.*, 2019). Thus, beer was previously put in contact with used oak wood chips at 30 °C (WB30) and 50 °C (WB50) during 48 h to obtain dissimilar accelerated aged beers with different concentrations of wood extractives. Due to the higher contact temperature, WB50 was characterized by a higher content in wood extractives, namely furan compounds and aldehydes, as presented in Table 7.1. Moreover, several other phenolic compounds can be extracted from wood, as for example phenolic acids such as syringic acid, ellagic acid and several other polyphenols (Coelho, Teixeira, *et al.*, 2019), which can impair yeast growth and metabolism. Nevertheless, as presented in Figure 7.5a), both yeasts were able to grow in both aged beers, even with the low sugar content available and the presence of ethanol and inhibitors. Both yeasts withstood higher concentrations of inhibitory compounds (furfural, 5-methylfurfural, vanillin and syringaldehyde) when compared with fermentations in synthetic medium with addition of oak extracts, presented in Table 7.1. However, ethanol concentrations in the aged beers were significantly lower, which led to lesser inhibitory stress and provided adequate conditions for growth. Competitive advantage of one species over another was not evident, as both species presented similar microbial growth in co-culture and single culture fermentations. As presented in Figure 7.5b), acetic acid production was only observed in pure and co-cultures including *D. bruxellensis* and absent in pure cultures of *S. cerevisiae.* Focusing secondary metabolites, formation and depletion of several compounds previously identified in the assays with synthetic media occurred in the fermentations of the accelerated aged beers either with pure or co-cultures. As visible in Table 7.1, several inhibitory compounds found in the accelerated aged beers, namely furfural, 5-methylfurfural, vanillin and syringaldehyde, were metabolized, corroborating the results obtained in the fermentations with wood extract. This bioconversion, performed by both yeasts, can be disadvantageous when envisaging incorporation of wood compounds in beer. These compounds are often associated with sweet desirable aromas, which are lost due to microbial growth and metabolism. The only compounds related with oak wood aroma that were maintained after fermentation of accelerated aged beers were *cis-* and *trans-*oak lactones, typically associated with coconut aroma descriptors, and 2,6-dimethoxyphenol. Also, concentrations of ethyl lactate, diethyl succinate and diethyl malate were maintained in the same range. These compounds were adsorbed from wine by wood in its previous contact and migrated to beer by reutilization of oak wood (Coelho, Teixeira, *et al.*, 2019). Therefore, microbial activity on accelerated aged beers did not affect significantly the occurrence of typical wine esters, preserving desired aromas. Regarding the compounds formed by metabolism of *S. cerevisiae* and *D. bruxellensis*, several previously identified compounds appeared in the fermented beers. For instance, isobutyl acetate appeared in beers fermented with *D. bruxellensis* and in higher concentrations in the co-culture. Again, several ethyl and acetate esters were formed mainly by *D. bruxellensis*, which contribute to the enhancement of fruity aroma in the accelerated aged beers. Also, production of volatile acids was more pronounced in cultures with *D. bruxellensis*, occurring at similar levels either in pure or co-cultures. Similar concentrations were also observed for the production of *D. bruxellensis* characteristic volatile phenols, 4-ethylphenol and 4-ethylguayacol either in pure or co-cultures of *D. bruxellensis* with *S. cerevisiae.* Also, concentration of ethylphenols was within the same range for the two tested beers implying that extraction of ethylphenol precursors was similar in both conditions using either 30 °C or 50 °C. On a broader analysis, production of *D. bruxellensis* key secondary metabolites was similar, either in pure or in co-culture of accelerated aged beers. This reinforces that in low sugar concentrations and in the presence of ethanol, the competition between the two yeasts for accessing to the substrate is modified.

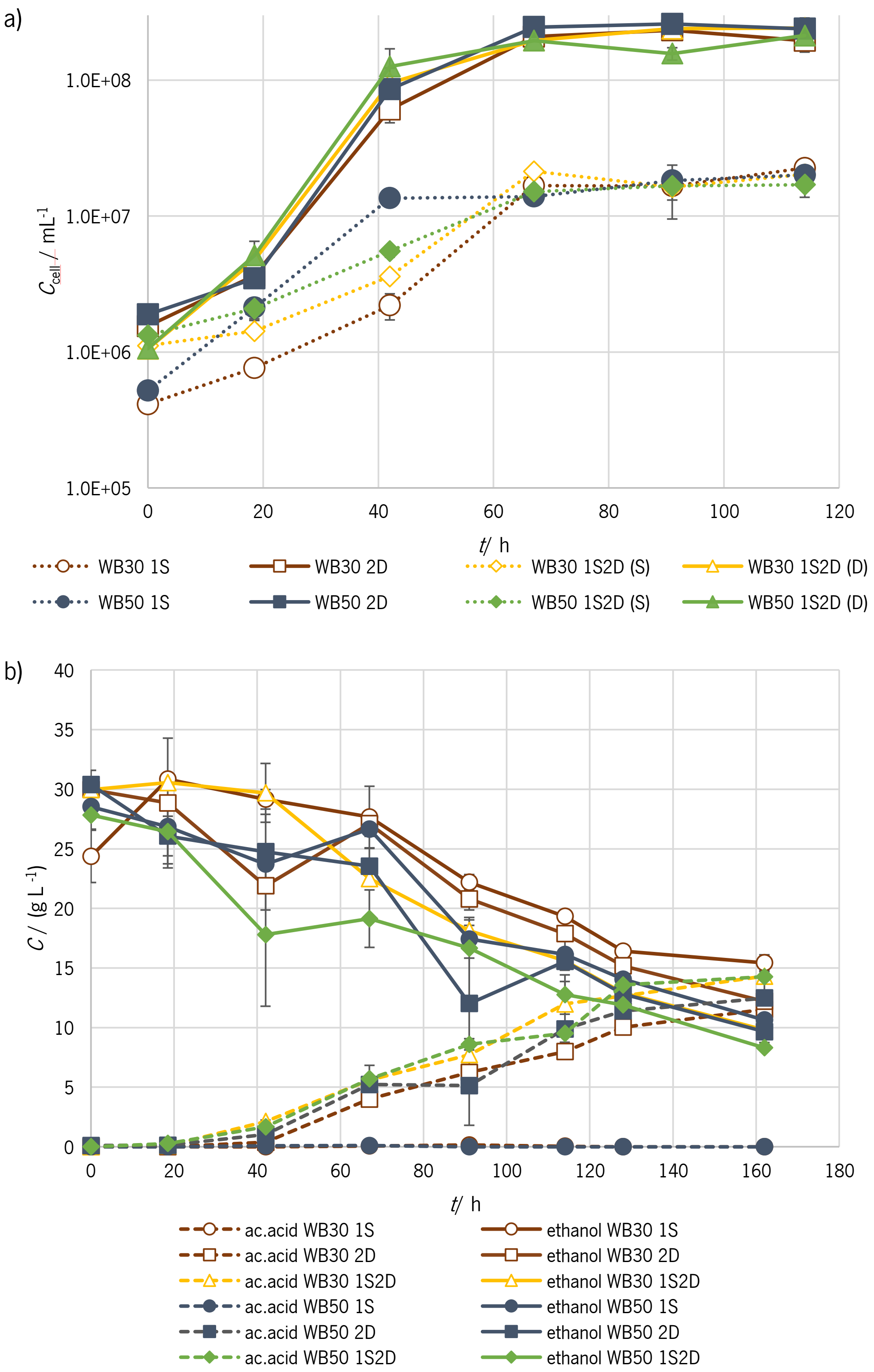

Figure 7.5: a) Cell concentration evolution (C_{cell}) of *S. cerevisiae* (S) and *D. bruxellensis* (D) and b) primary metabolite concentration (C) throughout time (t), in fermentations of accelerated aged beer put in contact with wood at 30 °C (WB30) and 50 °C (WB50), using pure cultures (2S and 2D) and co-cultures favoring *S. cerevisiae* (2SD) or *D. bruxellensis* (S2D). xSyD stands for *S. cerevisiae* cellular concentration of $x\times10^6$ mL^{-1} and yD stands for *D. bruxellensis* cellular concentration of $y\times10^6$ mL^{-1} in the starter inoculum. Error bars depict standard error of independent duplicates.

With ethanol as the main and abundant carbon source, *D. bruxellensis* is able to compete with *S. cerevisiae*, being its traits more easily displayed in the end-product. Scatterplots presented in Figure 7.3c) demonstrate clearly this mechanism, with co-culture fermentations appearing closely to *D. bruxellensis* pure culture fermentations. Also, fermentations including *D. bruxellensis* appeared closely with regard to the first component, which distinguishes them from *S. cerevisiae* pure culture fermentations, whereas the second extracted component distinguishes beers prepared with wood at 30 °C from beers prepared at 50 °C.

4. Conclusions

Both *Saccharomyces cerevisiae* and *Dekkera bruxellensis* are able to grow simultaneously in co-culture conditions. Medium composition strongly impacts the competition between *S. cerevisiae* and *D. bruxellensis*, which can be further influenced by pitching concentrations of each species depending on the medium. The differences in growth and competition are reflected in the metabolic profiles obtained in the end-product, being the use of co-cultures an effective strategy to amplify or minimize the production of target species metabolites. Ethanol and wood extract composition are inhibitory of *S. cerevisiae* and *D. bruxellensis* metabolism and production of *D. bruxellensis* related metabolites is favored over *S. cerevisiae* metabolites in low glucose/high ethanol conditions. Fermentation using multi-starter *S. cerevisiae*/*D. bruxellensis* cultures is a useful tool for the accelerated biological ageing of beer with targeted sensory profiles and furthermore suitable for integration with other accelerated ageing strategies. Therefore, the established hypothesis is confirmed.

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Chapter 8. General conclusions and work perspectives

1. General conclusions

Summing up the main conclusion of this thesis, several remarks can be made about the transformations occurring to cooperage wood during ageing of beverages, as well as its impact in subsequent ageing processes.

During contact with the beverage, wine penetrates the porous structure of wood, soaking it. The time needed for attaining equilibrium/wood saturation is dependent on wood particle size, as shown by mass transfer models outlining mass transfer rate and maximum wine uptake. Maximum retention capacity of wood is independent of particle size, as it is only related to wood specific pore volume. As a consequence of wine uptake, wood volatile compounds are also retained by wood, mainly esters, alcohols and volatile acids. Compound retention varies within different oak varieties, either French or American, with or without toasting. Along with the retained wine volatiles, wood still maintains significant content of characteristic wood extractives, which commonly contribute to aged beverages quality.

Apart from retaining wine in its porous structure, wood also adsorbs wine volatile compounds, which is chemically driven by hydrophobic interactions. Thus, adsorption affinity and selectivity is different for each volatile compound, depending both on wood and wine composition. Mathematical modeling demonstrated that extraction of wine volatile compounds adsorbed in wood was mainly affected by ethanol concentration, as it modifies polarity and therefore disrupts hydrophobic interactions. Along with adsorbed wine volatiles, characteristic wood compounds are also extracted. Selectivity is different for wood compounds as well, as extraction is affected not only by ethanol concentration in the hydroalcoholic matrix but also by contact temperature. Therefore, wood is a vector for recombination of aroma compounds, as it adsorbs compounds from wine and transfers them to the following matrix when reused. Furthermore, when applied in controlled, accelerated processes, incorporation of volatile compounds in beverages can be targeted towards specific compositions by selecting the most suitable extraction parameters. By manipulation of wood concentration, ethanol concentration and contact temperature, different volatile compositions are incorporated into the beverages. Differences in volatile composition lead to the perception of different sensory properties by trained panelists, affecting the final quality of the obtained products.

Transference of wine volatile compounds to beer by reuse of wood also occurs in traditional barrel ageing. Beer composition is influenced not only by the barrel's extractable pool but also by the ageing process applied, as aged beers are heterogeneous in their composition. Apart from transference of compounds between wood and beer, other phenomena occur during beer ageing that are imperative to its volatile composition. The action of endogenous barrel microbes in spontaneous fermentative processes further modifies volatile compounds in beer. Among the multiple microbes that can populate the barrel, *Dekkera bruxellensis* and *Saccharomyces cerevisiae* are the dominant species, with the most prominent impact in volatile composition of beer. Therefore, envisaging specifically the accelerated ageing of beer, spontaneous barrel re-fermentation of beer can be replaced by the application of multi-starter cultures of *D. bruxellensis* and *S. cerevisiae*, which is a successful strategy for controlling and accelerating the biological transformations occurring during beer ageing. When high sugar concentration is imposed, *S. cerevisiae* is dominant in co-culture with *D. bruxellensis*, whereas in high ethanol conditions *D. bruxellensis* is the dominant species. Concomitant growth and competition between both species strongly influences the production of specific metabolites, namely alcohols, characteristic of *S. cerevisiae,* or volatile phenols and acids, characteristic of *D. bruxellensis*.

The research aims proposed in this thesis were successfully reached, with significant contributions to the state of the art on wood reutilization in the ageing of beverages. Also, processes for the accelerated ageing of beverages reusing wood were successfully developed, providing a basis for further developments as well as tools for novel production and ageing processes.

2. Work perspectives

Commonly in scientific research, the creation of novel knowledge also creates novel questions. Even with all the research efforts regarding transformations occurring to wood and beverages during ageing processes, time restraints are inherent to the preparation of a thesis, being that much more scientific work could be performed to answer these questions and further contribute to this field.

Firstly, this thesis focused specifically on volatile compounds participating in the aroma fraction of beverages. However, several other compounds participate in the organoleptic characteristics of aged products, namely in gustatory and visual characteristics, which were only mildly explored in this work due to time restraints. Phenolic compounds and polyphenols could be the target of future studies, as they contribute to the color of beverages and gustatory characteristics, as for example astringency in the case of tannins.

Also, this work focused mainly oak wood and the sorption of wine compounds. However, several other wood varieties and beverages should be explored, in order to shed a broader light regarding the wide variety of tools and products available. Several other combinations could be target of similar studies, for example chestnut, cherry, maple or ash woods, as well as a multitude of beverages either regarding wine, beer or spirits, of different formulations and/or origins. Furthermore, the impact of alternative physical-chemical processes could be studied in the extraction of compounds from wood, as for example electric fields or ohmic heating.

Another area that could be target for further scientific studies is barrel ageing of beer. Several, additive, subtractive and biological ageing phenomena were identified, but chemical transformations, namely oxidative and condensation reactions occurring during barrel ageing of beer are still poorly understood. Knowledge about this type of reactions could further contribute to the fine tuning of accelerated ageing of beer, being that a micro-oxygenation strategy could be interesting to promote additional transformations. Also regarding biological transformations, a finer tuning could be performed either by performing additional works in *D. bruxellensis/S. cerevisiae* fermentative processes or by studying the incorporation of other species, for example lactic acid bacteria, in the multi-starter cultures.