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Production and Bioactivity Assessment of Beer Aged with Oak Wood Chips

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***Production and Bioactivity
Assessment of Beer Aged with Oak
Wood Chips***

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Abstract

Beer is widely reported to possess a multitude of biological properties strongly associated with phenolic compounds originated from hops and malt. Moreover, beer enjoys enormous plasticity in terms of sensorial attributes and chemical characteristics providing an interesting platform to the conception of new, attractive, and healthier products through beer enrichment strategies. In this work, the effect of the addition of oak wood chips to the beer was evaluated to determine the potential benefit, bioactivity wise, that this practice, increasingly more frequent in the brewing paradigm, can provide the final consumer.

Pale Ale and Stout beers were brewed in the laboratory. During the maturation stage, French and American oak wood chips were added to the beer and the effect on the global beer bioactivity was estimated. For the different beer samples, the total phenolics content ranged from 771.98 ± 18.91 to 1501.81 ± 41.62 mg.GAE.L⁻¹, the total flavonoids concentration varied between 54.12 ± 1.66 and 100.20 ± 5.02 mg.CE.L⁻¹, and the antioxidant activity varied from 333.10 ± 12.72 to 682.70 ± 55.92 mg.TE.L⁻¹ for the DPPH assay and from 1014.54 ± 64.59 to 3701.50 ± 38.58 mg.FSE.L⁻¹ for the FRAP assay. In all the assays, no differences were observed when comparing samples aged with wood and the control without wood addition, however, significantly higher values ($p < 0.01$) were observed for Stout beers. In the meantime, phenolic profiles were tentatively identified by HPLC-DAD where twelve of the thirty selected compounds were quantified, being gallic, 3,4-dihydroxyphenylacetic, vanillic, and syringic acids significantly higher ($p < 0.05$) in the samples treated with American oak wood. Finally, via cell cultures, the antiproliferative activity was assessed in the Caco-2 cell line with determined IC₅₀ values ranging from 16.45 ± 1.06 to 22.93 ± 1.06 (% beer in CM). Also, using the RAW 264.7 cell line, anti-inflammatory and antioxidant activities were evaluated with values ranging from 32.26 ± 2.25 to 24.25 ± 1.83 (%NO vs. LPS control) and from 53.71 ± 2.85 to 41.77 ± 2.49 (%RS vs. LPS control), respectively, for beer samples with a concentration of 12.5 (% beer in CM).

This work allowed us to report that, in the tested conditions, the beer samples present a significative antioxidant, antiproliferative and anti-inflammatory activity, despite no apparent increase being noticed with the added wood. Still, an increase of the concentration of some phenolic compounds was verified in samples matured with wood, with American oak wood displaying a more pronounced phenolic transfer rate for beer ageing.

Keywords

Beer; Bioactivity; Phenolic compounds; Wood; Beer enrichment.

Resumo

A cerveja é amplamente associada a diversas atividades biológicas associadas a compostos fenólicos provenientes do malte de cevada e do lúpulo. Ademais, desfruta de uma enorme plasticidade em termos de atributos sensoriais e químicos, providenciando uma plataforma interessante para a criação de produtos novos, saudáveis e atrativos através de estratégias de enriquecimento. Neste trabalho, a adição de aparas de madeira de carvalho à cerveja foi avaliada para determinar o potencial benefício, em termos de bioatividade, que esta prática, cada vez mais frequente no mundo da cerveja, pode trazer ao consumidor.

Cervejas Pale Ale e Stout foram produzidas no laboratório. Durante a fase de maturação foi-lhes adicionada madeira de carvalho (Francês e Americano) sob a forma de aparas e o seu efeito na bioatividade global das cervejas foi avaliado. Nas diferentes amostras de cerveja, o teor de compostos fenólicos totais variou entre 771.98 ± 18.91 e 1501.81 ± 41.62 mg.GAE.L⁻¹, o teor de flavonoides totais entre 54.12 ± 1.66 e 100.20 ± 5.02 mg.CE.L⁻¹ e a atividade antioxidante oscilou entre 333.10 ± 12.72 e 682.70 ± 55.92 mg.TE.L⁻¹ para o ensaio de DPPH e de 1014.54 ± 64.59 a 3701.50 ± 38.58 mg.FSE.L⁻¹ para o ensaio de FRAP. Em todos os ensaios, não foram verificadas diferenças entre amostras envelhecidas com madeira e o controlo, no entanto, valores significativamente maiores ($p < 0.01$) foram observados para as cervejas Stout. Paralelamente, o perfil de compostos fenólicos foi tentativamente identificado por HPLC-DAD com doze dos trinta compostos a serem quantificados, com aumentos significativos ($p < 0.05$) dos ácidos gálico e vanílico, entre outros, nas amostras tratadas com madeira de carvalho americano. Finalmente, a atividade antiproliferativa foi avaliada na linha celular Caco-2 com valores de IC₅₀ entre 16.45 ± 1.06 e 22.93 ± 1.06 (% cerveja em MC). Foram também avaliadas as atividades antioxidante e anti-inflamatória na linha celular RAW 264.7 com valores entre 32.26 ± 2.25 e 24.25 ± 1.83 (%NO vs. controlo LPS) e entre 53.71 ± 2.85 e 41.77 ± 2.49 (%ER vs. controlo LPS), respetivamente, para amostras de cerveja com concentrações de 12.5 (% cerveja em MC).

Este trabalho permitiu reportar que, nas condições testadas, as amostras de cerveja apresentam atividades antioxidante, antiproliferativa e anti-inflamatória significativas, embora não relacionadas com a adição de madeira. Todavia, foi verificado um aumento da concentração de alguns compostos fenólicos em amostras com madeira, tendo o carvalho americano apresentado resultados mais adequados para o envelhecimento de cerveja.

Palavras-chave

Cerveja; Bioatividade; Compostos fenólicos; Madeira; Enriquecimento de cerveja.

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Abbreviations list

AO – Antioxidant activity

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ABV – Alcohol by volume

ANOVA – Analysis of variance

BJCP – Beer Judge Certification Program

CE – Catechin equivalents

CM – Complete culture medium

COX – cyclooxygenase

CRP – C-reactive protein

DCF-DA – 2',7'-dichlorofluorescein diacetate

DMEM – Dulbecco's modified Eagle's medium

DMS – Dimethyl sulphate

DMSO – Dimethyl sulfoxide

DPPH – 2,2-diphenyl-1-picrylhydrazyl

EDTA – Ethylenediaminetetraacetic acid

EtOH – Ethanol

FAAS – Flame Atomic Absorption Spectroscopy

FBS – Fetal Bovine Serum

F-C – Folin-Ciocalteu

FDA – Food and Drug Administration

FRAP – Ferric Reducing Antioxidant Power

FSE – Ferrous sulphate equivalents

GA – Gallic acid

GAE – Gallic acid equivalents

GC – Gas chromatography

HPLC – High-performance liquid chromatography

IBU – International bitterness units

IC₅₀ – Half maximal inhibitory concentration

ICP-MS – Inductive coupled plasma mass spectroscopy

IL – Interleukins

iNOS – inducible isoform of the Nitric oxide synthetase enzyme

IX – Isoxanthohumol

LC-MS – Liquid chromatography with mass spectrometry detection

LOD – limit of detection

LOQ – limit of quantification

LPS – Lipopolysaccharide

MEM NEAA – Minimum essential medium non-essential amino acids

MLDs – Melanoidins

mRNA – messenger RNA

MRPs – Maillard reaction products

MS/MS – Tandem mass spectrometry

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NED – N-[naphth-1-yl]ethylenediamine dihydrochloride

NO – Nitric oxide

ORAC – Oxygen radical absorbance capacity

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PGs – Prostaglandins

PTFE – Polytetrafluoroethylene

PVPP – Polyvinylpolypyrrolidone

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

RS – Reactive species

SALLE – Salting out-assisted liquid-liquid extraction

SEM – Standard error of the mean

SRM – Standard reference method

TE – Trolox equivalents

TFC – Total flavonoids concentration

TNF- α – Tumour necrosis factor alpha

TPC – Total phenolic contents

TPTZ – 2,4,6-Tris(2-pyridyl)-s-triazine

VPCs – Volatile phenolic compounds

VSCs – Volatile sulphur compounds

XN – Xanthohumol

I. Introduction/Background

1. Beer

Traditionally, beer is a fermented alcoholic beverage brewed using water, malted barley, hops, and yeast. It is one of the most popular drinks all over the world and its consumption keeps trending due to its relatively low cost and potential health benefits when consumed in moderation. Its systematic production dates to 6,000 B.C. in ancient Mesopotamia, although the first brews date back to the Neolithic revolution back in 12,000 B.C. In 5,000 B.C., in Egypt, beer production turned into a common commercial activity and brewing started in Europe around 3,600 B.C. With the emerging presence of the Roman empire, beer became a drink relegated to the lower classes whilst wine became the luxury beverage of the upper ones. [1, 2].

With the uprising of the Holy Roman Empire in the 9th century, Monasteries in the north and central Europe became in charge of beer production. In the 12th and 13th centuries, monks started selling beer brewed in the monasteries. In 1492, the discovery of America and explorative voyages between 1500 and 1800 aided to establish new commercial routes that helped to cement the prominent role of this beverage in the worldwide panorama that still stands until the present day [1].

Nowadays, beer is reported to be the third most popular beverage in the world, only behind water and tea. In 2017, beer's global production was approximately 1.94 billion hectolitres with China being its leader [3]. When it comes to consumption, the world's *per capita* consumption leader is the Czech Republic with approximately 143 litres every year [4]. Furthermore, the global panorama has been significantly impacted by the craft beer movement since the craft breweries already account for a significant quote of the beer market all around the world. In 2016, craft breweries were responsible for 12.3 % of the US beer market with almost 5300 microbreweries [5].

This complex food matrix, rich in biologically active compounds from malt, hops and even yeast with relatively low alcohol content is adequate for bioactive enrichment performed through various processes/strategies aiming to generate new healthier beverages. To better design these enrichment strategies it is of capital importance to understand the raw materials and the brewing process of beer.

1.1. Raw materials

The *Reinheitsgebot* (German beer purity law) was adopted in Bavaria in the 16th century and is still governing commercial beer in Germany to this day. This law stated that the only ingredients allowed in the beer brewing process were water, barley, and hops (yeast's existence was yet to be discovered). Nowadays, a few centuries later, the key/core raw materials for the production of beer remain untouched. There are, however, a few ever-changing beer components: the adjuncts, preservatives, colourants, *etc.* These are controlled by each country's governmental guidelines [6, 7].

1.1.1. Water

This raw material is the major component of the beverage (about 90%). The importance of this raw material has been increasingly stressed through the history of beer. A few of the most relevant parameters concerning water are the absence of chlorine due to its antimicrobial properties and its pH value since its ions can interact with malt ions resultant from the mashing process affecting the quality and stability of the final product [7]. Water quality is determined by legislation in what concerns microbiological and physicochemical parameters. Additionally, there are some quality requirements for brewing purposes, often determined by the brewery itself, that play an important role in the final product [7].

Being the main ingredient in beer, water plays a relevant part in the cost of beer production. This is also an important factor in the selection of the brewing plant's location often being close to natural water sources [7].

1.1.2. Barley (malt)

Quantitatively speaking, barley is the second most important raw material for beer production. Some breweries still perform the malting process from barley grain, while others buy the malt from a supplier. It is the main cereal grain used in beer production being usually malted, although unmalted barley can be added. It provides the starch that will suffer enzymatic breakdown to provide simple sugar (mainly maltose and maltotriose) necessary for the yeast fermentative process [7]. Its role will be further explored in the malting process.

1.1.3. Hops

Hop (*Humulus lupulus* L.) cultivation is traced back to 200 A.D. in Babylon but it was only used in the brewing process from 1079 on. Although originally used as a beer preservative, nowadays hops are used mainly to confer bitterness and flavour characteristics to the final product [6].

The *Humulus* genus belongs to the Cannabaceae family, of which *Cannabis* is part. This genus comprehends three different species: *Humulus lupulus*, *Humulus japonicus* and *Humulus yunnanensis*, however only *H. lupulus* possesses industrial and medicinal potential. This climbing, dioic plant that can reach 10 meters high, spontaneously grows in temperate regions of the globe and can be classified into five subspecies: var. *lupulus* in Europe and western Asia, var. *cordifolius* in eastern Asia, var. *lupuloides*, *neomexicanus* and *pubescens* in North America. In beer production, only the female hop flowers are used. Inside the cones, in the base of the petals, are located the lupulin glands that synthesize and store the desired metabolites for hops usage (Figure 1) [5, 8].



Figure 1. Hop cone flower and its longitudinal cut showing yellow coloured lupulin glands (adapted from [8]).

The main desirable hop contents for beer brewing are resins (source of bitterness), essential oils (flavouring agents) and phenolic compounds (responsible for a considerable part of beer bioactivity) like xanthohumol and isoxanthohumol, depicted in figure 2. The content of these substances in the hop plants is highly influenced by the cultivar and the *terroir* [8].

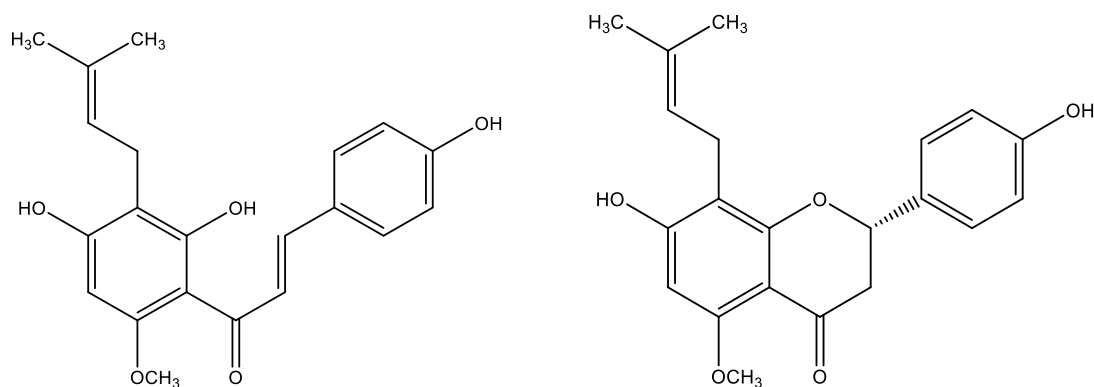


Figure 2. Chemical structure of xanthohumol and isoxanthohumol, respectively.

Hop resins include the bittering substances and represent 5–20% of the hop's dry weight. The principal compounds present in the resins are the α and β -acids (prenylated acylphloroglucinols), which have dissimilar bittering potential. During the wort boiling process (view section 1.3.5.), α -acids (humulones) are converted to more water-soluble iso- α -acids (Figure 3) which have substantial bittering potential. On the other hand, β -acids (lupulones) have low solubility in wort and beer and, consequently, have less impact in conferring bitterness [6, 7]. The content of α -acids in “Aroma hop” cultivars is about 3–4%, unlike “bitter hop” cultivars that can synthesize up to 20% of α -acids [5, 8].

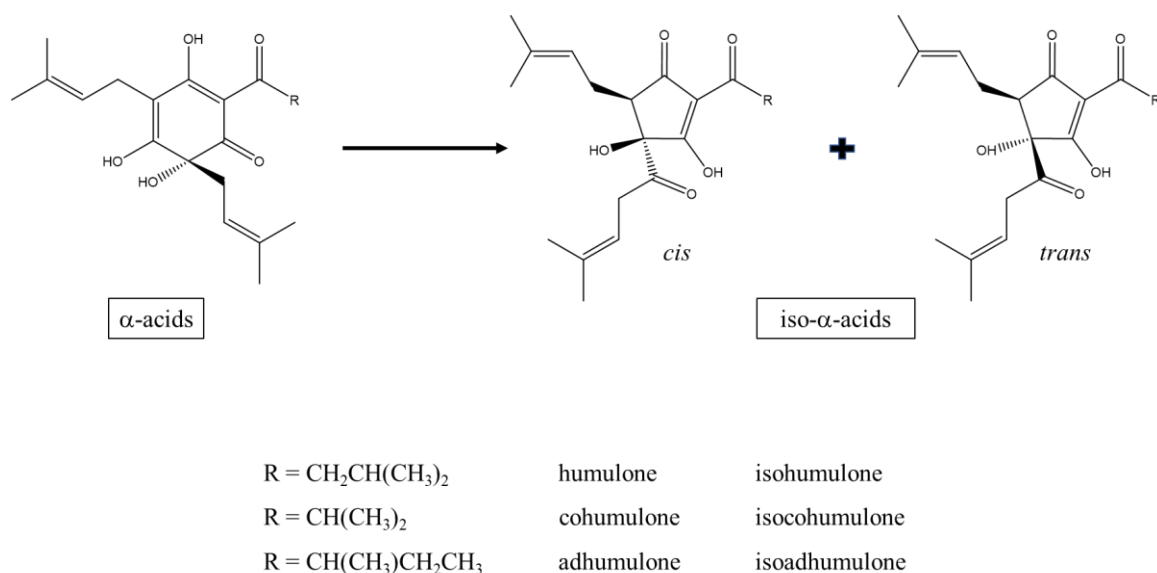


Figure 3. Isomerization of the hops' α -acids to their correspondent iso- α -acids (adapted from [7]).

In table 1, the concentration of the most relevant compounds from hops in beer is displayed. Further on, these compounds will be highlighted due to their impact in beer's bioactivity, especially through their antiproliferative potential.

Table 1. Concentration of the most relevant hop compounds in beer.

<i>α and β-acids</i>	<i>iso-α-acids</i>	<i>xanthohumol</i>	<i>isoxanthohumol</i>
0.4 – 5 $\mu\text{g/mL}$ ^[9-11]	1.4 – 40 $\mu\text{g/mL}$ ^[9-11]	$\leq 1 \mu\text{g/mL}$ up to 10 $\mu\text{g/mL}$ (Dry hopping) ^[12-15]	0.1 – 4 $\mu\text{g/mL}$ ^[9, 10, 16, 17]

Hop essential oils represent 0.4 to 2.0% of the dry weight of the cone and are responsible for conferring the aromatic attributes to the beer. They constitute a complex mixture of over 400 compounds essentially monoterpenes and sesquiterpenes [5-8]. Certain compound classes present in hops' essential oils are related to specific aroma attributes as displayed in table 2.

Table 2. Hops' essential oils compound classes and respective characteristic aroma attributes [5].

<i>Compound Class</i>	<i>Aroma attribute</i>
<i>Monoterpenes</i>	Citric, spicy, resinous, and herbaceous
<i>Sesquiterpenes</i>	Spicy, woods
<i>Esters</i>	Fruits, sweet-like
<i>Ketones</i>	Floral
<i>Aldehydes</i>	Grassy/green

Hop's phenolic compounds represent 4-14% of the dry weight of the cone flower and are responsible for a significant part of beer's bioactivity as it will be scrutinized further on. Phenolic compounds are represented in hops by five groups: chalcones (*e.g.* xanthohumol), flavanones (*e.g.* isoxanthohumol), flavonols (*e.g.* quercetin or kaempferol), flavan-3-ols (*e.g.* catechin or epicatechin), and phenolic acids (*e.g.* gallic or ferulic acids) [5, 8].

Hops have been used as a medicinal plant for more than two millennia due to its increasingly popular biological properties. Xanthohumol is often considered the most important polyphenol found in hops due to its reported sedative, antioxidant, anti-inflammatory, anti-proliferative and antimicrobial properties [5, 8]. Furthermore, the relation between hop compounds and biological properties are detailed in table 3.

Table 3. Hops' compounds associated with biological properties [5].

<i>Biological properties</i>	<i>Hop compound</i>
<i>Antioxidant activity</i>	Polyphenols, and Bitter acids
<i>Antimicrobial activity</i>	Polyphenols, Bitter acids, and Essential oils
<i>Sedative properties</i>	Resins, and Essential oils
<i>Neurodegenerative disease prevention</i>	Naringenin, and Xanthohumol
<i>Cardiovascular disease prevention</i>	Polyphenols
<i>Estrogenic activity</i>	8-prenylnaringenin
<i>Antiproliferative activity</i>	Xanthohumol, Kaempferol, and 8-prenylnaringenin
<i>Antiangiogenic activity</i>	Xanthohumol, and Isoxanthohumol
<i>Anti-inflammatory activity</i>	Xanthohumol, and 8-prenylnaringenin

Hop can be used in different preparations as detailed in table 4. Besides the traditional use of the whole cone, hops are nowadays used mostly in pellets and extract forms (Figure 4). Both latter options are the best solution for common problems like soaking liquid from the wort or having to separate the vegetative parts of the hop plant. The extract option is the most functional one since it can be pre-isomerized, and the resin-oil proportion can be personalized to obtain the desired effect on the final product. On the downside, the volatile fraction can be diminished due to loss during the extraction process [5, 6].

Table 4. Types of hops product and respective preparation process (adapted from [5]).

<i>Type of hops product</i>	<i>Preparation process</i>
<i>Fresh cones</i>	Pure state
<i>Dried cones</i>	Dried and pressed
<i>Pellets</i>	Dried, grounded and pressed
<i>Extract</i>	Cone or pellet extraction using supercritical CO ₂ , nitrogen-rich atmosphere, ethanol, or distillation methods



Figure 4. Different hop preparations for beer production.

1.1.3.1. Dry hopping

Hops are mainly added to beer during the wort boiling stage, although they can be added during or after fermentation. This last process, known as dry hopping, consists in a cold, aqueous extraction of hops and it allows the final product to retain as much flavour and aroma as possible since no volatile essential oils are boiled off. Regarding bitterness, this process does not improve this characteristic since boiling is vital to resin extraction and isomerization.

Even though dry hopping is trending in the craft beer panorama, it is still not a reality in most industrial-scale breweries due to the higher cost of hops in the form of dried cones or pellets [5].

1.1.4. Yeast

Yeasts are eukaryotic organisms used in the brewing fermentation process with the finality to produce carbon dioxide (CO_2), ethanol, and characteristic flavour compounds. They influence beer features such as turbidity, foam formation/stability, colour, aroma, or even flavour. During the yeast autolysis process, proteins, and polysaccharides (mainly glucans) are released and contribute to haze formation and turbidity. On the other hand, mannoproteins derived from the cell walls of the autolysed yeasts reduce haze formation [6, 7].

Beer's foam is also affected by yeast, mainly via the action of the two main products of the fermentative process – CO_2 and ethanol. The first increases foam formation while the

second destabilizes the foam. In the fermentation, sulphur dioxide (SO₂) is also produced by some yeast strains and is associated with colour bleaching of beer [6, 7].

Yeasts also impact beer flavour, especially aromatic characteristics (more pronounced in Lambic beers) via different metabolites produced through the different metabolic pathways, namely alcohols, aldehydes, acids, esters, ketones, volatile phenolic compounds (VPCs), terpenoids and volatile sulphur compounds (VSCs) [18]. These chemical compounds can impact the beer's aroma positively or negatively as exhibited in table 5.

Table 5. Different sensory attributes associated with yeast metabolites (adapted from [18]). Attributes that are commonly perceived negatively are underlined.

	<i>Sensory attributes</i>	<i>Examples</i>	<i>Ref.</i>
<i>Alcohols</i>	alcoholic, fruity, pungent, solvent-like and rose-like or floral.	ethanol, isoamyl alcohol, <i>n</i> -propanol, <i>etc.</i>	[19]
<i>Aldehydes</i>	green apple-like (acetaldehyde), malty (branched-chain aldehydes), worty (methional), flowery (2-phenylacetaldehyde) and almond- or cherry-like (benzaldehyde). <u>cardboard aroma</u> ((E)-2-nonenal), <u>cooked potato-like</u> (methional) and honey-like (2-phenylacetaldehyde)		[19, 20]
<i>Acids</i>	<u>cheesy, greasy and sweaty.</u>	acetic, caprylic, capric and lauric acids, <i>etc.</i>	[19, 21]
<i>Esters</i>	fruity and solvent-like (ethyl acetate), banana- and pear-like (isoamyl acetate), rose- and honey-like (2-phenylethyl acetate), or apple-like and sweet (ethyl hexanoate and ethyl octanoate).		[19]
<i>Ketones</i>	<u>Butter- or butterscotch like</u> (2,3-butanedione/diacetyl) and toffee like (2,3-pentanedione).		[19, 22]
<i>VPCs</i>	clove-like, spicy, smoky, medicinal or phenolic	guaiacol, 4-vinylsyringol, 4-vinylguaiacol, eugenol, vanillin, <i>etc.</i>	[23]
<i>Terpenoids</i>	rose-like/ floral (geraniol), fresh, coriander or lavender (linalool), lemon or lime (β-citronellol), floral, fresh, or green (nerol), and lilac (α-terpineol).		[19, 24]
<i>VSCs</i>	<u>rotten egg-like, cabbage-like, onion-like and garlic-like.</u> Accentuated fruitiness	methanethiol, ethanethiol, H ₂ S, dimethyl sulphide, dimethyl disulphide, methional, methionol, 3-mercaptohexanol, <i>etc.</i>	[19, 25]

The yeast strains are selected using different criteria such as their fermentation performance, behaviour during fermentation (bottom or top fermentation), flocculation, production or degradation of side products and propagation intensity. The most commonly

used yeasts belong to the genus *Saccharomyces*, especially the *S. cerevisiae* and *S. pastorianus* species although, due to such different criteria and demands, many brewers have their own yeast strains [6, 7].

1.1.5. Adjuncts

Adjuncts are used mainly due to economic motivation but can also be used to provide certain specific flavours or colours to the final product. This term is usually applied to ingredients that are not necessarily needed to achieve a certain beer type (*e.g.* unmalted cereals other than barley addition for economic purposes or the modification of sensory characteristics). This term also covers added sugars (*e.g.* dextrose, corn syrup, honey, *etc.*), fruits (especially relevant in the Lambic styles) or even spices [6].

The craft beer uprising has certainly changed the paradigm in terms of beer adjuncts tradition. Aiming to conceive new, alternative, bold beers, the use of ingredients that allow the brewer to achieve specific flavours and aromas has revolutionized the characteristics of numerous innovative beer styles.

Although the introduction of many adjuncts is relatively recent in the brewing industry, certain countries have established limits on the allowed percentage of adjuncts in beer brewing. In the USA, the limit for unmalted cereals in grist is 34% [7].

1.2. Brewing process

Despite the new technology and trends, the brewing process itself is very traditional, remaining fundamentally the same as during the Middle Ages. As previously mentioned, the same raw materials are used, and the brewing process is similar to that used centuries ago.

The brewing process of beer comprehends several stages. Although some can vary depending on the specific beer type/style, others are always used. The fundamental stages are malting, production of wort (mashing, lautering and wort boiling/clarification), fermentation, maturation, and downstream processing (Figure 5) [7, 26].

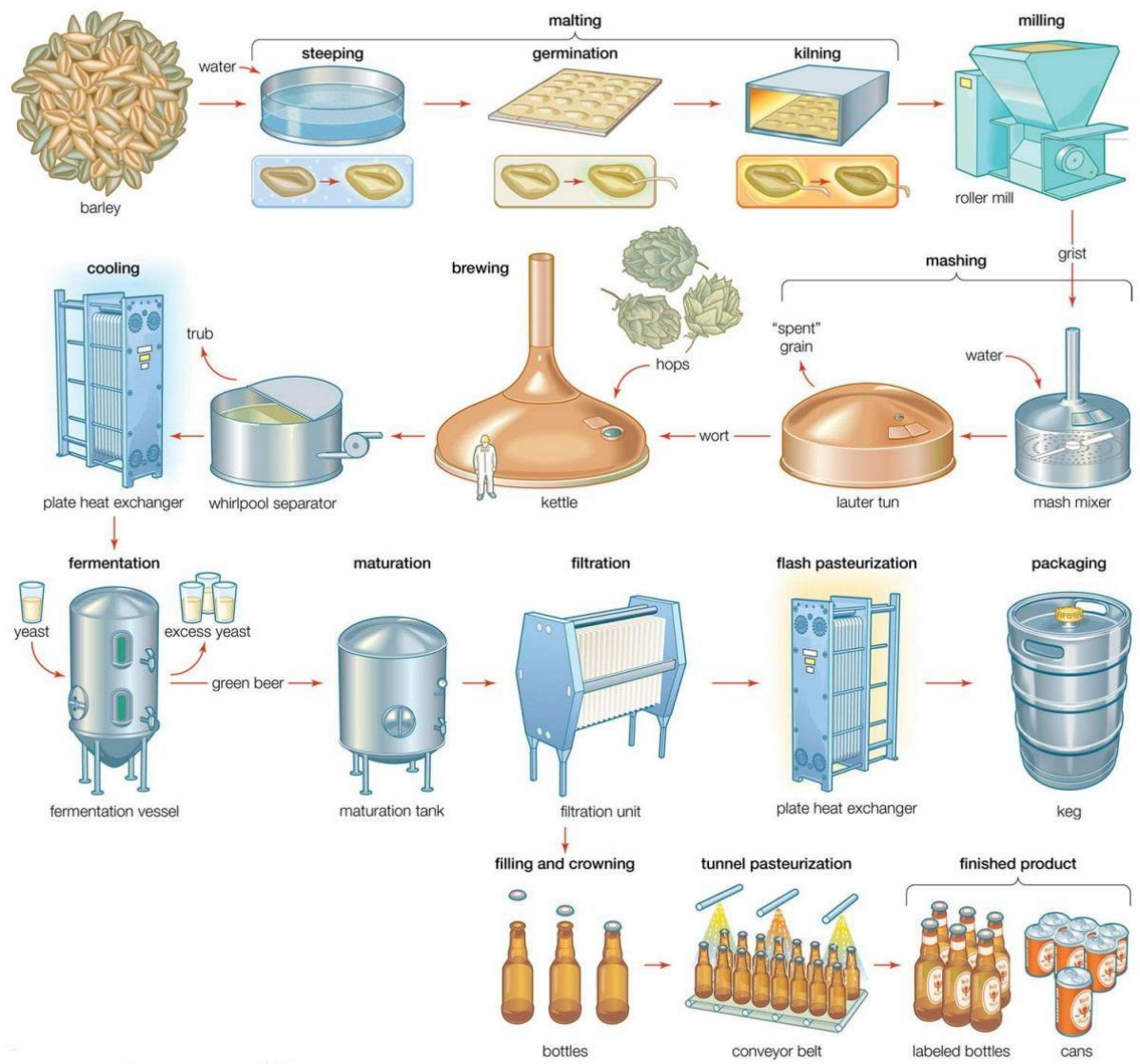


Figure 5. The brewing process of beer (adapted from [27]).

1.2.1. Malting

The malting process can be considered the artificially induced germination of a crop, more specifically barley in beer production. Its purpose is to produce/activate hydrolytic enzymes and to modify the barley kernel. The enzymes cause hydrolytic degradation of the cell wall, proteins, and starch making these constituents available for the yeast and also reduce the strength of the grain tissue [7, 26]. The process includes 3 steps: steeping, germination, and kilning.

Steeping is the initial step of the malting process. Its purpose is to provide humidity and oxygen needed for the germination of the raw barley seed. This stage consists of

“underwater periods”, where the grain is immersed in water, being uniformly hydrated, alternating with “air rest periods” where the water is drained from the grain and oxygen is provided. During the steeping, when the grain absorbs water, the embryo becomes active [7, 28, 29].

Germination is a process characterized by the growth of the embryo of the barley grain. The rootlets grow and the acrospires increase in length, with simultaneous modification of the endosperm and its constituents as represented in figure 6.

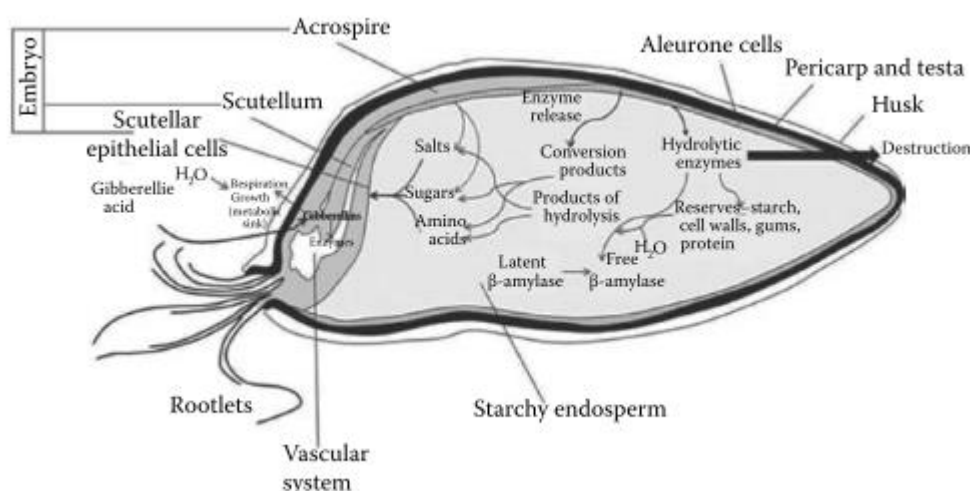


Figure 6. Scheme of the modifications that occur in the malting barley during the germination step [29].

The germinating grain is humified and, to ensure that the grain germinates homogenously, the barley is gently revolved. The main modification of the grain that occurs in this step is the large increase of the hydrolytic enzymes content. This increase causes the degradation of the energy storage substances such as starch, proteins, gums, and cell walls. As a result, the tissue strength is reduced as can be verified when analysing dry malt that, in contrast to dry barley, is friable and readily crushed, thus facilitating the milling. The optimal duration of the steeping and germinations steps is 6 days.

After the steeping and germination steps, the resulting green malt is dried in the kilning step. Typically, this step occurs in two different phases (drying and curing) aiming to increase the storage capacity of malt and to develop colour and flavour compounds.

Besides drying the malt, preventing further growth and modification, the kilning step also removes undesirable flavours, destroys some hydrolytic and other enzymes, and develops flavour and colour in the final product. While the enzyme activity decreases, the extractable bioactive compounds, such as polyphenols, present in the malt increases. The

kilning step originates diverse Maillard reaction products ($>95^{\circ}\text{C}$), including melanoidins. The resulting malt is a friable, readily milled stable product that can be stored for long periods [7, 29].

1.2.2. Milling

Previously to the mashing process, the malt is milled. Milling cracks the malt husk that surrounds the internal starches and sugar, increasing reactive surface for enzymes and, therefore, malt ingredients are easily available for the mashing process. Mashing and lautering processes are impacted by the milling quality and so is the final brewed product. The product obtained through the milling process is often called grist [7].

1.2.3. Mashing

The starch from the malt is essentially constituted by amylose (20-25%) and amylopectin (75-80%). During the mashing process, enzymes will dissolve these starch components [7].

The grist is mixed with hot water (approximately 60°C) and starch molecules are transformed from a crystalline structure to a disorganised one, more easily accessible by the amylase enzymes (gelatinizing). Gelatinized starch is digested by amylases. The α -amylase breaks the α -1,4 bonds of amylose and amylopectin producing dextrins (liquefaction). The dextrins are mainly broken down into maltose (saccharification) [7].

During mashing, proteolytic and cytolytic enzymatic reactions also take place interfering with the fullness of flavour, formation of foam, turbidity, acidity, and carbonation aspects of beer. Once finished, the mashing process originates a fermentable extract – Wort [7].

1.2.4. Lautering

In this process, the aim is to separate the liquid wort from the solid spent grist, extracting the maximum extent of fermentable materials. It is of great importance that the malt husk is cracked but still intact so it can operate as a filter [7].

First, the mash is transferred to the lauter tun and a resting period follows in order to form a grain bed. Afterwards, the liquid is drained (first wort), then the spent grist is

washed numerous times with hot water (last runnings). The spent grist washing must not be too extensive for it can excessively dilute the wort [7].

Temperature plays an important factor in this process. A high enough value must be achieved so that the viscosity is minimal, and the α -amylases are still intact [7].

1.2.5. Wort boiling and clarification

After lautering, the wort is transferred to a boiling device such as a kettle and is boiled for at least one hour. This process serves a multitude of purposes such as evaporation of unwanted flavour substances (*e.g.* DMS), the formation of colour and flavour substances, wort sterilization, enzyme inactivation, concentration of the wort, *etc* [7].

Normally, this stage is of great importance to the final product due mainly to the addition and posterior extraction of wanted compounds from hops, which will contribute to the beer's flavour, bitterness, and foam. Traditionally this process was done using whole cone hops, and nowadays this is still the reality of some breweries while others use hop pellets or liquid extracts as formerly stated (Figure 4) [7].

Hops can be added in different stages of the wort boiling. In the beginning, hop dosage mainly contributes to foaming control and bittering. When added later in the boil, hops provide characteristic aromas and flavours. Because of the boiling and addition of these bitter compounds with antimicrobial activity, beer was once considered safer to drink than tap water [7].

At the end of this stage, we encounter two products: wort and hot trub, consisting of precipitated proteins and polypeptides as well as spent hop particles [7].

Beer trub must be removed for it can aggravate yeast metabolism, clarification of green beer and filtration. The hopped wort is transferred to a whirlpool where the hot trub settles down in the centre by rotation and is collected as a bright, clarified wort almost ready to be fermented. The removed trub is often mixed in with the spent grain and is sold for cattle food [7].

Following this, the clarified wort must be cooled to prevent any contamination before the yeast is added. The desired temperature can vary depending on the type of yeast to be used. The wort is also aerated with oxygen to support yeast growth (oxygen concentration should be in the range of 8-9 mg/L) although the fermentative process is anaerobic [7].

1.2.6. Fermentation

This stage has a considerable impact on beer taste and aroma and consists of the conversion of the fermentable carbohydrates of the wort into alcohol, CO₂, and other by-products through the action of yeast in a process called alcoholic fermentation. The aerated and cooled wort must be quickly mixed with the “pitched” yeast to prevent or reduce bacteria development. The fermentative process rate is directly proportional to the temperature and the quantity of yeast that is used [7].

The resulting product of this stage is often denominated green beer, a rough and immature beer with 6-10% of residual sugars. As previously stated, the fermentative process is greatly influenced by the chosen yeast strain. The two major types of fermentation in the beer brewing process originate the most common types of commercial beer: Lager (bottom fermentation) and Ale (top fermentation) [7].

Top fermentation originates ale style beers. In this kind of fermentation, yeast *Saccharomyces cerevisiae* strains are used in a temperature range of 16 – 24 °C which allows a faster fermentation when compared to bottom-fermented beers. At the end of fermentation, top yeast becomes buoyant and tends to rise to the top of the beer due to CO₂ entrapment by the yeast flocs. Traditionally, in this type of fermentation, the yeast was removed by suction or skimming of the surface of the beer. However, with the development of cylindroconical vessels, bottom-cropping *S.cerevisiae* strains may be used [30].

Bottom fermentation generates lager-style beers. The typical lager yeast *Saccharomyces pastorianus* also known as *Saccharomyces carlsbergensis* is used in a lower temperature range (8 – 15 °C) when compared to ale yeasts. It forms a cloudy mass (floculates) near the end of the fermentative process usually being removed through the bottom of the vessel [31].

1.2.7. Maturation

The residual extract is fermented during the maturation stage to guarantee the complete absence of fermentable sugars in the final beer, as their presence would increase contamination risk and reduce digestibility. Maturation also aims to assure the production of enough CO₂, removal of oxygen, improvement of flavour and aromas. This second fermentation occurs at low temperatures with a low yeast count and it happens through the usage of the residual gravity in the green beer or through the addition of a priming sugar. During this stage, a CO₂-wash is often performed to remove undesired volatiles such as

aldehydes or sulphur compounds. Diacetyl is also degraded in this stage and the achievement of its minimum possible level defines the end of the maturation stage [6, 7].

1.2.8. Downstream processing

After maturation, a series of processes may follow aiming to enhance the shelf-life and to obtain the desired final product. These processes focus on the main identified threats to beer quality such as staling, hazing or infection. Although it is performed routinely for industrial beer, craft beer breweries normally skip some of these steps, improving certain flavours but compromising the products' shelf-life [6, 7].

The first process is, usually, cold conditioning. It takes a minimum of 3 days and it aims to allow precipitation of undesired beer solids (*e.g.* yeast, protein-polyphenol complexes, *etc.*) at refrigeration temperatures. It is not unusual the use of collagen natural sources to help sedimentation phenomena [6, 7].

It is followed by the filtration process. The most common practice uses a *kieselguhr* (diatomaceous earth) filter and alone can extend the beer's shelf-life for 2 months. The filtration allows the removal of undesired substances, but it often eliminates most of beer polyphenols and proteins too, which is not always wanted. Next in line, the stabilization process is performed. It often uses PVPP (polyvinylpyrrolidone) and/or silica gel. The former removes polyphenols and the latter removes proteins from the beer reducing its haziness. Finally, microbiological quality is assured using pasteurization techniques. Usually, flash pasteurization is used in kegged beer and tunnel pasteurization in bottled beer [6, 7].

1.3. Biological properties of beer

Information about beer's bioactivity is not so commonly found in literature, as it is for other widely consumed alcoholic beverages, for instance, wine. A search in the Scopus bibliographic database (www.scopus.com) using the terms "bioactivity" and "beer" included in the titles, abstracts or key-words retrieved 40 papers, by October 2020, whilst for wine 189 papers were found. Although the literature provides valuable information about the beer bioactive properties as a whole matrix, most studies are related to the individualized role of beer constituents reason why in the next sections both individual compounds found in beer and the beverage as a complex matrix will be detailed for each of the most important health-promoting properties described.

1.3.1. Antioxidant activity

As it is widely known, the oxidative stress caused by an increased reactive oxygen and nitrogen species (ROS and RNS, respectively) formation is associated with deleterious effects in various cellular components and has been recognized to be involved in the pathology of various diseases such as neurodegenerative diseases, diabetes, cancer, and atherosclerosis [32]. Different dietary antioxidants like the ones present in beer can prevent, minimize, or even repair the oxidative stress events [33]. In table 6, different beverages and their respective antioxidant capacity are displayed. Beer's overall antioxidant capacity, assessed by two different *in vitro* assays, is closest to white wine thus may constitute a healthier alternative due to its lower ethanol content [34, 35].

Besides their biological activity, these compounds are of particular interest due to technological roles they play such as physicochemical stability, maintenance of foam and extension of the end-product shelf-life [36]. The increase of endogenous antioxidant activity (AO) and the reduction of added antioxidants are the ultimate goal to solve the technological challenges and to provide the consumer with a beverage with potential health benefits [37].

The AO of beer mainly comes from endogenous compounds such as phenolic compounds, both from barley and hops, Maillard reaction products (MRPs), sulphites and other added antioxidant compounds [33, 38].

Table 6. Overview of the antioxidant capacity of different beverages assessed by two *in vitro* assays [37].

Beverage	FRAP ($\mu\text{mol.TE} / 100 \text{ ml}$)	ABTS ($\mu\text{mol.TE} / 100 \text{ ml}$)	Ref.
Lager beer	139.6 – 149.5	220.0 – 305.6	[34, 35]
Dark beer	278.8	259.0 – 536.5	[34, 35]
Alcohol-free beer	75.6 – 91.2	155.8 – 175.3	[34, 35]
<i>Coffee</i>	2267 $\pm$ 18.9	1328 $\pm$ 5.1	[39, 40]
<i>Tea</i>	601 $\pm$ 5.5	631 $\pm$ 8.0	[39, 40]
<i>Red wine</i>	1214 $\pm$ 24.5	1093 $\pm$ 54.2	[39, 40]
<i>White wine</i>	154 $\pm$ 36.8	181 $\pm$ 22.2	[39, 40]
<i>Rose wine</i>	286 $\pm$ 39.2	261 $\pm$ 23.7	[39, 40]
<i>Orange juice</i>	515 $\pm$ 41.5	249 $\pm$ 3.4	[39, 40]
<i>Cola</i>	20.7 $\pm$ 0.7	≤ 10	[39, 40]

1.3.1.1. Phenolic compounds

Several epidemiological studies suggest that there is a correlation between the intake of dietary polyphenols and a decrease in the incidence of oxidative stress-related diseases [36]. The most commonly found polyphenols in beer (table 8) are simple phenols, phenolic acids, benzoic acid derivatives, cinnamic acids, flavanols, flavonols, isoflavones and flavones [41]. The most abundant, displayed in figure 7, are ferulic acid, sinapic acid (hydroxycinnamic acids) and gallic acid (hydroxybenzoic acid) [36, 42]. Beer polyphenols can exhibit their AO through chelation of metal ions, inhibit fatty acid oxidation or preventing the formation of staling aldehyde precursors [43]. The polyphenol profile from barley doesn't necessarily overlap with the one obtained from the final product due to technological processes such as malting or filtration [44].

The polyphenols found in beer originate from malt (≈ 70 -80%) and hops (≈ 20 -30%) [42, 44], with Zhao *et al.* [37] reporting ferulic acid, syringic acid, (+)-catechin, caffeic acid, protocatechuic acid and (-)-epicatechin being the main contributors, with approximately 55.0-88.1%, to the AO of beer.

Several studies reported the total phenolic content (TPC) varied broadly between different beer samples. In table 8, the most relevant phenolic compounds obtained from beer's raw materials are displayed. It should be noted that there is only a partial recovery of those phenolic compounds in the final product [38].

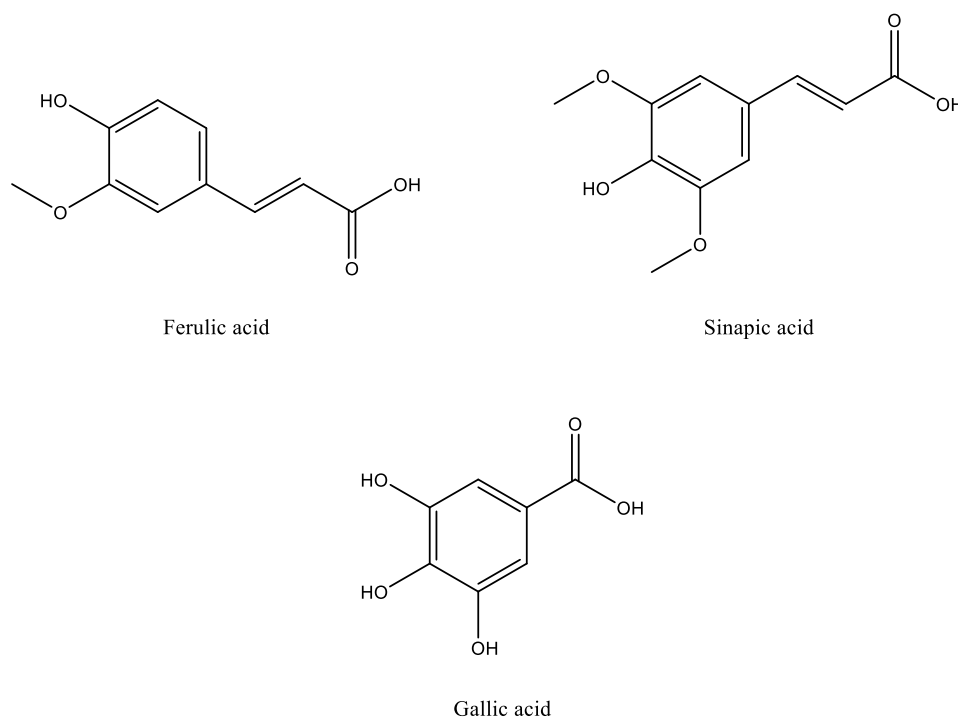


Figure 7. The most abundant phenolic acids found in beer [33, 36, 42].

There is also an established relationship between TPC, beer style and AO with various studies reporting darker beers to have higher TPC and higher AO while dealcoholized beer present the lowest values of TPC and AO [36, 42]. Piazzon *et al.* [42] reported that the TPC increased in the following order: dealcoholized < lager < pilsner < wheat < ale < abbey < bock, and even stated that a studied bock beer presents a 3-fold higher AO than a dealcoholized beer. The noteworthy differences found between the AO of the dealcoholized and the traditional beers are often justified with the low original extract content, limited fermentation time, short contact with brewing yeast and poor solubility of phenolic compounds [41, 42].

Moreover, Zhao *et al.* [37] stated that beers with higher original gravity also presented higher TPC, backing that phenolic compounds originate, mainly, from barley and hops, although the TPC is impacted by the brewing process. In table 7, we encounter the extremely variable TPC in different lager beers. The differences can be explained by the technological process and the original gravity of the wort.

Table 7. TPC in Lager beers described by different authors, assessed through the Folin-Ciocalteu method.

TPC (mg.GAE L⁻¹)	Ref.
88.47 – 238.33	Zhao <i>et al.</i> (2013) ^[45]
156.49 – 201.15	Obruča <i>et al.</i> (2009) ^[46]
152.01 – 339.12	Zhao <i>et al.</i> (2010) ^[37]
270 – 470	Lugasi <i>et al.</i> (2003) ^[47]
452	Piazzon <i>et al.</i> (2010) ^[42]

It is also important to highlight that besides the increasingly contested *in vitro* assays based studies [48] there are also different articles, whose methodologies use human plasma, that sustain the hypothesis that beer increases AO and focus on the polyphenols role [49, 50]. In one of these studies, Ghiselli *et al.* [49] determined that 500 mL of lager beer increased the plasma antioxidant capacity by up to 17%, 1 hour after ingestion.

Table 8. Comparison between concentrations of the most relevant phenolic compounds from barley, before and after the malting process, and from hop dry samples.

	Barley mg/100 g	Ref	Malt mg/100 g	Ref	Hops mg/100 g	Ref
Benzoic acid derivatives						
<i>p</i> -hydroxybenzoic acid	0.59	[51]	0.42	[51]	0.8	[52]
vanillic acid	0.16-0.76	[53]	0.29-0.94	[53]	2.9	[52]
gallic acid	0.03-0.09	[53]	0.01-0.03	[53]	0.3	[52]
syringic acid	0.03-0.09	[53]	0.03-0.16	[53]	0.5	[52]
Cinnamic acids						
<i>p</i> -coumaric acid	0.01-0.24	[53]	0.15-1.0	[53]	0.7	[52]
caffeic acid	0.03-0.21	[53]	0.03-0.19	[53]	0.6	[52]
ferulic acid	1.44-2.19	[53]	0.73-5.61	[53]	6.4	[52]
Flavanols						
(+)-catechin	1.10-1.70	[53]	0.09-1.21	[53]	4.9	[52]
(-)-epicatechin	1.02-1.59	[53]	0.01-0.06	[53]	1.1	[52]
Flavonols						
quercetin	0.03-0.08	[53]	0.01-0.11	[53]	8.1	[52]
rutin	0.01-0.09	[53]	0.01-0.09	[53]	2.0	[52]

1.3.1.2. Maillard reaction products

The Maillard reaction is named after the French scientist Louis Maillard, who first described this type of non-enzymatic browning in 1912. It involves the reaction of carbonyl groups, mainly from reducing sugars, and compounds with a free amine group, such as proteins, amines or amino acids with amine groups in their side-chains (lysine, arginine, tryptophan, and histidine), and it usually requires heat [54, 55].

Melanoidins (MLDs) stand out from the Maillard reaction products (MRPs) due to their antioxidant potential and complexity. While the chemical structure of these compounds is broadly unknown, MLDs are usually defined as a group of high molecular weight (>3500 Da), brown-coloured, nitrogenous compounds [38, 55]. In beer, they mainly originate in the malting process during the kilning stage where the content of MLDs is proportional to the colour and the kilning degree of malt [38].

Various studies showed that MLDs display AO through chain breaking, oxygen scavenging, free radical scavenging, and metal chelating mechanisms without cytotoxic effects [33]. Although their promising AO potential, MLDs have shown little bioavailability

in the upper gastrointestinal tract and, in some conditions, even presented pro-oxidant activity [33, 38].

1.3.1.3. Sulphites

In beer, sulphites are produced by yeast and originate as a by-product of the synthesis of sulphur-containing amino acids during fermentation. They act as antioxidants and prevent beer staling phenomena due to the reaction with aroma compounds transforming them into non-volatile sulphite adducts. The beer's sulphites content depends on the used brewer's yeast and the global brewing process with lager yeast strains producing higher concentrations of SO₂ [38].

2.4.2. Chemopreventive and antiproliferative activity

Regarding the anti-cancer activity of beer, most studies available in literature highlight the role played by hops, particularly emphasising one flavonoid: xanthohumol (XN). In table 9, various studies about XN's anti-cancer activities are shown.

In addition to XN, other compounds present in hops have shown similar biological activity towards cancer prevention or proliferation, although being less effective. Different authors have exalted IX's antiproliferative effect in several types of cancer cells, namely prostate or colon cancers [56-59]. 8-prenylnaringenin is also frequently cited in literature as a compound with anticarcinogenic potential in gliomas and other types of cancer cells [60, 61]. Despite their biological activities being less reported when compared to the prenylflavonoids, the hops' bitter acids (humulones and lupulones) have also been reported as biologically active towards cancer development [59, 62].

Table 9. XN anti-cancer activities (adapted from [59]).

<i>Cancer type</i>	<i>Cell lines</i>	<i>Dose/IC₅₀ (μM)</i>	<i>Result</i>	<i>Ref.</i>
<i>Respiratory system</i>	NSCLC	A549	5-25 ↓ Cell viability ↓ Proliferation ↑ Apoptosis	Slawinska-Brych <i>et al.</i> (2016) [63]
			5-30 ↑ Apoptosis	Zhang <i>et al.</i> (2015) [64]
			14, 28, 42 ↓ Proliferation ↑ Apoptosis	Yong <i>et al.</i> (2015) [65]
<i>Lymphatic and hematopoietic</i>	Leukemia	L1210	2.5, 5, 10 ↓ Cell viability ↑ Apoptosis ↓ Migration ↓ Invasion	Benelli <i>et al.</i> (2012) [66]
		KBM-5	50 ↑ Apoptosis ↓ Invasion	Harikumar <i>et al.</i> (2009) [67]
		K562	2,5, 5, 10 ↓ Cell viability ↑ Apoptosis ↓ Invasion ↓ Adhesion	Monteghirfo <i>et al.</i> (2008); Vakana <i>et al.</i> (2011) [68, 69]
<i>Digestive system</i>	Hepatocellular carcinoma	Huh-7, HepG2, Hep3B and SK-Hep-1	5 ↓ Cell viability ↓ Colony formation ↓ Confluence ability	Kunnimalaiyaan <i>et al.</i> (2015) [70]
		HepG2	10 None	Plazar <i>et al.</i> (2007) [71]
		HA22T/VGH and Hep3B	IC ₅₀ = 108, 166 None	Ho <i>et al.</i> (2008) [72]
	Cholangiocarcinoma	KKU-M214 and KKU-M139	20,50 ↓ Proliferation	Dokduang <i>et al.</i> (2016) [73]

Table 9. XN anti-cancer activities (cont.).

<i>Cancer type</i>	<i>Cell lines</i>	<i>Dose/IC₅₀ (μM)</i>	<i>Result</i>	<i>Ref.</i>		
<i>Digestive system</i>	Pancreatic cancer	PANC-1 and BxPC-3	5-100	↓ Cell viability ↓ Colony formation ↑ Apoptosis	Jiang <i>et al.</i> (2015) [74]	
		AsPC-1, PANC-1, L3.6pl, MiaPaCa-2	10	↓ Proliferation	Kunnimalaiyaan <i>et al.</i> (2015) [75]	
	Colorectal cancer	HT-29	0.1-100	↓ Cell viability	Miranda <i>et al.</i> (1999) [76]	
		HCT-115	IC ₅₀ = 10.2	↓ Proliferation	Lee <i>et al.</i> (2007) [77]	
		HT-29 and SW620	IC ₅₀ = 1.2 ± 0.9, 2.5 ± 0.2	↓ Proliferation	Hudcová <i>et al.</i> (2014) [78]	
		CACO-2	0.313-20 μg/mL	None	Machado <i>et al.</i> (2017) [59]	
		SW480, SW620 and CACO-2	IC ₅₀ = 3.6-7.3	↓ Proliferation	Ambrož <i>et al.</i> (2019) [79]	
		<i>Genitourinary organ</i>	Breast cancer	MDA-MB-231 and Hs578T	IC ₅₀ = 6.7, 4.78	↓ Proliferation ↓ Cell invasion
MDA-MB-231	10, 20			↓ Cell viability	Yoo <i>et al.</i> (2014) [81]	
MCF-7	10			↓ Proliferation	Guerreiro <i>et al.</i> (2007) [82]	
MCF-7/ADR	10			↓ Cell viability ↑ Apoptosis ↓ Stemness ↑ Chemo-sensitizing ↑ Radio-sensitizing	Kang <i>et al.</i> (2013) [83] Liu <i>et al.</i> (2016) [83]	
Prostate cancer	LNCaP, PC-3 and DU145			20, 40	↓ Cell viability ↑ Apoptosis	Deeb <i>et al.</i> (2010) [84]
	PC-3			2.5-20	↓ Cell viability ↑ Apoptosis	Colgate <i>et al.</i> (2007) [85]

Table 9. XN anti-cancer activities (cont.).

<i>Cancer type</i>	<i>Cell lines</i>	<i>Dose/IC₅₀ (μM)</i>	<i>Result</i>	<i>Ref.</i>
<i>Genitourinary organ</i>	Prostate cancer	PC-3 and DU145	2.5, 5, 10	↓ Proliferation ↓ Cell migration ↓ Cell invasion Vene <i>et al.</i> (2012) [86]
	Cervical cancer	Ca Ski	IC ₅₀ = 59.96 + 1.95, 34.01 + 1.13, 20.08 + 1.12	↓ Proliferation ↑ Apoptosis Yong <i>et al.</i> (2015) [87]
	Ovarian cancer	A-2780	IC ₅₀ = 0.52, 5.2	↓ Proliferation
SKOV3, OVCAR3		10, 20, 30	↓ Proliferation	Drenzek <i>et al.</i> (2011) [88]
<i>Head and neck cancers</i>	Glioblastoma	U87	25	↓ Cell viability ↑ Apoptosis Chen <i>et al.</i> (2016) [89]
		T98G	20	↓ Cell viability ↑ Apoptosis Festa <i>et al.</i> (2011) [90]
		Thyroid cancer	MTC	10, 20, 30
	TPC-1		10-100	↓ Proliferation
	Laryngeal squamous cell carcinoma	RK33 and RK45	IC ₅₀ = 12.3, 22.5	↓ Cell viability ↑ Apoptosis Slawinska-Brych <i>et al.</i> (2015) [93]
		SCC4	20, 30, 40	↓ Proliferation ↑ Apoptosis
	<i>Other cancers</i>	Melanoma	B16	0.5, 1, 5, 10
SK-MEL-2			IC ₅₀ = 14.4	↓ Proliferation

2.4.3. Other bioactive properties

Information related to other bioactive properties of beer is rather scarce, as previously stated. Nevertheless, there is a multitude of information regarding the individualized role of beer constituents, detailed in table 10.

Table 10. Beer components and their biological activities (adapted from [100]).

Beer component	Biological activity	Model	Refs.
XN	Anti-inflammatory	– Male senescence-accelerated prone mice (SAMP8). – BALB/c mice.	Dorn <i>et al.</i> (2013) ^[96] Rancan <i>et al.</i> (2017) ^[97]
	Anti-microbial	– In vitro assays against <i>B. fragilis</i> , <i>C. perfringens</i> and <i>C. difficile</i> strains.	Cermak <i>et al.</i> (2017) ^[98]
	Hypoglycaemic	– Diet-induced obese C57BL/6J mice.	Miranda <i>et al.</i> (2016) ^[99]
	Anti-obesity	– Murine 3T3-L1 adipocytes and primary human subcutaneous preadipocytes.	Samuels <i>et al.</i> (2018) ^[100]
8- <i>prenyl</i> naringenin	Phytoestrogen	– Prospective, double-blind, placebo-controlled study, human trial.	Heyerick <i>et al.</i> (2006) ^[101]
		– Rat calvarial osteoblasts.	Erkkola <i>et al.</i> (2010) ^[102]
		– Osteoclasts from the bone marrow of femora and tibiae of rabbits.	Aghamiri <i>et al.</i> (2016) ^[103] Ming <i>et al.</i> (2013) ^[104]
	Antidiabetic	– Type 2 diabetes mellitus mice model.	Costa <i>et al.</i> (2017) ^[105]
Bitter acids	Anti-inflammatory	– Mouse fibrosarcoma L929SA cells.	Van Cleempunt <i>et al.</i> (2009) ^[106]
	Anti-obesity	– Diet-induced obese rodent model	Yajima <i>et al.</i> (2005) ^[107]
	Antidiabetic	– C57BL/6N Mice.	Yajima <i>et al.</i> (2004) ^[108]

2.5. Nutritional value

Regarding nutritional value, one average beer (350 mL) accounts for about 2.6% of the daily requirement of carbohydrates and 2.1% for proteins in a diet according to FDA recommendations. Based on those recommendations, beer can provide almost 5% of the energy requirement for males and about 4% for females. Besides macronutrients, beer is also a source of multiple vitamins and minerals mainly potassium, calcium, magnesium, and phosphorous [109]. In table 11, beer's content regarding the above-mentioned parameters is detailed, as well as compared to red wine.

Table 11. Macronutrients, vitamins and minerals of beer and wine (adapted from [113]).

<i>Parameters</i>	<i>Daily requirement Male/Female (19-50)*</i>	<i>Beer 4 % (ABV) 350 mL</i>	<i>Red wine 350 mL</i>
<i>Energy (Kcal)</i>	2140/2676	105	266
<i>Carbohydrate (g)</i>	300 †	7.7	0.7
<i>Protein (g)</i>	50 †	1.05	0.35
<i>Fat (g)</i>	65 †	Traces	0
<i>Alcohol (g)</i>	----	10.15	37.45
<i>Calcium, Ca (mg)</i>	700	28	24.5
<i>Magnesium, Mg (mg)</i>	270/300	24.5	38.5
<i>Iron, Fe (mg)</i>	14.8/8.7	0.35	3.15
<i>Copper, Cu (mg)</i>	1.2	0.035	0.21
<i>Zinc, Zn (mg)</i>	7.0/9.5	0.35	0.35
<i>Manganese, Mn (mg)</i>	2.0 †	0.105	0.35
<i>Selenium, Se (mg)</i>	60/75	Traces	Traces
<i>Phosphorous, P (mg)</i>	550	49	45.5
<i>Potassium, K (mg)</i>	3500	112	385
<i>Sodium, Na (mg)</i>	1600	21	24.5
<i>Thiamin (mg)</i>	0.8/1.0	Traces	Traces
<i>Riboflavin (mg)</i>	1.1/1.3	0.105	0.07
<i>Niacin (mg)</i>	13/17	0.7	0.35
<i>Pantothenic acid (mg)</i>	10 †	0.175	0.14
<i>Vitamin B6 (mg)</i>	1.2/1.4	0.245	0.105
<i>Vitamin B12 (µg)</i>	1.5	Traces	Traces
<i>Biotin (µg)</i>	300 †	3.5	7
<i>Folate, DFE (µg)</i>	200	17.5	3.5

† - FDA recommendations

*Mean of 19 to 54 years old

2. Wood

The application of wood in ageing processes is a common technological procedure and has been used for centuries to mature alcoholic beverages such as wine and spirits. While mostly used to improve colour, structure, and certain flavours, wood also seems to contribute to an increase in the antioxidant capacity due to the important amount of extractable phenolic compounds [110, 111]. Nowadays, with the uprising of the craft beer movement, wood has been exploited to obtain specific flavours and create beer styles recognized and grouped by the *Beer Judge Certification Program (BJCP) 2015 style guidelines* under the category named wood-aged beer [112].

This frequent extraction of wood constituents to the beverage is known as additive ageing and it's mostly a desirable outcome, although it should be taken into account that extractable compounds such as polyphenols could negatively impact beer colloidal stability by prompting haze formation due to protein–polyphenol complexes [113-115]. In addition to the beneficial effects on the aged beverages, the consequences of the wood ageing process are also associated with subtractive ageing, the loss of compounds due to wood adsorption or evaporation [114, 115].

2.1. Types and preparations

The interest in bioactivity increment from wood aged beverages has benefited from a recent change in the paradigm of wood-aged beverages. The technological process has begun to shift from immersing the beverage in wood to the other way around. By adding wood chips, spirals, *etc.* (figure 8) to the beverage, extractable surface area from the wood greatly increases when compared to wooden barrels (where, approximately, only 40% of the wood surface is used). Although allowing to achieve a more efficient extraction of phenolic compounds it may impart some changes to the products mostly due to diminished micro-oxygenation [110].

The extraction of phenolic compounds and consequently increased antioxidant capacity depends on many factors such as wood type, origin, degree of toasting, and format. The most popular type of wood selected for this purpose is Oak (*Quercus* L.) wood from different origins, such as France or America, that is reported to possess significant amounts of phenolic compounds, detailed in table 12. The toast degree of the wood is also crucial for

the amounts of individual phenolics as different compounds are impacted differently by the toasting process.



Figure 8. Different wood preparations (oak wood chips and spiral) for the maturation of beverages.

Table 12. Concentration of phenolic compounds ($\mu\text{g}\cdot\text{g}^{-1}$) in natural and toasted oak wood from different origins (adapted from [111]).

Compound	American oak wood		French oak wood		Hungarian oak wood		Romanian oak wood		Russian oak wood	
	<u>Natural</u>	<u>Toasted</u>	<u>Natural</u>	<u>Toasted</u>	<u>Natural</u>	<u>Toasted</u>	<u>Natural</u>	<u>Toasted</u>	<u>Natural</u>	<u>Toasted</u>
gallic acid	396.1 $\pm$ 10.6	249.6 $\pm$ 6.1	442.8 $\pm$ 5.4	343.5 $\pm$ 8.0	392.8 $\pm$ 6.6	146.3 $\pm$ 7.7	220.2 $\pm$ 0.7	195.5 $\pm$ 10.9	149.4 $\pm$ 2.0	110 $\pm$ 6.0
protocatechuic acid	221.5 $\pm$ 4.7	81.7 $\pm$ 8.7	256.0 $\pm$ 9.3	130.2 $\pm$ 9.6	242.8 $\pm$ 12.3	82.9 $\pm$ 4.0	231.5 $\pm$ 11.4	66.1 $\pm$ 5.6	135.1 $\pm$ 8.0	23.9 $\pm$ 1.5
protocatechuic aldehyde	nd	112.3 $\pm$ 2.0	nd	116.0 $\pm$ 5.9	9.9 $\pm$ 1.8	32.4 $\pm$ 4.2	nd	11.2 $\pm$ 0.6	nd	34.8 $\pm$ 4.7
vanillic acid	91.6 $\pm$ 8.5	129.9 $\pm$ 3.2	87.1 $\pm$ 14.8	140.2 $\pm$ 5.2	70.8 $\pm$ 0.7	96.8 $\pm$ 1.9	45.7 $\pm$ 1.0	43.2 $\pm$ 0.3	39.6 $\pm$ 2.7	70.6 $\pm$ 9.4
caffeic acid	101.0 $\pm$ 2.6	26.4 $\pm$ 0.9	89.5 $\pm$ 1.1	18.3 $\pm$ 1.1	36.6 $\pm$ 2.0	4.6 $\pm$ 0.5	46.9 $\pm$ 2.1	6.1 $\pm$ 0.5	46.0 $\pm$ 0.9	7.5 $\pm$ 0.5
vanillin	106.4 $\pm$ 10.8	210.2 $\pm$ 3.7	94.7 $\pm$ 10.2	204.0 $\pm$ 10.1	76.7 $\pm$ 7.7	192.0 $\pm$ 0.1	45.6 $\pm$ 2.7	133.5 $\pm$ 1.1	37.0 $\pm$ 1.8	208.9 $\pm$ 9.9
syringaldehyde	97.9 $\pm$ 7.3	523.8 $\pm$ 4.2	41.8 $\pm$ 0.5	597.4 $\pm$ 7.5	57.6 $\pm$ 1.7	633.8 $\pm$ 8.3	19.7 $\pm$ 0.2	471.6 $\pm$ 14.0	55.5 $\pm$ 0.6	657.5 $\pm$ 10.4
p-coumaric acid	27.5 $\pm$ 1.0	20.4 $\pm$ 1.8	31.5 $\pm$ 0.7	25.9 $\pm$ 3.3	32.7 $\pm$ 2.5	26.5 $\pm$ 0.4	18.8 $\pm$ 0.3	16.2 $\pm$ 0.7	20.5 $\pm$ 0.8	15.6 $\pm$ 0.6
scopoletin	54.6 $\pm$ 2.5	35.0 $\pm$ 2.0	25.0 $\pm$ 3.0	21.1 $\pm$ 0.5	27.5 $\pm$ 1.5	18.2 $\pm$ 1.5	22.9 $\pm$ 1.0	15.4 $\pm$ 1.5	30.4 $\pm$ 2.5	16.1 $\pm$ 1.5
ferulic acid	59.0 $\pm$ 3.6	85.9 $\pm$ 3.0	54.1 $\pm$ 0.6	74.2 $\pm$ 4.8	24.6 $\pm$ 1.5	29.9 $\pm$ 0.9	20.5 $\pm$ 1.0	28.0 $\pm$ 1.2	20.4 $\pm$ 1.5	32.0 $\pm$ 2.2
sinapic acid	41.2 $\pm$ 2.8	61.1 $\pm$ 3.5	25.1 $\pm$ 1.0	53.9 $\pm$ 3.5	17.5 $\pm$ 3.5	46.5 $\pm$ 3.7	26.2 $\pm$ 1.1	48.6 $\pm$ 3.0	10.4 $\pm$ 0.5	39.1 $\pm$ 3.8
coniferaldehyde	111.2 $\pm$ 9.3	352.1 $\pm$ 9.1	112.5 $\pm$ 12.2	298 $\pm$ 2.4	17.3 $\pm$ 3.9	198.3 $\pm$ 11.5	50.3 $\pm$ 0.8	221.9 $\pm$ 8.6	39.4 $\pm$ 0.7	220.8 $\pm$ 12.4
sinapaldehyde	261.2 $\pm$ 9.6	1112 $\pm$ 19.4	305.7 $\pm$ 8.5	1043.0 $\pm$ 15.7	68.4 $\pm$ 3.1	605.7 $\pm$ 13.4	154.6 $\pm$ 3.1	590.0 $\pm$ 8.7	87.3 $\pm$ 12.4	780.5 $\pm$ 11.9
ellagic Acid	199.1 $\pm$ 8.7	236.0 $\pm$ 3.55	206.6 $\pm$ 9.1	267.0 $\pm$ 8.0	126.1 $\pm$ 6.9	199.3 $\pm$ 6.1	85.8 $\pm$ 6.1	247.5 $\pm$ 4.5	99.4 $\pm$ 3.7	154.8 $\pm$ 4.4

II. Research work

This work aims to clarify the effects of a wood-ageing process in beer bioactivity, through the addition of oak wood chips in the maturation stage of the brewing process. From a bioactivity standpoint, analysis regarding the total phenolic compounds content (*in vitro* analysis) and related biological activities in human cells (antiproliferative, antioxidant and anti-inflammatory activities) were carried out to better understand the potential enrichment process. Furthermore, the phenolic profile of the beer samples was tentatively identified through HPLC-DAD analysis.

1. Chemicals and materials

1.1. Beer production

The beer ingredients consisting of Pale Ale (Germany), chocolate (USA), and black (USA) malts, hops from the Centennial variety (USA), and the yeast Fermentis SafAle S-04 (Marcq-en-Barœul, France) were acquired from a local homebrew store. The wood chips used in the wood-ageing process were obtained from J. Dias & C^a, SA (Espinho, Portugal).

1.2. *in vitro* bioactivity assessment

Aluminium chloride hexahydrate ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$), (+)-catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH $\cdot$), gallic acid ($\geq 98\%$ purity), hydrochloric acid (HCl), iron (III) chloride (FeCl_3), iron (II) sulphate heptahydrate (FeSO_4), methanol (MeOH), sodium carbonate (NaCO_3), sodium hydroxide (NaOH), sodium nitrite (NaNO_2), 2,3,5-Triphenyltetrazolium chloride (TPTZ), and Trolox (97% purity) were acquired from Sigma-Aldrich Corp. (St. Louis, MO, USA). The absolute ethanol (EtOH), Folin-Ciocalteu reagent (F-C) was purchased from Merck (Darmstadt, Germany). The sodium acetate buffer solution (CH_3COONa) was obtained from Fisher Scientific (Hampton, NH, USA). The 96-well clear bottom microplates were bought from Corning (New York, USA).

1.3. HPLC-DAD phenolic compounds analysis

The solvents (HPLC grade) acetonitrile (ACN), ethyl acetate and methanol (MeOH) were obtained from Honeywell, Riedel-de-Haën (Seetze, Germany), whereas the acetic acid (LC-MS grade) was obtained from Biosolve Chimie (Dieuze, France). The sodium chloride (NaCl) was purchased from Sigma-Aldrich corp. (St. Louis, MO, USA) whereas the 0.45 µm PTFE membrane filter was bought from Agela Technologies (Wilmington, DE, USA). Ultrapure (up) water of 0.055 µS.cm⁻¹ was obtained through a Seralpur Pro 90CN system (Seral, Ransbach-Baumbach, Germany).

The standards (-)-epicatechin, (+)-catechin, 3,4-dihydroxybenzyl alcohol, 3,4-dihydroxyphenylacetic acid, 4-hydroxycoumarin, caffeic acid, carvacrol, chlorogenic acid, ellagic acid, gallic acid, gentisic acid, kaempferol, *m*-hydroxybenzoic acid, myricetin, naringenin, naringin, *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, quercetin, salicylic acid, sinapic acid, syringic acid, *trans*-cinnamic acid, *trans*-ferulic acid, vanillic acid, vanillin were purchased from Sigma-Aldrich corp. (St. Louis, MO, USA). Isoxanthohumol, syringaldehyde, and xanthohumol were purchased from Extrasynthese (Lyon, France).

1.4. Cellular assays

The human colorectal adenocarcinoma Caco-2 cell line (passages 82–87) was supplied by the “Molecular Physical-Chemistry” Research Group of the University of Coimbra, Portugal, whereas the murine macrophage RAW 264.7 cell line (passages 6–7) was obtained from the American Type Culture Collection (LGC Standards S.L.U., Barcelona, Spain).

High glucose Dulbecco’s modified Eagle’s medium (DMEM), Minimum essential medium non-essential amino acids (MEM NEAA) 100x, GlutaMAX™ 100x, fetal bovine serum (FBS), 0.25% Trypsin-EDTA solution and Penicillin/Streptomycin 100x solution (10,000 Units.ml⁻¹/10,000 µg.ml⁻¹) were all purchased from Gibco/Life Technologies corporation (Paisley, United Kingdom). 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (DCF-DA), dimethyl sulfoxide (DMSO), the phosphoric acid (H₃PO₄), Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), *N*-[naphth-1-yl]ethylenediamine dihydrochloride (NED),

phosphate-buffered saline (PBS), and the sulphanilamide were acquired from Sigma-Aldrich Corp. (St. Louis, MO, USA).

The T-75, T-25 flasks and 96 well plates for cell assays were obtained from Sarstedt (Nümbrecht, Germany) whereas the 96-well black plates with clear bottom for the fluorescence assays were obtained from Greiner Bio-One (Frickenhausen, Germany). The 0.22 μm nylon syringe filters were purchased from CHM (CHMLAB GROUP, Terrassa-Barcelona, Spain).

2. Methods

2.1. Brewing process

The brewing process took place in the laboratory using common homebrewing equipment, namely two Klarstein electric boilers. To start the brewing process, theoretical calculations of the ingredient's quantity were made using an online platform called *Brewer's friend* [116] as depicted in table 13. The calculated quantities of each malt grain were milled, preserving the husk's integrity, and, simultaneously, the calculated quantity of water for the mashing step was added to the boiler and heated to approximately 75 °C. Then, in the mashing step, the grist (milled malt grain) was added to the boiler and frequently revolved for 60 minutes at approximately 65 °C. At the end of the mashing step, the absence of starch was assessed using an iodine solution, the temperature was raised to around 78 °C for enzymatic inactivation, and, finally, the wort was obtained. Next, the wort clarification process was done through recirculation of the wort through the boiler and, subsequently, filtration through the malt husks. Hot water was added until the calculated pre-boil quantity of wort was obtained. Furthermore, the wort was transferred to the boiler and was boiled for 60 minutes. Around the 30-minute mark, hops were added to the wort. After cooling down, the wort was transferred to a fermenter and mixed with the yeast. It was then fermented for 7 days at 20 °C, matured for 5 days at 4 °C, divided into 1.5 L plastic bottles, and aged with French and American HiVan wood chips at 2 g.L⁻¹, for 21 days at 22 °C, according to supplier instructions (figure 9). Wood chips were selected considering their uniformity in order to increase the reproducibility of the wood addition to replicates. The final beer was aliquoted in 15 and 50 mL falcon tubes (according to the sampling scheme

presented in figure 10) and stored at -20 °C, except for the samples destined to the cell culture assays, which were frozen at -80 °C and then lyophilized for ethanol removal.

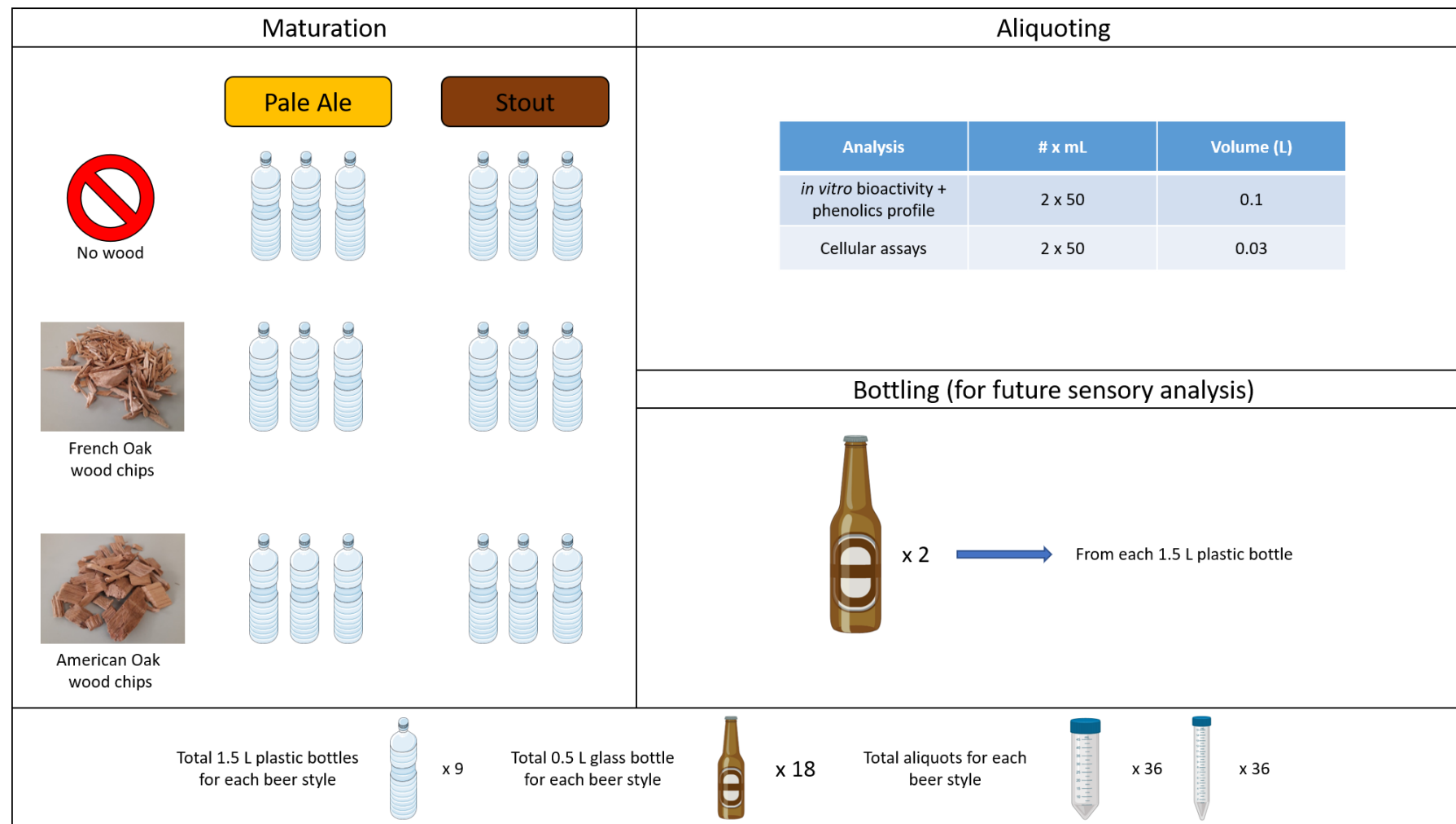
Table 13. *Brewer’s friend* theoretical calculations for the brewing process [116].

	<i>Pale ale</i>		<i>Stout</i>	
<i>Malts</i>	German Pale ale	4.4 kg	German Pale ale	3.90 kg
			American chocolate	415 g
			American black	260 g
<i>Hops</i>	Centennial	1 oz/28 g	Centennial	1 oz/28 g
<i>Yeast</i>	Fermentis SafAle S-04		Fermentis SafAle S-04	
<i>Batch size</i>	20 L		20 L	
<i>Original gravity</i>	1.050		1.049	
<i>Final gravity</i>	1.014		1.02	
<i>ABV</i>	4.99%		4.99%	
<i>IBU</i>	43.71		43.72	
<i>SRM</i>	4.01		39.49	



Figure 9. Oak wood chips from different origins. On the left, French oak wood chips and, on the right, American wood chips.

Figure 10. Sampling scheme for the beer analysis.



2.2. *in vitro* bioactivity assessment

As it was previously stated, it has been reported that beer possesses a myriad of biological activities. To assess the effect that the addition of oak wood chips, during the maturation stage, exerts on different biological activities of beer, various *in vitro* assays were carried out with the finality of establishing comparisons between the different analysed samples and validate the results previously described in available bibliography.

The TPC, DPPH and FRAP assays were performed in microplates and were carried out according to the schemes depicted in figure 11.

All measurements were performed in triplicate, and data was reported as mean $\pm$ standard error of the mean (SEM). One-way ANOVA followed by Dunnett's multiple comparisons test against controls and Tukey's test to determine the statistical differences between the treatment groups, and Pearson correlation analysis were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

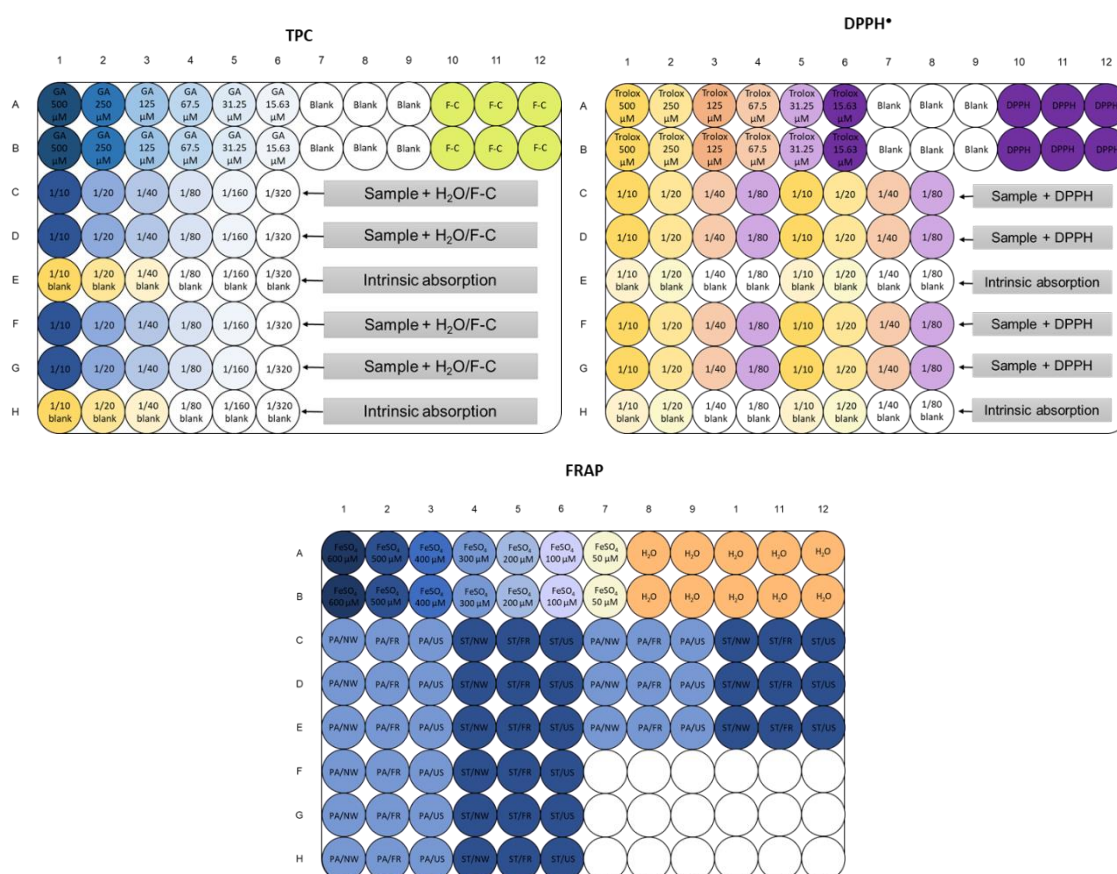


Figure 11. Schematic representation of the microplate layout for the TPC, DPPH, and FRAP assays.

2.2.1. Total phenolic contents (TPC) quantification

The total phenolic contents quantification was performed using the Folin-Ciocalteu (F-C) method, which is based on a transfer of an electron, in alkaline conditions, from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes from the F-C reagent. The electron transfer reaction shifts the colour of the reaction medium to intense blue colour, increasing absorbance values measured at $\lambda = 760$ nm [117].

The assay was carried out according to the conditions previously described by Magalhães *et al.* [118] with minor modifications. The F-C reagent 4:10 (v/v) and the NaCO₃ 6% (% m/v) were diluted in ultrapure H₂O. Using 96-well clear bottom microplates for absorbance measuring, the standards, reactants, and samples were added according to the scheme displayed in figure 11. In the lines A and B, successive dilutions of the gallic acid (GA) standard (75 μ L of ultrapure H₂O + 75 μ L of GA standard + 25 μ L of F-C) with concentrations ranging from 15.63 to 500 μ M, reagent blanks (100 μ L of ultrapure H₂O) and F-C controls (100 μ L of F-C reagent) were added. In the lines C-D and F-G, successive dilutions of the beer samples (75 μ L of ultrapure H₂O + 75 μ L of beer sample + 25 μ L of F-C) were added. Finally, the lines E and H were selected for the evaluation of the intrinsic absorption of the beer sample (100 μ L of ultrapure H₂O + 75 μ L of beer sample).

After the addition of the F-C reagent, the microplate was shaken in the microplate reader for 1 minute before 10 min of incubation. Subsequently, 100 μ L of NaCO₃ were added to every well. The consequent reduction of the phosphomolybdate-phosphotungstate complex in alkaline media operated by the phenolic compounds was measured, at 765 nm, after 120 minutes using a Synergy HT plate reader from BioTek (Vermont, USA).

2.2.2. Total flavonoid concentration (TFC) assay

The evaluation of the total concentration of flavonoids was performed through the methodology originally described by Zhishen *et al.* [119]. This colourimetric method is based on the formation of coloured complexes upon the reaction between Al(III) and the carbonyl and hydroxyl groups of flavonoids, preceded by a nitrosation of the flavonoids, whose absorbance can be measured at $\lambda = 510$ nm [120].

The total flavonoid concentration was assessed according to the method described by Herald *et al.* [121], with small modifications. In the extraction process, the samples (200 μ L) were extracted with methanol (800 μ L) in 2 mL microtubes, shaken for 5 min and centrifuged for 2 min at 5,000 *g*. Next, the assay was carried out according to the figure 12:

in a 10 mL plastic tube, 250 μL of the methanolic extract were mixed with 75 μL of a 50 g.L^{-1} NaNO_2 solution, 150 μL of a 100 g.L^{-1} AlCl_3 solution, and, after shaking vigorously and waiting 6 min, 500 μL of 1 mol.L^{-1} NaOH and 500 μL of ultrapure water were added. Finally, the tubes were centrifuged for 5 min at 3220 g and their absorbance was read at 510 nm against a reagent blank, using a UV-1800 UV spectrophotometer from Shimadzu (Kyoto, Japan). A calibration curve was made using (+)-catechin as a standard (5-250 mg.L^{-1}).

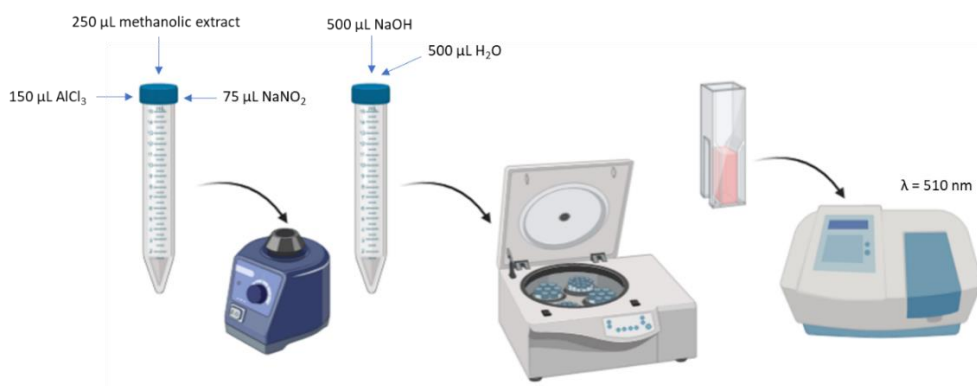


Figure 12. Schematic representation of the total flavonoid concentration assay procedure.

2.2.3. Antioxidant activity

To assess the antioxidant potential of a given sample, *in vitro* assays that evaluate radical scavenging activity using free radicals like 2,2-diphenyl-1-picrylhydrazyl ($\text{DPPH}\cdot$) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($\text{ABTS}\cdot^+$), as well as FRAP (Ferric Reducing Antioxidant Power), and ORAC (Oxygen Radical Absorption Capacity) are commonly used, providing a standard assay platform to compare results and validate conclusions between different laboratories. Therefore, we chose the first two to compare the different antioxidant potentials between the different beer samples.

2.2.3.1. DPPH assay

Possessing an unpaired valence electron at one of its nitrogen atoms, 2,2-diphenyl-1-picrylhydrazyl ($\text{DPPH}\cdot$) is a stable free radical that can be scavenged by an antioxidant species. As presented in figure 13, upon reduction by an antioxidant or a radical species, the $\text{DPPH}\cdot$ original absorption at $\lambda = 595 \text{ nm}$ diminishes [122, 123].

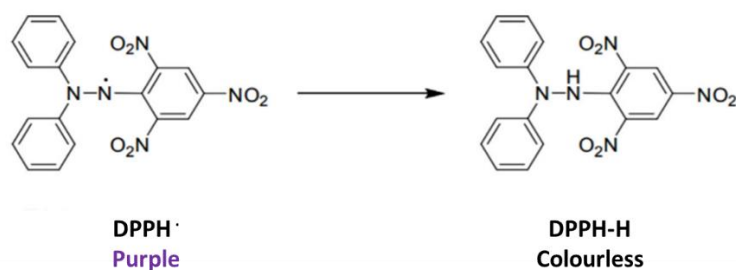


Figure 13. Reaction mechanism of the reduction of DPPH• to DPPH-H, through the action of an antioxidant molecule (adapted from [124]).

The assay was carried out according to conditions previously described by Magalhães *et al.* [125] and Viegas *et al.* [126]. In the day of the assay, the Trolox (1 mM) standard and the DPPH• (1 mM) stock solutions were prepared in absolute ethanol and kept in 4 °C, protected from the light. Then, the DPPH• solution was diluted with EtOH 50% (%v/v) until an absorbance value of 0.800 ± 0.020 was obtained. Finally, the assay was performed rendering the schematic representation in figure 11: in the lines A and B, successive dilutions of the Trolox standard (100 µL of EtOH 50% (%v/v) + 100 µL of Trolox standard + 100 µL of DPPH•) with concentrations ranging from 15.63 to 500 µM, reagent blanks (200 µL of EtOH 50% (%v/v) and DPPH• controls (100 µL of EtOH 50% (%v/v) + 100 µL of DPPH•) were added. In the lines C-D and F-G, successive dilutions of the beer samples (100 µL of EtOH 50% (%v/v) + 100 µL of beer sample + 100 µL of DPPH•) were added. Lastly, the lines E and H were selected for the evaluation of the intrinsic absorption of the beer sample (200 µL of EtOH 50% (%v/v) + 100 µL of beer sample).

After the addition of the DPPH• solution, the absorbance was measured at $\lambda = 595$ nm after 120 minutes using a Synergy HT plate reader from BioTek (Vermont, USA).

2.2.3.2. Ferric Reducing Antioxidant Power (FRAP) assay

Upon the reduction from Fe^{3+} to Fe^{2+} , as a result of the antioxidant action (figure 14), the Fe^{2+} -TPTZ (ferrous-tripyridyltriazine) complex develops an intense blue colour with an absorption maximum at $\lambda = 593$ nm. The absorbance can be measured to determine the amount of iron reduced from its ferric to its ferrous form, and correlate that information with the antioxidant activity [124, 127].

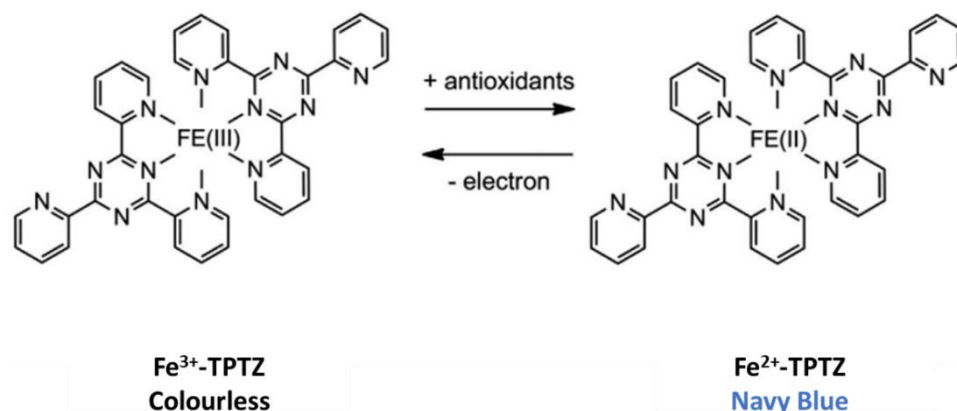


Figure 14. Mechanism of the reduction of the iron in the Fe-TPTZ complex (adapted from [124]).

The assay was performed as stated by Benzie *et al.* [127] with minor modifications. In the day of the assay, the FRAP reagent is prepared by mixing 50 mL of sodium acetate buffer (0.3 M), 5 mL TPTZ (10 mM), and 5 mL iron chloride (20 mM) solutions. In a 96-well clear bottom microplate, the samples, standards, and reagents are added rendering the scheme depicted in figure 11: in the lines A and B, successive dilutions of the FeSO_4 standard (35 μL of FeSO_4 standard + 265 FRAP reagent) with concentrations ranging from 50 to 600 μM , and reagent blanks (35 μL of ultrapure H_2O + 265 μL of FRAP reagent) were added, whereas in lines C-H, beer samples in the selected dilution of 1:10 (35 μL of beer sample + 265 μL of FRAP reagent) were added.

After the addition of the FRAP reagent, the absorbance was read after 30 min at $\lambda = 593$ nm using a Synergy HT plate reader from BioTek (Vermont, USA).

2.3. HPLC-DAD analysis of phenolic compounds

2.3.1. Extraction of phenolic compounds

A salting-out assisted liquid-liquid extraction (SALLE) procedure was performed according to Zhao *et al.* [37] with some adjustments. For each treatment, beer was degassed through intensive stirring and sonication for a combined time of 30 min. Then, 5 g of NaCl were added to 12.5 mL of the degassed beer sample. The resulting sample was extracted three times using 12.5 mL of ethyl acetate, centrifuging the sample (10,000 g , 10 min) between extractions to assist phase separation. The ethyl acetate extracts were then concentrated using a nitrogen sample evaporator. Finally, the extract was redissolved in MeOH and filtered through a 0.45 μm PTFE membrane (figure 15).

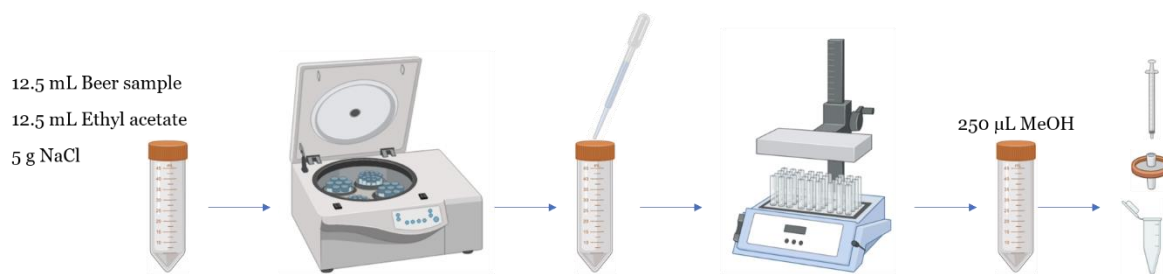


Figure 15. Schematic representation of the SALLE procedure for beer phenolic compounds.

2.3.2. HPLC-DAD conditions

A reverse-phase high-performance liquid chromatography (HPLC) analysis with a diode array detector (DAD) was performed to establish the phenolic compound profile of the tested beer samples. The separation was performed with an Acclaim™ PolarAdvantage C16 (3 µm, 3 x 150 mm, 120 Å) column (ThermoFisher Scientific, MA, USA) with the aid of a SecurityGuard ULTRA C18 guard column and a KrudKatcher ULTRA HPLC In-Line Filter (Phenomenex inc., CA, USA) in a UltiMate™ 3000 UHPLC system (ThermoFisher Scientific, MA, USA). The Chromeleon 7 chromatography data system (ThermoFisher Scientific, MA, USA) was used for data processing.

Regarding mobile phase composition, solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile) were selected according to Zhao *et al.* [37]. Chromatographic separation conditions (further detailed in table 14) were optimized to separate the thirty selected phenolic compounds, presented in table 17, with appropriate resolution (view figures 25 and 26) and achieved with the gradient as follows: 0 min, 3% B; 8 min, 3% B; 30 min, 35% B; 37 min, 75% B; 40 min, 75% B; 45 min, 3% B; 50 min, 3% B. Runtime was 50 min, the solvent flow rate was 0.4 ml.min⁻¹, and the injection volume was 5 µl. Regarding the detection of the phenolic compounds, four wavelengths (260, 280, 320, and 360 nm) were selected based on Sz wajgier *et al.* [128] to encompass the hydroxybenzoic and hydroxycinnamic acid derivatives and the flavonoid compounds. Phenolic compounds were quantified according to an external standard calibration curve based on the peak area for the selected wavelengths.

Calibration plots and respective parameters were determined according to Miller and Miller [129] and limits of detection (LOD) and quantification (LOQ) were calculated for the standards based on the standard deviation of y-residuals of the regression line ($S_{y/x}$):

$$\text{LOD} = \frac{3 \times S_{y/x}}{\text{slope}} \quad \text{LOQ} = \frac{10 \times S_{y/x}}{\text{slope}}$$

Table 14. Selected chromatographic conditions for the analysis of the selected phenolic compounds.

Runtime	50 min
Flow	0.400 mL.min ⁻¹
Mobile phase	A 0.1% acetic acid / H ₂ O up
	B 0.1% acetic acid / acetonitrile
DAD selected λ (nm)	280 Hydroxybenzoic acid derivatives
	260
	320 Hydroxycinnamic acid derivatives
	360 Flavonols
T sampler	4 °C
T oven	30 °C
Sample volume	5 µL

Moreover, one-way ANOVA followed by Dunnett's multiple comparisons test against controls and Tukey's test to determine the statistical differences between the treatment groups was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

2.4. Cellular assays

Many biological properties of beer constituents, namely the isolated activities of hop prenylflavonoid compounds, and even the whole beer matrix have been assessed using various cell lines, as previously mentioned. In this work, Caco-2 and RAW 264.7 cell lines were used to evaluate the antiproliferative, anti-inflammatory and antioxidant activities of the different beer samples.

The MTT assay and the determinations of the levels of NO and RS were performed in microplates and were carried out according to the schemes depicted in figure 16.

All measurements were performed in triplicate in at least two independent experiments, and data was reported as mean $\pm$ standard error of the mean (SEM). One-way ANOVA followed by Dunnett's multiple comparisons test against controls, and Tukey's test to determine the statistical differences between the treatment groups was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com). For the Caco-2 cell line, the IC₅₀ values were determined by curve fitting using the equation log (inhibitor) vs. response – Variable slope (four parameters). Statistical significance was set for $p < 0.05$, where the null hypothesis claims that there is no difference between the compared samples.

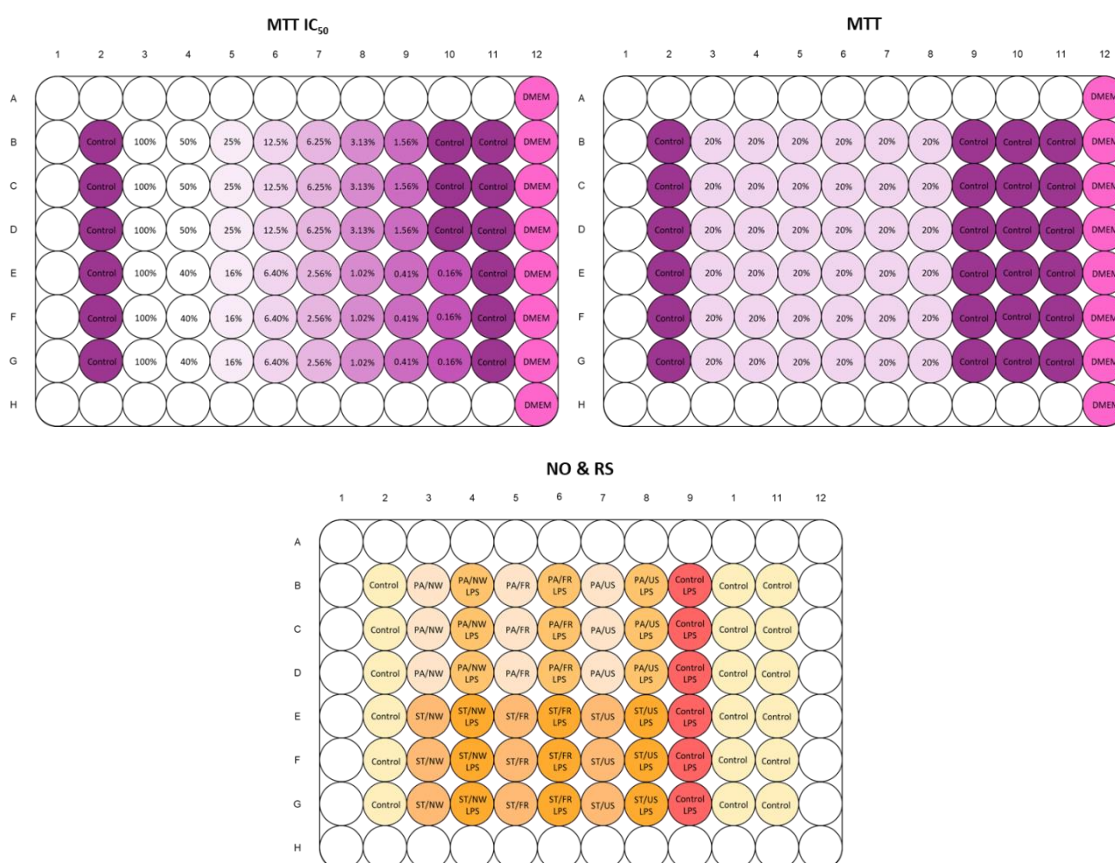


Figure 16. Schematic representation of the microplate layout for the MTT, NO, and RS assays.

2.4.1. Cell maintenance

2.3.1.1. Human colorectal adenocarcinoma Caco-2 cell line

Complete culture medium (CM), consisting of DMEM supplemented with 10% fetal bovine serum (FBS, heat-inactivated), 1% GlutaMAX™, 1% non-essential amino acids solution, 100 Units.mL⁻¹ penicillin, and 100 mg.mL⁻¹ streptomycin, was used for Caco-2 cell maintenance. Cells were incubated at 37 °C and 5% CO₂. The medium was changed every two days and cells passed at 70% confluence. For cytotoxicity assays, cells were used between passages 82-87. Contamination with Mycoplasma was assessed by PCR every four weeks using the protocol described by Van Kuppeveld *et al.* [130].

2.3.1.2. Murine macrophage RAW 264.7 cell line

Complete culture medium (CM), consisting of DMEM supplemented with 10% fetal bovine serum, 100 Units.mL⁻¹ penicillin, and 100 mg.mL⁻¹ streptomycin, was used for RAW 264.7 cell maintenance. Cells were incubated at 37 °C and 5% CO₂. The medium was changed every two days and cells passed at 70% confluence. For NO and ROS level determination assays, cells were used between passages 6-7. Contamination with Mycoplasma was assessed by PCR every four weeks using the protocol described by Van Kuppeveld *et al.* [130].

2.4.2. MTT assay

The MTT assay is a colourimetric assay commonly used to assess cellular metabolic activity working as an indicator of cytotoxicity, cell viability, and proliferation. The fundament of the assay consists of the ability of metabolically active cells to reduce the yellow MTT to the purple formazan crystals through the NAD(P)H-dependent oxidoreductase enzymes (figure 17). The crystals are then dissolved using DMSO and their absorbance is measured at $\lambda = 570 \text{ nm}$ [131].

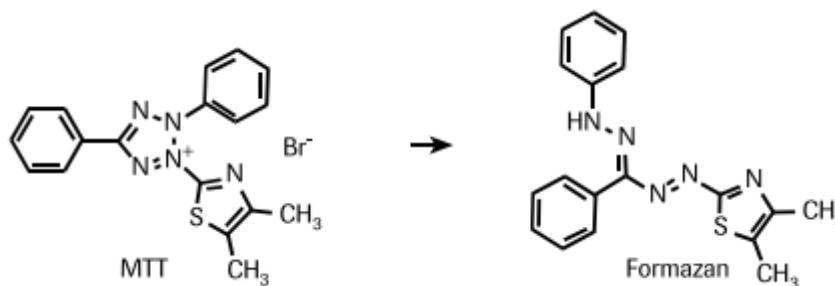


Figure 17. Mechanism of the MTT reduction to Formazan crystals, operated by viable cells during the MTT assay (adapted from [132]).

To assess cytotoxicity in Caco-2 cells for the exposition period of 48h, an MTT assay was carried out according to the scheme presented in figure 16. Cells were seeded in 96 well plates (Sarstedt, Nümbrecht, Germany) at a density of 1.25×10^4 cells.mL⁻¹ in 200 µl CM per well (2500 cells/well). After cell adhesion for 12 h, the medium was replaced with the test solutions containing serial dilutions of the beer matrixes and incubated at 37 °C with 5% CO₂ for the exposition period. The different beer lyophilizates were redissolved in CM, centrifuged at 5,000 *g* for 10 minutes, filtered through a 0.22 µm nylon filter and, subsequently, diluted in CM (concentrations ranging from 0.78 to 100% of beer in CM). A first assay was performed with one replicate for each of the 6 beer/wood treatments studied for the determination of the IC₅₀ values. Afterwards, all replicates were tested at concentrations near the IC₅₀. All solutions, as well as cell culture plates, were protected from light using aluminium foil. Plate readings were performed at $\lambda = 570$ nm in a BioTek Synergy HT plate reader (Vermont, USA).

2.4.3. Determination of extracellular nitric oxide levels

Nitric oxide (NO) is synthesized from L-arginine by the iNOS (inducible isoform of the nitric oxide synthetase enzyme), as a response to a stimulus (*e.g.* LPS and cytokines) that leads to the activation of immune cells. Thus, LPS is commonly used to induce an inflammatory phenotype in macrophage RAW 264.7 cells that results in the production of a myriad of inflammatory mediators, including NO. By evaluating the extracellular levels of NO produced by the macrophages, we can assess if the tested compounds can exert an anti-inflammatory effect.

The assay used for the determination of extracellular NO levels, by its conversion to nitrite, is based on the nitrite selective Griess reaction. Sulphanilamide, an aromatic amine undergoes diazotisation in the presence of nitrite and an acid catalyst (H_3PO_4). As a result, a reactive diazonium salt is formed and, upon the addition of NED (an electron-rich coupling agent), reacts to yield a highly coloured diazo dye whose absorbance is directly proportional to the nitrite concentration in the sample (figure 18) [133].

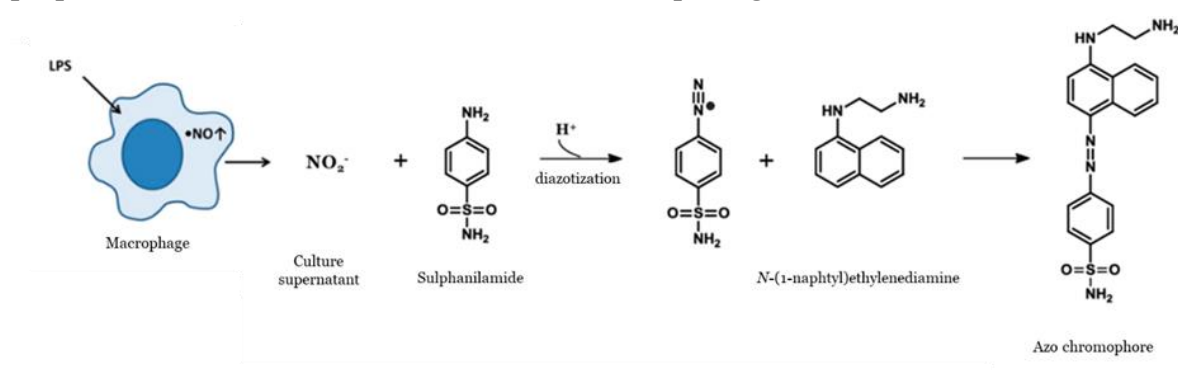


Figure 18. Mechanism of the Griess reaction (adapted from [134, 135]).

The murine macrophage cells were seeded in 96-well black microplates at a density of $200,000 \text{ cells.mL}^{-1}$ in $200 \mu\text{L}$ CM ($40,000 \text{ cells/well}$) and incubated for 24 h at 37°C and 5% CO_2 . Afterwards, the medium of each well was removed, and controls/samples were added according to the scheme displayed in figure 16. After 2 h, cells were stimulated with 1 mg.mL^{-1} of LPS for 22 h. The quantity of NO in cell culture medium was determined by its conversion to nitrite, by transferring $100 \mu\text{L}$ of culture medium to a clear bottom 96-well microplate and adding an equal volume of Griess reagent (1% sulphanilamide and 0.1% NED in 2% H_3PO_4). The plate was incubated for 10 min, in the dark, at room temperature and the absorbance was measured at 560 nm in a BioTek Synergy HT microplate reader (Vermont, USA).

The beer samples were added to the RAW 264.7 cells at a concentration of 12.5% (beer in CM). This concentration was selected after assessing cell viability through the MTT assay and verifying that, at this concentration, over 90% of the murine macrophage cells remained viable. The results correspond to the mean $\pm$ SEM of at least two independent experiments performed in triplicate and are expressed as a percentage of NO in cells exposed to LPS (positive control for NO production).

2.4.4. Determination of intracellular ROS and RNS levels

The determination of intracellular ROS and RNS levels is carried out based using 2',7'-dihydrochlorofluorescein diacetate (DCFH-DA), a cell-permeable fluorescence probe, that once de-esterified to 2',7'-dihydrochlorofluorescein (DCFH) and oxidised intracellularly by reactive oxygen and nitrogen species, forms a fluorescent product: dichlorofluorescein (DCF) whose fluorescence can be assessed by a fluorimetry-based approach (figure 19) [136].

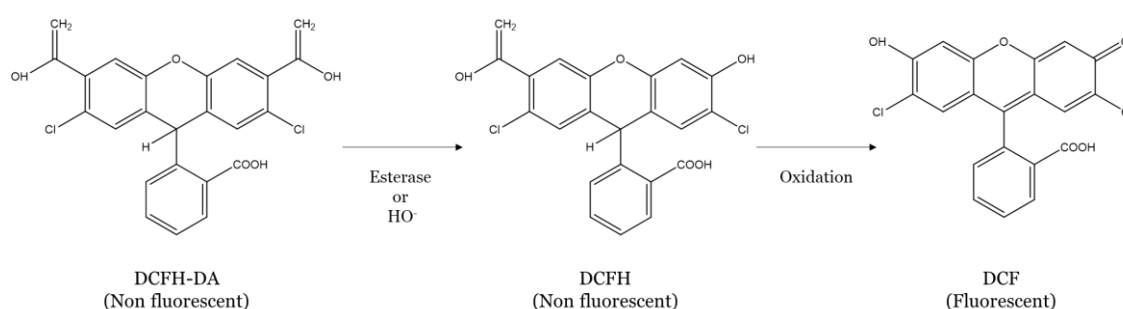


Figure 19. Mechanism of the de-esterification reaction of DCFH-DA to DCFH, and subsequent oxidation to the fluorescent product DCF, operated by ROS and RNS (adapted from [137]).

Following the transfer of 100 μ L of the culture medium from the original black microplate for extracellular NO level measurement, the rest of the culture medium was removed. Subsequently, the wells were gently washed twice with 100 μ L of PBS. Then, the cells were stained using 100 μ L of diluted 10 μ M of DCF-DA solution. After a 30 min incubation period (37 $^{\circ}$ C, in the dark), the DCF-DA solution was aspirated, and the wells were, once again, gently washed with 100 μ L of PBS. Finally, 100 μ L of PBS were added and the fluorescence was read immediately (λ_{exc} : 485nm / λ_{em} : 535nm) using a BioTek Synergy HT microplate reader (Vermont, USA) according to the scheme previously depicted in figure 16.

3. Results

3.1. *in vitro* bioactivity assessment

3.1.1. Total phenolic contents (TPC) quantification

In the figure 20 graph, the results from the TPC quantification assay are presented. The TPC values, expressed in mg of gallic acid equivalents per litre (mg.GAE.L⁻¹) of beer sample, varied between 771.98 ± 18.91 and 920.81 ± 34.76 for the pale ale style, and between 1495.16 ± 43.59 and 1501.81 ± 41.62 for the stout style.

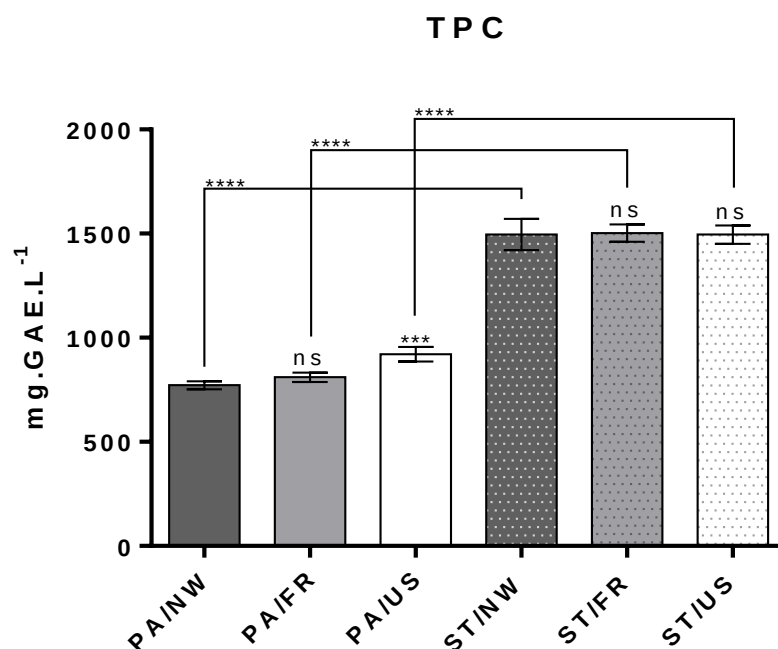


Figure 20. Total phenolic contents (TPC) quantification in mg of gallic acid equivalents per litre of beer sample (mg GAE.L⁻¹). ****Significant at $p < 0.0001$, and ***significant at $p < 0.001$ compared with control without addition of wood chips (NW). Values expressed as mean $\pm$ SEM ($n = 3$).

The obtained results are significantly higher than previously reported TPC values for commercial beers that range from 88.47 to 875 mg.GAE.L⁻¹ [37, 42, 45-47]. These differences can be justified by the common PVPP filtration process in commercial beer that, as previously mentioned, can remove a significant portion of the phenolic compounds. We

found no reports on the TPC of non-industrial non-filtered beer, which would probably be more similar to our results by the appointed reasons.

Following statistical analysis, concerning the pale ale style, when compared to the control beer without the addition of wood chips (PA/NW), beer aged with American Oak chips showed a statistically significant increase (***) $p < 0.001$ in TPC, whereas the beer aged with French Oak chips failed to exhibit any statistically significant increment in the concentration of phenolic compounds. Regarding the stout style, no statistically significant alterations in the total phenolic compounds content were observed. Furthermore, when comparing the samples of the two different beer styles, statistically significant differences were found for every comparison of the same ageing treatment between the samples of the two styles (****) $p < 0.0001$, highlighting the higher content of phenolic compounds in the darker, stout beer.

3.1.2. Total flavonoid concentration (TFC) assay

In the graph displayed in figure 21, the results from the assay concerning flavonoid quantification are presented. The results, expressed in mg of (+)-catechin equivalents per litre (mg.CE.L⁻¹) of beer sample, varied between 54.12 ± 3.52 and 60.01 ± 0.25 for the pale ale style, and between 83.51 ± 5.20 and 100.2 ± 5.02 for the stout style. The work by Nardini *et al.* [138] corroborates the findings with reported values ranging from 51.9 ± 1.1 to 73.2 ± 0.6 mg.CE.L⁻¹ in conventional commercial ale beers.

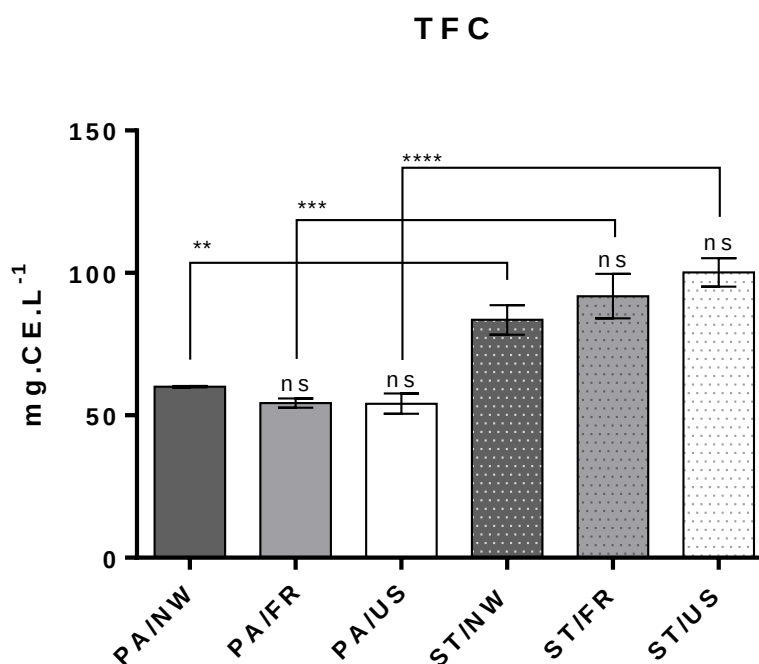


Figure 21. Total flavonoids content (TFC) presented in mg of (+)-catechin equivalents per litre of beer sample (mg.CE.L⁻¹). ****Significant at $p < 0.0001$, ***significant at $p < 0.001$, and **significant at $p < 0.01$ compared with control without addition of wood chips (NW). Values expressed as mean $\pm$ SEM ($n = 3$).

Following statistical analysis, neither beer style presented statistically significant differences in relation to the respective controls without the addition of wood. Nevertheless, when comparing the samples from the two distinct beer styles, once more, the stout beer exhibited a higher flavonoid content when compared to the pale ale. These results follow the tendency exhibited in the TPC assay.

3.1.3. DPPH assay

In figure 22, the results from the DPPH• assay for antioxidant activity evaluation are displayed. The results, expressed in mg of Trolox equivalents per litre (mg. TE.L⁻¹) of beer sample, varied between 333.10 ± 12.72 and 382.90 ± 19.21 for the pale ale style, and between 573.50 ± 55.98 and 682.70 ± 55.92 for the stout style.

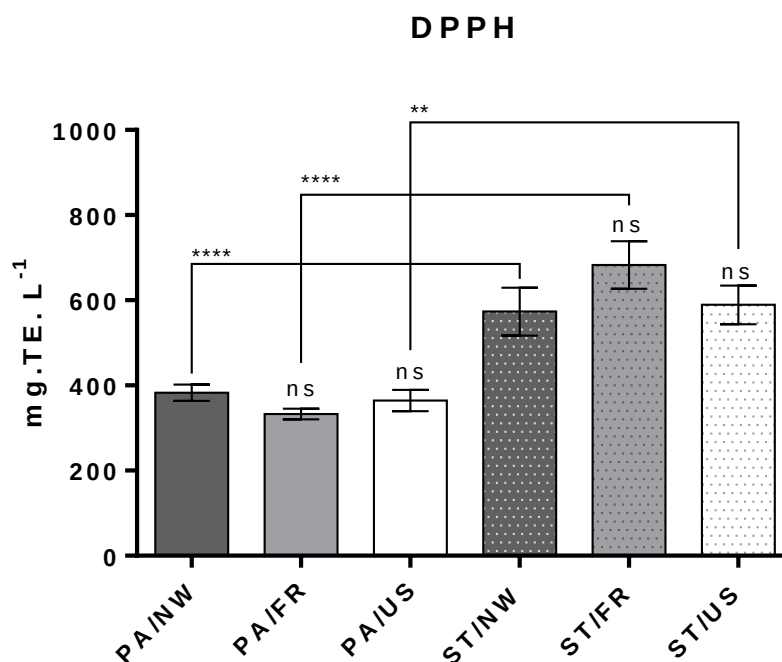


Figure 22. Antioxidant activity assessment through the DPPH• assay. Results presented in mg of Trolox equivalents per litre of beer sample (mg. TE.L⁻¹). ****Significant at $p < 0.0001$, and **significant at $p < 0.01$ compared with control without addition of wood chips (NW). Values expressed as mean $\pm$ SEM ($n = 3$).

The obtained results are, much like the ones obtained for the TPC assay, superior to previously reported values in the literature that range from 60 to 338 mg.TE.L⁻¹, for commercial beers [37, 45]. The justification for this disparity may be, yet again, the absence of a filtration process in the craft beers that, as stated before, can remove a significant portion of phenolic compounds, frequently associated with antioxidant activity.

After performing statistical analysis, both pale ale and stout beers unsuccessfully displayed any statistically significant differences between the wood-aged samples and the respective controls without the addition of wood chips (PA/NW and ST/NW, respectively). However, when compared to the pale ale beer, the stout beer exhibited a higher antioxidant activity. These results go along with the ones obtained in the TPC assay since phenolic compounds are heavily associated with the antioxidant activity as previously mentioned in this work.

3.1.4. FRAP assay

In figure 23, the results from the FRAP assay concerning antioxidant activity evaluation are displayed. The results, expressed in mg of ferrous sulphate equivalents per litre (mg. FSE.L⁻¹) of beer sample, varied between 1014.54 ± 64.59 and 1342.56 ± 66.51 for the pale ale style, and between 3190.87 ± 145.76 and 3701.50 ± 38.58 for the stout style. These results are significantly higher than the ones found in the bibliography with Piazzon *et al.* [42] reporting values ranging from 329 to 700 mg.FSE.L⁻¹. This disparity may be justified due to the lack of the filtration process in the craft beer samples used in this work, as observed in the other *in vitro* assays.

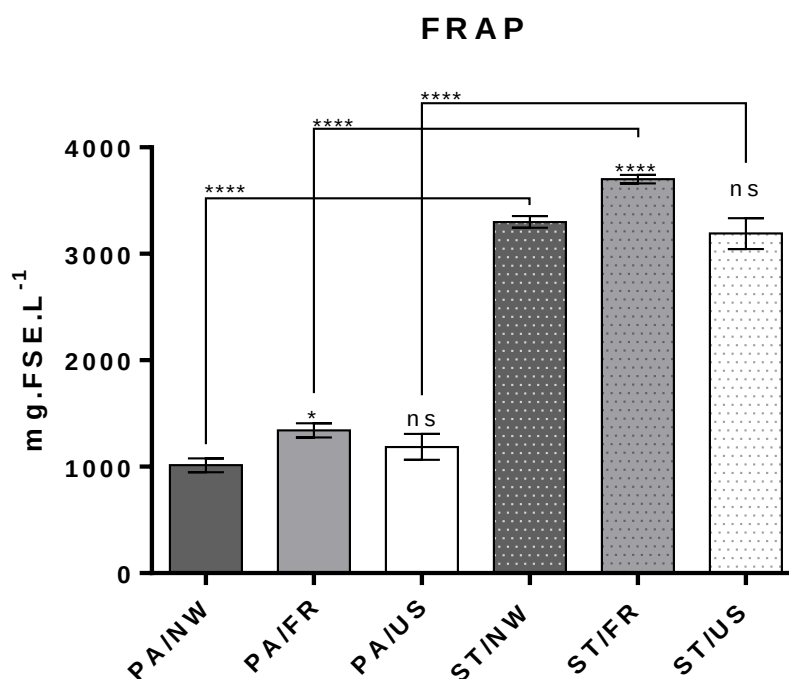


Figure 23. Antioxidant activity assessment through the FRAP assay. Results presented in mg of ferrous sulphate equivalents per litre of beer sample (mg.FSE.L⁻¹). ****Significant at $p < 0.0001$, and *significant at $p < 0.05$ compared with control without addition of wood chips (NW). Values expressed as mean $\pm$ SEM ($n = 3$).

Following statistical analysis, the beer treated with French oak wood chips presented statistically significant differences in relation to the respective controls with different degrees of significance (* $p < 0.05$, for the pale ale beer and **** $p < 0.0001$ for the stout beer), expressing an increase of antioxidant activity. Contrastingly, the beers treated with the American oak chips did not show any statistically significant differences when compared to the respective controls without the addition of wood. Finally, when comparing the

samples from the two distinct beer styles, once again, the darker, stout beer exhibited a higher antioxidant activity when compared to the pale ale. These results share the same propensity manifested in the DPPH• and the TPC assays.

3.1.5. Summary of results from the *in vitro* bioactivity assays

Concerning the total phenolic compounds quantification, the pale ale beer treated with American oak wood chips is the only one to manifest statistically significant differences when compared to the pale ale sample without the addition of oak wood chips but no improvements in antioxidant activity are verified for that sample, despite the supposed higher content in phenolic compounds, associated with a strong antioxidant effect.

In what respects the total flavonoid concentration, no differences were found between the wood chip-treated samples and the controls without the addition of wood. In all the above-mentioned assays, the stout beer samples exhibited significantly higher antioxidant activity, and a higher concentration of flavonoids and other phenolic compounds. Regarding antioxidant activity, despite the existence of statistically significant differences in the FRAP assay between the samples (pale ale and stout) treated with French oak chips and the control with no addition of wood, the DPPH• assay does not confirm that behaviour. These findings are summarized in table 15.

Table 15. Summary of results from the *in vitro* bioactivity assays. Results are expressed as mean $\pm$ SEM (n = 3).

		<i>TPC</i> <i>mg.GAE.L⁻¹</i>	<i>TFC</i> <i>mg.CE.L⁻¹</i>	<i>DPPH•</i> <i>mg.TE.L⁻¹</i>	<i>FRAP</i> <i>mg.FSE.L⁻¹</i>
<i>Pale Ale</i>	<i>No wood</i>	771.98 $\pm$ 18.91	60.01 $\pm$ 0.25	382.90 $\pm$ 19.21	1014.54 $\pm$ 64.59
	<i>French oak wood</i>	810.13 $\pm$ 21.81	54.34 $\pm$ 1.66	333.10 $\pm$ 12.72	1342.56 $\pm$ 66.51
	<i>American oak wood</i>	920.81 $\pm$ 34.76	54.12 $\pm$ 3.52	364.60 $\pm$ 25.25	1185.56 $\pm$ 121.6
<i>Stout</i>	<i>No wood</i>	1495.63 $\pm$ 75.45	83.51 $\pm$ 5.20	573.50 $\pm$ 55.98	3298.60 $\pm$ 54.92
	<i>French oak wood</i>	1501.81 $\pm$ 41.62	91.84 $\pm$ 7.80	682.70 $\pm$ 55.92	3701.50 $\pm$ 38.58
	<i>American oak wood</i>	1495.16 $\pm$ 43.59	100.20 $\pm$ 5.02	589.40 $\pm$ 45.27	3190.87 $\pm$ 145.76

Moreover, through the Pearson correlation analysis, a significant correlation was obtained when comparing both antioxidant activity assays (DPPH• and FRAP) with the total phenolic content of the beer samples (TPC), as depicted in figure 24, confirming the vastly described antioxidant activity of phenolic compounds. For the DPPH• assay, the Pearson r value was 0.9518 with **significant at $p < 0.01$, whereas for the FRAP assay, the Pearson r value was 0.9819 with ***significant at $p < 0.001$.

Additionally, the correlation between both assays (FRAP vs. DPPH•) was determined with a Pearson r -value of 0.9713 with **significant at $p < 0.01$. Also, the correlation of the total phenolic content and the total flavonoids concentration (TPC vs TFC) was calculated with a Pearson r -value of 0.9414 with **significant at $p < 0.01$.

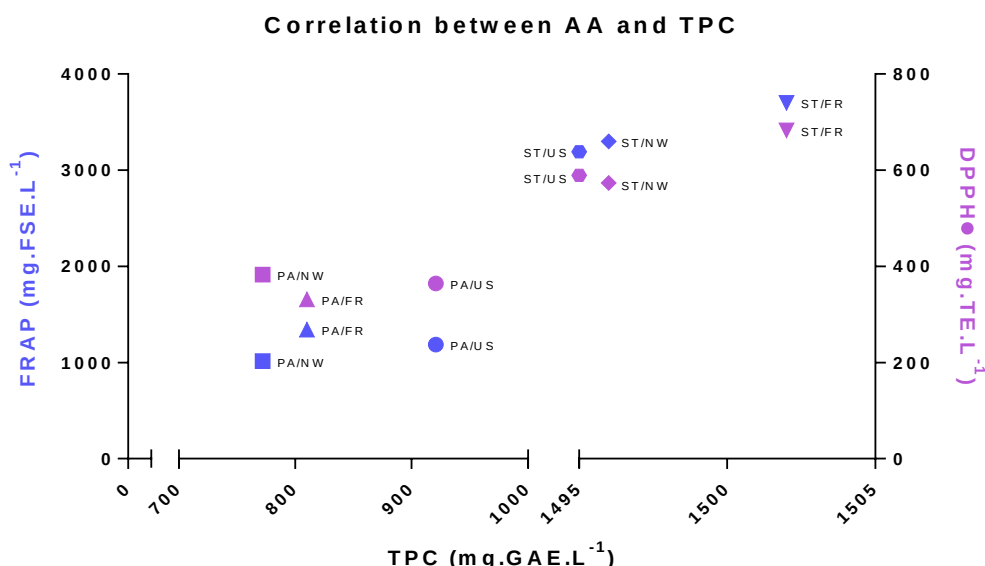


Figure 24. Correlation plot between the antioxidant activity assays (DPPH• and FRAP) and the total phenolic contents (TPC).

3.2. HPLC-DAD phenolic compounds analysis

3.2.1. Calibration plot and respective parameters

Table 17 presents the calibration parameters for the identification and quantification of the thirty phenolic compounds from different structural classes chosen to tentatively establish a phenolic profile for the different beer samples. The chromatographic separation of the said compounds is depicted in the chromatograms below.

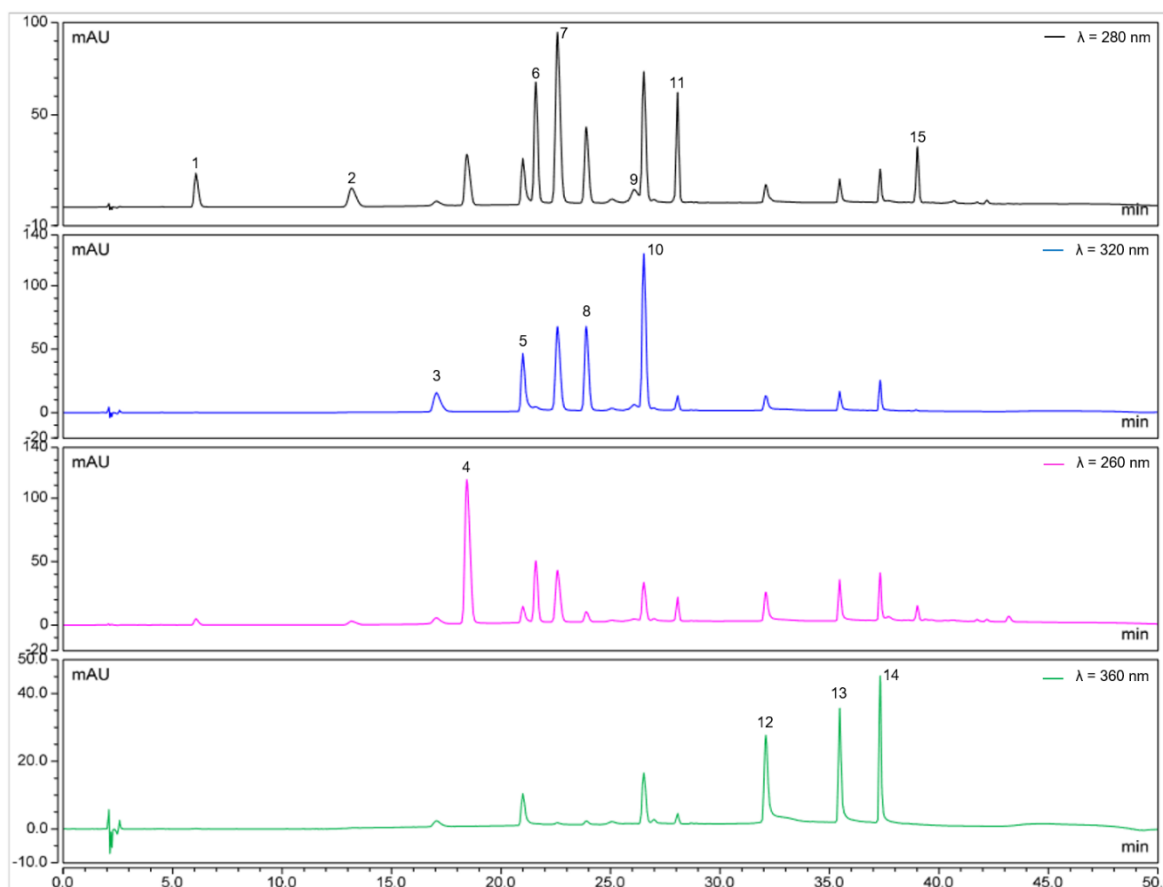


Figure 25. Chromatogram for the standard mix A with a concentration of 25 mg.L⁻¹.

1 – 3,4-dihydroxybenzyl alcohol, **2** – 3,4-dihydroxyphenylacetic acid, **3** – gentisic acid, **4** – *p*-hydroxybenzoic acid, **5** – chlorogenic acid, **6** – syringic acid, **7** – vanillin, **8** – syringaldehyde, **9** – salicylic acid, **10** – *trans*-ferulic acid, **11** – naringin, **12** – myricetin, **13** – quercetin, **14** – kaempferol, **15** – carvacrol.

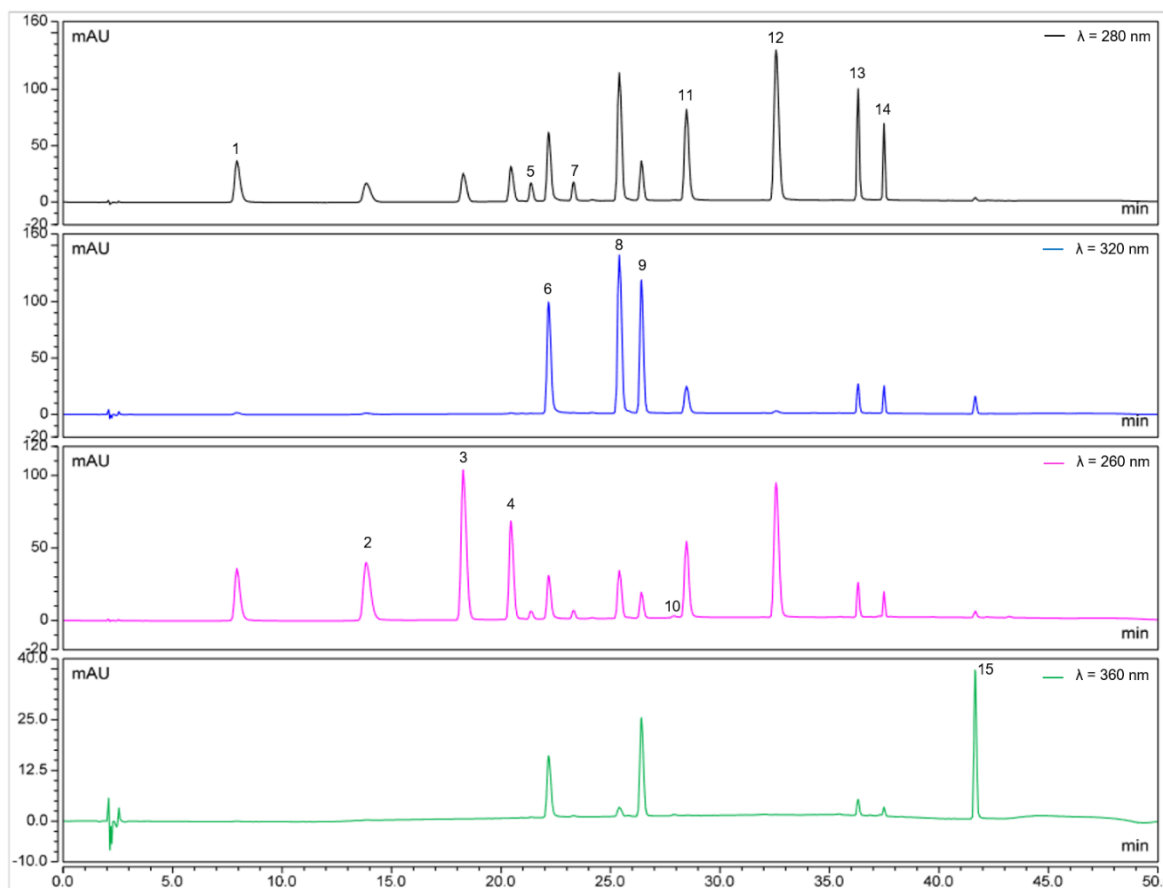


Figure 26. Chromatogram for the standard mix B with a concentration of 25 mg.L⁻¹.

1 – gallic acid, **2** – protocatechuic acid, **3** – *m*-hydroxybenzoic, **4** – vanillic acid, **5** – (+)-catechin, **6** – caffeic acid, **7** – (-)-epicatechin, **8** – *p*-coumaric acid, **9** – sinapic acid, **10** – ellagic acid, **11** – 4-hydroxycoumarin, **12** – *trans*-cinnamic acid, **13** – naringenin, **14** – isoxanthohumol, **15** – xanthohumol.

To evaluate the method's accuracy and reliability, recovery rates were determined. To achieve this, samples of both beer styles without wood addition (control) were spiked with four concentration levels of six phenolic compounds prior to the extraction procedure and quantitative analysis. The amounts determined were compared to the control beers with results, presented in table 16, within the acceptable range for recovery rate values (80-120%) [139], confirming the method's accuracy for beer phenolic compounds quantification.

Table 16. Recovery rates determined for selected phenolic compounds in Pale Ale and Stout beer samples ^a.

Pale Ale	GA	Pro	VA	Van	Q	IX
C/ mg.L⁻¹	%Rec	%Rec	%Rec	%Rec	%Rec	%Rec
25	107.74	100.90	101.06	95.93	118.11	93.44
12.5	114.13	109.39	108.99	104.32	118.17	97.11
7.5	81.06	105.99	103.11	102.24	113.66	109.18
2.5	86.60	111.51	113.76	117.86	107.60	106.15

Stout	GA	Pro	VA	Van	Q	IX
C/ mg.L⁻¹	%Rec	%Rec	%Rec	%Rec	%Rec	%Rec
25	94.76	95.87	97.56	94.62	99.77	108.28
12.5	97.30	100.90	110.64	106.58	110.06	116.60
7.5	93.08	101.71	114.18	111.98	98.17	103.96
2.5	113.23	115.06	111.60	95.18	95.27	116.75

^a **GA** – gallic acid, **Pro** – protocatechuic acid, **VA** – vanillic acid, **Van** – vanillin, **Q** – quercetin, **IX** – isoxanthohumol.

The determined calibration parameters, presented in the following table, allowed the quantification of twelve of the thirteen identified phenolic compounds. The determined values for LOD ranged from 0.131 to 6.924 mg.L⁻¹ and for LOQ from 0.436 to 23.080 mg.L⁻¹. All the calibration plots exhibited a r^2 value superior to 0.9900 which indicates a good fitness of the regression predictions for the phenolic concentration determinations.

Table 17. Calibration parameters for phenolic compound identification and quantification.

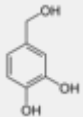
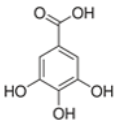
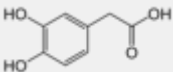
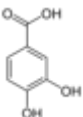
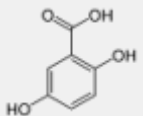
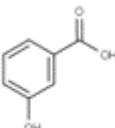
Phenolic compound	Structure	Retention time (min)	Selected $\lambda_{\text{máx}}$ (nm)	<i>b</i>	<i>a</i>	r^2	$S_{y/x}$	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
3,4-dihydroxybenzyl alcohol		6.01	280	0.154	0.039	0.999	0.027	0.519	1.729
gallic acid		6.97	280	0.435	-0.052	0.999	0.083	0.576	1.919
3,4-dihydroxyphenylacetic acid		13.12	280	0.164	0.033	0.999	0.019	0.344	1.146
protocatechuic acid		14.05	260	0.635	-0.025	0.999	0.115	0.545	1.815
gentisic acid		16.92	320	0.236	0.043	0.999	0.037	0.467	1.556
<i>m</i> -hydroxybenzoic acid (3-hydroxybenzoic acid)		18.41	260	1.148	0.032	0.999	0.117	0.306	1.018

Table 17. Calibration parameters for phenolic compound identification and quantification (cont.).

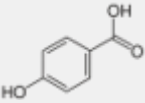
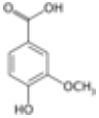
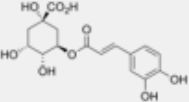
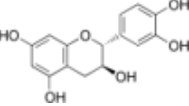
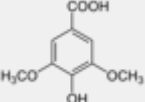
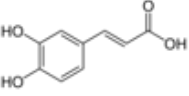
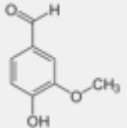
Phenolic compound	Structure	Retention time (min)	Selected $\lambda_{\text{máx}}$ (nm)	<i>b</i>	<i>a</i>	r^2	$S_{y/x}$	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
<i>p</i> -hydroxybenzoic acid (4-hydroxybenzoic acid)		18.42	260	1.201	0.326	0.999	0.190	0.474	1.581
vanillic acid		20.56	260	0.628	-0.001	0.999	0.055	0.263	0.875
chlorogenic acid		20.92	320	0.398	0.053	0.998	0.135	1.016	3.386
(+)-catechin		21.44	280	0.117	-0.010	0.999	0.018	0.469	1.562
syringic acid		21.55	280	0.492	0.155	0.999	0.092	0.560	1.867
caffeic acid		22.26	320	0.868	-0.138	0.999	0.154	0.532	1.775
vanillin		22.58	280	0.909	0.240	0.999	0.136	0.449	1.497

Table 17. Calibration parameters for phenolic compound identification and quantification (cont.).

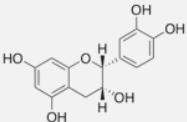
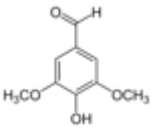
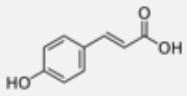
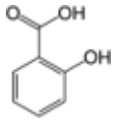
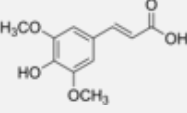
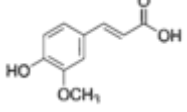
Phenolic compound	Structure	Retention time (min)	Selected $\lambda_{\text{máx}}$ (nm)	<i>b</i>	<i>a</i>	r^2	$S_{y/x}$	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
(-)-epicatechin		23.35	280	0.123	-0.100	0.998	0.053	1.287	4.289
syringaldehyde		23.87	320	0.573	0.153	0.999	0.094	0.494	1.648
<i>p</i> -coumaric acid		25.45	320	1.201	0.030	0.999	0.068	0.171	0.569
salicylic acid		26.05	280	0.087	-0.005	0.999	0.011	0.366	1.218
sinapic acid		26.46	320	0.906	-0.021	0.999	0.084	0.278	0.928
<i>trans</i> -ferulic acid		26.53	320	1.046	-0.447	0.999	0.188	0.539	1.795

Table 17. Calibration parameters for phenolic compound identification and quantification (cont.).

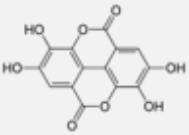
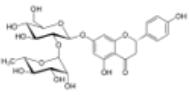
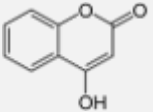
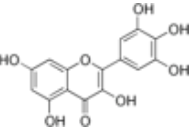
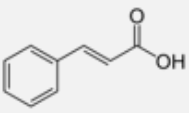
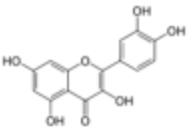
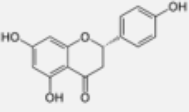
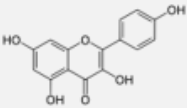
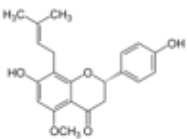
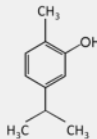
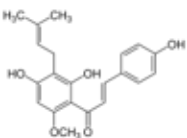
Phenolic compound	Structure	Retention time (min)	Selected $\lambda_{\text{máx}}$ (nm)	<i>b</i>	<i>a</i>	r^2	$S_{y/x}$	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
ellagic acid		27.87	260	0.009	0.087	0.997	0.022	6.924	23.080
naringin		28.09	280	0.326	0.083	0.999	0.051	0.467	1.555
4-hydroxycoumarin		28.53	280	0.761	0.013	0.999	0.059	0.234	0.780
myricetin		32.11	260	0.300	-0.259	0.991	0.245	2.445	8.148
<i>trans</i> -cinnamic acid		32.58	280	1.369	-0.002	0.999	0.117	0.255	0.851
quercetin		35.44	360	0.319	-0.119	0.997	0.113	1.390	4.635
naringenin		36.32	280	0.498	-0.033	0.999	0.066	0.399	1.329

Table 17. Calibration parameters for phenolic compound identification and quantification (cont.).

Phenolic compound	Structure	Retention time (min)	Selected $\lambda_{\text{máx}}$ (nm)	<i>b</i>	<i>a</i>	r^2	$S_{y/x}$	LOD (mg.L ⁻¹)	LOQ (mg.L ⁻¹)
kaempferol		37.28	360	0.253	0.347	0.999	0.033	0.394	1.315
isoxanthohumol		37.49	280	0.295	-0.328	0.997	0.142	1.444	4.813
carvacrol		39.03	280	0.148	-0.006	0.999	0.033	0.660	2.199
xanthohumol		41.60	360	0.216	0.001	0.999	0.009	0.131	0.436

b – slope; *a* – intercept; r^2 – correlation coefficient; $S_{y/x}$ – standard deviation of y-residuals of the regression line; LOD – limit of detection; LOQ – limit of quantification.

3.2.2. Phenolic compounds identification and quantification

In the selected chromatographic conditions, thirteen of the thirty selected standards were successfully identified through retention time comparison and matched with spectra library of pure phenolic standards (view item 1. in supplementary materials).

Regarding quantification, twelve phenolic compounds were quantified, as presented in table 18. In the two figures below, the chromatograms for the pale ale and stout beers aged with French oak chips are displayed with all quantified individual phenolic compounds represented.

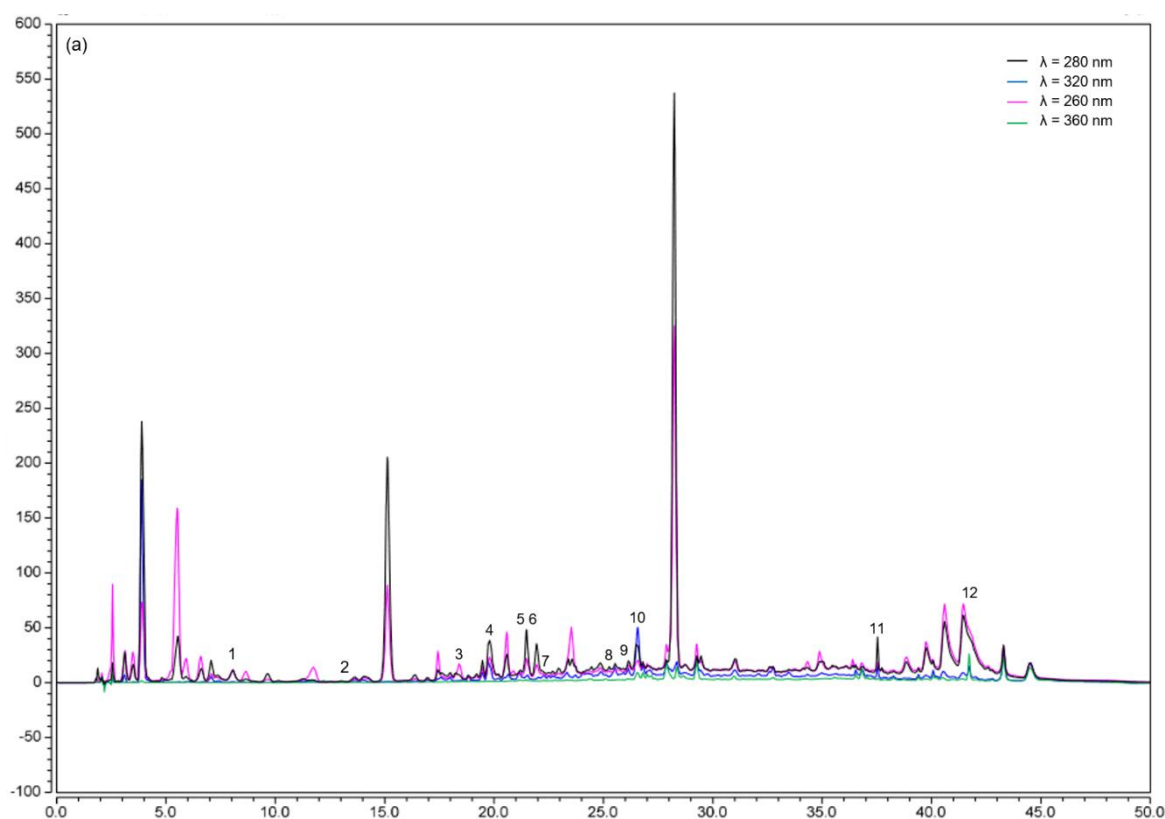


Figure 27. Chromatogram of the Pale Ale beer aged with French Oak Chips (overlaid wavelengths).

1 – gallic acid, **2** – 3,4-dihydroxyphenylacetic acid, **3** – *m*-hydroxybenzoic acid, **4** – vanillic acid, **5** – (+)-catechin, **6** – syringic acid, **7** – (-)-epicatechin, **8** – *p*-coumaric acid, **9** – salicylic acid, **10** – *trans*-ferulic acid, **11** – isoxanthohumol, **12** – xanthohumol.

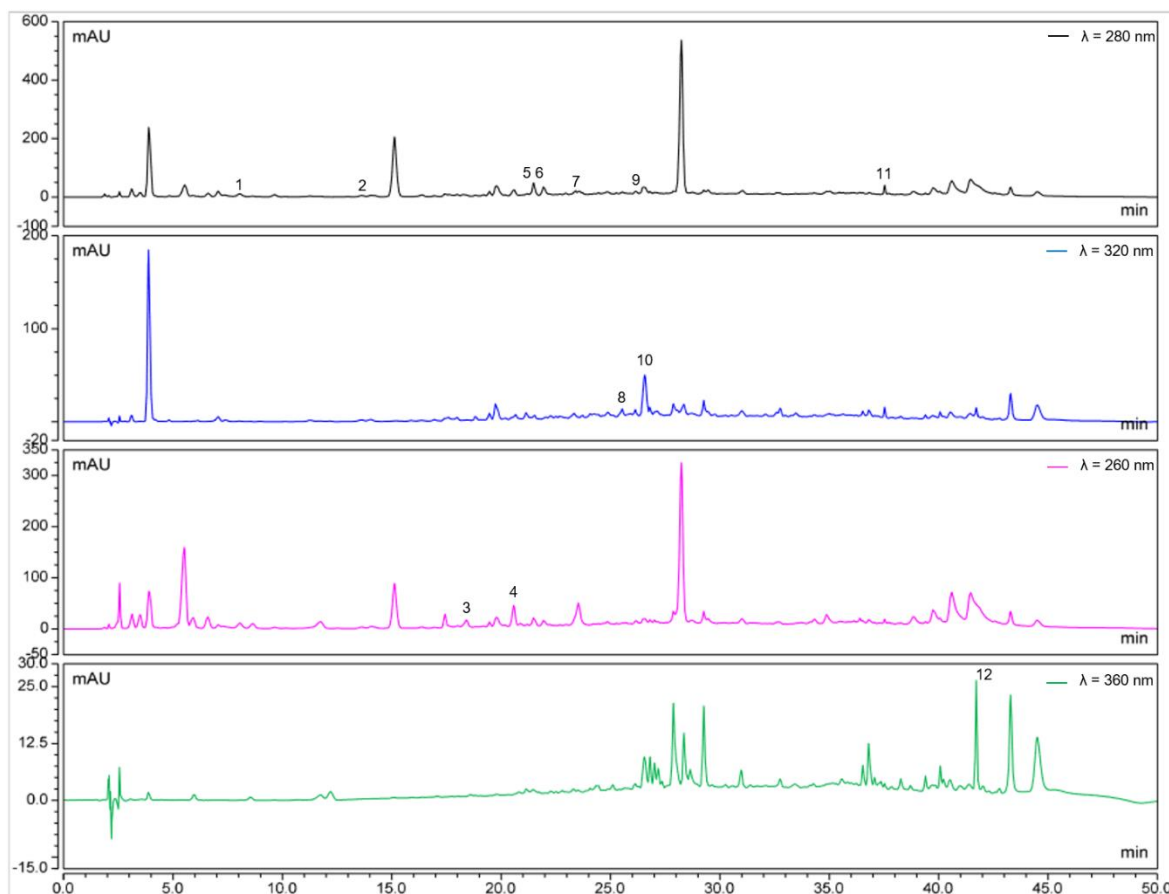


Figure 28. Chromatogram of the Pale Ale beer aged with French Oak Chips (stacked wavelengths).

1 – gallic acid, **2** – 3,4-dihydroxyphenylacetic acid, **3** – *m*-hydroxybenzoic acid, **4** – vanillic acid, **5** – (+)-catechin, **6** – syringic acid, **7** – (-)-epicatechin, **8** – *p*-coumaric acid, **9** – salicylic acid, **10** – *trans*-ferulic acid, **11** – isoxanthohumol, **12** – xanthohumol.

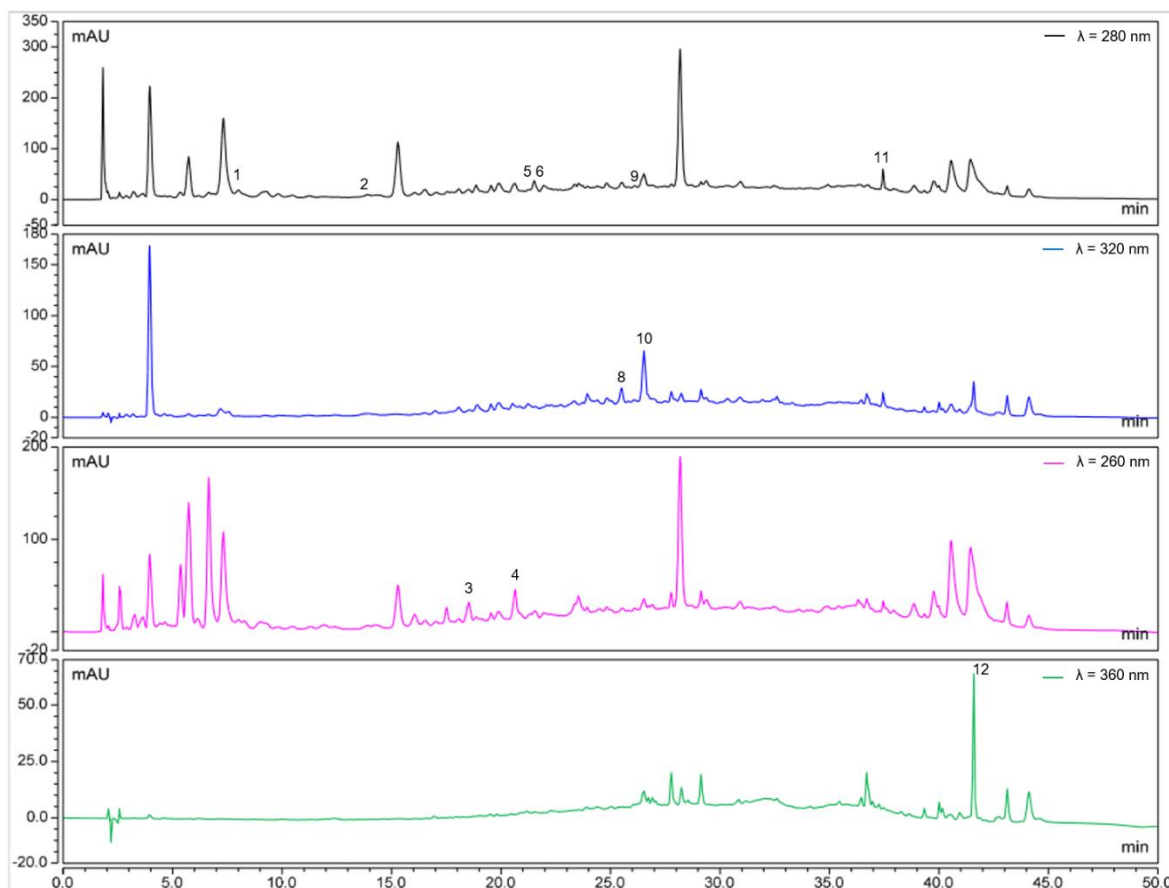


Figure 29. Chromatogram of the Stout beer aged with French Oak Chips.

1 – gallic acid, **2** – 3,4-dihydroxyphenylacetic acid, **3** – *m*-hydroxybenzoic acid, **4** – vanillic acid, **5** – (+)-catechin, **6** – syringic acid, **8** – *p*-coumaric acid, **9** – salicylic acid, **10** – *trans*-ferulic acid, **11** – isoxanthohumol, **12** – xanthohumol.

Table 18. Phenolic compounds concentration in the different beer samples ^a.

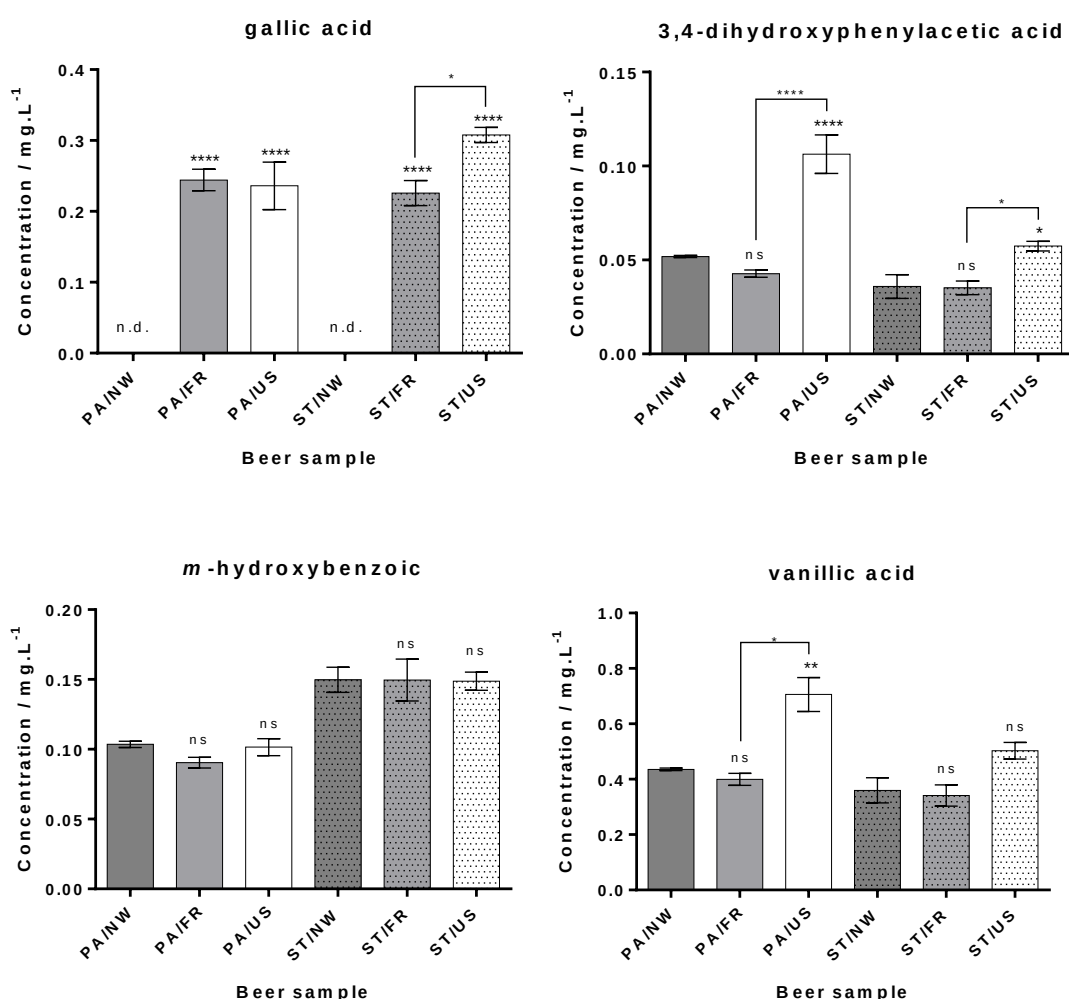
	Pale Ale					Stout				
	No wood	French Oak		US Oak		No wood	French Oak		US Oak	
GA	n.d.	0.244 ± 0.026	****	0.236 ± 0.058	****	n.d.	0.226 ± 0.031	****	0.309 ± 0.018	****
DHPA	0.052 ± 0.001	0.043 ± 0.003	ns	0.124 ± 0.032	****	0.036 ± 0.011	0.035 ± 0.006	ns	0.057 ± 0.005	*
m-HBA	0.104 ± 0.004	0.091 ± 0.007	ns	0.102 ± 0.011	ns	0.166 ± 0.030	0.150 ± 0.026	ns	0.149 ± 0.011	ns
VA	0.436 ± 0.008	0.400 ± 0.038	ns	0.706 ± 0.106	**	0.360 ± 0.079	0.341 ± 0.066	ns	0.503 ± 0.052	ns
C	1.139 ± 0.100	0.938 ± 0.088	ns	0.976 ± 0.120	ns	0.498 ± 0.047	0.511 ± 0.098	ns	0.480 ± 0.030	ns
Syr	n.d.	n.q.	ns	0.198 ± 0.030	****	n.q.	n.q.	ns	0.195 ± 0.024	****
Van	n.q.	n.q.		n.q.		n.q.	n.q.		n.q.	
E	0.353 ± 0.016	0.288 ± 0.021	**	0.294 ± 0.019	*	n.d.	n.d.		n.d.	
p-CA	0.050 ± 0.006	0.029 ± 0.009	ns	0.033 ± 0.006	ns	0.099 ± 0.019	0.090 ± 0.019	ns	0.095 ± 0.006	ns
Sal	0.688 ± 0.031	0.553 ± 0.039	*	0.574 ± 0.041	*	0.104 ± 0.032	0.090 ± 0.026	ns	0.127 ± 0.010	ns
t-FA	0.408 ± 0.011	0.282 ± 0.046	ns	0.359 ± 0.047	ns	0.384 ± 0.061	0.344 ± 0.066	ns	0.386 ± 0.024	ns
IX	0.458 ± 0.079	0.366 ± 0.028	ns	0.386 ± 0.026	ns	0.524 ± 0.088	0.441 ± 0.066	ns	0.352 ± 0.023	*
XN	0.488 ± 0.223	0.374 ± 0.028	ns	0.373 ± 0.036	ns	1.248 ± 0.137	1.079 ± 0.245	ns	0.955 ± 0.035	ns

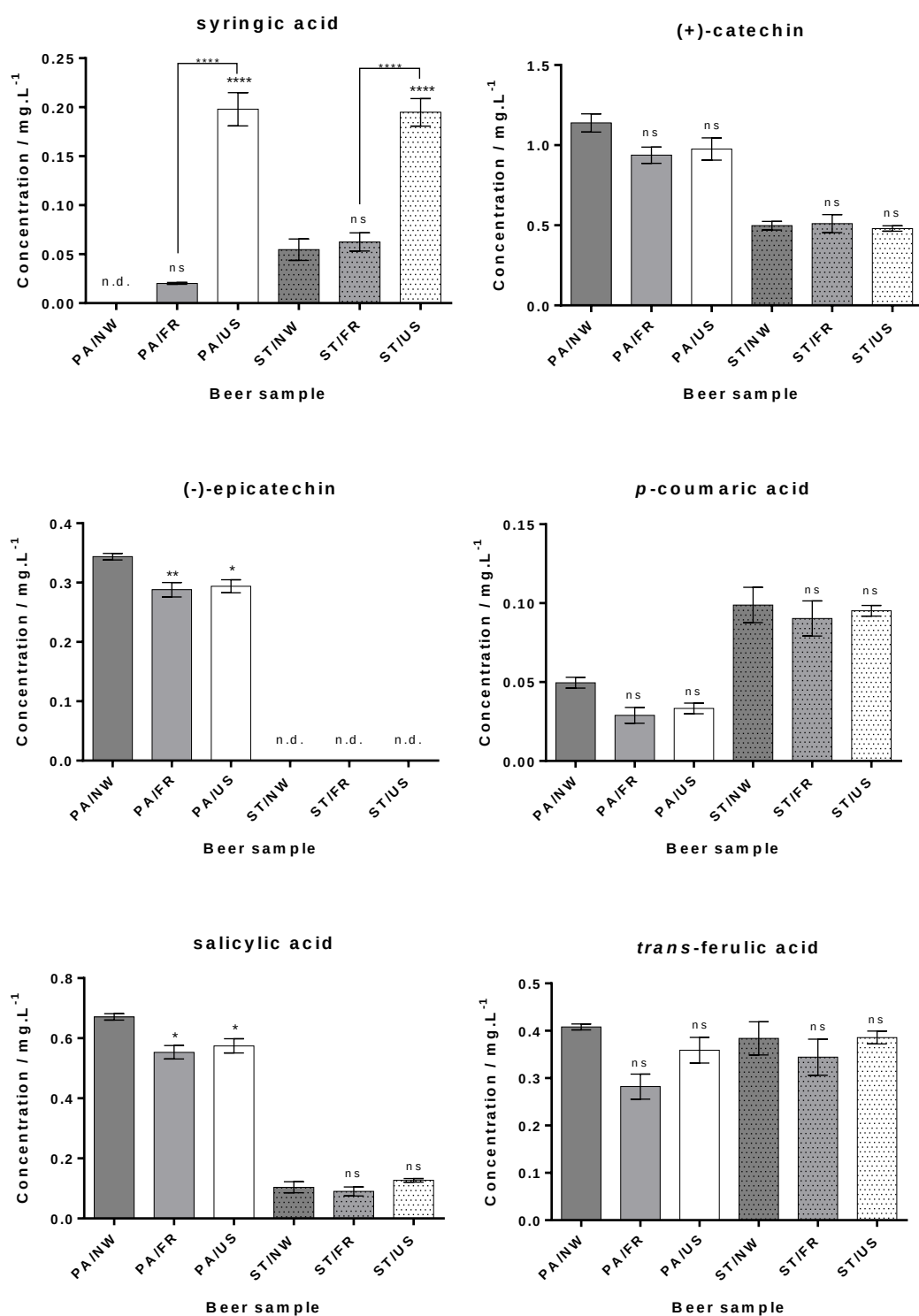
Results are expressed as mean ± SD (n = 3) of mg of phenolic compound per litre of beer sample (mg.L⁻¹). One-way ANOVA followed by Dunnett's multiple comparisons test against controls was performed with ****significant at $p < 0.0001$, **significant at $p < 0.01$, and *significant at $p < 0.05$.

^a **GA** – gallic acid, **DHPA** – 3,4-dihydroxyphenylacetic acid, **m-HBA** – *m*-hydroxybenzoic acid, **VA** – vanillic acid, **C** – (+)-catechin, **Syr** – syringic acid, **Van** – vanillin, **E** – (-)-epicatechin, **p-CA** – *p*-coumaric acid, **Sal** – salicylic acid, **t-FA** – *trans*-ferulic acid, **IX** – isoxanthohumol, **XN** – xanthohumol.

n.d. – not detected, **n.q.** – not quantified

Regarding the obtained results for the pale ale beer, in the samples treated with French oak wood chips, a statistically significant increase ($p < 0.0001$) in the concentration of gallic acid was verified whereas, in the samples treated with American oak wood chips, the concentration increased for the gallic, 3,4-dihydroxyphenylacetic, vanillic and syringic acids ($p < 0.05$). Concerning the stout beer, similar results were obtained with the samples treated with French oak wood chips presenting a statistically significant rise in the concentration of gallic acid ($p < 0.0001$), whereas for the samples treated with American oak wood chips the concentration of gallic, 3,4-dihydroxyphenylacetic, and syringic acids were significantly improved ($p < 0.05$), as depicted in the graphs presented below.





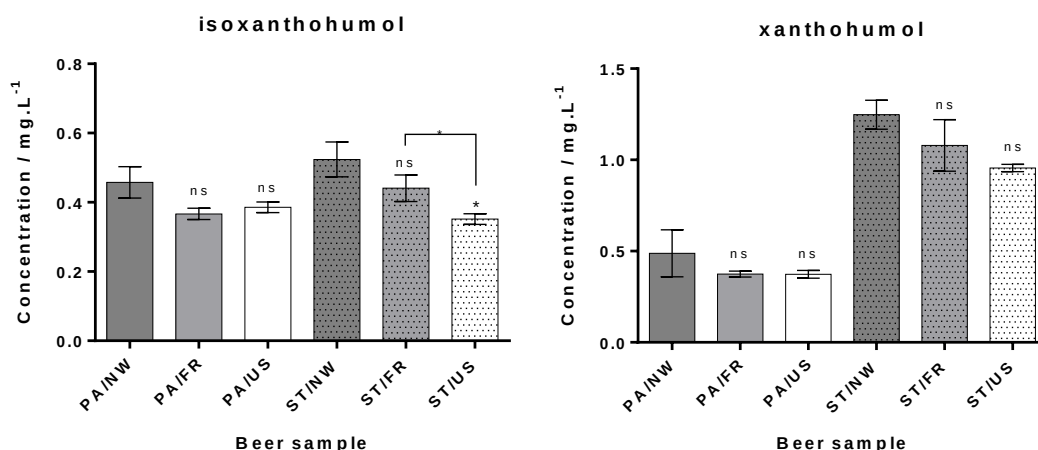


Figure 30. Concentration of the quantified individual phenolic compounds in the different beer samples. Results are presented as mean $\pm$ SEM ($n = 3$) with ****significant at $p < 0.0001$, **significant at $p < 0.01$, and *significant at $p < 0.05$.

After comparing the obtained results with the available literature for commercial beer, it is possible to observe that the concentration values of most quantified phenolic compounds concurs to what was previously described with values ranging from 0.14 to 5.77 mg.L⁻¹ for 3,4-dihydroxyphenylacetic acid [140], 0.01 to 0.13 mg.L⁻¹ for *m*-hydroxybenzoic acid [141], 0.06 to 2.98 mg.L⁻¹ for vanillic acid [37, 42, 141, 142], 0.03 to 4.00 mg.L⁻¹ for (+)-catechin [37, 141], 0.07 to 0.99 mg.L⁻¹ for syringic acid [37, 42, 142], 0.01 to 0.73 mg.L⁻¹ for (-)-epicatechin [37], and 0.01 to 1.83 mg.L⁻¹ for *p*-coumaric acid [36, 37, 42, 142].

The literature reports values for gallic acid (0.92 to 10.39 mg.L⁻¹ [36, 37, 141]), salicylic acid (3 mg.L⁻¹ [46]) and *trans*-ferulic acid (0.34 to 11.03 mg.L⁻¹ [37, 42, 142]), with higher concentrations compared to the studied beer samples. Some of these works were performed using a UV detector that only allows peak identification through retention time comparison possibly originating an overestimation of the reported concentration for the phenolic compounds. DAD generates an UV spectrum of each compound that can be matched with pure compounds' spectra to aid the identification and the determination of its purity. Additionally, the state-of-the-art detection technique, MS/MS, was scarcely found in literature and was mainly employed with the purpose of identification of the phenolic compounds.

Regarding the hops' prenylflavonoids, for isoxanthohumol, the obtained results are sustained by the available bibliography with reported values ranging from 0.1 to 4 mg.L⁻¹ [9, 16, 17, 143]. However, the obtained concentration values for xanthohumol are relatively higher than the values reported for commercial beers (up to 0.15 mg.L⁻¹). These results can

be, once again, explained by the absence of a filtration process. These technological processes, usually performed with PVPP or diatomaceous earth, can remove a significant part of the phenolic compounds, including xanthohumol [143]. Additionally, the use of different amounts of hops (either in the extract form or pellets) can justify the observed differences. In fact, high levels of xanthohumol ($>10 \text{ mg.L}^{-1}$) were reported for beer styles in which elevated amounts of hops are used, as is the case of India Pale Ales [14]. Also when comparing the pale ale and stout samples, the concentration levels of xanthohumol are significantly higher in the stout beer samples fact that can be explained by the formation of stable complexes with substances from roasted malts (possibly MLDs) affecting the isomerization process [143].

In order to demonstrate the reliability of the data provided, recoveries were performed in both pale ale and stout beers with values comprised between 80 and 120 % (table 16) [139].

Additionally, after analysing the phenolic extracts from both oak wood chips samples (chromatograms displayed in item 3. of supplementary materials) we concluded that ellagic acid is the major phenolic compound in both wood samples however, it is not efficiently extracted to beer since its detection was not viable.

3.3. Cellular assays

3.3.1. MTT assay

For the two beer styles tested, the control sample with no wood and both samples treated with oak wood chips from different origins managed to exhibit significant antiproliferative activity against human colorectal adenocarcinoma Caco-2 cells, after 48h of exposure. In the graph represented in figure 31, the log-dose vs response curve and the results of the calculation of the IC_{50} for the pale ale style beer are presented being lowest in the beer sample treated with American oak wood chips with a value of 18.70 ± 1.30 (% beer in CM).

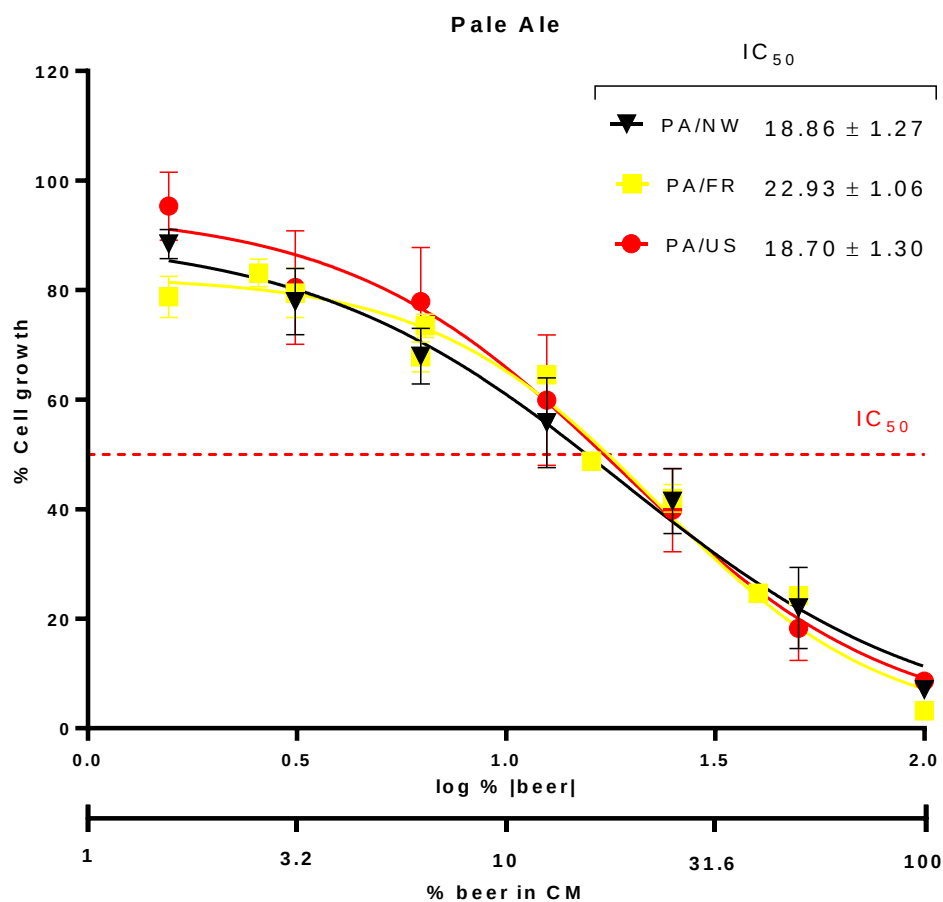


Figure 31. Log-dose vs. response curve for pale ale samples: control (PA/NW), treatment with French oak chips (PA/FR), and treatment with American oak chips (PA/US). Values obtained for beer samples with concentrations ranging from 1.56 to 100 % (beer in CM) on Caco-2 cell growth after 48h exposure.

For the stout style beer, the log-dose vs response curve and IC₅₀ values are represented in figure 32. The beer sample treated with French oak chips exhibited the lowest IC₅₀ value with 16.45 ± 1.06 (% beer in CM).

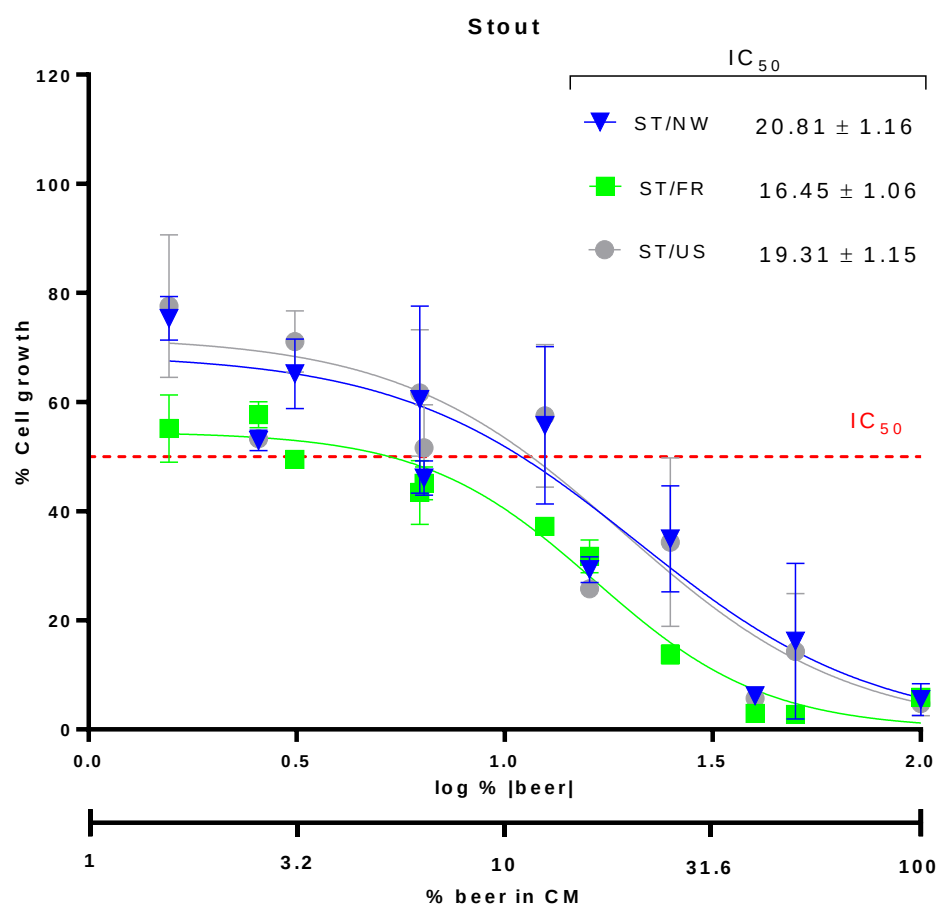


Figure 32. Log-dose vs. response curve for stout samples: control (ST/NW), treatment with French oak chips (ST/FR), and treatment with American oak chips (ST/US). Values obtained for beer samples with concentrations ranging from 1.56 to 100 % (beer in CM) on Caco-2 cell growth after 48h exposure.

For the pale ale beer, at a concentration of 20 % (beer in CM), the values concerning the inhibition of cell growth range from 29.36 to 37.05 % with no statistically significant differences when comparing the two samples with oak wood chips treatment (FR and US) with the control (NW). For the stout beer, the results range from 59.14 to 63.31 % of inhibition of cell growth. When comparing the antiproliferative activities of the different beer samples from the two beer styles at a common concentration, the results obtained are similar to the ones from the *in vitro* bioactivity assays, with the stout beer exhibiting a higher activity (figure 33). A noteworthy difference (**** $p < 0.0001$) between pale ale and stout beers was encountered, with the stout beer samples demonstrating superior antiproliferative activity with a maximum inhibition of 63.31% of cell growth for the stout beer treated with French oak chips.

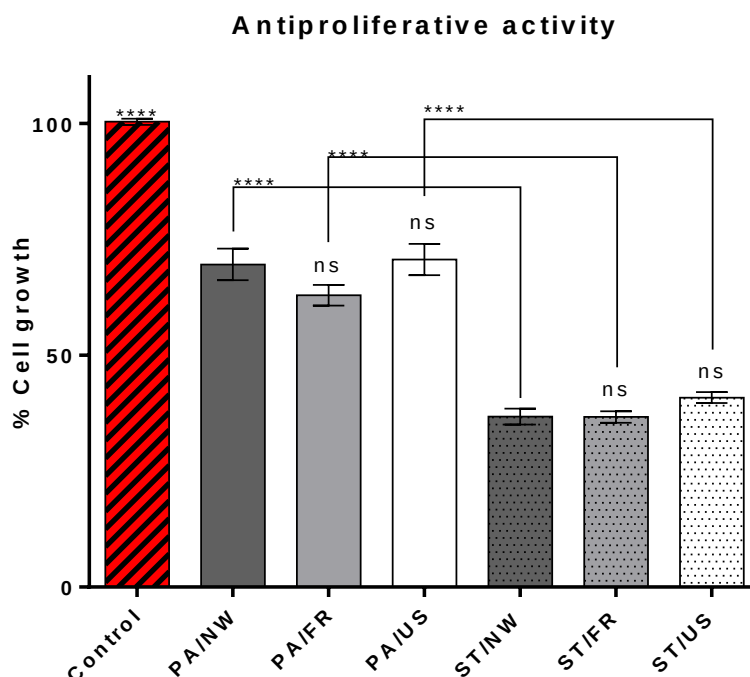


Figure 33. Antiproliferative activity of two different beer styles at a concentration of 20 % (beer in CM) and respective treatments [control (NW), French oak chips (FR), and American oak chips (US)] on Caco-2 cell growth after 48h exposure. **** Significant at $p < 0.0001$.

3.3.2. Determination of extracellular NO levels

After the evaluation of the extracellular levels of NO, it was verified that the beer samples increased the final absorbance signal in the cells without LPS stimulation, compared to the control without beer addition. That occurrence can be explained either through colour interference in the absorption of the diazo dye, through stimulation of the inflammatory process by the beer complex matrix, or through the possible presence of nitrites in beer. To clarify that, tests were carried out to evaluate the possibility of the absorption interference and to quantify the nitrites level in the beer samples. Overall, it was observed that the beer NO/NO₂ content could be considered negligible thus the verified signal could be due to the beer basal pro-inflammatory effect (data not shown).

Therefore, the results are presented in figure 34 as the %NO production observed in the macrophage cells treated with the different beer samples and LPS stimulation vs. the LPS stimulated RAW 264.7 controls. Considering the basal pro-inflammatory effect of the

beer samples, and to facilitate the comparison between the different treatments, the %NO production values corresponding to basal beer pro-inflammatory effect (without the LPS stimulation) were subtracted from the %NO corresponding to cells treated with beer and LPS-stimulated.

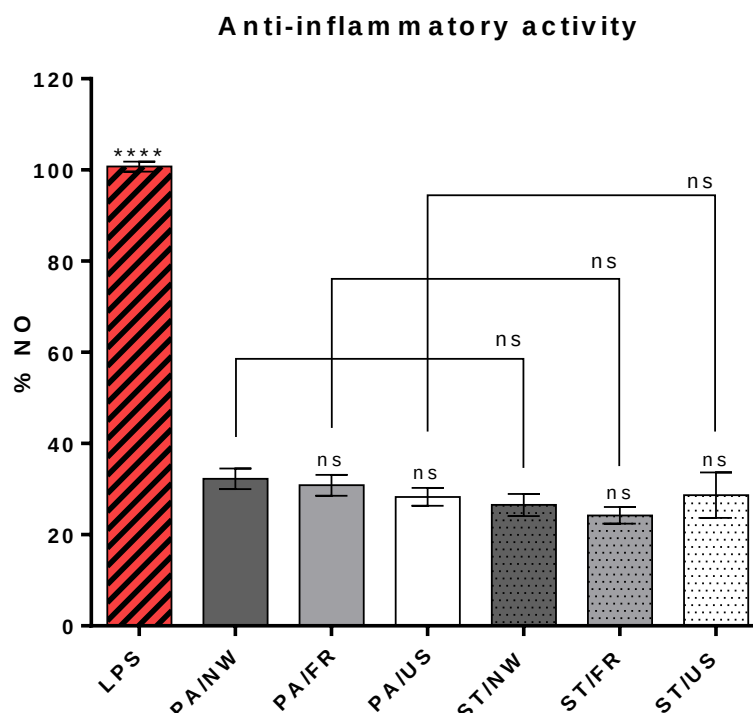


Figure 34. Anti-inflammatory activity assessed through the determination of extracellular NO levels, via Griess reaction mechanism. Beer samples vs. LPS control. ****significant at $p < 0.0001$.

After statistical analysis, it was observed that beer presented a notorious inhibition of the increase in NO production after stimulation by LPS. All beer samples presented significant anti-inflammatory activity when compared to the LPS stimulated control (**** $p < 0.0001$). The inhibition of NO production in RAW 264.7 cells ranged from 67.74 ± 2.25 to 71.70 ± 1.96 (% inhibition of NO production) for the Pale Ale beer samples and from 71.34 ± 4.99 to 75.75 ± 1.83 for the Stout beer samples. Furthermore, no statistically significant differences were observed between the samples aged with wood chips and the samples without the addition of wood. Additionally, when comparing the two different beer styles, no statistically significant differences were observed between respective sample treatments.

Kanuri *et al.* [144] reported that pilsner beer tested in RAW 264.7 cells, stimulated with LPS, managed to attenuate the LPS effect in iNOS and TNF- α mRNA expression, therefore exerting a significant anti-inflammatory activity, concurring with the obtained

results for the tested beer samples in this work. However, the authors also reported that Stout beer failed to display any activity compared to the LPS control, contradicting the verified tendency in this work, for the stout beer samples. It was also previously reported that ferulic acid, present in substantial amounts in beer is also able to diminish NO production in RAW 264.7 cells, attenuating TNF- α production [145].

To better understand the anti-inflammatory activity of the tested beer samples, it would be ideal to proceed with more fine studies to evaluate with more detail the mechanisms of the anti-inflammatory effect noticed in this assay. For instance, it would be very informative to evaluate the capacity of the samples to decrease iNOS or COX-2 expression, inducible isoforms of nitric oxide synthetase and cyclooxygenase, respectively (*e.g.* through western blot methodology, enzyme quantification using a biochemical test, or qPCR gene expression measurement) that are responsible for the production of NO and prostaglandins (PGs), correspondingly. Moreover, the evaluation of commonly cited diet associated inflammatory biomarkers such as interleukins (IL-1 β , IL-4, IL-6, and IL-10), tumour necrosis factor (TNF- α), and C-reactive protein (CRP) would also be very elucidative [146].

3.3.3. Determination of intracellular ROS and RNS levels

The results for the determination of intracellular ROS and RNS levels are presented, much like to the determination of NO extracellular levels assay, as the %NO production signal of the LPS-stimulated RAW 264.7 cells treated with beer samples vs. the macrophage cells stimulated with LPS. Once more, to simplify treatment comparison between the samples, the values of %NO production of the RAW 264.7 cells treated with the different beer samples, without LPS stimulation were subtracted.

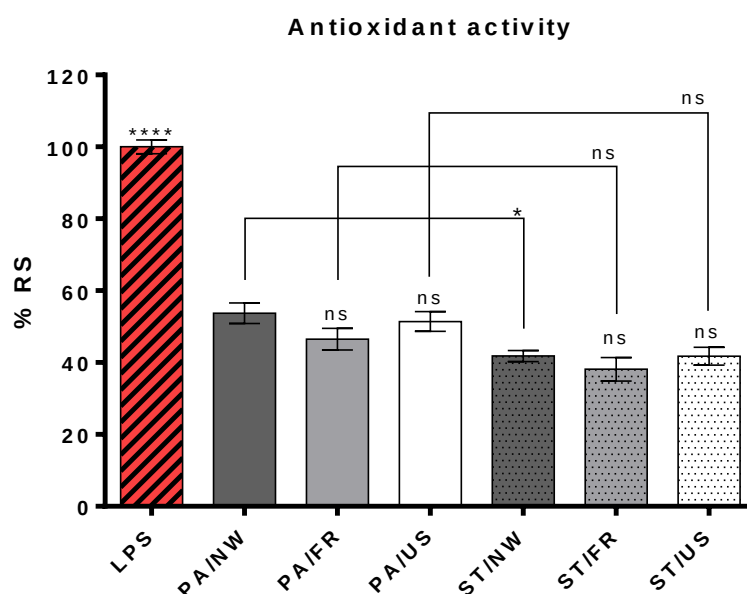


Figure 35. Antioxidant activity assessed through the determination of intracellular reactive species (RS) levels, via DCFH oxidation. Beer samples vs. LPS control. ****significant at $p < 0.0001$, and *significant at $p < 0.05$.

After statistical analysis, all beer samples presented significant antioxidant activity when compared to the LPS stimulated control (**** $p < 0.0001$). The inhibition of RS production in RAW 264.7 cells ranged from 46.29 ± 2.85 to 53.50 ± 3.03 (% inhibition of RS production) for the Pale Ale beer samples and from 58.19 ± 1.52 to 61.86 ± 3.26 for the Stout beer samples. Furthermore, no statistically significant differences were observed between the samples treated with wood chips and the samples without the addition of wood. Additionally, when comparing the respective treatments from the two beer styles, only the samples without the addition of wood presented statistically significant differences (* $p < 0.05$). Again, the study of the mechanism involved in the ROS production, including the expression of cytokines associated with the oxidative stress in cells should be exploited.

This correlation of the increase in NO and ROS production could be due to the contribution of NO to the ROS level, as referred, however, it is also well-known that both ROS and NO are involved in the inflammatory process and highly related.

3.3.4. Summary of the results from the cell cultures assays

In summary, as detailed in table 19, it was demonstrated that all beer samples exerted significant activity in all the assays carried out. However, no statistically significant differences were established between the beer samples aged with wood and the samples without wood addition. Moreover, in the cell viability assays performed using Caco-2 cell line, higher antiproliferative activity was demonstrated by the stout beer samples, accordingly to what was previously described for the *in vitro* assays. However, the RAW 264.7 cell line assay did not permit to establish the same differences between the two beer styles.

Table 19. Summary of the results from the Caco-2 and RAW 264.7 cell lines assays. The concentration of the beer samples was 20% and 12.5% (beer in CM) for the two cell lines, respectively. Results are expressed as mean $\pm$ SEM (n = 3).

		Caco-2	RAW 264.7	
		Antiproliferative activity (% cell growth)	Anti-inflammatory activity (% NO)	Antioxidant activity (% RS)
Pale Ale	No wood	69.57 $\pm$ 3.41	32.26 $\pm$ 2.25	53.71 $\pm$ 2.85
	French Oak	62.95 $\pm$ 2.22	30.83 $\pm$ 2.27	46.5 $\pm$ 3.03
	American Oak	70.64 $\pm$ 3.38	28.3 $\pm$ 1.96	51.42 $\pm$ 2.76
Stout	No wood	36.76 $\pm$ 1.72	26.52 $\pm$ 2.43	41.81 $\pm$ 1.52
	French Oak	36.69 $\pm$ 1.25	24.25 $\pm$ 1.83	38.14 $\pm$ 3.26
	American Oak	40.86 $\pm$ 1.17	28.66 $\pm$ 4.99	41.77 $\pm$ 2.49

III. Final remarks and future work

The experimental research work started with the production of two beer styles (Pale Ale and Stout) that, further on, were aged with the addition of oak wood chips. Subsequently, *in vitro* assays were carried out to analyse antioxidant activity, total phenolic, and total flavonoids concentration. After statistical analysis, we concluded that the beer samples possess a noteworthy antioxidant activity and higher phenolics and flavonoids concentrations when compared to the bibliography. Nonetheless, globally, no statistically significant differences were observed between beer samples aged with wood and the control beer without the addition of wood. Furthermore, it was verified that the darker, stout beer exhibited a higher phenolic and flavonoids concentration and, accordingly, superior antioxidant activity.

Next, twelve individual phenolic compounds were identified and quantified through an HPLC-DAD analysis with concentration values corroborated by the available literature for most of the selected compounds. The beer samples aged with the American oak wood chips exhibited the more promising results for phenolic extraction, namely for compounds such as gallic, 3,4-dihydroxyphenylacetic, vanillic and syringic acids.

Followingly, antiproliferative, antioxidant and anti-inflammatory activities of craft beer aged with oak wood chips were assessed via cell culture assays, using human Caco-2 and murine RAW 264.7 cell lines. Using the Caco-2 cell line, we observed a substantial antiproliferative activity and successfully determined the IC₅₀ value of the different beer samples, after assessing cell viability through the MTT assay, with values nearing 20% (beer in CM) for all the tested beer samples. Next, we chose the 20% concentration and performed a new MTT assay with the purpose of sample comparison and, after statistical analysis, no significant differences were found between the samples aged with oak wood chips and the control beer without the wood addition. Additionally, the Stout beer samples exhibited a significantly higher antiproliferative activity than the Pale Ale samples as noticed in previous biochemical assays.

Finally, via the murine RAW 264.7 cell line, the anti-inflammatory activity and the antioxidant activities of the beer samples were evaluated after cell stimulation with LPS. Once again, for the two mentioned biological properties, beer exhibited remarkable activity, however, yet once more, no statistically significant differences were verified between the beer samples aged with oak wood chips and the control beer without the addition of wood.

In conclusion, in the tested experimental conditions, no significant increment in bioactivity was observed through the addition of oak wood chips. It is of pivotal importance to highlight that this work reported, for the first time, the assessment of craft beer anti-inflammatory and antioxidant activities in the RAW 264.7 cell line whilst also providing valuable information regarding craft beer phenolic and flavonoid concentrations, a topic with relatively scarce bibliography support. This reported bioactive effects should be further exploited at the mechanism of action level by studying qPCR gene expression of the proinflammatory cytokines associated with the inflammatory and oxidative cellular process (e.g. IL-1 β , IL-6, and TNF- α).

In upcoming studies, it could be also important to experiment a new approach regarding the %ABV of beer, the quantity, time of exposure, and toast degree of the oak wood chips and their respective effect in the amount of extractable phenolic compounds and expected global bioactivity augmentation. Particularly the contact time between the beer and the wood chips followed in the present study, recommended by the wood chips supplier, could be increased in order to verify if this could result in more phenolics transfer with a consequent increase in bioactivity.

Next in line, it would be ideal to evaluate the impact of the addition of oak wood chips in the potential organoleptic improvement of beer through sensory analysis and via GC methodologies to focus on the volatile fraction of beer compounds. Furthermore, the confirmation of the obtained profile and further identification of phenolic compounds in the beer samples through LC-MS methodologies and a mineral profile through FAAS and ICP-MS methodologies would be beneficial to fully understand, with greater detail, changes operated by the ageing process with oak wood chips.

IV. References

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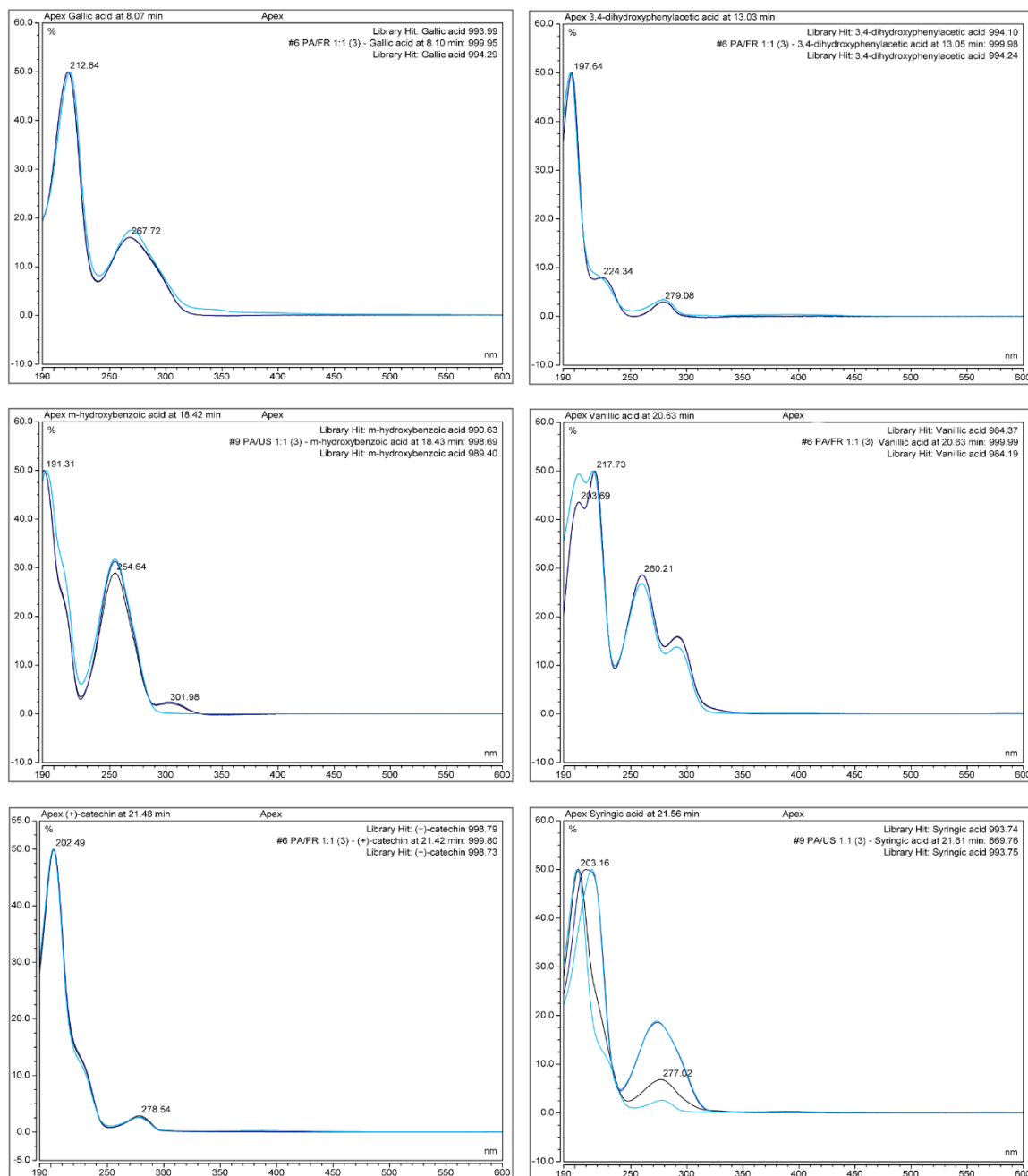
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Supplementary materials

1. Spectral comparison results for chromatographic peak identification.



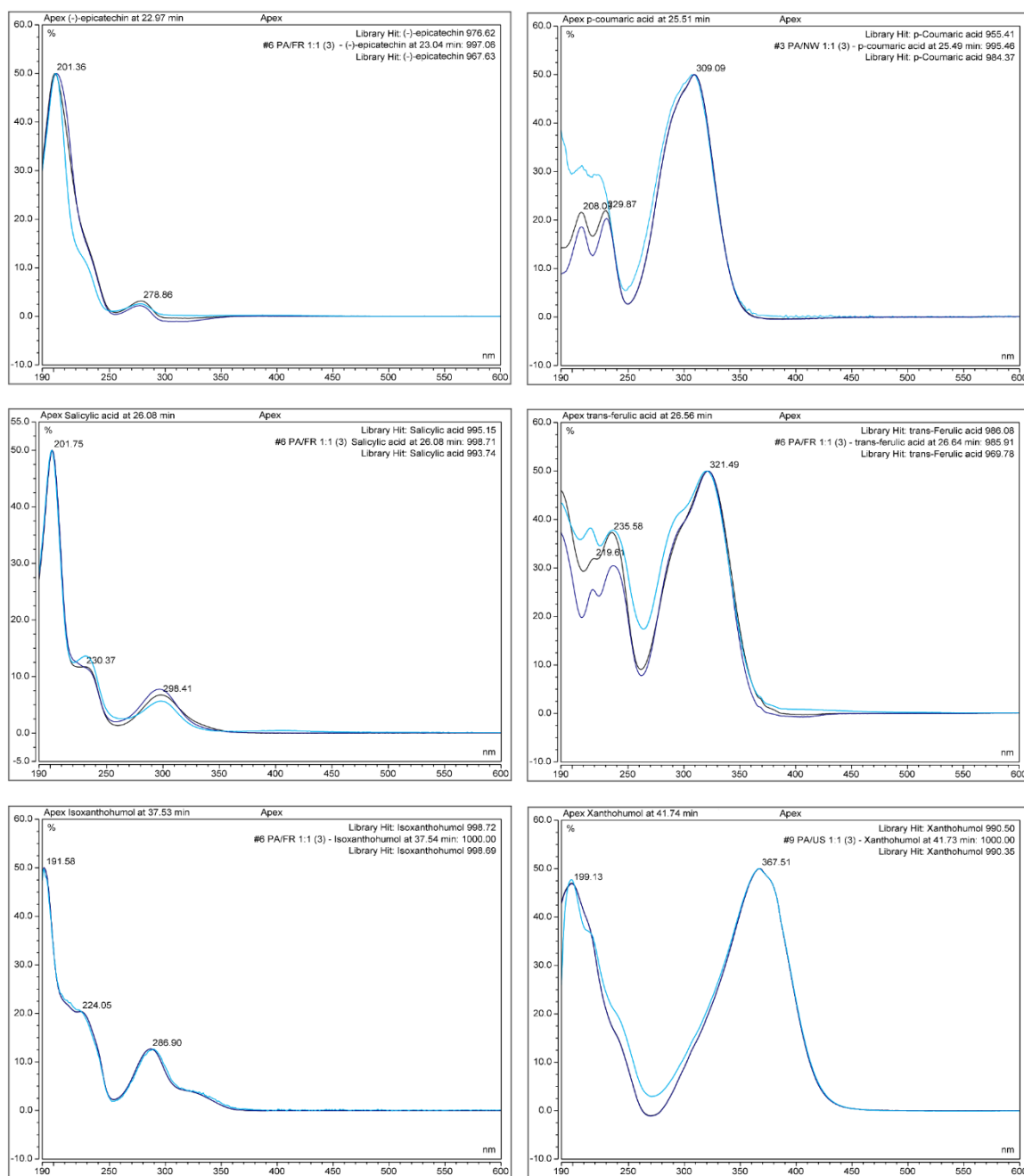


Figure 36. Spectral comparison analysis for the identification of phenolic compounds.

2. HPLC-DAD chromatograms of the selected wood samples phenolic compounds

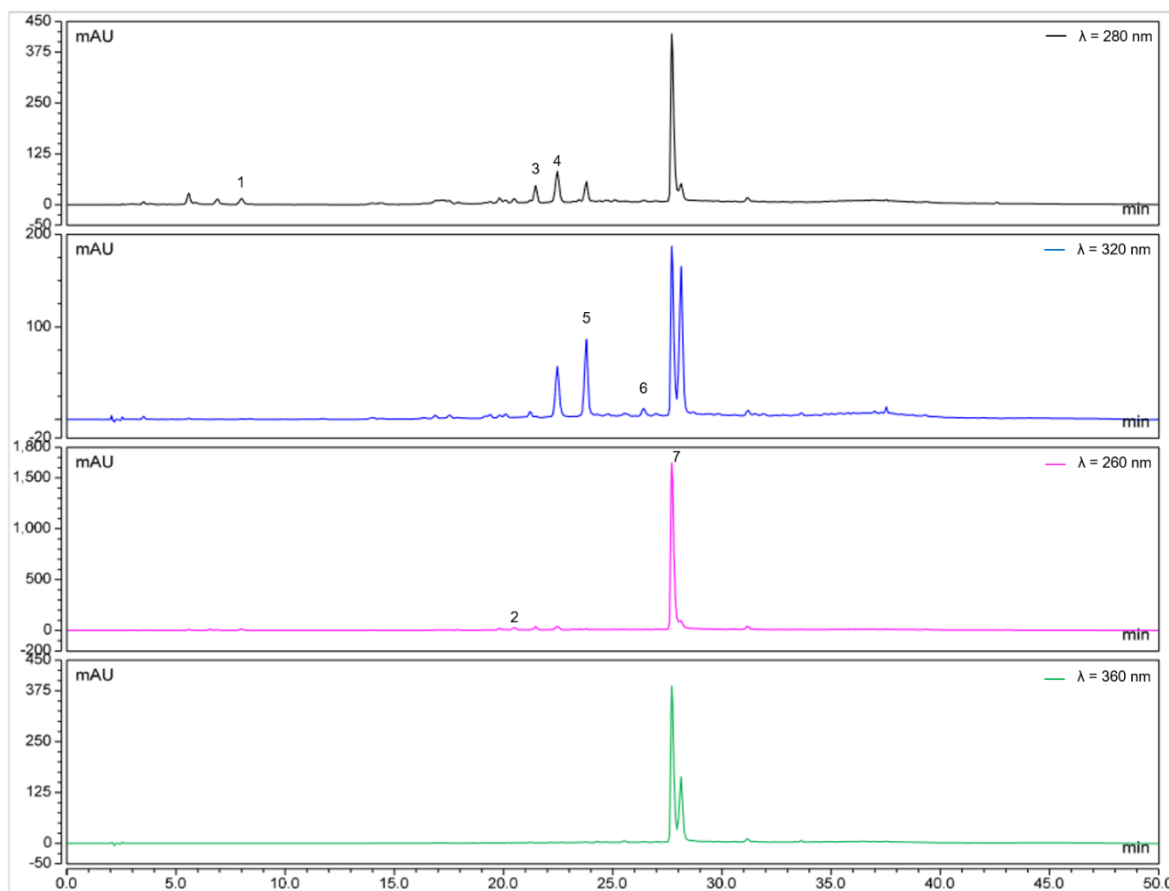


Figure 37. Chromatogram for the American oak wood chips analysis.

1 – gallic acid, **2** – vanillic acid, **3** – syringic acid, **4** – vanillin, **5** – syringaldehyde, **6** – sinapic acid, **7** – ellagic acid

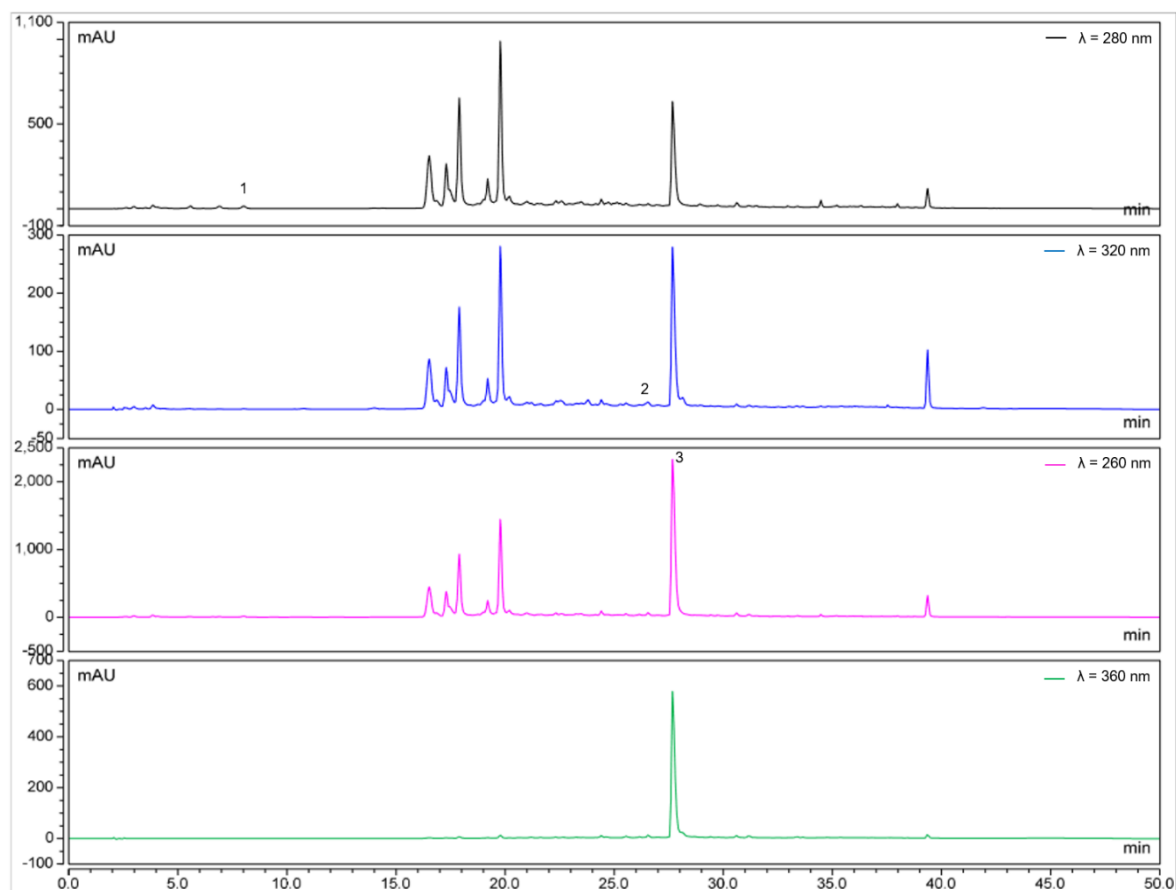


Figure 38. Chromatogram for the French oak wood chips analysis.

1 – gallic acid, **2** – syringaldehyde, **3** – ellagic acid