Further insights into the utilization of a by-product from the chestnut production chain as a source of functional ingredients for beverages: the beer challenge

Lavinia Silva Veríssimo

Final dissertation submitted to Escola Superior de Tecnologia e Gestão of Instituto Politécnico de Bragança to obtain the Master's degree in Chemical Engineering in the ambit of the double diploma with Instituto Federal do Espírito Santo.

Supervised by:
Dra. Daniele Bobrowski Rodrigues (CIMO/IPB, Portugal)
Dra. Tiane Cristine Finimundy (CIMO/IPB, Portugal)
Prof. Dr. Juliano Souza Ribeiro (IFES, Brazil)

Bragança
2023

The authors are grateful to the European Regional Development Fund (ERDF), through the research and technological development incentive system (SI I&DT), notice 13/si/2020 corporate R&D projects in co-promotion for inland territories, within the scope of Project Bio4Drinks (NORTE-01-0247-FEDER-113508). Moreover, they thank the Foundation for Science and Technology (FCT, Portugal) for financial support through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2020).

Acknowledgments

I would like to express my heartfelt gratitude to all those who have supported me throughout my journey in completing this thesis. This work would not have been possible without the invaluable assistance and inspiration from a multitude of individuals and organizations.

I owe a debt of gratitude to my family for their unwavering support, understanding, and encouragement throughout this challenging endeavor. Your belief in my abilities and the sacrifices you made have been the cornerstone of my success. My parents, Mário Sérgio and Ana Paula, who always taught me good things and all the support they gave me to complete this phase in the best way possible, always believing in my potential. Also, to my sister Gabrielli, my best friend and biggest supporter, who has been my safe haven all these years.

I would like to extend my deepest appreciation to my thesis advisor, Dra. Daniele Bobrowski Rodrigues. Your guidance, unwavering support, and insightful feedback were instrumental in shaping this thesis. Your dedication to excellence and passion for the subject matter have been truly inspiring.

I would like to acknowledge the research participants and the Bio4Drinks project, in special my co-advisor Dra. Tiane Finimundy, without whose cooperation and willingness to share their insights, this thesis would not have been possible. Your contributions are deeply appreciated.

I am also thankful to the faculty members and mentors at Polytechnic Institute of Bragança and Federal Institute of Espírito Santo who have provided me with a strong academic foundation and the resources to pursue my research. Their commitment to fostering intellectual curiosity and growth has been invaluable.

In advance I acknowledge the jury members for their willingness to participate and provide my thesis with their valuable feedback.

To my friends and colleagues, I am grateful for the camaraderie, discussions, and shared experiences that enriched my academic journey. Your encouragement and companionship made the long hours of research and writing more manageable. Special thanks to Nathalia Borba for advising me during these 2 years.

Last but not least, I would like to express my gratitude to the countless individuals who may not be mentioned here but have played a vital role, no matter how small, in my academic and personal growth. This thesis stands as a testament to the collective support and efforts of all those who have contributed to its realization. Your contributions have made a lasting impact on my academic journey, and for that, I am truly thankful.

ABSTRACT

Beer is one of the oldest and most appreciated alcoholic beverages worldwide. The industrial beer market is already established as an important factor for the national economy, however microbreweries have conquered their space, by offering craft products with distinct sensory attributes, with varied production styles and a wide range of flavours. Because they are artisanal, these beers have generally less stability and a shorter shelf life compared to large-scale processed beers. Aiming at greater conservation of these beverages, there is great interest in the inclusion of natural ingredients with bioactive potential to add quality to the product. In this sense, the present work aims to produce a functional ingredient from extracts rich in phenolic compounds from the chestnut flower, to apply it in the production of craft beers to stabilize the product by preventing its rapid oxidation. For this, flowers and burs of *Castanea sativa* collected in the region of Bragança were lyophilized, ground and subjected to an extraction process using food grade solvent. Chestnut by-products extracts were characterized and added to prototype craft beers produced by a local microbrewery. Beers with incorporated and control extracts were monitored using volatile oxidation markers by solid phase microextraction with dynamic headspace coupled to gas chromatography with flame ionization detector and gas chromatography coupled to mass spectrometry, in addition to monitoring the composition of phenolic compounds by high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass. All samples exhibited the ability to inhibit the oxidative process. Two bur extractions (maceration and ultrasound using water as a solvent) had the lowest half maximal effective concentration (EC_{50}) value of 0.002 mg/mL. This was followed by ultrasound extraction of flowers (0.003 mg/mL) and maceration of flowers and ultrasound with ethanol:water of burs (0.004 mg/mL). Samples with high phenolic compound content demonstrated better results, suggesting a positive correlation between phenolic composition and antioxidant activity. *C. sativa* extracts exhibited antibacterial activity against various strains of bacteria and caused death of the bacterial strains. Minimum inhibitory concentration (MIC) values for Gram-positive bacteria were lower than those for Gram-negative bacteria. The extracts also showed similar antifungal potential, with MIC values of 10 mg/mL. A total of 32 different compounds were identified in the extracts of *C. sativa* by-products. Incorporating a natural ingredient from chestnut flowers into Indian Pale Ale (IPA) craft beers was effective in preserving the beer's flavour profile during the storage months. This natural extract acted as a preservative agent, inhibiting reactions that could result in the formation of undesirable, off-flavour compounds. These findings highlight the potential of utilizing chestnut flower extract as an alternative to enhance the flavour stability and overall quality of craft beers, contributing to extending the shelf life of the product.

Keywords: craft beers; oxidation; phenolic compounds; *Castanea sativa*

RESUMO

A cerveja é uma das bebidas alcoólicas mais antigas e apreciadas em todo o mundo. O mercado de cervejas industriais já está consolidado como fator importante para a economia nacional, porém as microcervejarias têm conquistado seu espaço, trazendo produtos artesanais com atributos sensoriais afetivos, com estilos de produção variados e ampla gama de aromas e sabores. Por serem artesanais, essas cervejas têm menos estabilidade e menor prazo de validade em comparação com as cervejas processadas em larga escala. Visando uma maior conservação dessas bebidas e considerando indícios de riscos à saúde associados aos conservantes sintéticos, há grande interesse na inclusão de adjuntos naturais com potencial bioativo para agregar qualidade ao produto. Nesse sentido, o presente trabalho visa produzir um ingrediente funcional a partir de extratos ricos em compostos fenólicos da flor do castanheiro, para aplicá-lo na produção de cervejas artesanais para estabilizar o produto evitando sua rápida oxidação. Para isso, flores e ouriços de *Castanea sativa* coletadas na região de Bragança foram liofilizadas, moídas e submetidas a um processo de extração com solvente grau alimentício. Extratos de subprodutos da castanha foram caracterizados e adicionados a protótipos de cervejas artesanais produzidas por uma microcervejaria local. Cervejas com extratos incorporados e controle foram monitoradas utilizando marcadores voláteis de oxidação por microextração em fase sólida com *headspace* dinâmico acoplado a cromatografia gasosa com detector de ionização de chama e cromatografia a gás acoplada à espectrometria de massas, além do monitoramento da composição de compostos fenólicos por cromatografia líquida de alta eficiência acoplada à detecção de arranjo de diodos e massa em tandem de ionização por eletrospray. Todas as amostras exibiram a capacidade de inibir o processo oxidativo. Duas extrações de ouriço (maceração e ultrassom usando água como solvente) tiveram o menor valor de metade da concentração efetiva máxima (EC_{50}) de 0,002 mg/mL. Seguiu-se extração ultrassônica das flores (0,003 mg/mL) e maceração das flores e ultrassom com etanol:água dos ouriços (0,004 mg/mL). Amostras com alto teor de compostos fenólicos apresentaram melhores resultados, sugerindo uma correlação positiva entre composição fenólica e atividade antioxidante. Os extratos de *C. sativa* exibiram atividade antibacteriana contra várias cepas de bactérias e causaram a morte das cepas bacterianas. Os valores de concentração inibitória mínima (MIC) para bactérias Gram-positivas foram menores do que para bactérias Gram-negativas. Os extratos também apresentaram potencial antifúngico semelhante, com valores de MIC de 10 mg/mL. Um total de 32 compostos diferentes foram identificados nos extratos de subprodutos de *C. sativa*. A incorporação do ingrediente natural de flores de castanheiro em cervejas artesanais Indian Pale Ale (IPA) foi eficaz em preservar o perfil de sabor da cerveja durante os meses de armazenamento. Este extrato natural atuou como agente conservante, inibindo reações que poderiam resultar na formação de compostos indesejáveis e com sabor desagradável. Esses achados destacam o potencial da utilização do extrato de flor de castanheiro como uma alternativa para melhorar a estabilidade do sabor e a qualidade geral das cervejas artesanais, contribuindo para prolongar a vida útil do produto.

Palavra-chave: cervejas artesanais; oxidação; compostos fenólicos; *Castanea sativa*

LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgram
ABTS	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ATCC	*American Type Culture Collection*
DAD	Diode Array Detector
DMSO	Dimethyl sulfoxide
DPPH•	2,2-diphenyl-1-picrylhydrazyl radical
dw	Dry weight
EC50	Half-maximal effective concentration
ESI	Electrospray ionization
EtOH	Ethanol
EU	European Union
fw	Fresh weight
g	Gram
GAE	Gallic Acid Equivalent
GC-FID	Gas chromatography with flame ionization detector
GC-MS	Gas chromatography coupled to mass spectrometry
H_2O	Water
HPLC	High-Performance Liquid Chromatography
HPLC-DAD-MSn	High-Performance Liquid Chromatography coupled to Diode Array Detector and tandem Mass Spectrometry
HS	Headspace
INE	National Statistics Institute
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
MAC	Maceration
MAE	Microwave-assisted extraction
MBC	Minimum Bactericidal Concentration
MeOH	Methanol
MFC	Minimum Fungicidal Concentration
mg	Milligram
MIC	Minimum Inhibitory Concentration
min	Minutes
MS	Mass spectrometry
m/z	Mass to charge ratio

Nc	Not calculated
Nd	Not detected
Nq	Not quantified
O_2	Oxygen
PDO	Protected Designation of Origin
PUFA	Polyunsaturated Fatty Acids
RDI	Reference Daily Intake
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	Rotations per minute
Rt	Retention time
SD	Standard deviation
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
TBARS	Thiobarbituric Acid Reactive Species
TIC	Total Ion Chromatogram
tr	Traces
UAE	Ultrasound-Assisted Extraction
UV-Vis	Ultraviolet-Visible light
v/v	Volume to volume ratio
VOC	Volatile Organic Compounds
w/v	Weight to volume ratio

TABLE OF CONTENTS

FIGURE INDEX ..... **ix**
TABLE INDEX ..... **xi**
1. INTRODUCTION **1**
2. LITERATURE REVIEW **4**
2.1 BEERS AND CRAFT BEERS **4**
2.1.1 History of beer, definition, and legislation 4
2.1.2. Market overview 6
2.1.3. Ingredients, production process and quality of craft beers 8
2.2 CRAFT BEER FLAVOUR **11**
2.2.1 Beer flavour stability 12
2.3. PHENOLIC COMPOUNDS **15**
2.3.1. Phenolic compounds in beers 18
2.4. *CASTANEA SATIVA* AS A PHENOLIC COMPOUND SOURCE **19**
3. OBJECTIVE **21**
3.1 MAIN OBJECTIVE **21**
3.2 SPECIFIC OBJECTIVES **21**
4. MATERIAL AND METHODS **23**
4.1 CHEMICALS **23**
4.2 PLANT MATERIAL **23**
4.3 EXTRACTION OF PHENOLIC COMPOUNDS FROM CHESTNUT BY-PRODUCTS **24**
4.3.1 Maceration (MAC) 24
4.3.2 Ultrasound-assisted extraction (UAE) 24
4.3.3 Microwave-assisted extraction (MAE) 24
4.4 ANALYSIS OF PHENOLIC COMPOUNDS IN THE EXTRACTS **25**
4.5 *IN VITRO* BIOACTIVITY ASSAYS OF POLYPHENOL-RICH EXTRACTS **26**
4.5.1 Antioxidant activity 26
4.5.2 Antimicrobial activity 27
4.6. INCORPORATION OF PHENOLIC-RICH EXTRACT INTO CRAFT BEER **28**
4.7 BEER STABILITY **29**
4.6.1 Monitoring of volatile organic compounds (VOCs) in beer 29
4.6.2 Monitoring of phenolic compounds from a *C. sativa* by-product in beer 31
4.7 STATISTICAL ANALYSIS **32**

5. RESULTS AND DISCUSSION 33
5.1 EXTRACT CHEMICAL CHARACTERIZATION 33
5.2 EXTRACT BIOACTIVITIES 42
5.2.1 Antioxidant activity 42
5.2.2 Antimicrobial activity 44
5.3 EXTRACT SELECTION AFTER INITIAL SCREENING 46
5.4 EFFECT OF THE EXTRACT ON THE CHEMICAL STABILITY OF CRAFT BEERS 46
5.4.1 Volatile compounds 46
5.4.2 Chestanin monitoring in the beer containing the extract 51
6. CONCLUSION 54
7. FUTURE PERSPERCTIVES 55
BIBLIOGRAPHIC REFERENCES 56
PUBLICATIONS 67

FIGURE INDEX

Figure 1. Number of microbreweries in Europe in the period between 2008 and 2021...6

Figura 2. Examples of craft beers produced and commercialized in Portugal 7

Figure 3. Examples of craft beers produced with chestnuts and commercialized in Portugal.....8

Figure 4. Flow chart of the operations of the production process of the craft and industrial beers.....9

Figure 5. Conversion of glucose into alcohol and CO_2 by fermenting yeast 11

Figure 6. Structure of *trans*-2-nonenal, one of the most recognised compounds associated with off-flavours in beers 14

Figure 7. Analysis of the number of manuscripts published by year since the year 2010, related to the topic of oxidative change in beers. 15

Figure 8. Different classes of phenolic compounds with their backbone structures 16

Figure 9. Delocalization by phenolic radical resonance 17

Figure 10. Chestnut A. burs containing the nuts and B. male flowers. 20

Figure 11. Overview of the experiments carried out in the present work 22

Figure 12. Thermo Scientific HPLC (Dionex UltiMate 3,000 series, Thermo Scientific - San Jose, CA, USA) equipped with a diode array detector (DAD) and connected in series to an Orbitrap mass spectrometer (MS, Exploris 120, ThermoFinnigan - San Jose, CA, USA). 26

Figure 13. IPA craft beers with chestnut by-product extract and a control sample.....28

Figure 14. SPME manual system set up for extraction and concentration of volatile compounds from beers.....30

Figure 15. Clarus 580 GC/Mass Spectrometer (GC/MS). 31

Figure 16. Chromatograms, obtained by HPLC-DAD, of chestnut flower and bur extracts. The chromatograms were processed at 280, 330, 370nm (presented in this order), and the peak identification can be found in Table 2.....34

Figure 17. Relative contribution (percentage) of the different classes of phenolic compounds from the total content of these compounds found in the eight extracts of chestnut by-products. TPA: Total phenolic acids, TF: Total flavonoids; THT: Total hydrolysable tannins; MAC-HE: maceration carried out with hydroethanolic solvent (80% EtOH); UAE-HE: Ultrasound-Assisted Extraction carried out with hydroethanolic

solvent (80% EtOH); UAE-W: Ultrasound-Assisted Extraction carried out with water; MAE-W: Microwave-Assisted Extraction carried out with water. 39
Figure 18. TIC chromatograms, obtained by GC-MS, of the volatile compounds of beers added with chestnut flower extract (FLB1) and control (CB1), analysed in the first month of storage. 47
Figure 19. TIC chromatograms, obtained by GC-MS, of the volatile compounds of model beer system (A, control) and of the model system added with chestnut flower extract (B). 49
Figure 20. GC-MS (TIC) chromatograms of volatile compounds from craft beers prepared without (control, upper chromatogram) and with the incorporation of chestnut flower extract (chromatogram at the bottom) analysed in the sixth month of storage....50
Figure 21. Zoom of GC-MS (TIC) chromatograms of volatile compounds from craft beers prepared without (control, red line) and with the incorporation of chestnut flower extract (blue line) analysed in the sixth month of storage, after derivatization, highlighting the difference in levels of compound at Rt 22.4 min.....51
Figure 22. MS/MS spectrum of the molecular ion $[M-H]^-$ at *m/z* 937 corresponding to chestanin, as visualized in two mass analysers.....52
Figure 23. Chromatograms, obtained by HPLC-DAD at 280 nm of craft IPA beers incorporated with chestnut flower extract and control beer, analyzed in the first month of storage (FL1 and C1, respectively). Below the chromatogram of each sample processed at 280nm, there is the respective total ion chromatogram (TIC, MS) acquired monitoring the ion at *m/z* 937 as target and fragments at *m/z* 467 or 305 as base peaks, which show the presence of the compound through a strong signal in its retention time in samples containing extract. 52
Figure 24. Total ion chromatograms (TIC, MS) acquired with the ion at *m/z* 937 as target and fragments at *m/z* 305 as base peak, demonstrating the presence of the chestanin compound through a signal in its retention time (10.8 min) in the beer incorporated with the extract, in the last month of evaluation (May/2023).....53

TABLE INDEX

Table 1. Types of beer according to Portuguese regulation* 5
Table 2. Chromatographic and spectroscopy characteristics of phenolic compounds identified in different extracts from *C. sativa* by-products 35
Table 3. Quantification of phenolic compounds in different extracts from *C. sativa* by-products 40
Table 4. Antioxidant activity of *C. sativa* by-product extracts.....43
Table 5. Antibacterial (MIC and MBC, mg/mL) and antifungal (MIC and MFC) activity of extracts of *C. sativa* flowers and burs 45
Table 6. Volatile compounds of IPA craft beers in the first month of storage.....45

1. INTRODUCTION

The consumption of fermented alcoholic beverages has extended over centuries. Among these beverages is beer, a product of cereal malt fermentation that is still one of the most appreciated beverages worldwide (MAICAS, 2020). The growth of the beer market is accompanied by greater demand for the quality of products and innovation. More specifically, the craft beer segment has been gaining attention and is considered a movement, comprising more than the beverage itself, moving other facets of society, and stimulating the local economy (CALLEJO et al., 2019). Craft beers, known for their unique sensory attributes, are typically brewed by independent, regional microbreweries in small batches, which allows for flexibility in ingredient selection and the production of a variety of beer styles (MACHADO, 2019).

Beers are considered complex matrices in terms of their composition of volatile and non-volatile compounds and the reactions and interactions among them (RETTBERG et al., 2018). This composition is responsible for the beer flavour, one of the main, if not the main, factors related to beer quality and, consequently, to its acceptance by consumers (ROSSI et al., 2014). Because of their complex and delicate flavours and aromas, craft beers are overall highly susceptible to alterations, as they are not filtered and pasteurized (VIEIRA ARAÚJO, 2019). Those products undergo chemical reactions and physical modifications during both the production process and storage that may negatively affect their original flavour and are responsible for their relatively short shelf life (BAIANO, 2021). In addition, craft beers are generally not subjected to pasteurisation, which may contribute to their shorter shelf life compared to industrial beers (CERQUEIRA, 2016; DENG et al., 2018). Craft breweries face the challenge of maintaining the quality of their product for a longer period. Thus, control of all stages of production and adequate storage of these beverages are necessary to prevent, minimise, or delay physicochemical and, thus, sensory alterations of beers, maintaining their stability and quality (ESSLINGER, 2009). In addition, one of the strategies that could be useful to control beer alterations is the addition of natural antioxidants.

Not differently from the other food segments, the beverage industry is increasingly looking for natural alternatives to synthetic additives, such as potassium sorbate, which are often linked to potentially harmful effects on consumers' health (NAZIR et al., 2019). Even in segments that do not use synthetic additives, efforts and investments have been directed at finding natural ingredients that can enhance the quality of the product while guaranteeing its safety. These natural

products are commonly prepared from plant extracts rich in phytochemicals with interesting properties, with an emphasis on phenolic compounds (CAROCHO et al. 2015).

Phenolic compounds are a large family of plant secondary metabolites generally associated with antioxidant and antimicrobial effects (HU et al., 2021). Studies indicate that the antioxidant capacity of phenolic compounds can have a marked effect on the organoleptic quality of beer, reducing changes in flavour and aroma during storage (WANNENMACHER et al., 2019). Interestingly, extracts rich in polyphenols can also be obtained from by-products or residues of plant foods' cultivation and processing, and with this strategy, it is possible to reduce the waste and the environmental impact generated by activities in the agri-food sector, increase producers' earnings by adding value to these materials, and thus, implement a sustainable-based circular economy. In this sense, the by-products of the sweet chestnut (*Castanea sativa*) production chain, one of the most traditional and economically important cultures for the North region of Portugal, may represent a locally available and low-cost source of phytochemicals. Several botanical parts of *C. sativa* have been studied and identified as important natural sources of polyphenols, which can be exploited for their bioactivities (CAROCHO et al., 2015).

An ingredient based on *C. sativa* male flowers was recently developed by our research group and successfully applied to preserve wines, with questions being raised about the potential application of this and other chestnut by-products as a source of functional ingredients for other beverages. Whereas "chemical" additives are not used in craft beers, it was hypothesized that natural extracts from different parts of chestnut plants, rich in polyphenols that have been associated with antimicrobial and antioxidant activities, could be useful to extend the shelf life of craft beers, for which oxidation-related alterations also represent economical and safety concerns. However, beer is a distinct and complex matrix in terms of composition and the chemical and biochemical reactions it is subject to, and it is more susceptible to oxidation than wine (RADONJIC et al., 2020), which places greater demands on ingredient technology and sensitive chemical evaluations to detect the micro-oxidation. Moreover, obtaining natural-based ingredients depends first on the efficient extraction of the compounds, and it is crucial to explore the quality of the final extract in terms of composition and bioactivities, without losing sight of the sustainable character of the process. In this context, this project aimed to face these challenges by screening the composition and bioactivities of green and conventionally prepared polyphenol-rich extracts from chestnut flowers and burs, selecting one of them for scale-up extraction and incorporation into a craft beer. This product was monitored during storage against a control beer sample -

produced without the extract and following the usual production method, to assess the potential application of the extract as a natural antioxidant.

2. LITERATURE REVIEW

2.1 BEERS AND CRAFT BEERS

2.1.1 History of beer, definition, and legislation

The first records of the production of fermentation-based beverages date back to around six thousand years ago, when the Sumerians developed the manufacturing process of beer, product of the fermentation of malted cereal grains, traditionally barley, although it is assumed that the beer "creation" was accidental. Then, those responsible for spreading the beer across the Mediterranean and Europe were the Egyptians (SICARD; LEGRAS, 2011; FERREIRA et al., 2014). The introduction of hops to the list of main ingredients of beer occurred in the Middle Ages, and at that time, the monks were the biggest beer producers. They produced beverages with improved quality and conservation by replacing the *gruit*, a mixture of herbs previously added to impart specific flavours to beer, by hops (SILVA; PINHEIRO, 2018). As the beer production around the world evolved, many laws related to this process were created and lasted through time, with emphasis on the German Purity Law (*Deutschen Reinheitsgebot*) established in 1516. According to this law, beer should be produced only with barley malt, hops, and water. This law was later reformulated to add yeast as the fourth permitted raw material. This law is in effect until the present day in some parts of the world and is followed by several brewers (PAES, 2015).

In legal terms, the Ordinance nº 91/2022 (PORTUGAL, 2022) establishes the characteristics and guidelines for the production and marketing of beer, adapting Portuguese legislation to European standards, and defines beer as a beverage obtained by alcoholic fermentation from a must prepared from potable water and from cereal malt, by the action of yeasts, to which hop flowers and/or their derivatives are added, and whose cereal malts must correspond to at least 50% by mass of the total raw materials used as sources of sugar. Also, according to this Ordinance, other starchy or sugary raw materials, other ingredients intended for human consumption, and microbiological cultures with a non-alcoholic fermentative profile are allowed during the beer production process, as long as the maximum quantities of addition contained and specific legislation are observed. This document also establishes technical norms regarding the definitions, classifications, and compositions of different beers, conservation, and labelling standards, as well as analysis and sampling methods. The types of beer admitted in the document according to their actual or estimated alcoholic content are described in Table 1.

Table 1. Types of beer according to Portuguese regulation*

Type of beer [a]	Alcoholic content (%vol) [b]	Plato Degrees (°P) [c]
Non-alcoholic beer	≤ 0.5% vol.;	-
Low-alcohol beer	> 0.5% vol. but ≤ 1.2% vol.;	-
Beer or regular beer	> 1.2% vol.	< 13º
Special beer	> 1.2% vol	>13º but ≤ 15º
Extra beer	> 1.2% vol	> 15º

* Ordinance nº 91/2022; [a] Apart from these types of beer based on the alcoholic content, this official document also comprises "Beer from [cereal]" as a beer predominantly made from a specified cereal with less than 50% barley malt, besides "Refermented beer" and "Bottle-refermented beer" as products that undergo a secondary fermentation in the packaging phase or in the bottle, respectively, using the same or different yeast strains. [b] %Vol: Refers to "alcohol by volume" and is a standard measure of how much alcohol (ethanol, in volume) is contained in a volume of an alcoholic beverage, in percentage. [c] Plato degree measures the percentage by weight of the original extract (dissolved solids, mainly fermentable sugars, derived from malt) in the wort, being useful to estimate the potential alcohol content of the final beer.

Although this official document mentions the strong technical innovation and creativity in beer production, particularly with the advent of artisanal beer production, as one of the reasons for which the previous legal regime needed to be changed, a standardized legal definition for the terms artisanal or craft beers is not provided in it or other current legislation.

According to the European Brewers Association (2019), the definition of craft beer is still subjective; however, two criteria are generally considered for craft beer production: being independent and small. Independent in the sense that the capital participation of alcoholic beverage companies is restricted to 25%. Small because its annual production, according to Reid and McLaughlin (2014), needs to be equal to or less than 6 million barrels of beer. In this way, the main difference between craft beer and industrial beer according to the Association would lie in their production scale; while industrial beer is the result of large-scale production, craft beer is produced in a more individualized way, and has generally more complex formulations and flavours, and higher commercial value for investing (CENTRAL BREW, 2022). Besides the production by small independent breweries, In Italy the law concerning the regulation of beer production and commercialization refers craft beers as that not subjected to pasteurization and filtration processes (BAIANO et al., 2020).

In accordance with the provisions of Decree-Law nº 110/2002, Ordinance nº 1193/2003, and Ordinance nº 1085/2004 of Portugal, to be labelled with the mention “craft”, a beer must be produced by a producer with prior registration at CEARTE (Professional Training Centre for

Handicrafts and Heritage), with the artisan and craft production unit charters, and with recognition as a craft producer by the Institute of Employment and Professional Training (CERVEJEIROS DE PORTUGAL, 2022).

2.1.2. Market overview

Mainstream breweries are large-scale operations that produce beer for a wide consumer base. These breweries focus on producing beers that serves to a broad range of preferences, aiming at capturing a significant market share. They often have well-established brand names and extensive distribution networks, allowing their products to reach consumers on a national or even international level. One characteristic of mainstream breweries is their emphasis on consistency and uniformity of the final product. They produce beers with a consistent flavour profile, ensuring that each batch tastes the same regardless of when and where it is produced (ARAÚJO, 2022).

Even though mainstream breweries dominate the global market, it has been witnessed a remarkable rise of craft beer market during the past 15 years, with an increasing number of independent microbreweries (Figure 1, BREWERS OF EUROPE, 2022). The transition towards craft beer consumption can be attributed to various factors, including the rising demand for diverse options, a preference for rich flavours, and a focus on certain parameters of quality provided by artisanal processes rather than product uniformity. Furthermore, there is a noticeable trend towards the consumption of low-alcohol beverages driven by health-conscious consumers that seek for superior-tasting options and a wider selection of new styles of beer. The expansion of the craft beer market is intrinsically linked to the increasing consumer demand for a broader range of choices (CRAFT BEER MARKET, 2023).

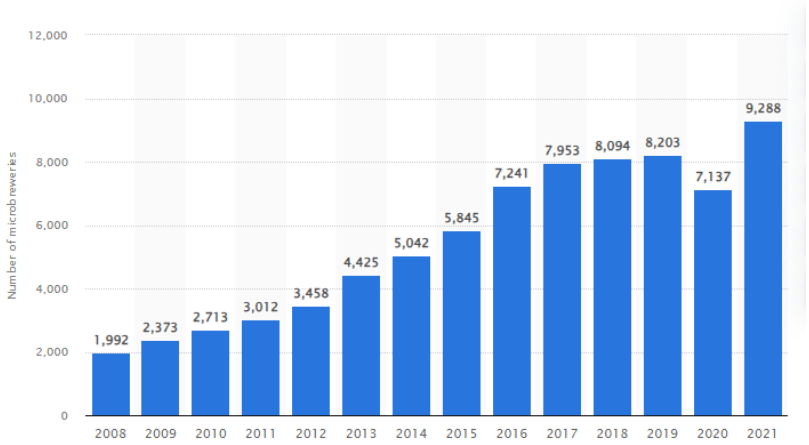

Figure 1. Number of microbreweries in Europe in the period between 2008 and 2021. Source: Brewers of Europe association (2022)

The global craft beer market size reached US$ 117.1 billion in 2022, being expected to reach US$ 221.5 billion by 2028, with a growth rate (compound annual growth rate, CAGR) of 10.8% during 2023-2028 (CRAFT BEER MARKET, 2023). In Europe, the craft beer market is estimated to register a CAGR of 8.62% in the next 4 years. According to the Brewers of Europe report for 2022, the number of microbreweries has increased significantly from 1,992 in 2008 to more than 9,000 in 2021, as shown in Figure 1. Several countries in Europe, including France, the United Kingdom, Germany, Italy, and Spain, have experienced a significant rise in the presence of active microbreweries, contributing to the overall expansion of the industry (EUROPE CRAFT BEER MARKET, 2023).

In Portugal, the craft beer movement is still considered relatively new when compared to other countries in Europe, between 2010 and 2015 the national craft beer market grew exponentially (FREITAS, 2021). In 2020, according to studies carried out by Marktest, only 7% of the Portuguese population over 18 years old consumed craft beers. Currently, there are about 130 craft brewery brands (Figure 2) in the country, in addition to the growth of stores, bars and festivals that move this market (FIGUEIREDO, 2020).

Figure 2. Examples of craft beers produced and commercialized in Portugal
Source: Cerveja Artesanal Portuguesa, 2020. Access at July 1st, 2022.

Currently, there are beers available in the local market produced with chestnuts (Figure 3). These chestnut-infused beers present the traditional chestnut flavours as incorporate the nuts as an ingredient in the production process, imparting a distinct nutty and earthy character to the final beverage. Of note, the addition of chestnuts in those beers are different than the addition of chestnut-based extracts, rich in phenolic compounds, as a technological additive.

Figure 3. Examples of craft beers produced with chestnuts and commercialized in Portugal. *Source: Author, 2023.*

2.1.3. Ingredients, production process and quality of craft beers

Craft beer composition and processes can vary widely depending on the style and the specific brewery. They are traditionally produced from historical ingredients, namely water, fermentable carbohydrates (predominantly malted barley), hops, and yeast, along with unconventional ones (blends of grains, fruits, flowers, flavouring compounds etc). Unlike its commercial counterpart, these beers often incorporate not only unusual but high-quality ingredients and different formulations and production techniques, enhancing the sensory attractiveness, while synthetic additives are not used (VILLACRECES et al., 2022). The addition of different varieties of hops, grains and local fruits, herbs and spices, not only impart unique characteristics and distinct local flavours to these beers but may also improve their nutritional and functional value (VILLACRECES et al., 2022; TIRADO-KULIEVA et al., 2023).

From the point of view of beverage technology, the brewing process is divided into four main stages, namely: malting, wort preparation, fermentation, and final processing (MIGNANI et al., 2013), with the main differences between the craft and industrial beer production process being shown in Figure 4. The production process of craft beers is dynamic and adjustable, with some stages being omitted, included, or rearranged into the flow depending on the type of production carried out in the microbrewery or even the style of beer that is produced. Basically, the malting process involves germination and controlled drying of barley or other cereal grains. It aims to activate enzymes that afterwards mobilize the carbohydrates of the natural grain, break compounds in cell walls, increase grain permeability, and develop aromatic compounds (CERQUEIRA, 2016). Different types of malt, such as pale malt, caramel malt, or roasted malt, can be used in craft beers to achieve desired flavours, colours, and aromas.

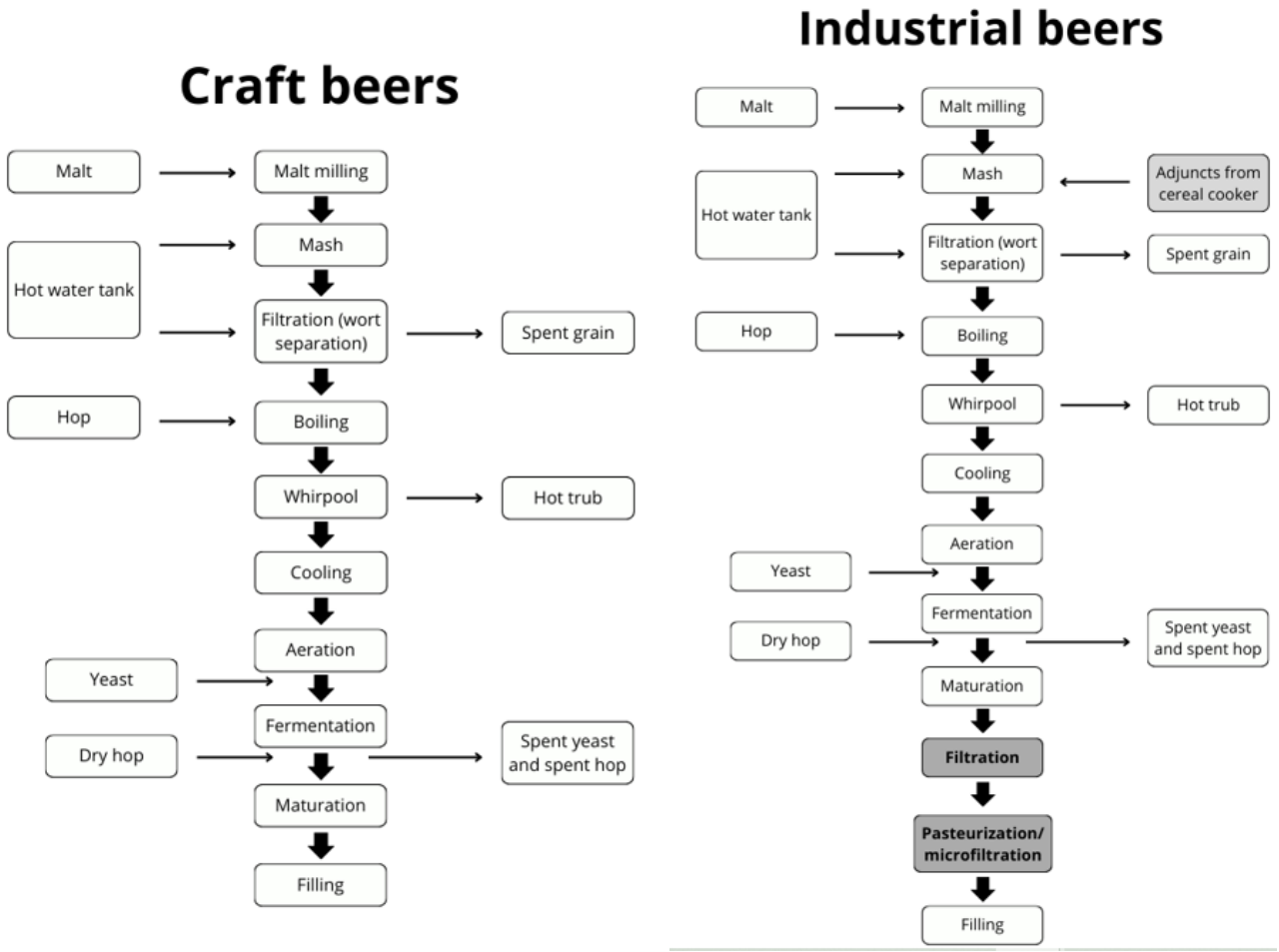

Figure 4. Flow chart of the operations of the production process of the craft and industrial beers
Source: Villacreces et al., 2022.

The wort preparation includes the steps of physical milling that exposes internal starch by breaking cereal grains, the mashing that converts starch into fermentable sugars and extracts compounds including proteins, vitamins, phenolic compounds from malted and unmalted cereals, and separation of the wort that will be boiled from the spent grains, process known as lautering (DRAGONE; ALMEIDA; SILVA, 2010; REITENBACH, 2010). Boiling denatures proteins and enzymes, eliminates sulphur compounds, and extract bitter and other flavour and aromatic compounds from hops that are typically added during this process (MEGA; NEVES; ANDRADE, 2013). Compounds from hops, including phenolic compounds, act as stabilizing and preservatives in those beers (DE KEUKELEIRE, 2000). The mixture is cooled to decant hops and coagulated materials, such as particles of proteins and polyphenols. Whirlpool technique aids in the quick and efficient decantation process. Cooling must be done rapidly to avoid oxidation (MATOS, 2011). In the sequence, fermentation starts with yeast inoculation into the wort. Different yeast species (*S. cerevisiae* and *S. pastorianus*) produce beers with varying characteristics. Alcoholic fermentation results in alcohol and carbon dioxide, as shown in Figure 5, besides esters and other compounds contributing to beer sensorial characteristics (MATOS, 2011). Also known as secondary fermentation, maturation occurs at low temperatures for weeks to months. It helps develop sensory characteristics, clarify the beer, and reduce undesirable compounds like diacetyl. During or after the fermentation process, the dry hopping technique has been often employed to increase the hop aroma without an increasing the bitterness. This process consists of a cold extraction of hop compounds. Indeed, although hops are usually added during the wort boiling, in the craft beer production the late addition of hops has been explored to produce more aromatic and flavoured beverages (LAFONTAINE; SHELLHAMMER, 2019). Finally, the final processing for beers in general may involve filtration, carbonation, aroma and flavour modification, colour standardization, and pasteurization (REITENBACH, 2010). Overall, craft beers are not subjected to pasteurization and microfiltration processes (MASCIA et al., 2016). The beer production process involves a series of carefully controlled steps, each contributing to the final product's flavour, aroma, and stability. Proper execution of these stages is vital for achieving the desired beer characteristics and quality (HANSEN, 2011).

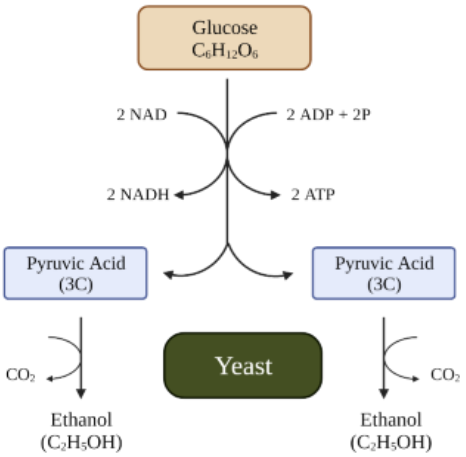

Figure 5. Conversion of glucose into alcohol and CO_2 by fermenting yeast
Source: Adapted from Fermentec, 2022. Access at: July 10th, 2022

The production of a quality beer, be it industrial or artisanal, requires attention to several aspects from the choice of ingredients, which must be of high quality, to the types of processes to which it is submitted. Apart from the microbiological stability, the quality parameters of beers encompass a range of factors that determine the physicochemical and sensorial characteristics of this beverage. These parameters include aspects such as alcohol content, foam stability, turbidity, colour, clarity, flavour profile, aroma, bitterness, and mouthfeel, among others. For the purposes of verifying the characteristics of beer set out in the ordinance 91/2022 currently in force in Portugal (PORTUGAL, 2022), the analysis methods adopted by the European Brewery Convention are used. Moreover, the American Society of Brewing Chemists (ASBC) also provides a set of scientific resources and methods used by industry professionals to assess beer quality.

2.2 CRAFT BEER FLAVOUR

For craft beers specifically, the quality is closely related to its flavour, that is, the integration of sensory perceptions formed by gustatory and olfactory chemical sensations of taste and aroma from volatile compounds (ROSSI et al., 2014). The volatile compounds of beer are responsible for the aroma and flavour of the product. According to Bamforth and Lentini (2009), in the wort, hops and yeast used in the production of beer there may be about 700 compounds present that contribute to the final flavour and odour of the beverage. The final flavour is influenced by the choice of raw material, the type of process it is subjected to, and the yeasts used, in addition to the compounds that are produced in the fermentation and maturation stages.

Some of the common volatile compounds found in beer include esters, alcohols, aldehydes, ketones, phenols, and hydrocarbons (OLANIRAN et al., 2017).

Esters are fruity or floral aromas that are produced by yeast during fermentation. They are responsible for the fruity aroma found in beers like Belgian Saisons and Hefeweizens. Alcohols such as ethanol and isoamyl alcohol are often found in beer, and they contribute to the overall aroma of the beer. Aldehydes are a group of volatile compounds that produce a variety of aromas, including nutty and fruity aromas. They are found in beers like stouts and porters. Ketones are an important component of beer aroma, and they produce aromas like butter or caramel. They can be found in beers like Scotch ales. Phenols are compounds that produce aromas like smoke, spice, and medicine. They are found in beers like Rauchbier and Belgian farmhouse ales. Hydrocarbons are a group of volatile compounds that are responsible for the hoppy aroma of beer. They are found in beers like IPAs and pale ales (ALVIM et al., 2017; GASINSKI et al., 2020).

The diverse array of volatile compounds and their interactions contribute to the complex and multi-dimensional aroma and flavour characteristic of a good beer. Therefore, the analysis of this fraction is crucial. Research on beer volatile constituents dates to the 1960s, and today more than 800 compounds have been identified and measured in beer (PINHO et al. 2006). The volatile fraction of beer presents a challenge to analyse due to its diverse range of polarities, volatilities, and concentrations (Malherbe et al. 2009).

Gas chromatography (GC) is the preferred method for detecting and identifying volatile compounds in beer, given their low concentrations and nature (DA SILVA et al. 2008). Over the years, headspace solid phase microextraction (HS-SPME) coupled with GC has become the most used headspace sampling technique for beer analysis (PINHO et al. 2006; SAISON et al. 2008). Compared to traditional extraction methods like solvent extraction and steam distillation, SPME has several benefits, including speed, automation simplicity, and small sample volume requirements. However, it also comes with drawbacks, such as coating damage due to scraping and needle bending during agitation, limitations on fibre length flexibility, and a thin coating that results in low polymer coating quantities.

2.2.1 Beer flavour stability

Stability can be defined as the ability to preserve this quality, to preserve the properties of the product without major changes, from the moment the beer is bottled until consumption. What is sought is that the beer presents chemical, colloidal, microbiological, colour and foam

stability, in addition to the most important thing, which is the stability of aroma and flavour, closely related to the oxidative stability of the compounds present in this matrix.

Because of their complex flavours and aromas, craft beers are overall considered delicate beverages highly susceptible to alterations. Those products undergo chemical reactions, including the enzymatic ones, and physical modifications during both the production process and storage that may negatively affect their original flavour and are responsible for their short shelf-life (ARAÚJO, SILVA & MINIM, 2003). Craft breweries face the challenge of maintaining the quality of their product for a longer period. In addition, craft beers are generally not subjected to pasteurization, as most of them are conditioned in the bottle and this process increases the level of oxidation in the beer, resulting in the loss of antioxidants and changes in protein amino acids (CERQUEIRA, 2016). Exposure of beer to heat can also trigger colour and flavour changes, decreased foam stability and increased turbidity due to the formation of new protein and tannin complexes with denatured proteins (DENG et al., 2018). However, the unpasteurized product has a shorter shelf-life compared to industrial beers.

Beer shelf life is generally associated with some factors, such as the presence of excessive amounts of oxygen in the beverage, so that if there is contact with oxygen after the fermentation stage, chemical changes are accelerated. This condition can cause the oxidation of the beverage's natural compounds - hop resins, amino acids, fatty acids, among others, and these compounds can be transformed, affecting the product (ESSLINGER, 2009). The presence of oxygen in artisanal beer in the period between fermentation and bottling depends on factors such as the quality of the bottling machine, how the operation is carried out, the way in which the packaging is closed, in addition to preventive measures to prevent this contact (ESSLINGER, 2009). Oxidation reactions are considered the main reason for the degeneration of beer flavour.

While all beers do not age the same, they generally decrease in bitterness over time, have fruity, sulphites and floral notes and are perceived as "harsh". Furthermore, if the intensity of the flavours decreases, the beer can develop staling, resulting in an unpleasant aftertaste. Staling is a complex set of organic chemical changes that occur in beer over time, transforming its flavour and causing it to deviate from the desired and expected flavour and appearance (DE CARVALHO et al., 2007).

Oxidation reactions that degenerate the beer flavour begin as soon as fermentation is finished where the yeasts lose their natural reducing effect. In addition to time, high temperatures also accelerate reactions, aging the beverage faster (FILHO; CEREDA, 2001). One of those

responsible for the unwanted flavour in beer is a compound formed by *trans*-2-nonenal (Figure 6) oxidation, which is an acetaldehyde, which is decisive in the organoleptic quality of a beer (SANTOS, 2002).

Figure 6. Structure of *trans*-2-nonenal, one of the most recognised compounds associated with off-flavours in beers

The *trans*-2-nonenal compound produces a "cardboard" flavour in beer and is formed by the degradation of trihydroxyoctadecanoic acid, which is formed in the oxidation of C18 unsaturated fatty acids. This degradation is favoured by high temperatures, presence of light and presence of metallic ions (HANSEN, 2011).

The production of carbonyl compounds is generally associated with storage time; however, in some cases, they can be identified in the brewery. These compounds are formed from several reactions, the main ones being the oxidation of alcohols to aldehydes, the auto-oxidation of fatty acids, the enzymatic degradation of lipids, and the secondary oxidation of long-chain unsaturated aldehydes. Beer with a high concentration of oxygen contains a considerably higher level of carbonyls (TORTORA, 2000).

One of the purposes of the maturation stage described above, in addition to changes that directly impact the quality of the beer, such as the ripening of the flavour by reducing the concentration of hydrogen sulphide, acetaldehyde and diacetyl (2,3-butadione), is that the beer is kept in its reduced state, in order to avoid oxidations that alter its flavour. Also in maturation, esters are originated that characterize the aroma and flavour of beer, among them ethyl acetate, isoamyl acetate, ethyl caproate and ethyl caprylate. The fatty acids formed in fermentation do not undergo modification at this stage (DE CARVALHO et al., 2007).

Industrial beer producers can add antioxidants or preservatives to the beverage, in addition to those produced naturally in the fermentation stage, in order to reduce the negative effects of oxidizing compounds. The most used antioxidants are ascorbates and sulphites (SANTOS, 2002). The growing consumer demand for clean-label products, associated with evidence that synthetic antioxidants widely used in industrial processes, such as sulphites, BHA and BHT, could trigger allergies or pose health risks (BRANEN, 1975; ITO et al., 1986 ;

WHYSNER et al., 1994) have led to an increase in investigations in the field of natural antioxidants (ABDALLA & ROOZEN, 1999; MOLYNEUX, 2004) which are potentially safer. Whereas no synthetic additives are used in craft beers, the use of natural molecules have been studied (De Francesco et al., 2020).

A search in scientific database (Science Direct, 22 setembro 2023) examining the publications using the terms "craft beer" and "oxidation" in scientific articles is indicative of the growing interest in this parameter of craft beers (Figure 7). This trend within the scientific community reflects a concern in the brewing community on the flavour quality and stability of craft beers, given that oxidation is a critical factor that can influence product shelf life and value.

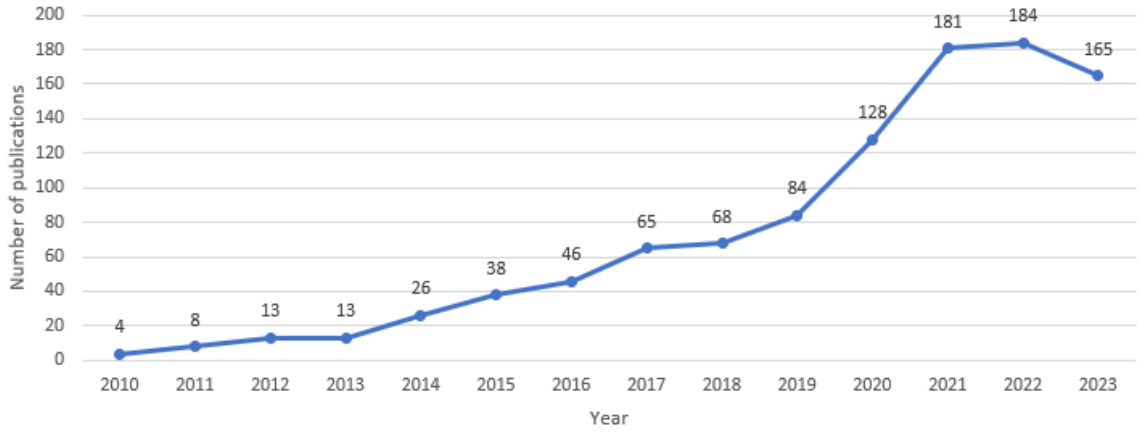

Figure 7. Analysis of the number of manuscripts published by year since the year 2010, related to the topic of oxidative change in beers.

2.3. PHENOLIC COMPOUNDS

Phenolic compounds are products of plant secondary metabolism. Chemically, they are characterized by having a basic structure formed by aromatic rings with one or more hydroxyl substituents and can vary from simpler compounds such as phenolic acids to highly complex polymerized molecules included in the different classes of these compounds (Figure 8). They are usually found linked to other molecules, forming derivatives such as glycosides and esters, but they are also found in their free form (LANDRAULT et al., 2002). These compounds have grounded great interest as functional and technological ingredients for incorporation into foods

and beverages due to the bioactive properties demonstrated by some of them, such as antioxidant and antimicrobial capacity.

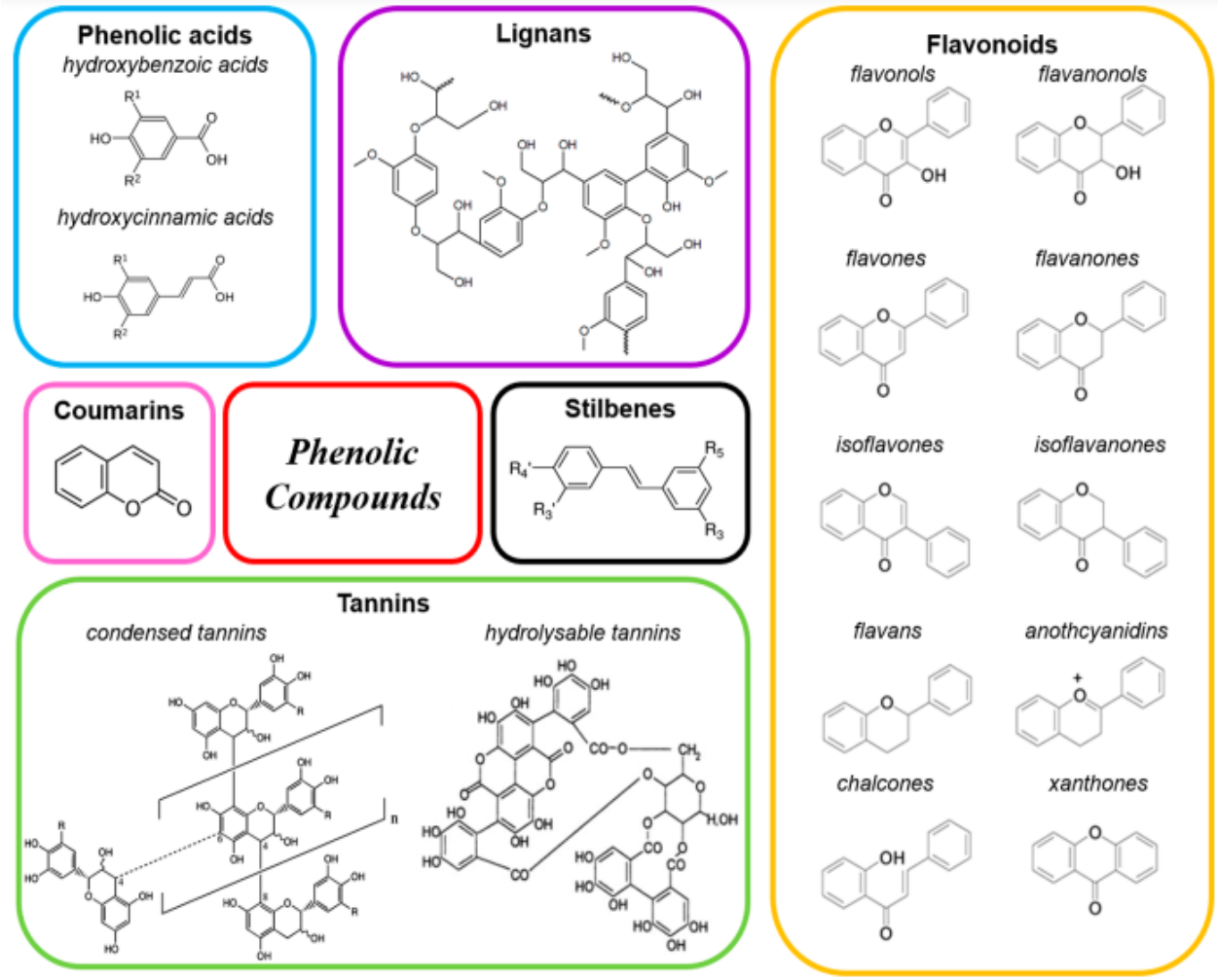

Figure 8. Different classes of phenolic compounds with their backbone structures
Source: Ali Redha, A. (2021).

In the area of Food Science, antioxidants can be considered compounds that inhibit, delay, or control the oxidation of food components, consequently preventing deterioration and extending the shelf life of food (ALAM, 2020). Due to their structure, phenolic compounds are generally good donors of electrons and hydrogen from their hydroxyl groups, and act as antioxidants in the deactivation of reactive oxygen species (ROS) and nitrogen (RNS). The chemical properties of these free radical deactivating compounds are a result of their acidic nature (ability to donate hydrogen). The breakage of the oxygen-hydrogen bond leads to the formation of a stable phenoxide anion, through resonance delocalization among the carbons of the aromatic

ring that stabilize the formed ion (Figure 9) (FENNEMA, 2018). The degree of ionization of polyphenols and, consequently, their antioxidant efficiency depends on the presence of substituents on the phenolic ring, even though they all have acidic properties, explaining the different chemical nature of these compounds. Generally, the antioxidant effectiveness increases by the action of substituents on the phenolic ring, which increase the hydrogen donation or stabilization capacity of the formed ion (FENNEMA, 2018). Besides that, the antioxidant activity of some polyphenols is also directly linked to their ability to chelate metal ions, which in turn is related to their acidity and negative charge delocalization. In foods, the antioxidant efficiency of phenolics also depends on other factors, such as their volatility, pH sensitivity, and polarity (FENNEMA, 2018).

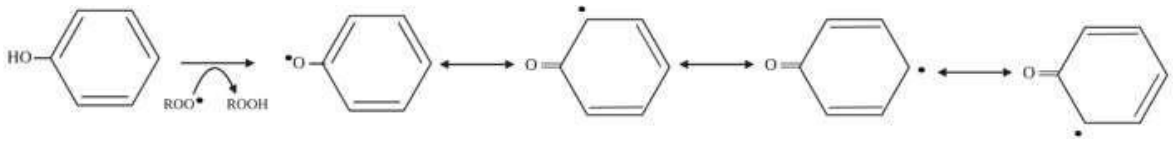

Figure 9. Delocalization by phenolic radical resonance
Source: Adapted from Shahidi and Wanasundara (1992).

Another positive factor that has been related to phenolic compounds is the antimicrobial activity, mainly to the class of tannins. The antimicrobial action mechanisms of these compounds are still not fully elucidated, but it is considered that they can act in different ways depending on their structure, for example causing changes in the permeability or damage to the cell membrane of bacteria and inhibiting enzymes when making binding of hydrogen with them, which can alter intracellular functions (CUSHNIE and LAMB, 2011; BOUARAB-CHIBANE et al., 2019).

In view of the exposed above, it is hypothesized that both the antioxidant activity and the antimicrobial activity of phenolic compounds could improve the stability and consequently the shelf life of food and beverage products to which they are naturally present or added, since the recommended shelf life for craft beers is a maximum of six months (BAIANO, 2021). Nonetheless, for these compounds to be added into foods, they need to be isolated from their plant matrices, and in this sense, a point that deserves attention is how to obtain extracts rich in phenolic compounds from plants. It is a challenge to find a universal extraction solvent, since the percentage of recovery of certain classes of compounds and, therefore, of the profile of compounds in the extract is linked to the type of solvent used, in addition to the nature of the compound and its location in plant tissues, and to the extraction variables, such as the extraction

technique and conditions used, such as time and temperature. In general, the most used solvents are water and organic solvents such as ethanol, methanol and acetone (WIJEKOON et al., 2011). Besides the extraction yields, it is crucial to focus on the sustainability of the extraction process. Considering the incorporation into foods and in order to reduce the environmental impact, it is important to use solvents that are food grade and recognized as "green", as well as move towards the application of green alternative methods of extraction that use the available resources more efficiently to extract phytochemicals from plant matrices considered source of these compounds. These innovative extraction techniques include those based on microwave, sonication, pressurized liquid technologies that represent an opportunity to recover biomolecules while minimizing the environmental impact.

2.3.1. Phenolic compounds in beers

The profile of phenolic compounds in beers mainly reflects that of the plant raw materials used in these beverages, such as cereals and hops, but also the compounds modified and formed during the processing and storage of this product. Cortese et al. (2019) performed a quantitative analysis of six different types of craft beers by HPLC-MS/MS, identifying phenolic acids and flavonoids from malt, and bitter acids and prenylflavonoids from hops. Marova et al. (2010) used a chromatograph with a DAD and MS detectors to analyze characteristic phenolic compounds in different lager beers from the Czech Republic, having identified 49 compounds in the samples. Out of the total, 11 compounds (gallic acid, (-)-catechin, epicatechin, ferulic acid, chlorogenic acid, morphine, rutin, quercetin, kaempferol, naringenin, and luteolin), predominantly from malt, were quantified. Petrucci et al. (2020) also used an HPLC-DAD-(ESI)-MS/MS method to determine the profile of free polyphenols in three craft beers, and identified 14 compounds, including hydroxybenzoic acids, hydroxycinnamic acids, and flavonols.

In analytical terms, chromatography and mass spectrometry are sensitive and robust techniques, considered the choice for identifying phenolic compounds in different matrices, and the same is valid for beers. However, the beer matrix is quite complex, and it is noted that many studies needed to perform pre-chromatographic procedures to improve the detection of polyphenols in this matrix. Quifer-Rafa et al. (2014) applied the solid-phase extraction (SPE) procedure in order to increase sensitivity and decrease the matrix effect. High resolution mass spectrometry was used to identify phenolic compounds, confirmed by fragment ion scanning experiments and high precision mass fragments. Forty-seven phenolic compounds were identified, including simple phenolic acids, flavonoids, hydroxyphenylacetic acids and prenyl

flavonoids. In 2019, Cheiran and collaborators identified phenolic and nitrogen compounds from different types of craft beers. In this study, they performed a pre-chromatographic SPE procedure to increase the sensitivity of the analysis, and subsequently the phenolic compounds were separated and identified by HPLC-DAD-ESI-MS/MS. In the end, 57 phenolic compounds were identified, 12 of which were found for the first time in beers.

In addition to the contribution of phenolic compounds from beer raw materials, the beneficial properties associated with these compounds in terms of food stability have led to recent studies on the feasibility of incorporating these compounds from natural sources as functional and technological ingredients in beers. De Francesco et al. (2020) evaluated the effect of adding different phenolic-rich extracts on beer flavour stability and noted that phenolic extracts contributed to better product stability in terms of turbidity, colour, and foam quality. The beers added with phenolic compounds also showed greater flavour stability. The authors concluded that extracts of phenolic compounds, mainly condensed ones, showed a protective effect on beer quality and would be a possible solution to counteract the effects of beer aging.

2.4. *CASTANEA SATIVA* AS A PHENOLIC COMPOUND SOURCE

The genus *Castanea* belongs to the Fagaceae family and includes 12 species, among them is *C. sativa*, the European chestnut tree. The chestnut tree is a large and deciduous tree that grows well above 500 meters of altitude and low temperatures (BARREIRA, 2008). In Portugal, the chestnut tree is distributed in the North and Center of the country, with the largest production concentrated in the Trás-os-Montes region, where around 12,500 hectares are used for its cultivation, contributing with approximately 85% of the national production. The city of Bragança is a producing region with Protected Designation of Origin (PDO), the fruit known as "Castanha da Terra Fria" (RIBEIRO et al., 2007). This chestnut culture has a great impact on the Portuguese economy (INE, 2014). Both in the leaves of *C. sativa* and in its other botanical parts (Figure 10) it is possible to identify a high concentration of polyphenols, which have been associated with multifunctional properties. Several studies point out the antioxidant potential of extracts obtained from by-products of chestnut production chain, which is a promising source of bioactive compounds (DELGADO et al., 2013; VASCONCELOS et al., 2010; RODRIGUES et al., 2014). In this way, a possible antioxidant action could motivate the use of by-products and bioresidues from chestnut cultivation and processing as raw material for the extraction of natural antioxidants. As a deciduous tree, chestnut leaves, as well as flowers and burs, fall on the ground at specific period. The extraction of natural antioxidants from by-products is considered attractive

due to their low cost and availability in large quantities (KONG et al., 2010), with the possibility of producing high added value ingredients and reducing the impact environment by making use of waste.

Figure 10. Chestnut A. burs containing the nuts and B. male flowers.
Source: Conedera et al. (2016)

Recent studies show that chestnut flowers or extracts obtained from them have a high amount of phenolic compounds, and that they can be applied in food preservation due to their ability to inhibit the oxidation of food biomolecules and microbial proliferation (CAROCHO et al., 2014a). Caleja et al. (2019) optimized the conditions for the recovery of phenolic compounds from chestnut flowers using heat-assisted extraction, with the aim of applying this extract in the food industry as a natural additive. The same authors showed the feasibility of using the aqueous extract of chestnut flowers as a natural preservative in *pasteis de nata*, as an alternative to synthetic potassium sorbate (CALEJA et al., 2020). Carocho et al. (2015) also reported the use of dried flowers and extracts rich in phenolic compounds derived from chestnut flowers in Portuguese pastry products as functional agents. The results showed that this strategy increased the antioxidant activity and phenolic content in the added products without causing major changes in their appearance. The preservative effects observed through the use of flowers or their extracts are attributed to the phenolic profile of these by-products, which have chesnatin as the major compound, in addition to other tannins and quercetin-3-*O*-glucuronide (CAROCHO et al., 2014b).

3. OBJECTIVE

3.1 MAIN OBJECTIVE

The primary objective of this study was to assess the applicability of a polyphenol-rich extract from a chestnut by-product incorporated into craft beers as a natural antioxidant in enhancing the chemical stability of this beverage.

3.2 SPECIFIC OBJECTIVES

To achieve the main objective, the following steps were performed (Figure 11):

- **i.** Extraction of phenolic compounds from *C. sativa* by-products, namely male flowers and burs, by using different extraction techniques and conditions;
- **ii.** Identification and quantification of phenolic compounds present in the extracts;
- **iii.** Evaluation of the antioxidant capacity of the extracts;
- **iv.** Evaluation of the antimicrobial activity of the extracts;
- **v.** Selection of the best extraction conditions for incorporation of the extract into craft beer;
- **vi.** Evaluation of the stability of craft beers incorporated with the extract against control samples during storage through monitoring of volatile compounds and presence of marker compounds of the extract.

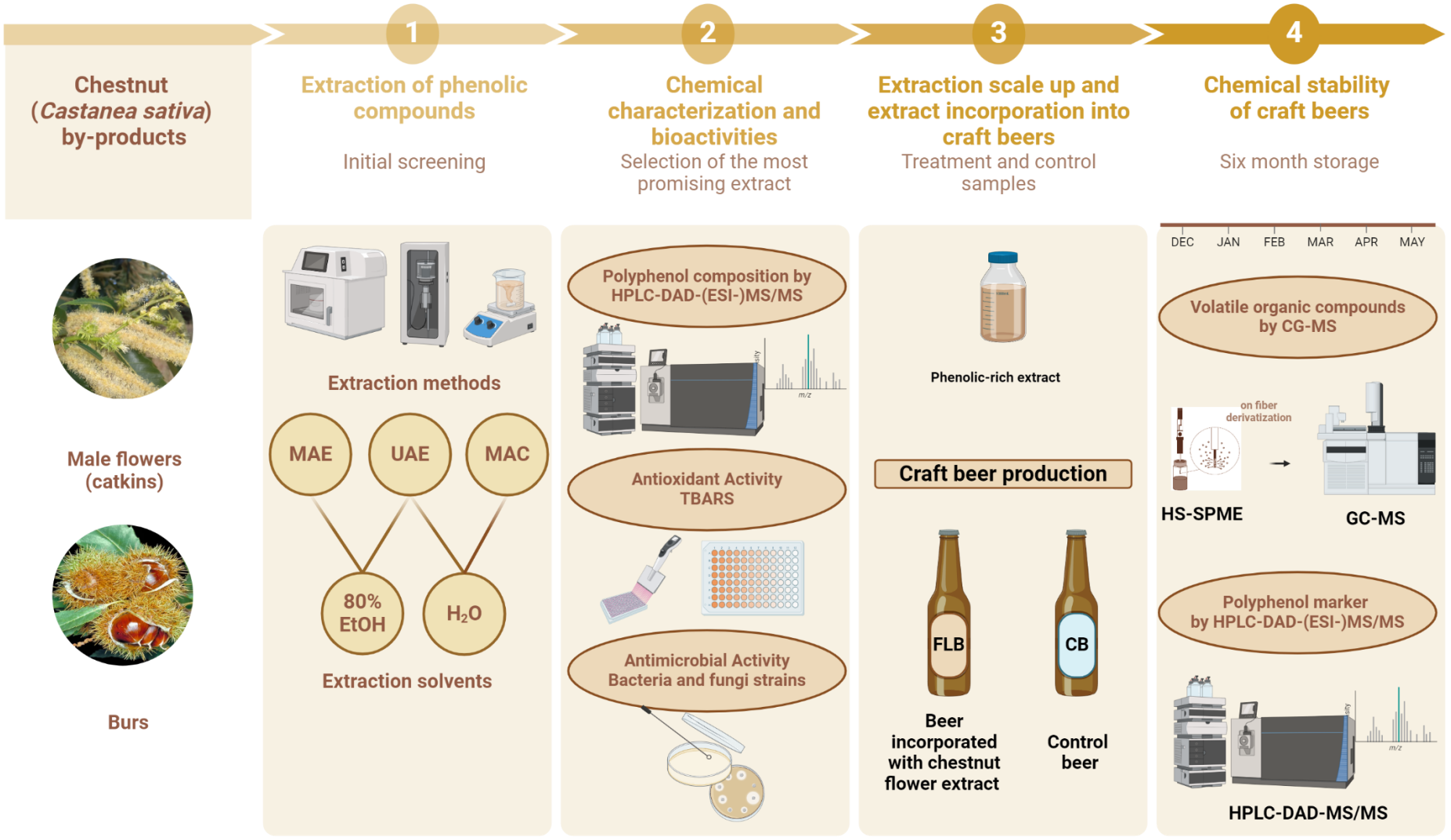

Figure 11. Overview of the experiments carried out in the present work

4. MATERIAL AND METHODS

4.1 CHEMICALS

Standards of phenolic compounds were purchased from Extrasynthése (Genay, France) and Sigma-Aldrich (St. Louis, MO, USA). Analytical grade reagents trichloroacetic acid (TCA), sodium chloride, trolox, tris, ascorbic acid, ellipticine and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiobarbituric acid (TBA), Tryptone Soy Broth (TSB), *p*-iodonitrotetrazolium, chloride sodium sulphate, calcium chloride and magnesium chloride were purchased from Panreac Applichem (Barcelona, Spain), whereas iron (II) sulphate was acquired from ACROS Organics (Geel, Belgium). Potassium dichromate, sodium bicarbonate and magnesium sulfate were supplied by Merck (Darmstadt, Germany), and potassium chloride was purchased from Pronalab (Lisbon, Portugal). Porcine (*Sus scrofa*) brain used in antioxidant activity was obtained from official slaughtering animals. Microbiology supplies such as Malt extract broth (MEB), and blood (sheep blood 7%), and MacConkey agars were purchased by LiofilChem S.R.L (Roseto d. Abruzzi, TE, Italy). Antibiotics methicillin, streptomycin, and ampicillin used for control assays were supplied from Fisher Scientific (Janssen Pharmaceutical, Belgium), whereas the antifungal ketoconazole was provided from Frilabo (Porto, Portugal). For cell culture, Dulbecco's modified Eagle's (DMEM) and Roswell Park Memorial Institute (RPMI 1640) media, hank's balanced salt solution (HBSS), fetal bovine serum (FBS), trypsin–EDTA, L-glutamine, penicillin and streptomycin were acquired from Hyclone (Logan, Utah, USA). HPLC-grade methanol, ethanol, acetonitrile, and extra pure formic acid were provided by Fisher Scientific (Leicestershire, UK). Ultrapure water was obtained through a Milli-Q system (TGI Pure Water Systems, Greenville, SC, USA). HPLC solvents and samples were filtered through 0.45 and 0.22 μm membranes, respectively, prior to chromatographic analysis. Oasis MAX solid phase cartridges (30 mg) were obtained from Waters (Milford, MA).

4.2 PLANT MATERIAL

Samples of by-products of *C. sativa* Mill. (male flowers and burs) were collected in the year 2022 from a chestnut orchard of the Longal variety, located in the Northeast of Portugal, at Vinhais municipality (coordinates 41º50'11.98"N, 7º9'23.695"W). Batches of at least two kg of each sample were collected already dried in the field and transported to the laboratory. They were reduced to a fine powder to produce a composite sample of each by-

product, which was stored at room temperature, protected from light, until extraction procedures.

4.3 EXTRACTION OF PHENOLIC COMPOUNDS FROM CHESTNUT BY-PRODUCTS

4.3.1 Maceration (MAC)

Dynamic maceration (MAC, under agitation) was employed in this study as a traditional solid-liquid extraction technique. Aliquots of 2 g of each dried sample were weighed and mixed with 60 mL of 80% ethanol (ethanol:water, 80:20, v/v) at room temperature. The mixture was continuously stirred (500 rpm, 1 hour at room temperature), using a magnetic bar and stirrer (Multimatic 9-N, Selecta- Barcelona, Spain). Upon completion of this process, the extract was filtered (filter paper, Ø 125 mm, CMHLAB - Barcelona, Spain). The sample residue was reextracted under the same conditions, and both extracts were combined. Extracts of chestnut flowers and burs obtained by maceration with hydroethanolic solvent are referred to as MAC-HE throughout this text.

4.3.2 Ultrasound-assisted extraction (UAE)

UAE, a promising alternative to traditional extraction techniques, was carried out in an ultrasonic device (model CY-500, Optic Ivymen System - Barcelona, Spain) equipped with a titanium probe. The extraction procedure was performed as previously described by López et al. (2018). A portion of 3 g of each sample was added to 100 mL of either 80% ethanol or ultrapure water and subjected to ultrasound for 27.3 ± 2.6 min at a power of 235.8 ± 36.8 W using an ice bath to avoid overheating of extractive mixtures. UAE extracts of chestnut by-products prepared using either hydroethanolic solution or water as extraction solvents are henceforth called UAE-HE and UAE-W, respectively.

4.3.3 Microwave-assisted extraction (MAE)

Also considered an innovative extraction approach, MAE was conducted using an analytical, enclosed microwave system (Speedwave Xpert, Berghof—Eningen, Germany). The process involved the extraction of 3 g of each sample, separately, in 100 mL of ultrapure water, in a closed vessel for 2 min at 80 ◦C (microwave power, P, 50–1000 W). The extracts obtained from different parts of the *C. sativa* plant are referred to as MAE-W in this thesis.

After each extraction procedure described above, aqueous or hydroethanolic extracts of phenolic compounds were collected by filtration (Ø 125mm, CMHLAB, Barcelona, Spain). Ethanol was evaporated under vacuum (T < 38 °C, rotatory evaporator Büchi R-210 - Flawil,

Switzerland) and the remaining aqueous portion was frozen and subjected to freeze-drying (Freeze Dryer Telstar LyoQuest-55, Milan, Italy) for 48 hours at -55 ± 0.5 °C (LÓPEZ et al., 2018).

4.4 ANALYSIS OF PHENOLIC COMPOUNDS IN THE EXTRACTS

Phenolic compounds in extracts were analysed in a Thermo Scientific HPLC (Dionex UltiMate 3,000 series, Thermo Scientific - San Jose, CA, USA) equipped with a diode array detector (DAD) and connected in series to an Orbitrap mass spectrometer (MS, Exploris 120, ThermoFinnigan - San Jose, CA, USA, Figure 12). Phenolic compounds were separated in a Spherisorb S3 ODS-2 C_{18} column (4.6 x 150 mm, 3 µm – Waters, Milford, USA) kept at 35 ºC, under a gradient of formic acid (0.1%) and acetonitrile. UV-Visible (UV-Vis) spectra were acquired between 180 to 700 nm and the chromatograms processed at 280, 330, and 370 nm for the different classes of phenolic compounds. The HPLC eluate was analysed by tandem mass spectrometry, and the compounds were ionized using an electrospray ionization (ESI) source operating in negative mode. The system was operated with a spray voltage of 2.5 kV, a source temperature of 325 ºC, and a vaporizer temperature of 300 ºC. The normalized HCD collision energy was 30%. Nitrogen served as the sheath gas (50 psi); Full MS and MS/MS spectra were acquired in the range from 110 to 1800 charge-to-mass ratio (*m/z*). Data acquisition and processing were performed with Xcalibur® (ThermoFinnigan, San Jose, CA, USA). For compound identification, elution order on the C_{18} column and characteristics of the UV-Vis and mass spectra (molecular ion ($[M-H]^-$) and MS/MS fragments) were interpreted and compared with standards, when available, and literature data. Quantification was performed using 9-point external calibration curves of authentic standards. The results of

phenolic compounds were expressed as mg per g of dry sample (mg/g dw) (Rodrigues et al., 2023).

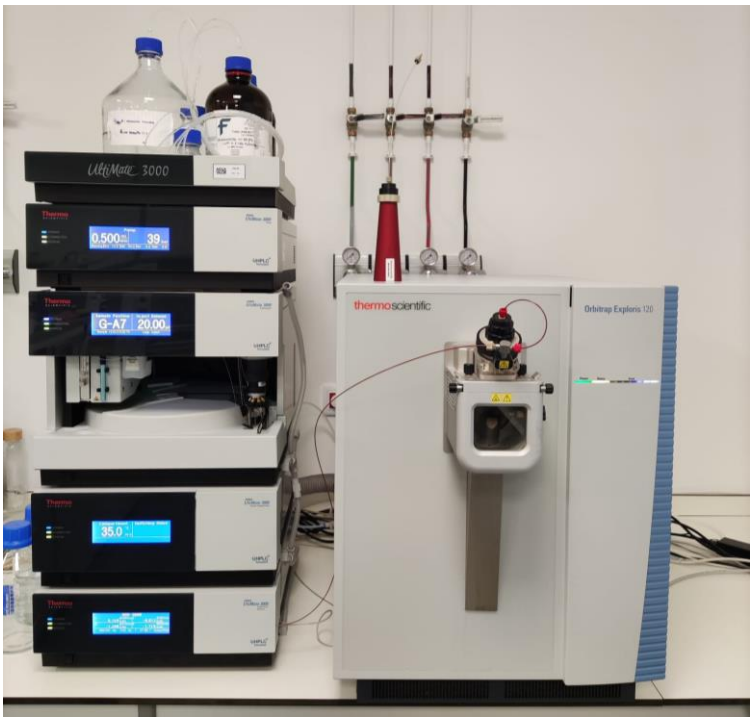

Figure 12. Thermo Scientific HPLC (Dionex UltiMate 3,000 series, Thermo Scientific - San Jose, CA, USA) equipped with a diode array detector (DAD) and connected in series to an Orbitrap mass spectrometer (MS, Exploris 120, ThermoFinnigan - San Jose, CA, USA).

4.5 *IN VITRO* BIOACTIVITY ASSAYS OF POLYPHENOL-RICH EXTRACTS

4.5.1 Antioxidant activity

A given mass of pig brain was weighed into a falcon tube, and twice this mass of Tris-HCl buffer (20 mM; pH = 7.4) was added. After shaking, the tube was taken to the centrifuge at 3,500 rpm for 10 minutes. As performed by Pinela et al. (2012), in 48-well microplates, 200 µL of extract solution in hydroethanolic mixture used in the extraction was added and serial dilution was performed to obtain 8 distinct concentrations, depending on the tested extract and in triplicate. The extraction solvent was used as negative control. In the sequence, 100 µL of ascorbic acid (0.1 mM), 100 µL of iron sulphate (10 mM), and 100 µL of the pig brain suspension supernatant were added to the wells. The plate was incubated at 37±0.5 °C for 1 hour. 500 µL of freshly prepared trichloroacetic acid (28%, w/v) and 380 µL of thiobarbituric acid (2%, w/v) were added. The plate was incubated at 80±0.5 °C for 20 minutes. The contents of each well were transferred to Eppendorfs, which were centrifuged at 3000 rpm for 5 minutes. The supernatant was transferred to a 96-well plate and taken for absorbance reading

in SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenberg, Germany) at 532 nm wavelength. From Equation 1, the percentage of lipid peroxidation inhibition (I) was determined.

$$I(\%) = \frac{Abs_{control} - Abs_{ext}}{Abs_{control}} \; x \; 100 \qquad \text{Eq. (1)}$$

where the absorbance presented by the blank assay (negative control) is referred to as $Abs_{control}$ and that presented by a given extract concentration is referred to as Abs_{ext}. By analysing the relation between the concentrations tested and their respective percentage of inhibition, the results were expressed as EC_{50} values, i.e., the effective concentration that provides a half-maximal antioxidant response (µg/mL), or in other words, the concentration of extract able to inhibit lipid peroxidation by 50%.

4.5.2 Antimicrobial activity

The antimicrobial activity was determined according to the method described by Pires et al. (2018) with microorganisms, namely food fungi and bacteria purchased from Frilabo, Porto, Portugal. The assay included the determination of five Gram-negative bacteria, namely, *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923), which came from the company Frilabo, Porto, Portugal. Prior to each assay, the microorganisms need to be incubated at 37°C for a period of 24 hours, which is necessary for them to reach a state of exponential growth using appropriate media for each different bacterial strain, of which *Enterobacter cloacae*, *Escherichia coli*, *Yersinia enterocolitica* and *Pseudomonas aeruginos*a grow on MacConkey agar and *Salmonella enterica*, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* grow on Blood agar.

The procedure starts by dissolving the samples using a maximum concentration of 5% (v/v) DMSO and 95% autoclaved distilled water to give a final concentration of 20 mg/mL for the stock solution. Then add 90 µL of TSB (96-well microplate) in all wells and 100 µL of the extract solution in the first well (in duplicate) thus performing a successive dilution to obtain different concentrations finishing with the addition of 10 µL of inoculum ensuring the presence of 1.5×10^5 CFU (standardized to 1.5×10^6 Colony Forming Unit (CFU)/mL). Finally, they were incubated for 24 hours) at 37°C. The next day, the minimum growth inhibitory concentration (MIC) was determined by adding (40 µL) 0.2 mg/mL *p*-iodonitrotetrazolium

chloride and incubating at 37°C for 30 min. In the presence of MIC, 10 µL of liquid from each well that showed no colour change was plated onto solid medium, blood agar and incubated at 37°C for 24 h for determination of the lowest concentration required to cause death of the bacteria.

As for fungi, two different strains were used, *Aspergillus fumigatus* (ATCC 204305), *Aspergillus brasiliensis* (ATCC 16404), by using the methodology described by Heleno et al. (2013). The provenance of the samples follows the same as previously described. However, the fungi need to be incubated at 25°C for 72 hours to reach their exponential growth state for subsequent analysis, which is done by washing them on the surface of the agar plates with 0.85% sterile serum containing 0.1% Tween 80 (v/v), adjusting the spore solution to a concentration of 1.0 × 105, in a final volume of 100 µL per well. MEB culture medium was used for the assay using the microdilution method, and MICs were determined at 72 h of assay under binocular microscope. Presenting MICs, fungicidal concentrations were determined by serial subculture of 2 µL of tested compounds dissolved in medium and inoculated for 72 h into microplates containing 100 µL of MEB per well, and subsequent 72 h incubation at 26 °C.

4.6. INCORPORATION OF PHENOLIC-RICH EXTRACT INTO CRAFT BEER

The most promising extract from the chemical and bioactive screening performed was incorporated into craft beers produced in the facilities of Letra Cervejaria (Vila Verde - Portugal). Indian Pale Ale (IPA) craft beers with and without the extract (treatment and control samples, Figure 13) were produced following the recipe and production process defined by the microbrewery. Information regarding the processing stage at which the extract was incorporated, as well as its final concentration in the product, is confidential and falls under the responsibility of the Bio4Drinks project.

Figure 13. IPA craft beers with chestnut by-product extract and a control sample.

4.7 BEER STABILITY

To assess the potential of the chestnut by-product extract to preserve the stability and quality of the beers, the volatile compounds of control and treatment beers were monitored during the storage months (December/2022-May/2023) and analysed more specifically in month 1 (December/2022), month 4 (March/2023) and month 6 (May/2023). Moreover, the presence or consumption of a compound marker of the chestnut by-product extract was also monitored.

4.6.1 Monitoring of volatile organic compounds (VOCs) in beer

4.6.1.1 Extraction of volatiles

The HS-SPME (headspace-solid-phase microextraction) procedure was employed for the extraction of VOCs in beers as previously performed by Rodríguez-Bencomo et al. (2012) with adaptations. An aliquot of 6 g of recently opened beer sample was weighted into a 20-mL SPME vial containing 1.8 g of NaCl and a magnetic bar and rapidly sealed with a cap with a PTFE/Silicon septum (Supelco, Bellefonte, PA, USA). At a first moment, the samples were incubated under agitation for 10 min at 44.8 °C, then the extraction was performed in the headspace of the vial by exposing the fibre for 47 min at 44.8 °C. The extraction was performed using a 50/30-µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre of 2-cm length (Supelco, Bellefonte, PA, USA) (Figure 14). This procedure was performed in duplicate and the compounds in the fibre were readily injected into chromatograph.

Figure 14. SPME manual system set up for extraction and concentration of volatile compounds from beers.

4.6.1.2 On-fibre derivatization

During the experiments it was noticed that aldehydic compounds, important to monitoring the oxidation of compounds in beer, were not satisfactorily detected by the previous methodology. Therefore, a derivatization procedure was performed as described by Vesely et al. (2003). In a 20-mL glass vial, a mixture was prepared by combining 100 µL of *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine (PFBOA) solution with a concentration of 6 g/L, along with 10 mL of deionized water. The vial was sealed, and a DVB/CAR/PDMS SPME fibre was introduced into the headspace of the PFBOA solution and left for 10 min at 50 °C. The SPME fibre loaded with PFBOA was then exposed to the headspace of 10 mL of beer placed in a 20-mL glass vial.

4.6.1.3 Identification of volatile compounds

Volatile compounds were analysed in a gas chromatograph coupled to mass spectrometer (GC-MS, Clarus 580, Perkin Elmer, MA-USA) (Figure 15) with the software Turbo Max. For separation, a Supra-Wax fused silica capillary column (60-m×0.25-mm i.d.×0.5-µm film thickness) from Konik (Barcelona, Spain) was used. The desorption was performed in the injector of the gas chromatograph in splitless mode for 1.5 min at 270 °C. Helium was the carrier gas (1 mL/min). The oven temperature was programmed as follows: 40 °C as initial temperature, held for 5 min, followed by a ramp of temperature at 4 °C/min to 240 °C and then held for 15 min. For the MS system, the temperatures of the transfer line, quadrupole and ion source were 270, 150 and 230 °C, respectively; electron impact mass

spectra were recorded at 70 eV ionization voltages and the ionization current was 10 μA. The MS spectra acquisitions were performed in Scan mode from 35 to 450 mass-to-charge (*m/z*) range (RODRÍGUEZ-BENCOMO et al., 2012). Compounds were identified based on their spectral characteristics compared with NIST® libraries and literature data.

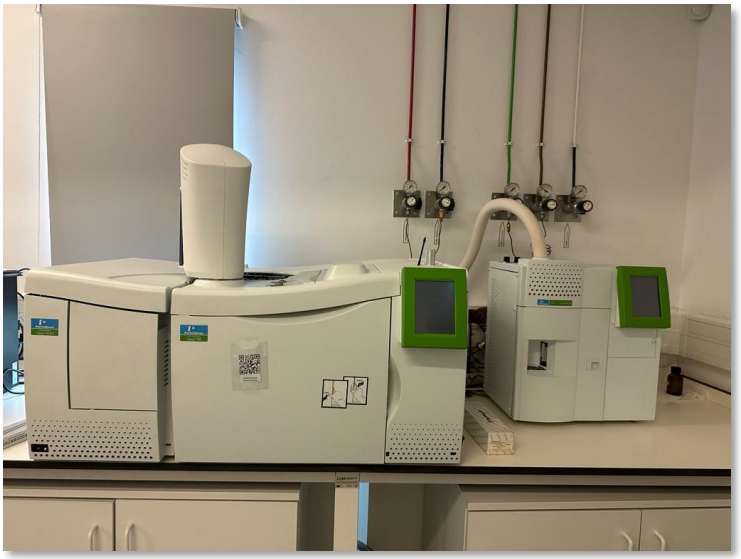

Figure 15. Clarus 580 GC/Mass Spectrometer (GC/MS).

4.6.2 Monitoring of phenolic compounds from a *C. sativa* by-product in beer

4.6.2.1 Extraction of phenolic compounds from beer

Aliquots of approximately 50 mL of beers were removed from the bottles and degassed in an ultrasound bath (JP Selecta Ultrasonic Cleaning Baths, Spain) for 30 min. Phenolic compounds from all beer samples were extracted by solid-phase extraction (SPE) according to the procedure described by (Quifer-Rada et al., 2015b) with adaptations. Briefly, the ethanol content of the beer was reduced under N_2 flux. Oasis MAX cartridges (Waters - MA, USA) were activated with 1 mL of methanol and conditioned with 1 mL of sodium acetate buffer (50 mM, pH 7). Then 2 mL of partially dealcoholized beer was acidified with 34 μL of hydrochloric acid (38%) and loaded into the cartridges. The content was washed with 1 mL of sodium acetate buffer (50 mM, pH 7) containing 5% methanol, to remove interfering compounds. Phenolic compounds were eluted with 1800 μL of methanol with 2% formic acid. Prior to HPLC analysis, samples purified by SPE (1800 μL) were concentrated under a stream of nitrogen and reconstituted with acidified water (0.5% formic acid), filtered through a PVDF membrane (0.22 μm) and stored until HPLC analysis.

4.6.2.2 Phenolic compound analysis

Analysis of phenolic compounds in beers by HPLC-DAD-(ESI-)MS/MS was performed according to the procedure described in the item 4.4, with some modifications in the MS method for detection of specific marker compounds of the extracts in beer. The deprotonated ion of the marker compound of the extract (parent ion) and its product ions were monitored through target MS^2 experiments.

4.7 STATISTICAL ANALYSIS

For screening and ranking purposes, the means of either total phenolic contents or antioxidant capacity of all extracts were thoroughly compared by analysis of variance (ANOVA), followed by Tukey's post hoc test (α = 5%), in the Statistica 7.0 software. Regression analysis for the construction of external calibration curves of phenolic compound standards were also carried using this software, while the construction of chromatograms was carried out using Origin 8.5 Software.

5. RESULTS AND DISCUSSION

5.1 EXTRACT CHEMICAL CHARACTERIZATION

The analysis of phenolic compounds in the extracts of *C. sativa* by-products by HPLC-DAD-(ESI)MS/MS showed the presence of a total of 33 different compounds, including regioisomers (Table 2, Figure 16). It is possible to notice that specific portions of the chromatograms, and therefore of the profile of flower and bur phenolic compounds are similar. Twenty-eight compounds were identified in flowers and 20 in burs, being 13 compounds common to both by-products. This is consistent with the data provided by Barros et al. (2013) that characterized the phenolic compounds of *C. sativa* male flowers by HPLC–DAD–ESI/MS and found 20 different compounds, being 18 compounds the same as those identified in the present study. Silva et al. (2020) found a total of seven different compounds in chestnut burs, all of them also identified in the bur extracts analysed in this study, but accounted for less than a half of the number of compounds found in the present study.

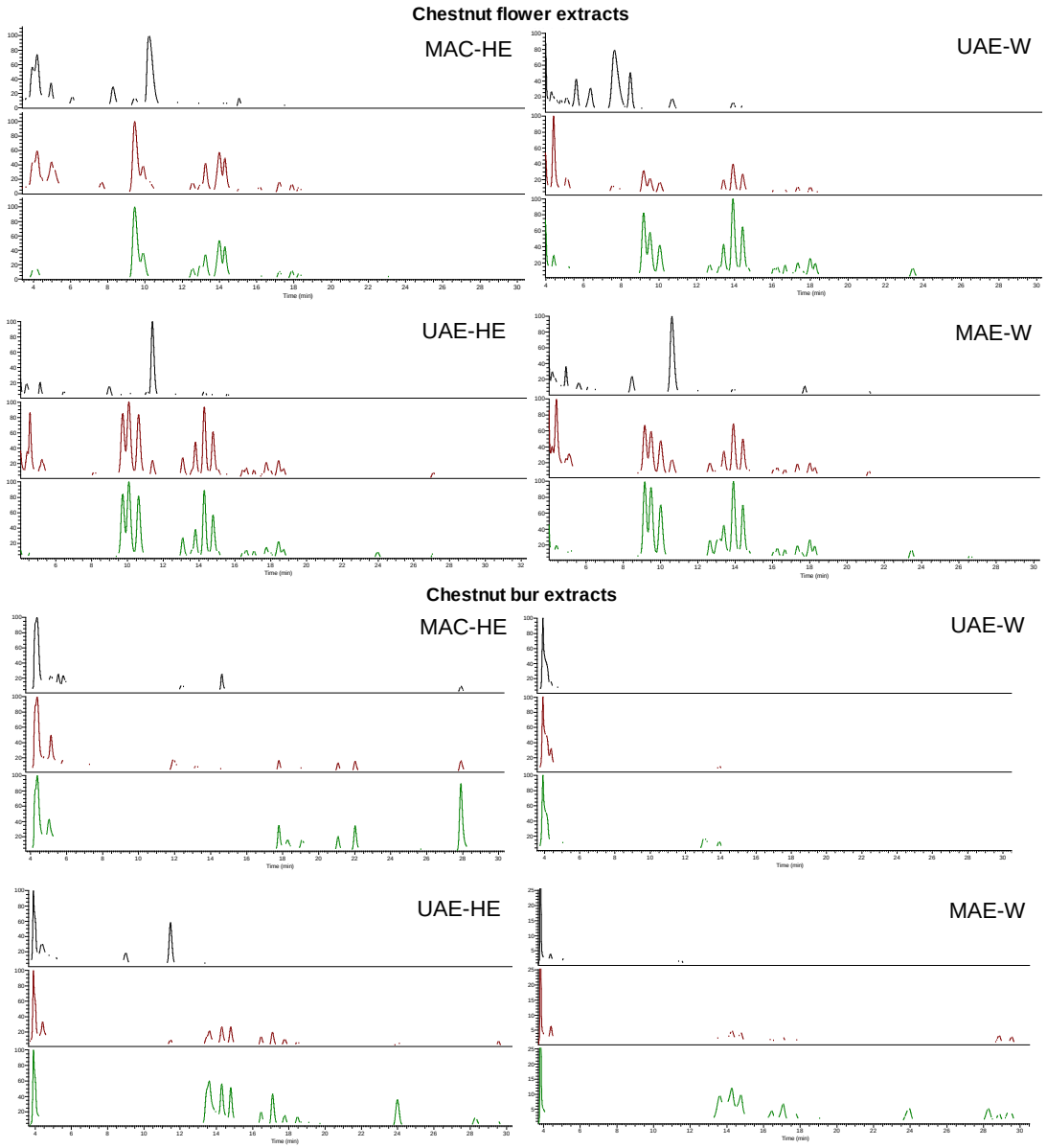

Figure 16. Chromatograms, obtained by HPLC-DAD, of chestnut flower and bur extracts. The chromatograms were processed at 280, 330, 370nm (presented in this order), and the peak identification can be found in Table 2.

Table 2. Chromatographic and spectroscopy characteristics of phenolic compounds identified in different extracts from *C. sativa* by-products

Peaks[a]	Tentative identification	Rt (min)[b]	λmax (nm)[c]	[M-H]⁻ (*m/z*)	MS² fragments (*m/z*)[d]	Chestnut by-products	
						F	B
1	Gallic acid	3.9	270	169	125(100)		
2	Bis-HHDP-glucose (pedunculagin I) (isomer 1)	4.38	278	783	481(13), 301(45)		
3	Chesnatin (isomer 1)	4.91	270	637	467(100),305(23)		
4	Trigalloyl-HHDP-glucose	5.11	274	937	937(100),637(20),301(12)		
5	Galloyl-bis-HHDP-glucose (isomer 2)	5.45	275	935	633(100),301(18)		
6	Pentagalloyl glucose (isomer 1)	5.61	276	939	631(31),469(66),169(100)		
7	Chesnatin (isomer 2)	6.13	272	637	467(100),305(23)		
8	Pentagalloyl glucose (isomer 2)	6.42	276	939	631(31),469(66),169(100)		
9	Pentagalloyl glucose (isomer 3)	7.81	276	939	631(31),469(66),169(100)		
10	Cretanin	8.38	274	469	169(100)		
11	Myricetin-*O*-glucuronide	9.15	356	493	317(100)		
12	Myricetin-3-*O*-glucoside (isomer 1)	9.49	350	479	317(100)		
13	Myricetin-3-*O*-glucoside (isomer 2)	10.01	350	479	317(100)		
14	Chestanin (isomer 1)	10.47	274	937	637(6),467(100),305(7),169(17)		
15	Isorhamnetin-*O*-hexoside	12.34	353	477	315(100)		
16	Ellagic acid	12.53	366	301	229 (88),257 (55)		
17	Quercetin-3-*O*-rutinoside	12.67	347	609	301(100)		
18	Quercetin-*O*-hexoside	13.39	354	463	301(100		
19	Quercetin-3-*O*-glucuronide	13.77	352	477	301(100)		
20	Quercetin-*O*-hexoside	14.71	354	463	301(100)		
21	Chestanin (isomer 2)	15.12	274	937	637(6),467(100),305(7),169(17)		
22	Quercetin dirhamnoside	15.95	334	593	301(100)		
23	Kaempferol-3-*O*-rutinoside	16.25	334	593	285(100)		
24	Isorhamnetin-3-*O*-rutinoside	16.62	355	623	315(100)		
25	Kaempferol-3-*O*-glucoside	17.23	348	447	285(100)		
26	Quercetin-*O*-deoxyhexoside	17.87	349	447	301(100)		

Peaks[a]	Tentative identification	Rt (min)[b]	λmax (nm)[c]	[M-H]$^-$ (*m/z*)	MS^2 fragments (*m/z*)[d]	Chestnut by-products	
						F	B
27	Isorhamnetin-*O*-hexoside (isomer 1)	18.17	353	477	315(100)		
28	Methyl ellagic acid hexoside	18.46	362	477	301(100)		
29	Isorhamnetin-*O*-hexoside (isomer 2)	18.75	353	477	315(100)		
30	Methyl ellagic acid deoxyhexoside (isomer 1)	18.88	368	461	315(100),301(32)		
31	Methyl ellagic acid deoxyhexoside (isomer 2)	19.89	367	461	315(100),301(32)		
32	Trimethyl-ellagic acid hexoside	23.97	234/355	551	343(100)		
33	Isorhamnetin-*O*-acetylhexoside	27.08	350	519	477(6),315(81)		

[a] Peaks according to the retention times on the chromatograms shown in Figure 16. [b] Retention time on C_{18} column. [c] Gradient of 0.1% formic acid and acetonitrile. [d] MS/MS fragments are followed by their relative abundance in the spectrum in parentheses. Grey cells in the F and B columns indicate the detection of the compound in chestnut flower and and burs.

Among the polyphenols found in the extracts, 6 were identified as phenolic acids and their derivatives. Gallic acid (peak 1) and ellagic acid (peak 16) were identified as their retention time and spectroscopic characteristics matched to the standards available. Gallic acid presented a UV-Vis spectrum with two ranges of maximum absorbance, around 210 and 270 nm, besides the molecular ion $[M-H]^-$ at *m/z* 169, which generated fragment ion $[M-H-CO_2]^-$ at *m/z* 125 in the MS^2 spectrum. Ellagic acid absorbed maximally at around 365 nm and presented desprotonated molecule $[M-H]^-$ at *m/z* 301 and typical MS^2 fragments at *m/z* 257 and 229 (BOWERS et al., 2018). This phenolic acid occurs in nature mainly in its esterified form, either as ellagitannins or glycosides, with nuts and their by-products being primary dietary sources (CLIFFORD; SCALBERT, 2000). Compounds from peaks 28, 30 and 31 were identified as mono-methyl ellagic acid glycosides since presented a similar pattern of light absorption and generated the same MS^2 ions at *m/z* 315, due to the neutral loss of a sugar moiety (hexosyl residue (-162 u) for peak 28 and deoxyhexosyl residue (-146) for peaks 30 and 31), and at *m/z* 301, corresponding to the further loss of a methyl group (-15 u) and to the ellagic acid molecule. On the other hand, peak 32 displayed the ion at *m/z* 343 as their base peak in MS/MS spectra, consistent with a three-methylated ellagic acid structure after losing a hexosyl moiety (-162 u) from the molecular ion $[M-H]^-$ at *m/z* 505. The most prominent ion detected in the MS spectra for this compound was the formic acid adduct $[M-H+HCOOH]^-$ at *m/z* 551, in line with the findings of Formato et al. (2022), so it was tentatively identified as trimethyl-ellagic acid hexoside.

Chestnut by-products contain not only gallic and ellagic acids but also gallotannins and ellagitannins, which belong to the group of hydrolysable tannins. These tannins can be easily hydrolysed to release gallic and/or HHDP acids, with the latter being spontaneously converted into ellagic acid. The gallotannins found in extracts from chestnut burs and flowers were primarily molecules composed of hexose, 3,4,5-trihydroxy benzyl alcohol, and gallic or dehydrodigallic acid units. First isolated in chestnut, this group of gallotannins was distinguished by fragment ions corresponding to gallic acid (*m/z* 169) and to the neutral loss of the terminal trihydroxy benzyl alcohol-hexoside residue (-300 u, dehydrated) (OSAWA, 1977a; OSAWA, 1978; FORMATO et al., 2022). Cretanin (peak 10) was identified based on its molecular ion $[M-H]^-$ at *m/z* 469, which yielded an intense fragment ion $[M-H-300]^-$ at *m/z* 169, consistent with a gallic acid molecule upon cleavage and released of the hexosyl-trihydroxy-benzyl alcohol unit (FORMATO et al., 2022; CERULLI et al., 2021). Additionally, chesnatin isomers (peaks 3 and 7) were detected in flowers and displayed a molecular ion $[M-H]^-$ at *m/z* 637 and fragment ion at *m/z* 467 corresponding to the galloyl-trihydroxybenzyl-hexoside structure. With a molecular ion $[M-H]^-$ at *m/z* 937, peaks 14 and 21 detected in burs were assigned as chestanin isomers. Indeed, the daughter ions $[M-H-300]^-$ at

m/z 637, [M-H-300-170]$^-$ at *m/z* 467 (base peak), [M-H-300-170-162]$^-$ at *m/z* 305, and [M-H-300-170-300]$^-$ at *m/z* 169 indicated the occurrence of one more hexosyl moiety and one more trihydroxybenzyl alcohol unit than chesnatin (OSAWA et al., 1988). In addition to this type of gallotannins, three pentagalloyl glucose isomers (peaks 6, 8 and 9) were detected exclusively in chestnut flowers. All these compounds, as simple glucosides or esters of gallic acid, presented UV-Vis spectra that resemble that of this acid.

Besides gallotannins, three ellagitannins were also tentatively identified in extracts of chestnut by-products based on their characteristic fragmentation pattern, with neutral losses of one or more units of HHDP (-302 u) and, in some cases, gallic acid (-170 u or -152 u for dehydrated galloyl moieties), often accompanied by the loss of sugar (usually glucose, loss of -180 u or -162 u for the dehydrated residue) (MOILANEN et al., 2013). The shape of the UV-Vis spectrum of these compounds provided information on the proportion of free and bound galloyl units in their structure and was also considered for the peak assignment. Peak 2 presented the molecular ion [M-H]$^-$ at *m/z* 783 and MS^2 ions [M-H-302]$^-$ at *m/z* 481, evidencing the loss of an HHDP, and [M-H-302-180]$^-$ at *m/z* 301 corresponding to the loss of an HHDP-glucose group (482 u) and to the ellagic acid. These characteristics, along with its UV-Vis spectra consistent with the absence of free galloyl units, allowed the identification as bis-HHDP-glucose (called pedunculagin I). Peak 5 presented the molecular ion [M-H]$^-$ at *m/z* 935 and fragment ions [M-H-302]$^-$ at *m/z* 633 and [M-H-302-332]$^-$ at *m/z* 301 in their MS/MS spectra. These neutral losses indicated the occurrence of one more galloyl unit than pedunculagin eliminated as a galloyl-HHDP-glucose unit (-634 u), thus these peaks were tentatively identified as galloyl-bis-HHDP-glucose isomers. Finally, the molecular ion [M-H]$^-$ at *m/z* 937 and MS^2 fragment ions at *m/z* 637 and 301 found in peak 4 were consistent with those of compounds previously identified in chestnut catkins as trigalloyl-HHDP-glucose (CALEJA et al., 2019; CAROCHO et al., 2016; CAROCHO et al., 2014).

Examining the percentage contribution of various polyphenol classes to each extract can yield valuable insights into the composition of different chestnut by-products and the effectiveness of different extraction techniques and solvents in recovering specific phenolic compounds. In this study, hydrolysable tannins (THT) were the most prevalent class of phenolic compounds in all chestnut flower extracts, regardless of the extraction method used (Figure 17). These extracts also showed higher proportion of flavonoids to the polyphenol profile in contrast to the bur counterparts. In is noticeable that all the flower extracts presented very similar relative contribution of each polyphenol class, which was considerably different from bur extracts, despite the overall polyphenol profile present some compounds in common as indicated at the beginning of the section (Figure 16). Bur extracts present a higher percentage of phenolic acids in most of its extractions, with exception

of the UAE-HE, which presented a relative proportion of phenolic classes similar to that found in flower extracts. Despite this, all extracts showed the same classes of phenolic compounds.

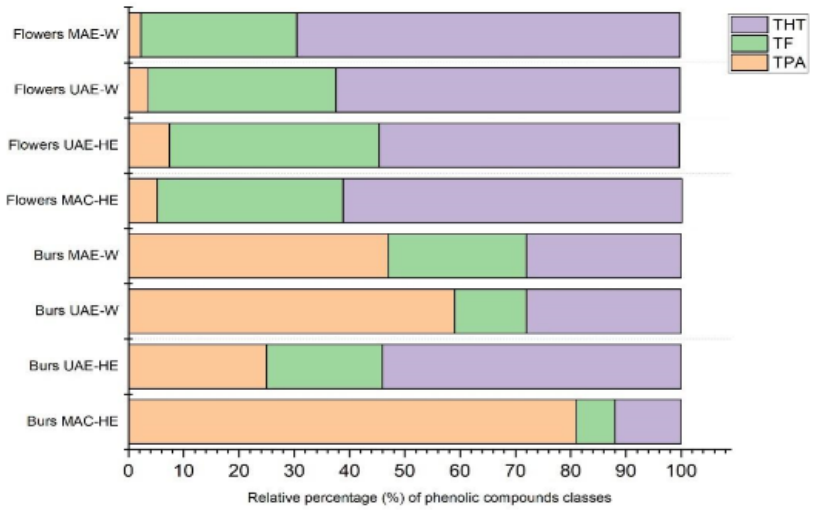

Figure 17. Relative contribution (percentage) of the different classes of phenolic compounds from the total content of these compounds found in the eight extracts of chestnut by-products. TPA: Total phenolic acids, TF: Total flavonoids; THT: Total hydrolysable tannins; MAC-HE: maceration carried out with hydroethanolic solvent (80% EtOH); UAE-HE: Ultrasound-Assisted Extraction carried out with hydroethanolic solvent (80% EtOH); UAE-W: Ultrasound-Assisted Extraction carried out with water; MAE-W: Microwave-Assisted Extraction carried out with water.

The data on total phenolic compounds (TPC, mg/g freeze-dried extract, dw), which were calculated by the sum of all individual compounds separated by HPLC, indicated that flower extracts presented consistently higher TPC than burs regardless the extraction procedure (p<0.05), ranging from 53 ± 2 to 137 ± 2 mg/g for UAE-W and MAC-HE extracts, respectively (Table 3). On the other hand, the TPC of extracts from chestnut burs varied from 37 ± 1 mg/g in the UAE-HE to 10 ± 0.1 mg/g in the MAE-W extracts, with MAC-HE and UAE-W presenting roughly half the highest total amount recovered for this by-product (22 ± 0.01 and 17 ± 0.2 mg/g dw, respectively). These values greatly surpassed that Silva et al. (2020) found for a conventional ethanolic extract of burs from the same chestnut variety (4.48 ± 0.01 mg/g dw). Therefore, the hydroethanolic extract of flowers obtained through maceration had the highest yield of phenolic compounds compared to the other seven extracts analysed (137 ± 2 mg/g dw), the MAE-W and UAE-HE extracts of flowers also showed satisfactory recoveries of polyphenols (124 ± 5 and 116 ± 2 mg/g dw, respectively), and twice that of UAE-W (53 ± 2 mg/g). In conjunction, these results demonstrated the efficiency of the simplest extraction method of maceration, although traditional, as well as the potential of UAE when associated with hydroethanolic solvent, as a green extraction procedure that can efficiently recover natural and bioactive molecules from agro-industrial materials.

Table 3. Quantification of phenolic compounds in different extracts from *C. sativa* by-products

Phenolic compound [1]	**Individual quantification (mg/g)** [2]							
	Flowers				**Burs**			
	MAC-HE	**UAE-HE**	**UAE-W**	**MAE-W**	**MAC-HE**	**UAE-HE**	**UAE-W**	**MAE-W**
Gallic acid	nd	nd	nd	nd	17.55±0.067	7.58±0.012	10.31±0.093	4.28±0.03
Bis-HHDP-glucose (pedunculagin I) (isomer 1)	21±0.1	5.9±0.1	2.4±0.1	9.8±0.2	2.04±0.02	5.67±0.14	2.23±0.03	0.5±0.02
Chesnatin (isomer 1)	2.07±0.02	2.4±0.1	1.26±0.03	7.8±0.5	nd	nd	nd	nd
Trigalloyl-HHDP-glucose	nd	nd	nd	nd	0.18±0.003	1.26±0.05	1.55±0.05	0.47±0.02
Galloyl-bis-HHDP-glucose (isomer 1)	nd	nd	nd	nd	0.065±0.004	1.71±0.11	nd	0.53±0.03
Pentagalloyl glucose (isomer 1)	3.1±0.1	5.5±0.2	2±0.1	6.4±0.3	nd	nd	nd	nd
Chesnatin (isomer 2)	2.16±0.01	1.99±0.04	3.7±0.2	2.8±0.2	nd	nd	nd	nd
Pentagalloyl glucose (isomer 2)	5.4±0.1	3.3±0.2	3.5±0.2	4.5±0.2	nd	nd	nd	nd
Pentagalloyl glucose (isomer 3)	2.09±0.01	1.47±0.03	12.7±0.2	4.7±0.2	nd	nd	nd	nd
Cretanin	6.3±0.1	5.8±0.1	4.2±0.2	8.1±0.3	0.186±0.002	3.66±0.17	0.79±0.03	0.52±0.02
Myricetin-*O*-glucuronide	5.4±0.2	6.8±0.1	2.3±0.1	5±0.4	nd	nd	nd	nd
Myricetin-3-*O*-glucoside (isomer 1)	7.4±0.2	8.5±0.1	2±0.1	4.8±0.3	nd	nd	nd	nd
Myricetin-3-*O*-glucoside (isomer 2)	7.2±0.1	7.1±0.1	0.75±0.02	4.3±0.2	nd	nd	nd	nd
Chestanin (isomer 1)	39±1	34±2	2.2±0.1	38±2	0.077±0.004	7.53±0.31	0.26±0	0.65±0.03
Isorhamnetin-*O*-hexoside	1.12±0.03	nd	1.02±0.02	1.8±0.1	nd	nd	nd	0.14±0.01
Ellagic acid	1.09±0.02	3.3±0.1	0.42±0.01	2.8±0.1	nd	nd	nd	nd
Quercetin-3-*O*-rutinoside	nd	nd	nd	nd	0.18±0.004	2.67±0.12	1.24±0.07	0.61±0.02
Quercetin-*O*-hexoside	2.65±0.02	1.09±0.01	1.4±0.03	2.9±0.2	0.063±0.001	1.18±0.05	nd	0.4±0.01
Quercetin-3-*O*-glucuronide	3.53±0.01	2.9±0.1	2.8±0.1	5.2±0.1	0.36±0.004	1.66±0.15	0.75±0.02	0.57±0.03
Quercetin-*O*-glicoside	2.2±0.02	6.6±0.3	2±0.1	3.8±0.2	nd	nd	nd	nd
Chestanin (isomer 2)	2.79±0.03	2.5±0.1	0.58±0.03	2±0.1	nd	nd	nd	nd
Quercetin dirhamnoside	5.1±0.2	3.6±0.3	0.75±0.03	0.93±0.05	nd	nd	nd	nd
Kaempferol-3-*O*-rutinoside	2.47±0.02	0.81±0.002	0.86±0.03	1.16±0.03	nd	nd	nd	nd
Isorhamnetin-3-*O*-rutinoside	1.09±0.02	1.3±0.1	0.85±0.04	1.15±0.03	0.10±0.005	0.83±0.07	0.29±0.04	0.27±0.01

Phenolic compound [1]	Individual quantification (mg/g) [2]							
	Flowers				Burs			
	MAC-HE	UAE-HE	UAE-W	MAE-W	MAC-HE	UAE-HE	UAE-W	MAE-W
Kaempferol-3-*O*-glucoside	1.07±0.02	1.13±0.02	0.9±0.04	1.2±0.1	0.1±0.002	0.41±0.01	nd	0.15±0.01
Quercetin-*O*-deoxyhexoside	1.6±0.1	0.729±0.001	0.84±0.05	1.1±0.1	nd	0.404±0.01	nd	0.123±0.01
Isorhamnetin-*O*-hexoside (isomer 1)	nd	nd	nd	nd	nd	nd	nd	nd
Methyl ellagic acid hexoside	3.52±0.02	2.6±0.01	0.141±0.005	0.7±0.05	0.11±0.004	0.4±0.01	nd	0.12±0.01
Isorhamnetin-*O*-hexoside (isomer 2)	1.22±0.04	1.4±0.04	0.66±0.04	nd	0.10±0.004	0.23±0.04	nd	nd
Methyl ellagic acid deoxyhexoside (isomer 1)	nd	nd	nd	nd	0.11±0.003	0.32±0.01	nd	0.17±0.01
Methyl ellagic acid deoxyhexoside (isomer 2)	nd	nd	nd	nd	nd	0.34±0.01	nd	nd
Ellagic acid 3,3',4-trimethoxy 4'-*O*-β-D-glucopyranoside	1.23±0.02	1.1±0.02	0.5±0.01	nd	nd	1.01±0.02	nd	nd
Isorhamnetin-*O*-acetylhexoside	nd	0.97±0.04	0.11±0.01	nd	nd	nd	nd	nd
	Total quantification (mg.g^{-1}) [b]							
	Flowers				**Burs**			
	MAC-HE	**UAE-HE**	**UAE-W**	**MAE-W**	**MAC-HE**	**UAE-HE**	**UAE-W**	**MAE-W**
Total phenolic acids (TPA)	7.2±0.1	8.6±0.1	1.9±0.1	2.8±0.1	17.8±0.1	9.3±0.1	10.3±0.1	4.4±0.1
Total flavonoids (TF)	46±1	44±1	18±1	35±2	1.5±0.1	7.8±0.3	2.3±0.1	2.4±0.1
Total hydrolysable tannins (THT)	84±1	63±3	33±1	87±4	2.6±0.1	19.8±0.8	4.8±0.1	2.7±0.1
Total phenolic compounds (TPC)	**137±2**^{a}	**116±4**^{b}	**53±2**^{c}	**124±5**^{b}	**21.85±0.01**^{e}	**36.87±1.09**^{f}	**17.42±0.16**^{g}	**9.56±0.1**^{h}

[1] Phenolic compounds tentatively identified according to the data shown in Table 1. [2] Quantitative data expressed as mg.g^{-1} of the freeze-dried extract are presented as mean ± standard deviation. nd: not detected. MAC-HE: maceration carried out with hydroethanolic solvent (80% EtOH); UAE-HE: Ultrasound-Assisted Extraction carried out with hydroethanolic solvent (80% EtOH); UAE-W: Ultrasound-Assisted Extraction carried out with water; MAE-W: Microwave-Assisted Extraction carried out with water. Different superscript letters in the same row indicate significant differences ($p < 0.05$, Tukey's test).

Moreover, the quantity of phenolic compounds, especially in flower extracts, further highlights the value of this chestnut by-product as a locally available, inexpensive, source of these natural, bioactive molecules.

Although the relative contribution (in percentage) of the phenolic classes was similar among all the four flower extracts (Figure 17), in absolute values different values of TPA, TF and THT can be noticed (Table 3). In addition, although the polyphenol content of flower extracts is much higher in contrast to the bur extracts, the latter showed higher absolute content of phenolic acids (from 4.4 to 17.8 mg/g dw). Vázquez et al. (2012) studied chestnut bur extracts and reported that gallic acid esters of glucose and ellagic acid contributed to the antioxidant activity of the extracts. Studies in literature reporting the use of chestnut flower extracts as a natural preservative in foods associate it mainly to their high TPC. Barros et al. (2013) investigated the phenolic compounds in wild medicinal flowers from Portugal by HPLC-DAD-ESI/MS and found that the *C. sativa* sample presented the highest amount of phenolic compounds (about 19 mg/g) and hydrolysable tannins (15 mg/g fw).

Individually, the tannin chestanin was the major compound found in three flower extracts (up to 39 ± 1 mg/g dw in MAC-HE), in exception to UEA-W extract that presented pentagalloyl glucose isomer as the major compound (12.7 ± 0.2 mg/dw in UAE-W). In bur extracts, high amounts of gallic acid were found in all the extracts (up to 12.55 ± 0.067 mg/g dw in MAC-HE), with UEA-HE one also presenting good amounts of chestanin (7.53 ± 0.31 mg/g dw).

5.2 EXTRACT BIOACTIVITIES

5.2.1 Antioxidant activity

The TBARS assay is widely used to measure lipid peroxidation. When lipids are oxidized, they generate a complex mixture of products, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and other aldehydes. These aldehydes react with TBARS to form a colored product that can be detected spectrophotometrically. The results regarding the antioxidant activity, evaluated by the TBARS assay, are presented in Table 4. All extracts analysed revealed the ability to inhibit the oxidative process with low EC_{50} values being observed, which indicates a high antioxidant activity. In particular, two bur extracts (MAC-HE and UAE-W) displayed the lowest absolute EC_{50} value (0.002mg/mL), followed by the UAE-HE extract of flowers (0.003mg/mL), and MAC-HE of flowers and UAE-HE of burs (0.004 mg/mL). Of note, all these extracts showed a

higher ability to inhibit lipid peroxidation than the antioxidant Trolox used as a positive control (Table 4).

Table 4. Antioxidant activity of *C. sativa* by-product extracts

	Castanea sativa by-products			Positive control
Antioxidant activity		**Flowers**	**Burs**	**Trolox (µg/mL)**
TBARS (EC_{50}, mg/mL) [1]	MAC-HE	0.004±0.0001[a]	0.002±0.0001[a]	0.0058 ± 0.0006
	UAE-HE	0.003±0.0001[a]	0.002±0.0001[a]	
	UAE-W	0.007±0.001[b]	0.004±0.0001[a]	
	MAE-W	0.007±0.001[b]	0.008±0.0002[b]	

All results were expressed in EC50 values (mg/mL, mean ± standard deviation). [1] EC_{50} value refers to the extract or standard concentration (mg/mL) corresponding to 50% of antioxidant activity. MAC-HE: maceration carried out with hydroethanolic solvent (80% EtOH); UAE-HE: Ultrasound-Assisted Extraction carried out with hydroethanolic solvent (80% EtOH); UAE-W: Ultrasound-Assisted Extraction carried out with water; MAE-W: Microwave-Assisted Extraction carried out with water. Different letters in the same assay indicate significant differences ($p < 0.05$, Tukey's test).

According to a study conducted with chestnut flowers and that also performed the TBARS assay, they reported EC_{50} value of 2.7 µg/mL, which are very close to the values shown herein (ALAYA et al., 2021). Another study that used samples of chestnut shells also using the ultrasonic extraction method obtained antioxidant activity values for DPPH and ABTS of 44.1 µg/mL and 65.6 µg/mL, respectively (LAMEIRÃO et al., 2020).

There are some studies about the antioxidant potential of extracts of *C. sativa* flowers, leaves, and shells presenting it as an interesting source of polyphenols (PINTO, 2021; CAROCHO, 2015; BARREIRA, 2008), whereas the chestnut burs are still relatively less explored. The chestnut flowers are one of the most studied by-products, with several investigations testing, in fact, the incorporation of extracts originated from the flowers into real foods and showing satisfactory results when compared to the control samples formulated without extract (CAROCHO et al., 2015; CALEJA et al., 2020).

Together these results suggest the overall lack of correlation between the total phenolic content and the antioxidant capacity of the extracts. This may be due to various factors such as the potency of individual phenolic compounds with different active groups, eventual interactions among the phenolic compounds or other plant metabolites concomitantly extracted and possibly all these events occurring simultaneously. The complexity of crude plant extracts makes it challenging to predict the net effect of synergistic, additive and antagonistic interactions among the compounds, but it is likely that such interactions play a role in their overall antioxidant capacity

5.2.2 Antimicrobial activity

The antibacterial and antifungal activities of extracts from *C. sativa* by-products were evaluated using various strains of bacteria and fungi of food relevance and the results obtained are presented in Table 5. The MIC values obtained for Gram-positive bacteria were generally lower than those obtained for Gram-negative bacteria, indicating that the bacteriostatic activity of these two chestnut by-product extracts may be higher against Gram-positive strains. Silva et al. (2020) evaluated the antibacterial activity of different parts of *C. sativ*a, and found that the inner shell extract presented the lowest MIC values against Gram-negative bacteria, while the leaf extract was more active against Gram-positive bacteria. In the present study, the bacterial strain that showed the highest sensitivity to *C. sativa* extracts, i.e., which recorded the lowest MIC values, was *S. aureus* (0.15 and 0.3 mg/mL for flower and bur extracts, respectively, and up to 2.5 mg/mL for both). In terms of MBC values, it is noticeable that the highest bactericidal activities were found for MAE-W extracts of both, chestnut burs and flowers. At the concentration of 5 mg/mL, the flower MAE-W extract was able to cause death of the Gram-negative *E. cloacae* and *E. coli* and Gram-positive *L. monocytogenes* and *S. aureus*, while the bur one killed the Gram-negative *E. cloacae* and *P. aeruginosa* and the Gram-positive *S. aureus*. None of the samples studied showed lower MIC/MBC values and therefore higher antibacterial activity than the positive controls used (commercial antibiotics, Streptomycin, Methicillin and Ampicillin).

Regarding to the antifungal activity, all the extracts of *C. sativa* flowers and burs presented the same antifungal potential against the two species of *Aspergillus* used in the experiment, with MIC values of 10mg/mL (Table 5). None of them was able to cause the death of the fungi at the maximum tested concentration. More promising results were obtained in the study of Alaya et al. (2021), in which the chestnut flower extract presented lower MIC values (between 0.25 and 2 mg/mL) against fungi.

Table 5. Antibacterial (MIC and MBC, mg/mL) and antifungal (MIC and MFC) activity of extracts of *C. sativa* flowers and burs

	Flowers				**Burs**				**Positive Control**		
	MAC-HE	**UAE-HE**	**UAE-W**	**MAE-W**	**MAC-HE**	**UAE-HE**	**UAE-W**	**MAE-W**	**Streptomicin (1mg/mL)**	**Methicilin (1 mg/mL)**	**Ampicillin (10 mg/mL)**
	MIC/MBC	**MIC/MBC**	**MIC/MBC**	**MIC/MBC**	**MIC/MBC**	**MIC/MBC**	**MIC/MBC**	**MIC/MBC**	MIC/MBC	MIC/MBC	MIC/MBC
Gram-negative bactéria											
Enterobacter cloacae	>10/>10	>10/>10	5/>10	2.5/5	>10/>10	>10/>10	5/>10	2.5/5	0.007/0.007	n.t/n.t	0.15/0.15
Escherichia coli	>10/>10	10/>10	10/>10	2.5/5	>10/>10	>10/>10	5/>10	10/>10	0.01/0.01	n.t/n.t	0.15/0.15
Pseudomonas aeruginosa	10/>10	10/>10	10/>10	2.5/>10	>10/>10	>10/>10	>10/>10	2.5/5	0.06/0.06	n.t/n.t	0.63/0.63
Salmonella enterocolitica	10/>10	10/>10	10/>10	5/>10	10/>10	10/>10	5/>10	1.25/10	0.007/0.007	n.t/n.t	0.15/0.15
Yersinia enterocolitica	10/>10	10/>10	>10/>10	>10/>10	>10/>10	>10/>10	>10/>10	>10/>10	0.007/0.007	n.t/n.t	0.15/0.15
Gram-positive bactéria											
Bacillus cereus	5/>10	2.5/>10	10/>10	5/>10	2.5/>10	2.5/>10	5/>10	1.25/>10	0.007/0.007	n.t/n.t	n.t/n.t
Listeria monocytogenes	5/>10	10/>10	2.5/>10	0.6/5	10/>10	5/>10	1.25/>10	0.6/>10	0.007/0.007	n.t/n.t	0.15/0.15
Staphylococcus aureus	1.25/>10	2.5/>10	2.5/>10	0.15/5	2.5/>10	1.25>10	0.6/10	0.3/5	0.007/0.007	0.007/0.007	0.15/0.15
Fungi	**MIC/MFC**	**MIC/MFC**	**MIC/MFC**	**MIC/MFC**	**MIC/MFC**	**MIC/MFC**	**MIC/MFC**	**MIC/MFC**	**Ketoconazole (1 mg/mL)**		
									MIC/MFC		
Aspergillus brasiliensis	10/>10	10/>10	10/>10	10/>10	10/>10	10/>10	10/>10	10/>10	0.06/0.125		
Aspergillus fumigatus	10/>10	10/>10	10/>10	10/>10	10/>10	10/>10	10/>10	10/>10	0.5/1		

Data are mean. MAC-HE: maceration carried out with hydroethanolic solvent (80% EtOH); UAE-HE: Ultrasound-Assisted Extraction carried out with hydroethanolic solvent (80% EtOH); UAE-W: Ultrasound-Assisted Extraction carried out with water; MAE-W: Microwave-Assisted Extraction carried out with water. MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration; MFC: minimal fungicidal concentration.

5.3 EXTRACT SELECTION AFTER INITIAL SCREENING

It is important to note that in this work, different extraction methods were employed, each one performed according to their established conditions within our laboratory framework. While this setup indeed presents challenges in drawing direct comparisons of the results, it was employed to get insights into the intrinsic potential and overall quality of the extracts generated from each method. The first aim of the present work was to evaluate the potential of chestnut by-product extracts obtained with these protocols to be used as natural food additives, as an initial screening. As such, in this case, it was more relevant to us to assess the overall quality of the extract obtained in terms of polyphenol composition and bioactivities, rather than to directly compare the extraction efficiency of the three methods in various conditions. In other words, the focus primarily centred on assessing the outcome of the extraction technique, rather than separating the individual effects of the technology, operation, or solvent to evaluate the extraction efficiency *per se*.

From this initial chemical and bioactive screening of various chestnut bur and flower extracts, it was noticed that the flower ones showed the highest content of polyphenols, particularly the hydroethanolic extract of flowers obtained through maceration (137±2 mg/g dw). Although it is true that the extraction yield and recovery are not the only aspects to be considered, the antioxidant activity of all the extracts was very similar and high (IC_{50} ranging from 0.003 to 0.007 mg/mL). Despite the extracts presented more differences regarding to their antimicrobial activity, the MAC-HE extract of flower was selected for the next part of the study based on its amount of polyphenols and antioxidant activity, considered the most important aspects for the final objective of this work.

5.4 EFFECT OF THE EXTRACT ON THE CHEMICAL STABILITY OF CRAFT BEERS

5.4.1 Volatile compounds

The HS-SPME method was used to characterize the volatile fraction composition of beer containing chestnut flower extract and of the control sample. The volatile profiles of the beer samples evaluated in the first month of monitoring are shown in Figure 18. In the control beer, a total of 18 volatile compounds belonging to different chemical classes were separated and identified in the sample (Table 6). The volatile profile of the beer was characterized by the presence of ten esters, four alcohols, two hydrocarbons, one

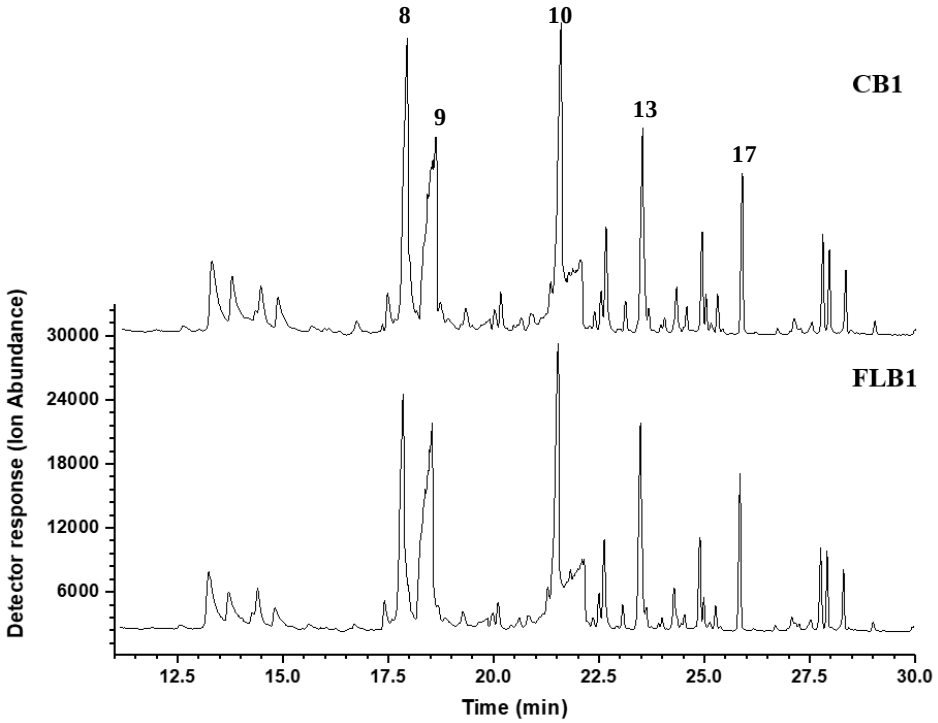

Figure 18. TIC chromatograms, obtained by GC-MS, of the volatile compounds of beers added with chestnut flower extract (FLB1) and control (CB1), analysed in the first month of storage.

Table 6. Volatile compounds of IPA craft beers in the first month of storage

Peak	Rt (min)	Tentative identification	Molecular weight	Target fragment *(m/z)*	Fragments (*m/z*)
1	8.610	Isoamyl acetate	130.19	43	55, 61, 70, 87
2	8.680	Ethyl acetate	88.11	43	61, 70
3	9.048	Unknown compound	-	104	40, 78
4	13.336	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	136.23	93	41, 69, 79
5	13.756	Ethyl 5-methylhexanoate	158.24	88	43, 60, 73, 115
6	14.438	Ethyl butyrate	116,16	71	43
7	14.876	4-Pentenoic acid, 3,3-dimethyl-, methyl ester	142.198	55	40, 67, 82, 93, 111, 127
8	17.904	Linalool	154.25	93	55, 71, 80, 121
9	18.411	β-Phenylethyl alcohol	122,16	91	39, 51, 65, 122
10	21.492	Ethyl undecanoate	214.34	88	41, 73, 101
11	22.629	Citronellyl propionate	212.33	69	41, 55, 81, 95, 109, 123
12	23.084	Phenylethyl alcohol	122.16	91	65
13	23.522	2-Phenylethyl ester	164.20	104	43, 51, 77, 91
14	24.275	Hexyl ester	144.21	43	55, 69, 73
15	24.887	Pentadecane	226.44	43	71, 85, 112
16	25.290	Unknown compound		73	45, 147

17	25.850	Nerol	154.25	69	41, 123
18	27.757	Ethyl 4-decenoate	198.30	69	41, 55, 88, 96, 110, 152
19	27.950	Unknown compound	-	55, 71	41, 67, 99, 108
20	28.318	Decanoic acid	200.32	88	41, 55, 73, 101
21	29.018	Unknown compound	-	91	44, 69, 79, 105, 120, 133
22	30.173	Humulene	204.35	93	67, 80, 107, 121, 147

carboxylic acid, and one terpene already described in IPA beers. Esters and alcohols are crucial chemical group in beers. Whereas esters are associated with the fruity flavour profile observed in beer, the presence of alcohols extends beyond ethanol's contribution of an alcoholic flavour. The fruity and solvent-like characteristics are due to the presence of more intricate alcohol types. Additionally, higher alcohols with a greater molecular weight than ethanol act as immediate precursors of flavour-active esters. Rodriguez-Bencomo et al. (2012) studied the effect of the experimental variables (volume of the sample and the extraction temperature) on the extraction of 28 representative volatile compounds for the beer flavour profile, and found several compounds also identified in this study like isoamyl acetate, ethyl acetate and nerol. In 2020, Gasinski et al. investigated the content of volatile compounds in beers with hawthorn using SPME/GC-MS and identified 53 volatile compounds, having also identified esters as the largest chemical group (25 compounds), besides alcohols and sesquiterpenes (8 compounds each). Esters were also the most abundant compounds in the volatile fraction of beer samples analysed by Castro et al. (2014). A total of twenty-eight different esters were identified in all the samples, four compounds, namely ethyl acetate, isoamyl acetate, ethyl hexanoate, and ethyl octanoate, being common to all of them (CASTRO et al., 2014).

When the chromatogram of the control beer is compared with that of the beer incorporated with extract in the first month of the storage, no differences can be found, i.e., exactly the same volatile profile was noticed (Figure 18). This indicate null or low interference of the extract in the flavour of the IPA beer, which is desirable for an ingredient intended to promote beer stability and not impart or influence the original product's flavour. However, volatile compounds may present different sensory thresholds or sensory activities, so it is necessary to contrast these data with the results of sensory evaluation.

Moreover, to investigate the transfer of volatile compounds from chestnut flower extract to beer, a rapid analysis using a model beer system was conducted. This system comprised tartaric acid at 11 g/L, ethanol at 6.5% v/v, and a pH of 4.5, following the

protocol of Rodriguez-Bencomo et al. (2012), with modifications to match the alcohol content typical of the craft IPA beers analysed. The extract was introduced into the model system at the same concentration used in the beer samples, and the volatile profile of this sample was analysed. As a control, the volatiles were also analysed in the model system without the extract. The chromatogram of the model system of beer containing the extract (Figure 19) revealed that the compounds present in the chestnut flower extract appeared in significantly lower concentrations (as seen by the signal intensity, detector response) compared to those in the craft beers where the extract was incorporated (Figure 18). Furthermore, these compounds were not detected in their respective retention times in chromatograms of the beers, suggesting a minimal or non-existent contribution of the volatile compounds of the extract to the final flavour profile of the highly aromatic IPA beer. This observation aligns with our earlier findings from the volatile profile comparison between control and extract-incorporated beers. It potentially indicates a negligible sensory interference from the added extract.

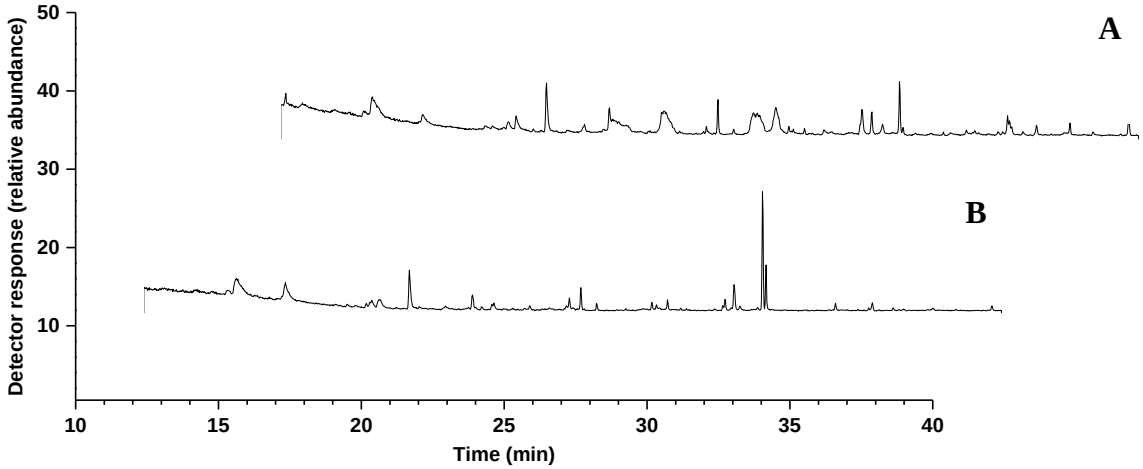

Figure 1919. TIC chromatograms, obtained by GC-MS, of the volatile compounds of model beer system (A, control) and of the model system added with chestnut flower extract (B).

At the final of the stability experiment (6 months), the chromatograms of the control and flower extract-incorporated craft beers from last month of monitoring showed a noticeable decrease in the signal of some important volatile compounds of characteristic beer flavour in the control sample in contrast to that containing the extract, which was perceived like a “flattening” in the chromatographic profile of control beer (Figure 20). This fact suggests a stabilizing effect of the chestnut flower extract on the beer's flavour.

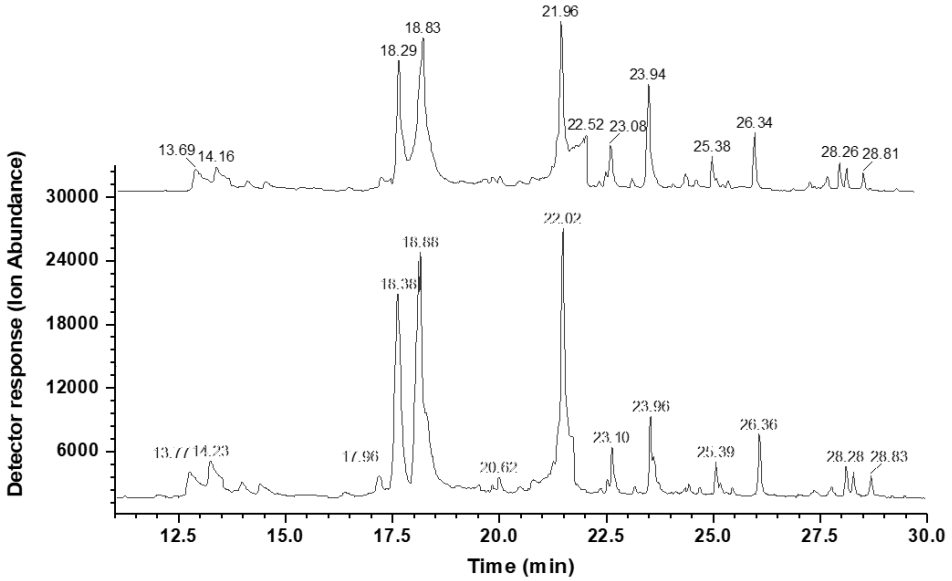

Figure 20. GC-MS (TIC) chromatograms of volatile compounds from craft beers prepared without (control, upper chromatogram) and with the incorporation of chestnut flower extract (chromatogram at the bottom) analysed in the sixth month of storage.

During the stability experiment it was noticed that the carbonyl compounds, primarily aldehydes, compounds widely related to beer off-flavours even in small quantities, were not detected. Indeed, these compounds are difficult to detect in commonly used methods because of their low relative concentration and high volatility and reactivity. To enhance the selectivity of extraction method and the properly detection of aldehydes, a derivatization step was required. This approach involved the derivatization of carbonyl compounds on the SPME fibre with PFBOA, a reagent often utilized in GC analysis of beers (VESELY et al., 2003; ROSSI et al., 2013; SILVA et al., 2015). This strategy allowed the monitoring of any possible carbonyl compound contributing to beer off-flavours.

Of particular relevance, at the completion of the storage time, the analysis of the volatile profile of the beers after the derivatization process revealed an interesting difference between the control and extract-incorporated beers (Figure 21). The signal captured of the 2-*trans*-nonenal compound, one of the main aldehydes related to off-flavours and decrease in the sensory quality of beers, was higher in the control beer than in the beer with extract. This promising finding suggest that the extract may have an effective role in protecting the beer from reactions that lead to its degradation, reducing oxidation or aging processes, which may contribute to a better stability of this beverage.

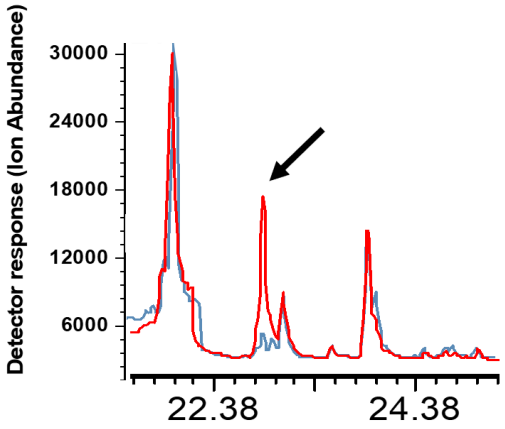

Figure 21. Zoom of GC-MS (TIC) chromatograms of volatile compounds from craft beers prepared without (control, red line) and with the incorporation of chestnut flower extract (blue line) analysed in the sixth month of storage, after derivatization, highlighting the difference in levels of compound at Rt 22.4 min.

5.4.2 Chestanin monitoring in the beer containing the extract

To get insights on the presence and consumption of the polyphenols from the chestnut flower extract in beers over the storage time, the marker compound (chestanin) of the chestnut flower extract was monitored monthly by LC-MS. Chestanin, a phenolic compound of great abundance in chestnut flower extract as aforementioned, has a maximum absorption in the UV region of the electromagnetic spectrum (~274 nm); this compound has a molecular mass 938u and therefore molecular ion [M-H]- de *m/z* 937 in the MS spectrum when ionized in the negative mode, and fragment ions of *m/z* 637, 467 and 305 in the MS/MS spectrum (Figure 22).

Beer is a complex product that contains several types of components such as sugars and proteins in addition to phenolic compounds, and their presence interferes in response of the chromatographic analysis when only a DAD is used, making it difficult to identify the major tannin of the extract in the beers. Due to differences in sensitivity between the DAD and MS detectors, in the chromatograms processed at 280 nm this compound is not clearly visualized, but it is unequivocally detected by MS (Figure 23), which makes the proper use of this technique essential for the present purpose. In the MS analysis, the analytical signal is evidenced in the retention time of the compound in the total ion chromatogram (TIC) processed with the ion *m/z* 937 as target and fragments *m/z* 467 and 305 as base peaks. This strategy, along with the use of SPE cartridge prior to extract injection into the HPLC, allowed detecting the compound over the first few months of the stability assay.

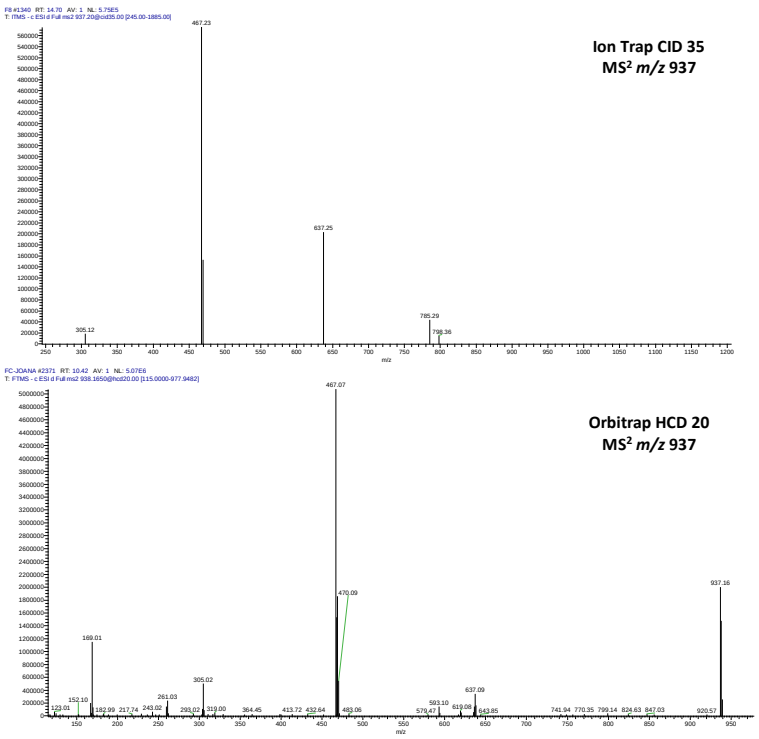

Figure 22. MS/MS spectrum of the molecular ion $[M-H]^-$ at *m/z* 937 corresponding to chestanin, as visualized in two mass analysers.

Chromatograms presented in Figure 23 show the strong detection of this compound by MS in the beer samples incorporated with chestnut flower extract evaluated in in the first month of storage, and its absence in the control sample, as expected.

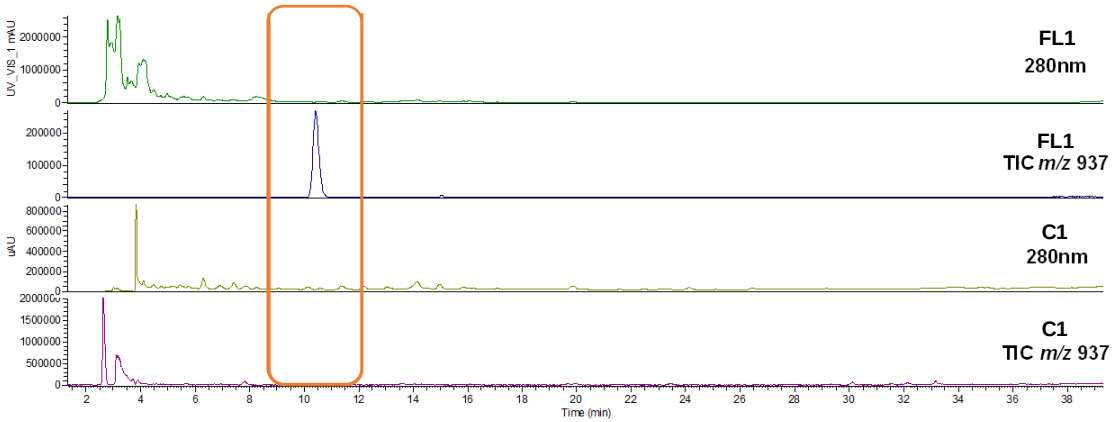

Figure 23. Chromatograms, obtained by HPLC-DAD at 280 nm of craft IPA beers incorporated with chestnut flower extract and control beer, analyzed in the first month of storage (FL1 and C1, respectively). Below the chromatogram of each sample processed at 280nm, there is the respective total ion chromatogram (TIC, MS) acquired monitoring the ion at *m/z* 937 as target and fragments at *m/z* 467 or 305 as base peaks, which show the presence of the compound through a strong signal in its retention time in samples containing extract.

The signal of major tannin of the chestnut flower extract slightly but progressively declined until month 4 (data not shown), probably due to its consumption in inhibiting oxidative mechanisms, preventing the degradation of the beer components, and acting as an antioxidant. Although in lower relative proportion, the chestanin was detected in FL beer until the sixth month of storage, the TIC chromatogram of the beer incorporated with the extract analysed in the last month of storage demonstrating the clear presence of a peak corresponding to the ion at *m/z* 937 (Figure 24).

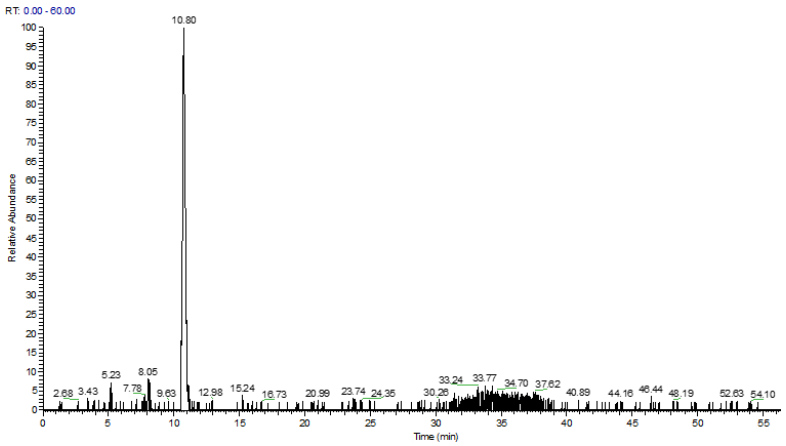

Figure 24. Total ion chromatograms (TIC, MS) acquired with the ion at *m/z* 937 as target and fragments at *m/z* 305 as base peak, demonstrating the presence of the chestanin compound through a signal in its retention time (10.8 min) in the beer incorporated with the extract, in the last month of evaluation (May/2023).

6. CONCLUSION

Chestnut by-products, and particularly chestnut flowers, have been supported as a locally accessible and economically viable source of phenolic compounds for several applications. The polyphenol distribution varied among the parts of chestnut plants, although flowers and burs shared some characteristic compounds. Notably, phenolic acids were the predominant polyphenol class in bur extracts, while hydrolysable tannins were the most abundant in flower extracts. The method and solvent used to prepare the extracts influenced the extraction yield and recovery of polyphenol classes from the different by-products. The hydroethanolic solution was generally more efficient in the extraction of total phenolic compounds and total tannins from chestnut by-products, especially when associated with ultrasound-assisted extraction. Nonetheless, water also showed potential in extracting satisfactory amounts of compounds, yielding extracts with interesting bioactivities, especially evident in the antimicrobial potential of microwave-assisted extraction aqueous extracts of flowers and burs. The antioxidant capacities of all evaluated extracts from burs and flowers were found to be high and comparable. Following initial screening, the extract from chestnut flowers prepared from maceration with hydroethanolic solvent was selected for incorporation into the product of interest, craft-beer.

The study´s findings regarding the flavour stability of craft beers incorporated with the polyphenol-rich extract obtained from chestnut flowers during storage are promising as indicated: (i) the initial similarity between the volatile profiles of control and treated samples, suggesting minimal interference arising from the extracts on the beer original flavour; (ii) better preservation of volatile compounds over the months of storage in treated beers compared to the control, suggesting the extract's role in maintaining the beer's aromatic profile; and (iii) a lower detection of 2-*trans*-nonenal, an aldehyde associated with beer off-flavors, in the extract-incorporated beer, implying the extract's ability in inhibiting oxidative and undesirable reactions. Consequently, the original quality of the craft beer with the extract was maintained for a longer time than the control sample. In conclusion, these findings suggest the utilization of chestnut flower extract as an alternative additive to enhance the flavour stability and overall quality of craft beers, contributing to extending the shelf life of the product.

7. FUTURE PERSPERCTIVES

The promising results obtained in this study with the incorporation of the crude chestnut flower extract in craft beers open a large avenue of research opportunities to improve the technological and functional properties of this extract as an ingredient for food and beverages and the design of clean-label products. Attention can be directed to the ingredient technology, for example with the encapsulation of polyphenols from the extract with cyclodextrins to improve the ingredient solubility in beers and possibly the antioxidant activity, aiming also at the reduction of the aftertaste and undesirable interactions of tannins with proteins, which may cause particle precipitation and haze. Other strategies that can be explored include the use of purified fractions of the extract, the extract standardization in terms of the main compound, the extraction of bound polyphenols, as well as the investigation of whether the use of tannase to hydrolyse the tannins may improve the performance of this ingredient and the antioxidant activity.

BIBLIOGRAPHIC REFERENCES

Ali Redha, A. (2021). Review on extraction of phenolic compounds from natural sources using green deep eutectic solvents. Journal of Agricultural and Food Chemistry, 69(3), 878-912.

Almeida, D. M. Mariano, R. O. Nascimento, K. L. Tavares, M. M. (2020). Análise técnica das etapas dos processos de produção para ampliação fabril de uma cervejaria de pequeno porte. Revista Cientifica Integrada, 4(4), 1-14

Almeida, I. F., Fernandes, E., Lima, J. L., Costa, P, Bahia, M. (2008). Protective Effect of *Castanea sativa* and Quercus Robur Leaf Extracts Against Oxygen and Nitrogen Reactive Species. *Journal of Photochemistry and Photobiology Biology*, 91(2), 87-95.

Alvim, R. P. R., de Cássia Oliveira Gomes, F., Garcia, C. F., de Lourdes Almeida Vieira, M., & de Resende Machado, A. M. (2017). Identification of volatile organic compounds extracted by headspace solid-phase microextraction in specialty beers produced in Brazil. Journal of the Institute of Brewing, 123(2), 219-225.

Angelo, P. M., Jorge, N. (2007). Compostos fenólicos em alimentos – Uma breve revisão. Revista Do Instituto Adolfo Lutz, 66(1), 1–9.

Araújo. F. B., Silva, P. H. A., Minim, V. P. R. (2003). Perfil Sensorial e Composição Físico Química de Cervejas Provenientes de Dois Segmentos do Mercado Brasileiro. Food Cience Technology, 23(2), 121-128.

Araujo, S. B. (2022). Cerveja e pandemia: um estudo sobre as estratégias de branding de cervejas mainstream e artesanais nos anos de 2020 e 2021 (Monografia). Universidade Federal do Rio Grande do Norte, Natal.

Baiano, A. (2020). Craft beer: an overview. Comprehensive Reviews in Food Science and Food Safety, 20(2), 1829-1856.

Bamforth, C.W., & Lentini, A. (2009). Beer: A Quality Perspective (1ª ed.). Academic Press.

Barreira. J, C., Ferreira, I. C, Oliveira, M. B. P., Pereira, J. A. (2008). Antioxidant activities of the extracts from chestnut flower, leaf, skins and fruit. Food Chemistry, 107(3),1106- 13.

Barros, L., Dueñas, M., Alves, C. T., Silva, S., Henriques, M., Santos-Buelga, C., Ferreira, I. C. F. R. (2013). Antifungal activity and detailed chemical characterization of Cistus ladanifer phenolic extracts. Industrial Crops and Products, 41(1), 41-45.

Bluml, S. (2009). Handbook of Brewing (1ª ed.). Freiberg: WILEY-VCH Verlag GmbH & Co.

Bosco, J., Pickett, J., Schaeffer, Z., Smith, S. (2019). Investigation of the Effects of Oxygen and Other Considerations on the Shelf-Life of Craft Beer (Dissertação de Mestrado). Instituto Politécnico de Worcester, Worcester.

Bowers, J. J., Gunawardena, H. P., Cornu, A., Narvekar, A. S., Richieu, A., Deffieux, D., Quideau, S., & Tharayil, N. (2018). Rapid screening of ellagitannins in natural sources via Targeted Reporter Ion Triggered Tandem Mass Spectrometry. Scientific Reports, 8(1).

Brand-Williams, W., Cuvelier, M. E., Berset, C. (1995). Use Of A Free Radical Method To Evaluate Antioxidant Activity. Lwt - Food Science And Technology, 28(1), 25-30.

Brewers Association. (2016). Brewers Association Releases 2016 Beer Style Guidelines. Disponível em: https://www.brewersassociation.org/press-releases/2016-beer-style-guidelines/.

Brewers Association. (2019). Craft Brewer Definition - Brewers Association. https://www.brewersassociation.org/statistics-and-data/craft-brewer-definition/

BUSCH, J. (2015) More Beer. Disponível em: https://www.morebeer.com/articles/advancedmashing . Access at: 15 jun. de 2022.

Caleja, C. Barros, L., Barreira, J. C. M., Sokovic, M., Calhelha, R. C., Bento, A., Oliveira, M. B. P. P., Ferreira, I. C. F. R. (2020). Castanea sativa male flower extracts as an alternative additive in the Portuguese pastry delicacy "pastel de nata". Food Funct., 11, 2208-2217.

Caleja, C., Barros, L., Prieto, M. A., Bento, A., Oliveira, M. B. P. P., Ferreira, I. C. F. R. (2019). Development of a natural preservative obtained from male chestnut flowers: optimization of a heat-assisted extraction technique. Food & Function, 10(3), 1352- 1363.

Callejo, M. J., Tesfaye, W., González, M. C., & Morata, A. (2019). Craft beers: Current situation and future trends. New advances on fermentation processes.

Câmara, J.S.; Albuquerque, B.R.; Aguiar, J.; Corrêa, R.C.G.; Gonçalves, J.L.; Granato, D.; Pereira, J.A.M.; Barros, L.; Ferreira, I.C.F.R. (2021). Food Bioactive Compounds and Emerging Techniques for Their Extraction: Polyphenols as a Case Study. Foods, 10(1), 37.

Carocho, M., Barreira, J. C. M., Barros, L., Bento, A., Cámara, M., Morales, P., Ferreira, I. C. F. R. (2015). Traditional pastry with chestnut flowers as natural ingredients: an approach of the effects on nutritional value and chemical composition. Journal Of Food Composition And Analysis, 44(1), 93-101.

Carocho, M., Barreira, J. C. M., Bento, A., Morales, P., Ferreira, I. C. F. R. (2014). Chestnut flowers as functionalizing agents to enhance the antioxidant properties of highly appreciated traditional pastry. Food Funct, 5(2989).

Carocho, M., Barros, L., Bento, A., Santos-Buelga, C., Morales, P., Ferreira, I. C. F. R. Castanea sativa Mill. (2014). Flowers amongst the Most Powerful Antioxidant Matrices: a phytochemical approach in decoctions and infusions. Biomed Research International, 2014(1), 1-7.

Carocho, M., Calhelha, R. C., Queiroz, M. J. R. P., Bento, A., Morales, P., Soković, M., Ferreira, I. C.F.R. (2014). Infusions and decoctions of Castanea sativa flowers as effective antitumor and antimicrobial matrices. Industrial Crops And Products, 62(1), 42-46.

Carvalho, D. (2008). Estudo de compostos fenólicos de bebidas com potencial actividade anti-tumoral. Efeitos biológicos in vitro (Dissertação de Mestrado). Universidade do Porto, Porto.

Castro, L. F., Ross, C. F., & Vixie, K. R. (2015). Optimization of a solid phase dynamic extraction (SPDE) method for beer volatile profiling. Food analytical methods, 8, 2115-2124.

Castro, T. L. A., Santos, M. S. M., Cardoso, C. A. L. (2022). Produção de cerveja artesanal com pimenta dedo-de-moça comercial. Revista Fitos, 15(1), 73-78.

Central Brew. (2020). Saiba qual é a diferença entre a cerveja artesanal e a cerveja industrial. Available at: https://centralbrew.com.br/blog/saiba-qual-e-a-diferenca-entre-a- cerveja-artesanal-e-a-cerveja-industrial/. Access at : 01 jul. 2022.

Cerqueira, J. F. O. (2016). Estratégias para Melhorar a Qualidade da Cerveja Artesanal – Análise de Pontos Críticos (Dissertação de Mestrado). Univerisdade do Minho, Braga.

Cerulli, A., Napolitano, A., Hošek, J., Masullo, M., Pizza, C., & Piacente, S. (2021). Antioxidant and in vitro preliminary anti-inflammatory activity of Castanea sativa (Italian cultivar "Marrone di Roccadaspide" PGI) burs, leaves and chestnuts extracts and their metabolite profiles by LC-ESI/LTQOrbitrap/MS/MS. Antioxidants, 10(2), 1–22.

Cervbrasil (Brasil). A Indústria cervejeira está conectada com o desenvolvimento do país. Disponível em: http://www.cervbrasil.org.br/novo_site/industria-cervejeira-conectada-desenvolvimento-pais/. Access at: 19 jul. 2022.

Cerveja Artesanal Portuguesa. (2020). Mean Sardine eleita Melhor Cervejeira de Portugal para o RateBeer. Disponível em: https://cervejaartesanalportuguesa.pt/post/61167848027550515 2/mean-sardine-eleita- melhor-cervejeira-de-portugal. Access at 01 de jul. de 2022.

Cheiran, K. P., Raimundo, V. P., Manfroi, V., Anzanello, M. J., Kahmann, A., Rodrigues, E., Frazzon, J. (2019). Simultaneous identification of low-molecular weight phenolic and nitrogen compounds in craft beers by HPLC-ESI-MS/MS. Food Chemistry, 286(1), 113-122.

Clifford, M. N., & Scalbert, A. (2000). Ellagitannins – nature, occurrence and dietary burden. J. Journal of the Science of Food and Agriculture, 80, 1118-1125.

Conedera, M., Tinner, W., Krebs, P., De Rigo, D., Caudullo, G. (2016). Castanea sativa in Europe: distribution, habitat, usage and threats. In: San-Miguel-Ayanz, J., de Rigo, D., Caudullo, G., Houston Durrant, T., Mauri, A. (Eds.), European Atlas of Forest Tree Species. Luxembourg: Publ. Off. EU.

Cortese, M., Gigliobianco, M. R., Peregrina, D. V., Sagratini, G., Censi, R; Martino, P. (2019). Quantification of phenolic compounds in different types of crafts beers, worts, starting and spent ingredients by liquid chromatography-tandem mass spectrometry. Journal Of Chromatography A, 1612(1), 1-39.

Craft Beer Industry Market Segments | Brewers Association. (2023). Brewers Association. https://www.brewersassociation.org/statistics-and-data/craft-beer-industry-market-segments/

Craft Beer Market Size & Share Analysis - Growth Trends & Forecasts (2023 - 2028). (2023). Mordor Intelligence. Available at: https://www.mordorintelligence.com/industry-reports/craft-beer-market

Cushnie, T. T., Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. Int. J. Antimicrob. Agents, 38, 99–107.

Day, L., Seymour, R., Pitts, K., Konczak, I., Lundin, L. (2009). Incorporation of functional ingredients into foods. Trends in Food Science & Technology, 20(1), 388- 395.

De Carvalho, G. B. M. Bento, C.V. Silva, J. B. A. Elementos biotecnologicos fundamentais no processo cervejeiro: 3º parte - A maturação. Revista Analytica, Lorena, v. 27, p. 69-74, mar. de 2007.

De Lima, A.C.; Aceña, L.; Mestres, M.; Boqué, R. (2022). An Overview of the Application of Multivariate Analysis to the Evaluation of Beer Sensory Quality and Shelf-Life Stability. Foods, 11, 1-15.

Decreto Lei no 28/2022 de 9 de Fevereiro da Economia e Transição Digital e Agricultura. Diário da República: série I, No 131 (2022). Acedido a 01 de jul. 2022. Disponível em www.dre.pt.

Delgado, T., Pereira, J., Casal, S., Ramalhosa, E. (2013). Efeito dos ultra-sons, temperatura e tempo na actividade antioxidante de cascas de castanha (Castanea sativa Mill.). In XIX Encontro Galego Português de Química. Vigo: Colegio Oficial de Quimicos de Galicia.

Deng, Y., Bi, H., Yin, H., Yu, J., Dong, J., Yang, M., et al. (2018). Influence of ultrasound assisted thermal processing on the physicochemical and sensorial properties of beer. Ultrasonics Sonochemistry, 40, 166–173.

Diário de Notícias. Cervejas artesanais perderam cerca de 50% das vendas em Portugal. (2022). Available at https://www.dn.pt/edicao-do-dia/15-jun-2022/cervejas- artesanais-perderam-cerca-de-50-das-vendas-em-portugal-14939793.html. Access at 01 de jul. de 2022.

Dragone, G.; Almeida E Silva, J. B. (2010). Cerveja. In: VENTURINI FILHO, W. G. Bebidas alcoólicas: ciência e tecnologia. São Paulo: Edgard Blücher.

Esposito, T., Celano, R., Pane, C., Piccinelli, A. L., Sansone, F., Picerno, P., Zaccardelli, M., Aquino, R. P., & Mencherini, T. (2019). Chestnut (Castanea sativa Miller.) burs extracts and functional compounds: UHPLC-UV-HRMS profiling, antioxidant activity, and inhibitory effects on phytopathogenic fungi. Molecules, 24(2).

Esslinger, H. M. (2009) Handbook of Brewing: Processes, Technology, Markets, Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA.

Europe Craft Beer Market Size & Share Analysis - Growth Trends & Forecasts (2023 - 2028). (2023) Mordor Intelligence. Available at: https://www.mordorintelligence.com/industry-reports/europe-craft-beer-market

Ezhilarasi, P.N.; Indranib, D.; Jenac, B.S.; Anandharamakrishnana, C. (2013). Freeze drying technique for microencapsulation of Garcinia fruit extract and its effect on bread quality. Journal of Food Engineering, 117(4), 513–520.

Fermentec. (2020). Controle bacteriano na fermentação alcoólica influencia diretamente na redução dos custos operacionais e perdas dos açúcares. Available at: https://fermentecnews.com.br/2020/09/04/controle-bacteriano-na-fermentacao- alcoolica-influencia-diretamente-na-reducao-dos-custos-operacionais-e-perdas-dos- acucares/. Access at: 10 de jul. 2022.

Ferrara, E., Pecoraro, M. T., Cice, D., Piccolella, S., Formato, M., Esposito, A., Petriccione, M., & Pacifico, S. (2022). A Joint Approach of Morphological and UHPLC-HRMS Analyses to Throw Light on the Autochthonous 'Verdole' Chestnut for Nutraceutical Innovation of Its Waste. Molecules, 27(24).

Ferreira, A. S., Benka, C. L. (2014). Produção De Cerveja Artesanal A Partir De Malte Germinado Pelo Método Convencional E Tempo Reduzido De Germinação (Tese de Doutorado). Universidade Tecnológica Federal do Paraná, Francisco Beltrão.

Figueiredo, M. S. L. (2020). Sovina: Um estudo de caso sobre a cerveja artesanal portuguesa (Projeto de Qualificação de Mestrado). Universidade do Porto, Faculdade de Belas Artes, Porto.

Filho, W. G. V. Cereda, M. P. (2001). Cerveja. In: Lima, U. A. Aquarone, E. Borzani, W. Schmidell, W. Biotecnologia Industrial: Biotecnologia na Produção de Alimentos. São Paulo: Edgard Blucher.

Flores, M. I. A., González, R. R.; Vidal, J. L. M.; González, F. J. E.; Frenich, A. G. (2014). Monitoring of phytochemicals in fresh and fresh-cut vegetables: A comparison. Food Chemistry, 142, 392-399

Forbes. (2022). The Craft Beer Industry Has Reason To Celebrate According To The Latest Data. Available at < https://www.forbes.com/sites/hudsonlindenberger/2022/05/25/the-craft-beer-industry- has-reason-to-celebrate-according-to-the-latest-data/?sh=6f2cbfef2837>. Access at 15 set. 2022.

Formato, M., Vastolo, A., Piccolella, S., Calabrò, S., Cutrignelli, M. I., Zidorn, C., & Pacifico, S. (2022). Castanea sativa Mill. Leaf: UHPLC-HR MS/MS Analysis and Effects on In Vitro Rumen Fermentation and Methanogenesis. Molecules, 27(24). https://doi.org/10.3390/molecules27248662

Freitas, G. L. (2006). Potencial Antioxidante e Compostos Fenólicos na Cerveja, Chopp, Cevada (Hordeum vulgare L.) e no Bagaço de Brassagem (Dissertação de Mestrado). Universidade Federal de Santa Catarina, Florianópolis.

Freitas, F. (2021). Consumo de cerveja artesanal cresce nos "países dos vinhos." Cervejar. Available at: https://cervejar.com/consumo-de-cerveja-artesanal-cresce-nos-paises-dos-vinhos/. Access at 15 set. 2022.

Gales, P.W. (2007). Brewing Chemestry and Technology in the America. AACC International.

Garcia-Cruz, C. H.; Foggetti, U.; Da Silva, A. N. (2008). Alginato bacteriano: aspectos tecnológicos, características e produção. Química Nova, 31(7), 1800-1806.

Gasiński, A., Kawa-Rygielska, J., Szumny, A., Gąsior, J., & Głowacki, A. (2020). Assessment of volatiles and polyphenol content, physicochemical parameters and antioxidant activity in beers with dotted hawthorn (Crataegus punctata). Foods, 9(6), 775

Gonçalves, R. M. F. (2007). Estudo Da Inibição De Tripsina Por Compostos Fenólicos Isolados De Fontes Naturais: Efeito Antinutricional De Bebidas Comuns (Tese de Doutorado). Universidade do Minho, Braga.

González-Gómez, D., Cardoso, V., Bohoyo, D., Ayuso, M.C., Delgado-Adamez, J. (2014). Application of experimental design and response surface methodology to optimize the procedure to obtain a bactericide and highly antioxidant aqueous extract from orange peels. Food Control, 35, 252-259.

Gorelik, S., Ligumsky, M., Kohen, R., Kanner, J. (2008). A novel function of red wine polyphenols in humans: prevention of absorption of cytotoxic lipid peroxidation products. Journal of the Federation of American Societies for Experimental Biology, 22, 41-46.

Hansen, M. F. (2011). Avaliação dos Processos de Oxidação em Cervekas Tipo Pilsner (Monografía de Licenciatura). Universidade Federal do Rio Grande do Sul, Porto Alegre.

Hu, M., Yang, X., & Chang, X. (2021). Bioactive phenolic components and potential health effects of chestnut shell: A review. Journal of Food Biochemistry, 45(4), e13696.

Jandera, P., Łkeříková, V., Řehová, L., Hájek, T., Baldriánová, L., Łkopová, G., Kellner, V., Horna, A. (2005). RP-HPLC analysis of phenolic compounds and flavonoids in beverages and plant extracts using a CoulArray detector. Journal Of Separation Science, 28(9), 1005-1022.

Jaskula-Goiris, B., De Causmaecker, B., De Rouck, G., Aerts, G., Paternoster, A., Braet, J., & De Cooman, L. (2018). Influence of transport and storage conditions on beer quality and flavour stability. Journal of the Institute of Brewing, 125(1), 60–68. https://doi.org/10.1002/jib.535

Junior, A. A. D; Vieira, A. G. (2009). Ferreira, T. P. Processo de Produção de Cerveja. Revista Processos Quimicos, 3(6), 61-72.

Junior, A. J. C. (2021). Se Há Brasileiros Cá, Há Cerveja Ó Pá! A Influência Da Migração Brasileira Na Cultura Da Cerveja Artesanal Em Portugal (Dissertação de Mestrado). Universidade de Coimbra, Coimbra.

Kleban, J.; Nickerson, I. (2011). The US craft brew industry. In: Allied Academies International Conference. International Academy for Case Studies: Proceedings, 18(1), 33– 38, Orlando.

Koppel, K.; Chambers, E. Iv.; Vázquez-Araújo, L.; Timberg, L., Carbonell-Barrchina, A. A.; Suwonsichon, S. (2014). Cross-country comparison of pomegranate juice acceptance in Estonia, Spain, Thailand, and United States. Food Quality and Preference, 31, 116- 123.

Leitao, C., Marchioni, E., Bergaentzlé, M., Zhao, M., Didierjean, L., Taidi, B., Ennahar, S. (2011). Effects of Processing Steps on the Phenolic Content and Antioxidant Activity of Beer. Journal Of Agricultural And Food Chemistry, 59(4), 1249-1255.

Lordan, R.; O'keeffe, E.; Tsoupras, A.; Zabetakis, I. (2019).Total, Neutral, and Polar Lipids of Brewing Ingredients, By-Products and Beer: Evaluation of Antithrombotic Activities. Foods, 8(5), 1-17.

Machado, S. (2019). Craft beer in Portugal – A study about consumers, perceptions, drivers and barriers of consumption (Dissertação de Mestrado). Universidade Católica Portuguesa, Lisboa.

Machado, W. R. C., Filippin, A. P., Silva, L. M., Silva, H. D. M., Hoffmann, R. M. M. (2021). Incorporation of phenolic compounds in food products: a review. Brazilian Journal of Development, 7(5), 46470-46499.

Maicas, S. (2020). The role of yeasts in fermentation processes. Microorganisms, 8(8), 1142.

Malherbe, S., Watts, V., Nieuwoudt, H. H., Bauer, F. F., & Du Toit, M. (2009). Analysis of volatile profiles of fermenting grape must by headspace solid-phase dynamic extraction coupled with gas chromatography−mass spectrometry (HS-SPDE GC-MS): novel application to investigate problem fermentations. Journal of Agricultural and Food Chemistry, 57(12), 5161-5166.

Malta, L. H. (2006). Estudos de parâmetros de propagação de fermento (*Saccharomyces cerevisiae*) para produção de cachaça de alambique (Dissertação de Mestrado). Universidade Federal de Minas Gerais, Belo Horizonte.

Marova, I., Parilova, K., Friedl, Z., Obruca, S., Duronova, K. (2011). Analysis of Phenolic Compounds in Lager Beers of Different Origin: a contribution to potential determination of the authenticity of czech beer. Chromatographia, 73(83), 83-95.

Matos, R. A. G. Cerveja: Panorama Do Mercado, Produção Artesanal, E Avaliação De Aceitação E Preferência (Monografia de Licenciatura). Universidade Federal de Santa Catarina, Florianópolis.

Mega, J. F., Neves, E., Andrade, C. J. (2011). A Produção Da Cerveja No Brasil. Revista de Ciência, Tecnologia, Inovação e Oportunidade, 1(1), 34-43.

Mignani, A.G., Ciaccheri, L., Mencagli, A.A., Ottevaer, H., Baca, E., Thienpont, H., (2013). Optical measurements and pattern-recognition techniques for identifying the characteristics of beer and distinguishing Belgian beers. Sensors and Actuators, 179, 140-149.

Moilanen, J., Sinkkonen, J., & Salminen, J. P. (2013). Characterization of bioactive plant ellagitannins by chromatographic, spectroscopic and mass spectrometric methods. Chemoecology, 23, 165–179.

Munekata, P. E. S., Franco, D., Trindade, M. A., & Lorenzo, J. M. (2016). Characterization of phenolic composition in chestnut leaves and beer residue by LC-DAD-ESI-MS. LWT-Food Science and Technology, 68, 52-58..

Nazir, M., Arif, S., Khan, R. S., Nazir, W., Khalid, N., & Maqsood, S. (2019). Opportunities and challenges for functional and medicinal beverages: Current and future trends. Trends in Food Science & Technology, 88, 513-526.

Olaniran, A. O., Hiralal, L., Mokoena, M. P., & Pillay, B. (2017). Flavour-active volatile compounds in beer: production, regulation and control. Journal of the Institute of Brewing, 123(1), 13-23.

Olaniran, A. O., Hiralal, L., Mokoena, M. P., & Pillay, B. (2017). Flavour-active volatile compounds in beer: production, regulation and control. Journal of the Institute of Brewing, 123(1), 13-23.

Ozawa, T., Arai, N., & Takino, Y. (1978). Structure of a new phenolic glycoside, chesnatin, from chestnut galls. Agricultural and Biological Chemistry, 42(10), 1907–1910.

Ozawa, T., Kobayashi, D., & Takino, Y. (1978). Isolation and Structure of a New Phenolic Glycoside, Neocretanin, from Chestnut Galls. Agricultural and Biological Chemistry, 42(6).

Ozawa, T., Kobayasiii, D., & Takino, Y. (1977). Structures of the New Phenolic Glycosides MP-2 and MP-10 from Chestnut Galls. Agricultural and Biological Chemistry, 41(7).

Ozawa, T., Raga, K., Kobayashi, D., Kamiyama, T., & Takino, Y. (1977). Isolation and characteristics of new phenolic glycosides of chestnut galls. Agricultural and Biological Chemistry, 41(7), 1249–1256. https://doi.org/10.1080/00021369.1977.10862638

Paes, R. F. (2015). A Construção da Qualidade: Estudo Sobre a Legitimação do Standard da Lei de Pureza Alemã no Mercado Catarinense de Cervejas (Monografia de Licenciatura). Universidade Federal de Santa Catarina, Florianópolis.

Pastore, G.M.; Bicas, J.L.; Junior, M.R.M. (2013). Biotecnologia de alimentos (Vol. 12). São Paulo: Atheneu.

Peña-Gómez, N., Ruiz-Rico, M., Pérez-Esteve, E., Fernández-Segovia, I., Barat, J. M. (2020). Microbial stabilization of craft beer by filtration through silica supports functionalized with essential oil components. LWT, 117, 108626.

Pereira, C. C. B. (2022). Desenvolvimento, Caracterização E Atividade Antioxidante De Cerveja Artesanal Contendo Amaranto (Tese de Doutorado). Universidade Federal de São Paulo, São Paulo.

Petrucci, R., Matteo, P., Francesco, G., Mattiello, L., Perretti, G., Russo, P. (2020). Novel Fast Identification and Determination of Free Polyphenols in Untreated Craft Beers by HPLC-PDA-ESI-MS/MS in SIR Mode. Journal Of Agricultural And Food Chemistry, 68(30), 7984-7994.

Pimenta, L. B., Rodrigues, J. K. L. A., Sena, M. D. D., Corrêa, A. L. A., Pereira, R. L. G. (2020). A história e o processo da produção da cerveja: uma revisão. Cadernos de Ciência & Tecnologia, 37(3), 26715.

Pinela, J., Barros, L., Dueñas, M., Carvalho, A. M., Santos-Buelga, C., Ferreira, I. C. F. R. (2012). Antioxidant activity, ascorbic acid, phenolic compounds and sugars of wild and commercial Tuberaria lignosa samples: effects of drying and oral preparation methods. Food Chemistry, 135(3), 1028-1035.

Pinho, O., Ferreira, I. M., & Santos, L. H. (2006). Method optimization by solid-phase microextraction in combination with gas chromatography with mass spectrometry for analysis of beer volatile fraction. Journal of chromatography A, 1121(2), 145-153.

Pires, T. C. S. P., Dias, M. I., Barros, L; Alves, M. J., Oliveira, M. B. P. P., Santos-Buelga, C., Ferreira, I. C. F. R. (2018). Antioxidant and antimicrobial properties of dried

Portuguese apple variety (Malus domestica Borkh. cv Bravo de Esmolfe). Food Chemistry, 240, 701-706.

Priest, F.G. Stewart, G. G. (2006). Handbook of Brewing, Boca Raton: Taylor & Francis Group.

Quifer-Rada, P., Vallverdð-Queralt, A., Martínez-Huélamo, M., Chiva-Blanch, G., Jáuregui, O., Estruch, R., Lamuela-Raventós, R. (2015). A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC–ESI-LTQ-Orbitrap-MS). Food Chemistry, 169, 336-343.

Radonjić, S., Maraš, V., Raičević, J., & Košmerl, T. (2020). Wine or beer? Comparison, changes and improvement of polyphenolic compounds during technological phases. Molecules, 25(21), 4960.

Rebello, F. F. P. (2009). Produção de Cerveja. Revista Agrogeoambiental, 1(3), 145-155.

Reid, N., Mclaughlin, R. B., Moore, M. S. (2014). From Yellow Fizz to Big Biz: american craft beer comes of age. Focus On Geography,. 57(3), 114-125.

Reitenbach, A. F. (2010). Desenvolvimento de cerveja funcional com adição de probiótico: Saccharomyces boulardii (Dissertação de Mestrado). Universidade Federal de Santa Catarina, Florianópolis.

Rettberg, N., Biendl, M., & Garbe, L. A. (2018). Hop aroma and hoppy beer flavor: chemical backgrounds and analytical tools—a review. Journal of the American Society of Brewing Chemists, 76(1), 1-20.

Ribeiro, B., Rangel, J., Valentao, P., Andrade, P. B., Pereira, J. A., Bölke, H. (2007). Organic acids in two portuguese chestnut (Castanea sativa Miller) varieties. Food Chemistry, 100(2), 504-508.

Rodrigues, F., Santos, J., Pimentel, F. B., Braga, N., Palmeira-De-Oliveira, A., Oliveira, M. B. P. P. (2015). Promising new applications of Castanea sativa shell: nutritional composition, antioxidant activity, amino acids and vitamin e profile. Food & Function, 6(8), 2854-2860.

Rodrigues, M. A., Morais, J. S., Castro, J. P (Eds.) (2015). Jornadas de lúpulo e cerveja: novas oportunidades de negócio: livro de atas. Bragança: Instituto Politécnico, CIMO.

Rodrigues, D.B.; Veríssimo, L.; Finimundy, T.; Rodrigues, J.; Oliveira, I.; Gonçalves, J.; Fernandes, I.P.; Barros, L.; Heleno, S.A.; Calhelha, R.C. Chemical and Bioactive Screening of Green Polyphenol-Rich Extracts from Chestnut By-Products: An Approach to Guide the Sustainable Production of High-Added Value Ingredients. Foods 2023, 12, 2596.

Rodriguez-Bencomo, J. J., Muñoz-González, C., Martín-Álvarez, P. J., Lázaro, E., Mancebo, R., Castañé, X., & Pozo-Bayón, M. A. (2012). Optimization of a HS-SPME-GC-MS procedure for beer volatile profiling using response surface methodology: application to follow aroma stability of beers under different storage conditions. Food analytical methods, 5, 1386-1397.

Rosa, C. G., Dellinghausen, C. B., Zambiazi, R. C., Rutz, J. K., Luz, S. R., Krumreich, F. D., Benventti, E. V., Nunes, M. R. (2014). Encapsulation of the phenolic compounds of the blackberry (Rubus fruticosus). LWT, 58(2), 527-533.

Rossi, S., Sileoni, V., Perretti, G., & Marconi, O. (2014). Characterization of the volatile profiles of beer using headspace solid-phase microextraction and gas chromatography–mass spectrometry. Journal of the Science of Food and Agriculture, 94(5), 919-928

Rutz, J. K., Zambiazi, R. C., Borges, C. D., Krumreich, F. D., Luz, S. R., Hartwing, N., Rosa, C. G. (2013). Microencapsulation of purple Brazilian cherry juice in xanthan, tara gums and xanthan-tara hydrogel matrixes. Carbohydrate Polymers, 98, 1256-1265.

Saison, D., De Schutter, D. P., Delvaux, F., & Delvaux, F. R. (2008). Optimisation of a complete method for the analysis of volatiles involved in the flavour stability of beer by solid-phase microextraction in combination with gas chromatography and mass spectrometry. Journal of chromatography A, 1190(1-2), 342-349.

Sant'anna, V., Christiano, F. D. P., Marczak, L. D. F., Tessaro, I. C., Thys, R.C.S. (2014). The effect of the incorporation of grape marc powder in fettuccini pasta properties. Food Science and Technology, 58(2), 497-501.

Santos, C. M. (2018). Caracterização do processo produtivo da cerveja artesanal Praxis (Relatório de Estágio). Instituto Politécnico de Coimbra, Coimbra.

Santos, J. R. S. (2002). Determinação do trans-2-nonenal em Cerveja por Cromatografia Líquida de Alta Eficiência com Detecção Espectrofotométrica (Dissertação de Mestrado). Universidade do Porto, Porto.

Santos, R. O. (2015). Phenolic Compounds And Antioxidant Activity Of Blueberry Wines From Different Cultivar (Tese de Doutorado). Universidade Federal de Santa Maria, Santa Maria.

SEBRAE. (2017). Microcervejarias ganham espaço no mercado nacional. Sebrae Nacional. Disponível em: https://respostas.sebrae.com.br/microcervejarias-ganham-espaco-no-mercado-nacional/. Access at: 15 jun. de 2022.

Sicard, D., Legras, J. (2011) Bread, beer and wine: yeast domestication in the Saccharomyces sensu stricto complex. Comptes Rendus Biologies, 332, 229-236.

Silva, G. C., da Silva, A. A., da Silva, L. S., Godoy, R. L. D. O., Nogueira, L. C., Quitério, S. L., & Raices, R. S. (2015). Method development by GC–ECD and HS-SPME–GC–MS for beer volatile analysis. Food chemistry, 167, 71-77..

Silva, R. N. P., Dias, J. F., Koblitz, M. G. B. (2021). Cervejas: relação entre estilos; compostos fenólicos e capacidade antioxidante. Research, Society And Development, 10(3), 1-17.

Silveira, A. L. C. (2013). Validação de métodos para a determinação de compostos fenólicos em melancia (Dissertação de Mestrado). Instituto Politécnico de Castelo Branco, Castelo Branco.

Siqueira, P. B. (2007). Estudo da cinética bioquímica e sensorial de diferentes tipos de cervejas brasileiras (Dissertação de Mestrado). Universidade Estadual de Campinas, Campinas.

Sousa, A. R. E. (2014). Estudo do Impacto de Variáveis de Processo nos Parâmetros de Qualidade da Cerveja Artesanal ao Longo do Tempo de Prateleira (Tese de Doutorado). Universidade do Minho, Braga.

Stewart, G.G. (2016). Shelf Life and Stability. In P. Subramaniam (Ed.), The Stability and Shelf Life of Food Beer (2ª ed, pp. 293-309). Woodhead Publishing.

Svobodova, B., Barros, L., Sopik, T., Calhelha, R. C., Heleno, S., Alves, M. J., Walcott, S., Kuban, V., Ferreira, I. C. F. R. (2017). Non-edible parts of Solanum stramoniifolium Jacq. – a new potent source of bioactive extracts rich in phenolic compounds for functional foods. Food & Function, 8(5), 2013-2021.

Takebayashi, J., Iwahashi, N., Ishimi, Y., Tai, A. (2012). Development of a simple 96-well plate method for evaluation of antioxidant activity based on the oxidative haemolysis inhibition assay (OxHLIA). Food Chemistry, 134(1), 606-610.

Tozetto, L. M. (2017). Produção E Caracterização De Cerveja Artesanal Adicionada De Gengibre (Zingiber officinale) (Dissertação de Mestrado). Universidade Tecnológica Federal do Paraná, Ponta Grossa.

Tirado-Kulieva, V. A.; Hernández-Martínez, E.; Minchán-Velayarce, H. H.; Pasapera-Campos, S. E.; Luque-Vilca, O. M. (2023) A comprehensive review of the benefits of drinking craft beer: Role of phenolic content in health and possible potential of the alcoholic fraction. Current Research in Food Science, 6, 100477.

Vargas, B. O. (2018). Desenvolvimento, Caracterização Físico-Química E Avaliação Do Potencial Antioxidante De Cervejas Tipo Ale (Ipa) (Tese de Doutorado).Universidade Federal de Uberlândia, Patos de Minas.

Vasconcelos, M., Bennett, R. N., Quideau, S., Jacquet, R., Rosa, E. A., Ferreira-Cardoso, J. V. (2010). Evaluating the potential of chestnut (Castanea sativa Mill.) fruit pericarp and

integument as a source of tocopherols, pigments and polyphenols. Industrial Crops and Products, 31(2), 301-311.

Vázquez, G.; Fernández-Agulló, A.; Gómez-Castro, C.; Freire, M.S.; Antorrena, G.; González-Álvarez, J. Response surface optimization of antioxidants extraction from chestnut (Castanea sativa) bur. Ind. Crops Prod. 2012, 35, 126–134

Venturini Filho, W. G., Cereda, M. P. (2001). Cerveja. In: Aquarone, E., Borzani, W., Schmidell, W., Lima, U. A. (Coord.). Biotecnologia na produção de alimentos (vol. 4). São Paulo: Edgard Blücher,

Vesely, P., Lusk, L., Basarova, G., Seabrooks, J., & Ryder, D. (2003). Analysis of aldehydes in beer using solid-phase microextraction with on-fiber derivatization and gas chromatography/mass spectrometry. Journal of Agricultural and Food Chemistry, 51(24), 6941-6944.

Viana, R. A. (2020). Métodos de controlo de qualidade nas diferentes etapas de produção de cervejas artesanais (Dissertação de Mestrado). Universidade do Porto, Porto.

Vieira Araújo, F. R. (2019). Impacto Sensorial da Microbiota no Off-flavour da Cerveja Artesanal Session IPA Durante o Armazenamento (Dissertação de Mestrado). Universidade Federal do Ceará, Fortaleza.

Villacreces, S.; Blanco, C. A.; Caballero, I. (2022) Developments and characteristics of craft beer production processes, Food Bioscience, 45, 101495.

Wijekoon, M. M. J. O., Bhat, R., Karim, A. A. (2011). Effect of extraction solvents on the phenolic compounds and antioxidant activities of bunga kantan (Etlingera elatior Jack.) inflorescence. Journal of Food Composition and Analysis, 24(4-5), 615-619.

PUBLICATIONS

Articles

1. Daniele Bobrowski Rodrigues†, **Lavínia Veríssimo**†, Tiane Finimundy, Joana Rodrigues, Izamara Oliveira, João Gonçalves, Isabel P. Fernandes, Lillian Barros, Sandrina A. Heleno· Chemical and bioactive screening of green polyphenol-rich extracts from chestnut by-products: an approach to guide the sustainable production of high-added value ingredients. *Foods* 2023, 12, 2596. https://doi.org/10.3390/foods12132596

†These authors contributed equally to this work and share first authorship

2. Jonata M. Ueda, Karoline Ribeiro Griebler, Tiane C. Finimundy, Daniele B. Rodrigues, **Lavínia Veríssimo**, Tânia Pires, João Gonçalves, Eliana Pereira, Lillian Barros, Sandrina Heleno. Polyphenol composition by HPLC-DAD-(ESI-)MS/MS and bioactivities of extracts from grape agri-food wastes. *Molecules Molecules* 2023, 28(21), 7368; https://doi.org/10.3390/molecules28217368

foods

MDPI

Article

Chemical and Bioactive Screening of Green Polyphenol-Rich Extracts from Chestnut By-Products: An Approach to Guide the Sustainable Production of High-Added Value Ingredients

Daniele Bobrowski Rodrigues [1,2,†], Lavínia Veríssimo [1,2,†], Tiane Finimundy [1,2], Joana Rodrigues [1,2], Izamara Oliveira [1,2], João Gonçalves [3], Isabel P. Fernandes [4], Lillian Barros [1,2], Sandrina A. Heleno [1,2,*] and Ricardo C. Calhelha [1,2]

1 Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; daniele@ipb.pt (D.B.R.); lavinia.verissimo@gmail.com (L.V.); tiane@ipb.pt (T.F.); joanapbrodrigues@ipb.pt (J.R.); izamara@ipb.pt (I.O.); lillian@ipb.pt (L.B.); calhelha@ipb.pt (R.C.C.)
2 Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
3 Tree Flowers Solutions, Lda, Edifício Brigantia Ecopark, Av. Cidade de Léon, 5300-358 Bragança, Portugal; joaogoncalves@treeflowerssolutions.com (J.G.); isabelfernandes@treeflowerssolutions.com (I.P.F.)
* Correspondence: sheleno@ipb.pt
† These authors contributed equally to this work.

Abstract: Opportunities for the valorisation of agro-industrial residues of the chestnut (*Castanea sativa* Mill.) production chain have been fostered with the production of multifunctional polyphenol-rich extracts with the potential to be introduced as natural additives or active components in several products. Nonetheless, it is crucial to explore the feasibility of different extracts from the various by-products for these applications through the exhaustive study of their composition and bioactivities without losing sight of the sustainable character of the process. This work aimed at the screening of the phenolic compound composition and bioactivities of different green extracts of chestnut burs, shells and leaves, as the first step to establish their potential application as natural ingredients, primarily as food preservatives. To this end, maceration (MAC) as a conventional extraction method besides ultrasound and microwave-assisted extractions (UAE and MAE) was employed to obtain the extracts from chestnut by-products using water (W) and hydroethanolic solution (HE) as solvents. Phenolic compounds were analysed by HPLC-DAD-(ESI-)MS/MS; the antioxidant capacity was assessed by colourimetric assays, and the antimicrobial activity was evaluated against several strains of food-borne bacteria and fungi. The leaf extracts obtained by MAC-HE and UAE-HE presented the highest concentration of phenolic compounds (70.92 ± 2.72 and 53.97 ± 2.41 mg·g^{-1} extract dw, respectively), whereas, for burs and shells, the highest recovery of total phenolic compounds was achieved by using UAE-HE and UAE-W (36.87 ± 1.09 and 23.03 ± 0.26 mg·g^{-1} extract dw, respectively). Bis-HHDP-glucose isomers, chestanin and gallic acid were among the most abundant compounds. Bur extracts (MAC-HE and UAE-HE) generally presented the highest antioxidant capacity as measured by TBARS, while the best results in DPPH and reducing power assays were found for shell extracts (MAE-W and MAC-HE). Promising antibacterial activity was noticed for the aqueous extracts of burs, leaves and hydroethanolic extracts of shells, with emphasis on the MAE-W extract of burs that showed bactericidal activity against *E. cloacae*, *P. aeruginosa* and *S. aureus* (MBC 5 mg·mL^{-1}). Overall, it can be concluded that chestnut by-products, including burs, shells and leaves, are sources of polyphenolic compounds with significant antioxidant and antimicrobial activities. The choice of extraction method and solvent greatly influenced the composition and bioactivity of the extracts. These findings highlight the potential of chestnut by-products for the development of natural additives, particularly for food preservation, while also emphasizing the importance of sustainable utilization of agricultural waste materials. Further research is warranted to optimize extraction techniques and explore additional applications for these valuable bioactive compounds.

check for updates

Citation: Rodrigues, D.B.; Veríssimo, L.; Finimundy, T.; Rodrigues, J.; Oliveira, I.; Gonçalves, J.; Fernandes, I.P.; Barros, L.; Heleno, S.A.; Calhelha, R.C. Chemical and Bioactive Screening of Green Polyphenol-Rich Extracts from Chestnut By-Products: An Approach to Guide the Sustainable Production of High-Added Value Ingredients. *Foods* **2023**, *12*, 2596. https://doi.org/10.3390/foods12132596

Academic Editor: Olga Gortzi

Received: 3 June 2023
Revised: 21 June 2023
Accepted: 27 June 2023
Published: 4 July 2023

molecules

MDPI

Article

Polyphenol Composition by HPLC-DAD-(ESI-)MS/MS and Bioactivities of Extracts from Grape Agri-Food Wastes

Jonata M. Ueda [1,2,†], Karoline Ribeiro Griebler [1,2,†], Tiane C. Finimundy [1,2], Daniele B. Rodrigues [1,2], Lavínia Veríssimo [1,2], Tânia C. S. P. Pires [1,2], João Gonçalves [3], Isabel P. Fernandes [3], Eliana Pereira [1,2], Lillian Barros [1,2], Sandrina A. Heleno [1,2,*] and Ricardo C. Calhelha [1,2]

1 Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal; jonata.ueda@ipb.pt (J.M.U.); karolinegriebler@alunos.utfpr.edu.br (K.R.G.); tiane@ipb.pt (T.C.F.); daniele@ipb.pt (D.B.R.); lavinia.verissimo@gmail.com (L.V.); tania.pires@ipb.pt (T.C.S.P.P.); eliana@ipb.pt (E.P.); lillian@ipb.pt (L.B.); calhelha@ipb.pt (R.C.C.)
2 Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Alameda Santa Apolónia, 5300-253 Bragança, Portugal
3 Tree Flowers Solution, Lda, Edifício Brigantia Ecopark, Av. Cidade de Léon, 5300-358 Bragança, Portugal; joaogoncalves@treeflowersolutions.com (J.G.); isabelfernandes@treeflowersolutions.com (I.P.F.)
* Correspondence: sheleno@ipb.pt
† These authors contributed equally to this work.

Abstract: Background: Grape agri-food wastes, such as skin, seeds, and other discarded by-products, contain phytochemical compounds that offer potential health benefits. Methods: This study aimed to investigate the polyphenol composition and bioactivities of different extracts obtained from grape marc and seeds, with the goal of exploring their potential for application as natural food additives. Results: Regardless of the extraction method used (dynamic maceration, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE)), all extracts exhibited relatively high concentrations of phenolic compounds. The chemical characterization of the extracts revealed the presence of specific compounds and chemical groups associated with each extraction methodology. Moreover, the extracts displayed satisfactory antioxidant activities, especially in inhibiting lipoperoxidation as assessed by the TBARS assay. Additionally, the extracts demonstrated effective inhibition against different strains of bacteria and fungi known as food contaminants. Taken together, these findings indicate that those extracts have the potential to be tested as natural antioxidants and preservatives with sustainable origins in food and beverage systems. Among the extraction methods evaluated, traditional maceration and UAE provided extracts with the highest antioxidant and antimicrobial activities. Conclusions: Our results suggest the opportunity to explore grape marc and seeds discarded by the winery industry in Portugal as natural sources of bioactive compounds, which could be employed as functional food ingredients or technological additives. The valorization of grape biowastes offers a promising strategy to reduce waste and harness their potential health benefits.

Keywords: grape marc; by-products; phenolic compounds; antioxidant; antimicrobial

check for updates

Citation: Ueda, J.M.; Griebler, K.R.; Finimundy, T.C.; Rodrigues, D.B.; Veríssimo, L.; Pires, T.C.S.P.; Gonçalves, J.; Fernandes, I.P.; Pereira, E.; Barros, L.; et al. Polyphenol Composition by HPLC-DAD-(ESI-)MS/MS and Bioactivities of Extracts from Grape Agri-Food Wastes. *Molecules* **2023**, *28*, 7368. https://doi.org/10.3390/molecules28217368

Academic Editor: Lucia Panzella

Received: 31 August 2023
Revised: 24 October 2023
Accepted: 30 October 2023
Published: 31 October 2023

1. Introduction

Wine, produced mainly from *Vitis vinifera*, is one of the most valuable and consumed alcoholic beverages throughout the world, with an annual production of approximately 27 billion litres [1,2]. Europe is recognised for its high-quality wines, and in this scenario, Portugal stands out for its traditional and expressive production of wines with Protected Designation of Origin (PDO) and Geographical Indication (PGI) labels, highlighting the distinctive wine production across the 14 different regions of the country [3,4]. To exemplify it, in a recent competition in Portugal, over 1300 Portuguese wines were sensorially evaluated by 151 experts and most received scores ranging between 80 and 90 on a 0–100 scale, corroborating the level of quality and diversity of the wines produced [5].

Work presented in Conferences

1. **Lavínia Veríssimo**, Daniele Bobrowski Rodrigues, Tiane Finimundy, Izamara Oliveira, Regina Soares, João Gonçalves, Isabel P. Fernandes, Lillian Barros, Sandrina A. Heleno. Assessment of the potential of green phenolic extracts of a chestnut by-product to be used as a natural preservative. 2023. *International Congress on Sustainable Solutions for the Agrifood Industry*, Portugal.

2. **Lavínia Veríssimo**, Daniele Bobrowski Rodrigues, Tiane Finimundy, Regina Soares, Diana da Costa, Isabel P. Fernandes, Lillian Barros, Sandrina A. Heleno. Antioxidant potential of ultrasound-assisted extracts rich in phenolic compounds obtained from a chestnut biowaste. 2023. *International Congress on Sustainable Solutions for the Agrifood Industry*, Portugal.

3. Regina Soares, Daniele Bobrowski Rodrigues, **Lavínia Veríssimo**, Tiane Finimundy, Neivaldo Murrube, Isabel P. Fernandes, Miguel D. Gonçalves, Lillian Barros, Sandrina A. Heleno. Effect of chestnut flower extract on the flavour stability of craft beers. 2023. *International Seminar "ArtiSaneFood: Bio-preservation and Risk Modelling Approaches"*, Portugal.

4. Cristiano Mateus, Tiane Finimundy, Daniele Bobrowski Rodrigues, **Lavínia Veríssimo**, Carlos Shiraishi, Regina Soares, Jonata M. Ueda, Isabel P. Fernandes, Diana da Costa, Lillian Barros, Sandrina A. Heleno. Stability studies of chestnut flower-based ingredients: prospecting incorporation into beverages. 2023. *International Seminar "ArtiSaneFood: Bio- preservation and Risk Modelling Approaches"*, Portugal.

5. Cristiano Mateus, Tiane Finimundy, Daniele Bobrowski Rodrigues, **Lavínia Veríssimo**, Regina Soares, Carlos Shiraishi, Isabel P. Fernandes, Lillian Barros, Sandrina Heleno. Incorporating chestnut flower extract into flavoured water for functional enrichment: evaluating bioactive potential and stability. 2023. *EuroFoodChem Congress*, Serbia.

6. Daniele Bobrowski Rodrigues, Tiane Finimundy, **Lavínia Veríssimo**, Regina Soares, João Gonçalves, Isabel P. Fernandes, Lillian Barros, Sandrina A. Heleno Assessment of the *in vitro* bioaccessibility of polyphenols from chestnut flower extract: a step towards exploring its functional potential. 2023. *Food Bioactive & Health Conference*, Czech Republic.