



Upcycling of brewer's spent grain: development of a biorefinery approach

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Summary

The predicted increase in population, together with the development of industrial activities, is leading to a significant rise in agricultural and food-processing waste. The management of this waste is increasingly becoming challenging and alternatives for the disposal of these residues are needed to limit their accumulation. A promising strategy is to look at these wastes not anymore as a biomass to be discarded, but as a source of biomass to valorise. This concept reflects the vision of a circular economy, which is the main focus of the international programs for sustainable development. Among the different possible approaches to attain this goal, the extraction of valuable compounds from agri-food by-products and their re-integration in industrial processes is the most sought-after up-cycling strategy.

One of these agri-food by-products, of which the generation is foreseen to increase in the near future, is brewer's spent grain (BSG). This poorly valorised biomass is the first and most abundant by-product of brewing process and consists of the insoluble part of the grain. It is a low-cost material, that is mainly exploited as cattle feed or disposed of to landfills. BSG has an heterogenous chemical composition and is rich in bioactive compounds with potential health-promoting properties. Based on these premises, the overall objective of the current thesis is to develop a biorefinery approach whereby the valorisation of BSG is maximized by recovering different bioactive fractions in an extraction cascade. The development primarily targets laboratory-scale experiments, with an outlook towards pilot-scale extractions. The four extracts are characterized and compared to studies that reported the optimized extraction of each of the individual fractions. The valorisation markets, that are aimed at, are the food, pharmaceutical and cosmetic sectors.

In a first stage a detailed characterization of BSG extracts was performed by using the most common treatments applied on this biomass. A metabolomic profile was defined through the

identification of the main compounds detected in the extracts. Considering that the antioxidant capacity and the total phenolic content of BSG extracts are two main parameters investigated in BSG studies, these factors were used to evaluate and discriminate the quality of the extracts. Moreover, a detailed study on the most common stabilization pre-treatment (oven-drying) of BSG biomass was conducted. The comparison between extracts from oven-treated and untreated BSG showed that extracts from fresh BSG have a higher antioxidant capacity and total phenolic content than those from oven-treated biomass. These results indicate that stabilization of the biomass, an energy-dense treatment, which is considered essential in most studies, can and perhaps should be avoided. To maximize the use of the complex composition of BSG, a biorefinery model was developed, whereby four different fractions with, based on scientific knowledge, high-added value are produced. Finally, the up-scalability of this process was tested. It has proven to be challenging once the increasing volumes necessitate the application of a new set of equipment and approaches.

The data presented here provide a scientific base for the further development of a biorefinery approach on BSG. A new class of compounds, previously hypothesized to exist, was identified and the details of the effect of drying as pre-treatment have been uncovered. Although the effective bio-activities of the extracts need to be confirmed experimentally, four fractions with potentially healthy properties, which are of interest in different industrial sectors, were produced. The proposed biorefinery model provides a framework process for the up-cycling of BSG and could contribute to the development of a more sustainable and circular economy. Nevertheless, further optimisation is needed, and the industrial-scale feasibility and the economic value this process can generate need to be assessed.

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List of abbreviations

AA	Amino acids
AAPH	2,2'-Azobis(2-amidinopropane)
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AX	Arabinoxylans
BSG	Brewer's spent grains
BSH	Brewer's spent hops
BSY	Brewer's spent yeasts
CE	Circular economy
CV	Column volume
DGDG	Digalactosyldiacylglycerol
DGMG	Digalactosylmonoacylglycerol
DHDMT	2,4-dihydroxy-2,5- dimethyl-3(2H)-thiophenone
DiHODE	Dihydroxyoctadecadienoic acid
DiHOME	Dihydroxyoctadecenoic acid
ESI	Electrospray ionization source
EtOH	Ethanol
FRAP	Ferric reducing antioxidant power assay
FSC	Food supply chain
GABA	Gamma-aminobutyric acid
GAE	Gallic acid equivalent
GC	Gas-chromatography
GHG	Greenhouse gas
Glu/Gln	Glutamic acid/Glutamine
Gly	Glycine
GRAS	Generally recognise as safe
HBA	Hydroxy-benzoic acids
HCA	Hydroxy-cinnamic acids
HILIC	Hydrophilic-interaction liquid-chromatography
His	Histidine
HODE	Hydroxyoctadecadienoic acid
HPLC	High performance liquid-chromatography
HRMS	High-resolution – mass spectrometry

Ile/Leu	Isoleucine/Leucine
LC-MS	Liquid-chromatography - mass spectrometry
LRMS	Low-resolution - mass spectrometry
Lys	Lysine
m/z	Mass over charge
MALDI	Matrix Assisted Laser Desorption/Ionization
MeOH	Methanol
Met	Methionine
MG	Monoacylglycerol
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PC	Phosphatidylcholine
PC	Phosphatidylcholine
pCA	p-coumaric acid
PE	Phosphatidylethanolamine
Phe	Phenylalanine
Pro	Proline
QC	Quality control
RPLC	Reverse-phase liquid-chromatography
RT	Retention time
SRM	Selected Reaction Monitoring
TE	Trolox equivalent
Thr	Threonine
TOF	Time-of-flight
TPC	Total phenolic content
TriHODE	Trihydroxyoctadecadienoic acid
TriHOME	Trihydroxyoctadecenoic acid
Trp	Tryptophan
Tyr	Tyrosine
UHPLC	Ultra-high performance-chromatography
Val	Valine

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GENERAL INTRODUCTION

In the context of a population increase and the consequent higher demand for food and beverages, the agricultural and industrial sector must increase their production. A direct consequence of this increase is the higher generation and accumulation of agri-food waste (Kumar et al. 2022). The disposal of these residues is becoming increasingly challenging for the society and new alternatives for the management of these residual biomasses are needed (Mostaghimi and Behnamian 2023). In addition, society is more and more sensitive to environmental protection, demanding to match economic development with increased sustainability. These demands can be matched by changing the linearity of economic production, from commodity to product to waste, into a circular model wherein waste from one activity is the commodity of another. Two of the challenges that such circular model has to face are reducing the generation of residues by limiting the avoidable waste, and re- or upcycling of the inevitable waste (Chen et al., 2020).

Brewer's spent grain (BSG) is an abundant by-product generated during the brewing of beer. As BSG is the most abundant residue brewing and since there is a projected higher production of beer, the amount of BSG is likewise expected to increase (Agrawal et al. 2023). BSG is mainly used as cattle feed and for biogas production, nonetheless about 20% is currently disposed of in landfills (Rachwał et al. 2020). BSG as such has a low commercial value and with the current ineffective management practises is considered an economic loss for the brewing sector (Buffington 2014). However, this residue contains many and diverse components giving BSG a broad valorisation potential (Bonifácio-Lopes et al. 2020). Together with its non-seasonality and its predictable production in amount and time, this biomass provides many possibilities for the development of valorisation plans (Jackowski, Niedzwiecki, and Trusek 2020).

Recently, several applications are being investigated for the upcycling of BSG in various industrial sectors (Jackowski et al. 2020; Mussatto 2009). The most frequently studied applications focus on using health-beneficial properties of compounds that can be extracted from BSG for applications in food, pharmaceuticals, cosmetics and biotechnological processes (Jackowski et al. 2020; Mussatto 2009; Rachwał et al. 2020). Therefore, the extraction of brewer's spent grain compounds represents a promising approach for the re-/upcycling of this residue to commodities thereby contributing to the development of a circular economic model.

Given the complex composition of BSG, various extraction techniques have been applied for the recovery of the different classes of compounds present in this biomass mainly targeting phenolic compounds, proteins and arabinoxylans (Hellebois et al. 2021; Lynch, Steffen, and Arendt 2016; Rachwał et al. 2020). Most of these studies focus on the recovery of one specific class of compounds and the study of the biological activities of the extracts. While this provides a knowledge-base on the possible applications for these extracts, this approach leaves most of the compounds present in BSG un-used (Zeko-Pivač et al. 2022). An emerging strategy, that aims at collecting more than one group of compounds from BSG and other residual biomasses, is the application of a biorefinery approach. Till recently biorefineries *de facto* fractionated lignocellulosic materials with a focus on their conversion into bioenergy (Mohanty et al. 2021; Yaashikaa, Senthil Kumar, and Varjani 2022). Today, the concept and the scope of biorefinery has expanded and the recovery of other high-added value products with applications in different industrial sectors is seen as a component in the economic balance of biorefining (Sarangi et al. 2023).

There are numerous studies on the extraction of valuable compounds from BSG both using traditional and more innovative extraction methods (Jha and Sit 2022), however little work has been done surpassing the optimisation on laboratory scale. Although the introduction of new extraction approaches resulted in an increased

yield with an increased sustainability, technical, economic and practical issues make that traditional extraction methods such as Soxhlet, maceration and hydro-distillation are still the workhorse for industrial scale extractions from biomass (Escobar-Avello et al. 2021; Gori et al. 2021). Therefore, despite all the limitations in terms of environmental and economic impact, the most applied extraction technique is maceration, considered the easiest and most cost-effective at industrial level (Daud et al. 2022), an approach that was used in this work.

The observation that BSG is currently under-valorised, the limited amount of studies on the development of an extraction cascade from BSG and the lack of knowledge on the up-scaling of this biomass are at the basis of the project of which the results are described in this thesis. Based on the hypothesis that BSG is a low-cost biomass, which is currently poorly valorised, although rich in valuable components, the main objective of my work was the development of a biorefinery approach to maximize its valorisation. This has been attained by the generation multiple high-added value outputs that can be applied in the food, pharmaceutical and cosmetic sectors. To achieve this goal, the balance between feasibility and maximum extraction yields with the available equipment was a constant factor considered for the selection of the applied approach. While there is more research needed, mainly to confirm the specific bio-activities of the generated fractions, that were attributed to them based on previous studies, this thesis represents some steps towards the economic viable upcycling of BSG. Furthermore, experience gained and data presented here can contribute to the valorisation of other biomasses.

Thesis outline

This manuscript is divided into six chapters. The first chapter provides an introduction of the work by describing the context of increasing societal demands for sustainability and the tension this causes with economic development. A description of the brewing process, the biomass residues generated in this process and the different ways

BSG, as main residue that is produced, is currently valorised is followed by a state-of-the-art on technical aspects of the current work. In chapter two an overview of the objectives of this work is presented.

Since the overall objective of this project is to increase the value of BSG, the chapters three to five described the results of the experimental steps undertaken towards the realisation of this aim. Chapter three focusses on the first extraction in the extraction cascade and is divided in three parts related to three outputs that are generated. After the description of the metabolomic of brewer's spent grain extracts, resulting in the identification of a new type of Amadori products, the impact of BSG pretreatment on the extracts was determined and preliminary data on the generation of a barley-specific extract described.

In chapter four a sequential extraction cascade is developed, a biorefinery approach, that results in the recovery of more than one valorizable fractions within the application of one process. As a first step towards industrial application and to provide initial data on the up-scaling of extractions from BSG, results from milligram to kilogram extractions are described in chapter five. This manuscript concludes with a general discussion and conclusion, in chapter six an overview of all the results, the achieved advances in the research context investigated, and the future perspectives in research and industrial applications were highlighted.

CHAPTER 1.

LITERATURE REVIEW

1 Overview

1.1 Current socio-environmental framework and emerging difficulties

During the last decades, the world population has remarkably risen reaching approximately 8 billion of people nowadays. This number is foreseen to increase up to 10 billion by 2050, and, therefore, this global growth is leading to a higher world food demand (FAO, 2023). To cope with this, the agricultural and industrial sectors must increase their production, causing an increase in the generation of waste that needs to be disposed. Furthermore, the consequent intensification of agricultural practices and the increase of industry-related activities are the principal reasons of land degradation, soil erosion, fertility and biodiversity reductions and the increment of greenhouse gas (GHG) emissions (Francaviglia et al., 2023). All these factors, already considered in the Sustainable Development Goals (SDGs) adopted by the United Nations in 2015 (www.undp.org/united-states/sustainable-development-goals), are currently attracting more and more attention of each individual, and they are becoming part of the major societal concerns.

A clear link, describing the relation between the growth of the population and the higher food waste production, is explained by the food supply chain (FSC) (Sinha & Tripathi, 2021). From the farmers, involved in the generation of raw products, through the industries, committed in food processing and packaging, to retail and purchasing, the entire society is responsible and contributes to this chain. However, beside the foreseen rise in the global population, 1/3 of the

food produced for human consumption, corresponding to 1.3-1.4 billion of tons, is lost as waste. Spurred by the predicted population growth, the situation is estimated to get worse with an increase to 2.6 billion of tons food wasted in 2025 (V. Kumar et al., 2022). There are many different causes of food losses and waste generation. Among them, poor economic interest, lower quality of the biomass and raw products, transport damage, bad storages, contamination, incorrect packaging or unsuitable purchasing in quantity and quality, are considered the most important (Sinha & Tripathi, 2021).

1.2 *New challenges for organic waste management*



Figure 1.2-1 Circular economy model.

To build an holistic response to the global challenge of food waste, there are mainly two strategies applicable, one is related to the development of innovative, efficient and sustainable plans to limit all these losses and waste, and the other one deals with the valorisation of this waste (Chen et al., 2020). In this context, researchers must be part of this mission and work “to create more resilient food systems” (C. Chen et al., 2020). This concept is currently being considered within the European Commission, which is actively analysing ways to reduce the environmental, health and social impacts, and set more sustainable and effective approaches to limit food waste and thus increase the resilience of our food system (Amicarelli et al., 2021). A concrete

example of a more constructive approach is shown in the “farm to fork” strategy aiming to develop an effective traceability along the FSC (Hassoun et al., 2023).

A good tracking procedure is part of the first strategy mentioned above, and it involves mainly the management and control within each stage of the FSC. This plan aims to monitor the preventable food waste (Morone et al., 2019), while the second strategy focuses mainly on the exploitation of the non-preventable residues, whose disposal has become an equally crucial issue (Belaud et al., 2019; Morone et al., 2019).

Looking at the near future, disposal of the increasing amount of food waste will be more problematic. From an economic perspective, the price of landfilling is estimated to rise because of the reduction of the physical area used for that, and in parallel, the relative transportation costs will increase as well. On the environmental side, waste disposal is risky due to the augmentation of GHG emissions with the consequent intensification of pollution. Moreover, the higher accumulation of waste in landfills will lead to an higher microbial decomposition and leachate production, thus modifying the ecosystem balance (Y. Chen et al., 2023; Nath et al., 2023).

While the current linear model of production, consumption and waste disposal creates a unidirectional path leading to a continuous generation and accumulation of residues, the desirable model for the future consists in a loop, wherein the last stage of the FSC represents the end of one cycle and the beginning of a new economic cycle. This vision is conceptualized as Circular Economy (CE) (Figure 1.2-1), nowadays considered as one of the main global objectives. A complete definition of CE is coined by Figge and co-workers: “The circular economy is a multi-level resource use system that stipulates the complete closure of all resource loops. Recycling and other means that optimise the scale and direction of resource flows, contribute to the circular economy as supporting practices and activities. In its conceptual perfect form, all resource loops will be fully closed. In its

realistic imperfect form, some use of virgin resources is inevitable” (Figge et al., 2023). In this frame, the main focus of CE reflects the strategy previously reported (Galanakis, 2022). In the book "Food waste recovery: Processing technologies and Industrial techniques" C.M. Galanakis promotes a waste-to-resource approach leading to a “win-win” condition (C. Chen et al., 2020). Within the European Union, the directive 2008/98/EC already promoted the concept of re-use and recycling instead of disposing of waste. It describes a waste management hierarchy where disposal is at the bottom, considered the very last option for waste treatment, and re-use and recycling are considered the best options just after the prevention of waste production (data.europa.eu/eli/dir/2008/98/2018-07-05). Looking at by-products as new resources and including them into production processes, may lead to a direct reduction of their accumulation. Furthermore, their integration in the industrial cycle and conversion into new products represent a earning opportunity rather than an expense for their disposal (Rashwan et al., 2023).

In the development of the above mentioned circular model, recycling thus assumes a crucial role. However, this concept can be applied through two main approaches: downcycling and upcycling. Differentiating these two concepts is the key to maximise waste exploitation. Downcycling refers to convert “something in such a way that the resulting product is of lower value than the original item” (Campbell-Johnston et al., 2020) and often leads to the degradation of the products and their functional properties. For example, energy and biogas production is deemed as a downcycling process, in particular if used for producing fuel. This process completely degrades the original material. In contrast, upcycling is defined as “reuse of discarded materials which results in an increase in ‘value’” (Aschemann-Witzel et al., 2023) and “it can be purely artistic, scientific, or anything simply useful” (Campbell-Johnston et al., 2020). For instance, considering the organic waste from food processing, up-cycling involves converting this waste into new food products or ingredients that can be reintegrated into various industrial sectors, thus creating a new source of value (Aschemann-Witzel et al., 2023). However, both

approaches aid to reduce the accumulation of waste and to address the economic and environmental challenges associated with waste management. Therefore, selecting the appropriate approach to apply must consider the overall benefits in terms of cost and value gained (Campbell-Johnston et al., 2020).

1.3 New alternative approaches and applications of organic waste

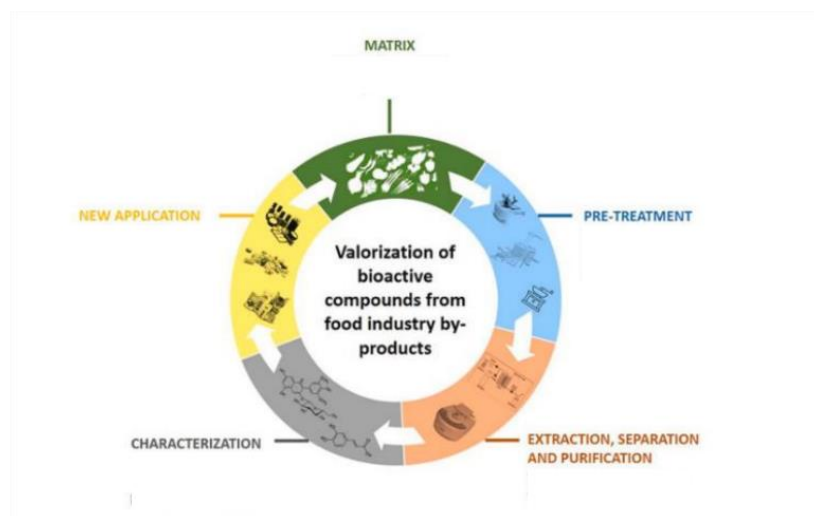


Figure 1.3-1 Description of a circular model of food industry-by product valorisation through the extraction and isolation of compounds, and the further re-integration of these in the manufacturing sector (Gil-Martín et al., 2022)

In the near future, the global waste generation (including all types of waste such as electronic, plastic, radioactive, municipal and food waste) is predicted to rise and its management, that is already a challenge, will become a more crucial issue (Mostaghimi & Behnamian, 2023). In particular, the incorrect handling and disposal of bio-waste, such as crop residues, municipal solid green waste, animal and plant waste, and effluents, sewage sludge, wastewaters, waste food items or left-over food (Lahiri et al., 2023; Srivastava et al., 2023) from municipal, agricultural, and industrial sectors can severely impact the environmental and economic preservation and quality. According to the composition, origin, use and conditions of

these residues, different recycling approaches can be considered. One of these approaches targets the recovery of the remarkable amount of nutrients and bioactive compounds still contained in residual biomass. These residues can thus be recycled into high-added value ingredients or products for human consumption (Srivastava et al., 2023). Conversely, certain bio-waste such as animal and plant effluents, more likely containing pathogens, cannot be exploited for human nutrition or health. However, being rich in minerals and other micronutrients, these wastes can be re-integrated in the production chain as biosolids or biofertilizers (Kaur et al., 2018). Furthermore, for the other agriculture biomasses, which are poor in bioactive compounds, or whose valorisation is not economically advantageous, other alternative approaches are designed. For example the production of green energy, biogas by microbial anaerobic activity, biofuel, biopolymers and biochemicals can be envisaged (Sarangi et al., 2023). Similarly, residues from the processing of dairy can be converted by microbial activity into glycerol, ethanol, or directly used as substrate for biosurfactant production (Usmani et al., 2022). Recently, research on the acoustic, thermal or mechanical properties of various plant food residues (banana bunch, hemp, sunflower, maize, rapeseed, rice straw or pineapple and palm leaves, ...) opened other valorisation possibilities in the construction sector (Badouard et al., 2021).

An innovative concept, that is currently attracting the societal interest, is the development of the sustainable biorefinery concept. This might represent a valid, holistic alternative to bio-waste disposal aiming to maximise the exploitation of organic by-products according to their potential applications and their economical outcomes (Figure 1.3-1) (Sarangi et al., 2023). One promising application of the biorefinery approach is the recovery of the potential bioactive compounds present in those biomasses by sequential extractions. The possible multiple outputs resulting from the application of a biorefinery approach lead to a further enhancement of the recycling of these bio-waste. From one process, different rich valuable fractions can be recovered and converted then into high-added value products. These by-products involve mainly waste derived from fruits, vegetables (including

legumes, seeds, and cereals) and animals (dairy, meat, and fish) both in their raw and processed forms. The products from animal origin, that are considered as having a high valorisation potential, are for instance protein and lipid hydrolysates, minerals (magnesium, potassium, sodium and chloride) from animal-derived waste (for example bones, skins and shells) (Rashwan et al., 2023), collagen from bovine stomach tissue (Udhayakumar et al., 2017) or keratin from pig hair (Cassoni et al., 2018; Sarangi et al., 2023). Alternatives to produce bulk products from vegetal waste are for instance the use of olive pomace, kernels of dates and grape seeds as source of oils because of their high content in lipids (Rashwan et al., 2023). As well, for example apple pomace and banana peels can be used for vinegar production (Guiné et al., 2021; Prisacaru et al., 2021).

The most attractive recycling method for plant and fruit by-products is the extraction and isolation of bioactive constituents. Indeed, plant by-products often contain high-quality compounds such as phenolics, saponins, carotenoids, fibers, lipids, and proteins, which might be used in their purified form or as a feedstock for further biotechnological processes. Phenolics and pectins for example can be extracted from sugar beet pulp (Alexandri et al., 2019). Other vegetables, such as tomato, pumpkin, carrot and pepper, are used for instance as source of carotenoids (Saini et al., 2019). A by-product that is largely used as a feedstock for biotechnological processes is sugarcane bagasse. This biomass can be exploited as a medium for the microbial production of enzymes, such as inulinases, alpha-amylases, pectinases, proteases, cellulases, lipases as well as organic acids like citric, succinic and butyric acids (Bustamante et al., 2020). The residues from the cultivation and manufacturing of cereals are also highly generated by-products. With a production of 2,8 billion of tons in 2023, cereals are one of the main crops cultivated (FAO, 2023), and about 30% of the total production is estimated to be lost and wasted during the FSC (Fărcaș et al., 2022). These by-products are rich in polysaccharides, fibers, and phenolic compounds. Wheat bran, rice husk, brewer's spent grain and corn processing waste are investigated to be used as medium for the *in-vitro* production of enzymes (Sarangi et al., 2023).

Likewise, rye is attracting interest because of its protein, phenolics and fatty acids content, as well as brewer's spent grain for its high content in fibers, proteins, unsaturated lipids and phenolics (Fărcaș et al., 2022).

1.4 High-value products and related bioactivities for the food sector

Among the wide array of bioactive phytochemicals that can be extracted from biomass residues, the most studied classes are polyphenols, fibers, lipids, and proteins. The main reason is that they provide activities that can be used across a wide range of applications and have the potential of creating a high-added value.

Polyphenols represent a group of intensively studied compounds mainly because of their antioxidant activity. Many of these compounds have also shown anti-inflammatory, anti-microbial and anti-diabetic properties (Montenegro-Landívar et al., 2021; Y. Zeng et al., 2023). According to epidemiological studies, a diet rich in polyphenols has a positive impact against the development of diseases involving oxidative stress, such as cancers, cardiovascular and neurodegenerative problems, and metabolic disorders (Sorrenti et al., 2023). Phenolic compounds are characterised by one or more phenol groups and are classified into 5 families based on their chemical structure: phenolic acids, flavonoids, stilbenes, lignans and tannins.

The first two families, phenolic acids and flavonoids, are the main phenolics present in the human diet. Phenolic acids are divided into two subcategories, hydroxy-benzoic acids (HBA) and hydroxycinnamic acids (HCA), and have the simplest structure of all phenolics. Flavonoids, a diverse group of molecules with a more complex structure, are sub-grouped into 6 classes according to the oxidation degree and substitutions on the base chemical structure: flavanols, flavones, flavanones, flavanols, isoflavones and anthocyanidins (Mir-Cerdà, Nuñez, et al., 2023; Rosales & Fabi, 2023).

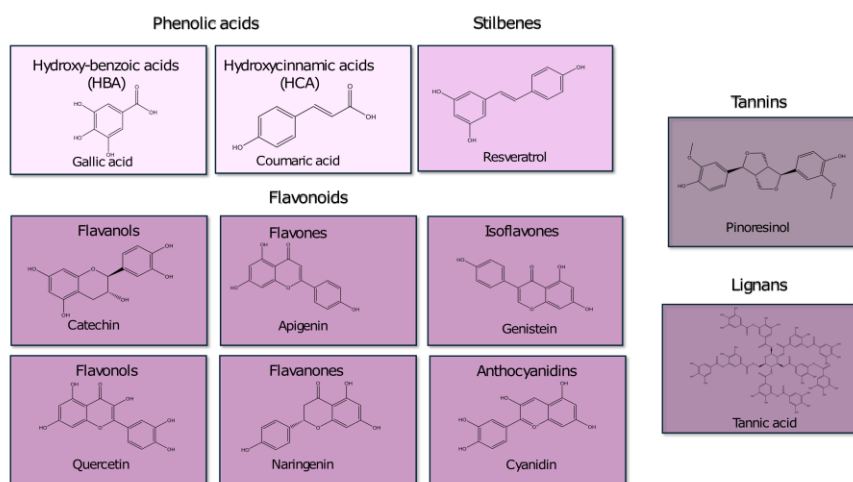


Figure 1.4-1 Main classes of phenolic compounds

The main agri-food residues explored for their content in phenolic compounds originate from the wine and olive oil industries. Grape pomace is rich in caftaric acid and astilbin (Mir-Cerdà, Carretero, et al., 2023), and the olive press cake after oil extraction contains mainly luteolin glycosides, oleuropein and hydroxytyrosol (Mir-Cerdà, Nuñez, et al., 2023; Tapia-Quirós et al., 2022). While valorisation of biomass residues from these two industries is well-developed, high concentrations in polyphenols have also been found in the residues from popular vegetables and fruits (Y. Zeng et al., 2023). Cyanidin (from grape seed, blueberry, cranberry pomace, carrot waste and apple peel), catechin/epicatechin (from citrus peel, grape skin and spent tea leaves), quercetin (from tomato, grape skin and apple pomace), resveratrol (from peanuts and grape residues), hesperidin (from orange peels) and ferulic acid (from citrus and potato peel, sugar beet pulp) are considered phenolics that can be extracted and valorised from plant residues (Liu et al., 2023).

Fibers represent another group of compounds with health-promoting properties that are abundant in fruit and vegetable residues. Plants are rich in fibers and accumulate them mainly in the cell walls to maintain the cellular structure (Timm et al., 2023). Fibers are classified according to their solubility. Water-soluble fibers include pectins

(found in fruits, vegetables and legumes), gums (in plant seeds, exudates, tubers (Mirhosseini & Amid, 2012)) and mucilage (in linseeds (Chand et al., 2024), aquatic plants and *Cactaceae* (Hussain et al., 2020)). Insoluble fibers include cellulose and hemicelluloses (Zannini et al., 2022) which are predominantly abundant in cereals (Hussain et al., 2020), as well as lignin, which is a polyphenol (Plakantonaki et al., 2023).

Fibers are considered as dietary fibers when they are present in plant-based foods, are not digested and adsorbed in the upper part of the human intestine (He et al., 2021) and are at least partially fermented in the large bowel (He et al., 2021). In the past, only polysaccharide chains with a polymerisation degree of more than 10 were considered dietary fibers. Recently, however, fibers with shorter chains, i.e. a polymerisation degree from 3 to 9, were shown to have prebiotic activities and are thus now considered dietary fibers as well. Cereal by-products contain significant amounts of dietary fibers. For example, the dietary fiber content found in maize bran accounts for 87% on a dry matter basis (Plakantonaki et al., 2023) and high amounts are found in brewer's spent grains (Olivares-Galván et al., 2022) and in rice seeds (Plakantonaki et al., 2023). Other rich sources of fibers are fruit residues such as apple, peach and grape pomace and in vegetable by-products such as tomato, onion and potato pulp (Plakantonaki et al., 2023).

Currently, there is an increasing interest in the health promoting properties of fibers. Beta-glucans, polysaccharides of β -(1,3) or β -(1,4) linked glucose subunits with β (1,6) branches, and pectins, polysaccharides composed of galacturonic acid units bound together by α -(1-4) glycosidic linkage, are involved in the maintenance of blood cholesterol levels (European Commission, 2012), and arabinoxylans are known to contribute to intestinal protection (Weng et al., 2023). The pre-biotic activities and the potential effect of lowering cholesterol levels, reducing cardiovascular diseases (McRae, 2017), obesity (Tucker & Thomas, 2009) as well as decreasing type 2 diabetes (Mazhar et al., 2023), cancer development (Turati et al.,

2023) and gastrointestinal disorders make dietary fibers sought-after phytochemicals that can be valorised in the food and pharmaceutical sectors (Hussain et al., 2020; Zannini et al., 2022).

The lipids are another group of molecules attracting the attention of the scientific community. Lipids represent a wide group of compounds, not only containing hydrophobic compounds but also numerous amphiphilic molecules. This feature, explained by the presence of both hydrophobic and hydrophilic groups in their chemical structure, allows lipids to have many different interactions and functions in the organism. Lipids play crucial roles in human metabolism, and their unbalances are involved in the development of inflammation, cancer, obesity, diabetes and other diseases related to cardiovascular and neurodegenerative problems (Leuti et al., 2020).

For the brewer's spent grain, the biomass investigated in this thesis, the most detected lipids are polyunsaturated fatty acids (PUFAs) and phospholipids. Although in the past, the consumption of lipids was not considered healthy, it is now known that the intake of sufficient lipids with a balanced fatty acid composition has a positive impact on human health (Vázquez et al., 2019). Phospholipids, the main constituents of eukaryotic cell membranes, contribute largely to cellular activities and are highly involved in human metabolic processes. For example, phosphatidylcholines (PC), one of the phospholipid subclasses, play a role in human fat tissue lipolysis and contribute to the intestinal barrier function and gastric protection (Park et al., 2021).

The active participation of lipids in human metabolism indicates that they have bioactive properties applicable in the medical, cosmetic and food sectors (Park et al., 2021). The food industry is currently putting a growing interest on these molecules, mainly for the development of functional foods and nutraceuticals (Akonjuen & Aryee, 2023). The main sources of lipids currently used in nutraceuticals are plants and animals. For the first category, lipids are largely found in seeds, for example from flax and chia plants; in beans such as soybean; in grains

from cereals and in algae. Animal lipids used in nutraceuticals mainly originate from fish, milk and dairy products (Akonjuen & Aryee, 2023; Bernal et al., 2011; Morrison & Coventry, 1985; Wang et al., 2021).

Human consumption in the Western world is currently shifting in the direction of plant-derived rather than animal-based food products. This and the will to become more independent from imported protein (the European protein strategy (www.europarl.europa.eu/RegData/etudes/BRIE/2023/751426)), make that the European demand for protein-rich plant-based food ingredients is rising. Many plant products, for instance canola, flax and hemp seeds, cereals, and legumes (peas, chickpeas, lentils, fava beans, ...) are interesting and their cultivation are increasing. However, proteins are also present in agri-food residual biomass streams, still owing remarkable nutritional values (Liu et al., 2023).

The main industries involved in the generation of protein-rich residues are the dairy, meat and seafood industry for animal protein sources, and soy, cereal and oilseed processing (Peydayesh et al., 2023; Zhou et al., 2023) for the plant-based protein sources. For example, the pulp of soybean (also known as okara) contains 20 to 39% of protein on a dry matter basis (Peydayesh et al., 2023). Bran of wheat contains about 15% of protein with a higher lysine and arginine content compared to the endosperm of wheat, while the protein content of brewer's spent grain ranges from 10 to 26% on a dry matter basis (Zhou et al., 2023). Furthermore, apart from providing protein intake, plant-based proteins have been investigated for re-integration in the food industry for their emulsifying, gelation, and textural potential, properties determined by their size, shape, chemical structure and composition (Du et al., 2020). In addition, due to the importance of protein intake in human metabolism, they are also studied for their beneficial impact on human diseases and disorders.

Proteins from soybeans exhibit activities in controlling metabolic disorders as diabetes, hyperlipidaemia and obesity. Wheat, rice,

rapeseed and sunflower protein isolates are exploited for food fortification and, rice isolates, being gluten-free, are used as an alternative for reducing intolerance to diet products (Peydayesh et al., 2023). Another interesting aspect of plant-based protein is that they represent an important source of bioactive peptides (Liu et al., 2023). The development of nutraceuticals based on these peptides is increasing, especially for those who have special dietary needs, such as babies, athletes and elderly (Singh et al., 2023).

2 Brewing process and by-products

2.1 Brewing process

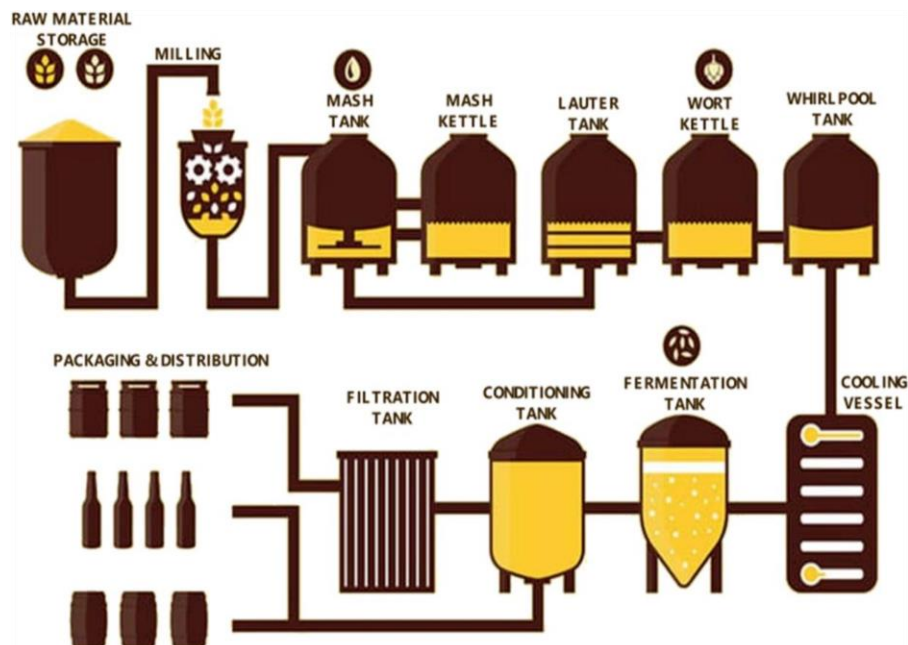


Figure 2.1-1 Brewing process (Mainardis et al., 2024)

To find innovative approaches for the development of a more sustainable future, the economic, social, and environmental sectors must be considered as one unit. On the industrial point of view, the agri-food sector is deeply involved in this global challenge. This includes all steps from agricultural production, food processing to the management of waste generated during these activities. With beer

being the most consumed alcoholic beverage in the world, the brewery industry contributes to the generation of residues. The global beer production accounted for 1.89 billion hectoliters in 2022 (www-statista-com.proxy.bnl.lu/statistics/270275/worldwide-beerproduction) with China as the largest producer with 360 million hectoliters, followed by the United States and Brazil. Germany is the first European producer, with 87.8 million hectoliters produced (www-statista-com.proxy.bnl.lu/statistics/270269/leading10countriesinworldwide-beer-production/).

To have a more concrete view on how brewing results in the generation of agri-food by-products, a short overview of the brewing process and its residues is given. The most common brewing process is composed of four steps: malting, mashing, wort boiling and fermentation, and it involves four principal ingredients: grains, water, hops, and yeasts. During the first step, grains are washed and steeped in water in order to start germination. Throughout this stage, the grains undergo an incomplete germination, in which hydrolytic enzymes are progressively released from the aleurone and degrade the cell walls, exposing the starch granules and solubilizing the proteins (Yin Tan et al., 2023). Known as malting, this step is defined as a controlled germination that guarantees specific physical and biochemical modifications within the grains. Afterwards, during kilning, grains are dried with hot air to stabilize the grains and stop the germination process. Therefore, three main actions are necessary to obtain a correct malting procedure: firstly, soaking grains with water for 24-48 h to reach a moisture content of about 42-48% within the grain; secondly, starting the germination, during which the temperature should be between 15 and 22°C; and finally, kilning which can occur at temperatures between 80 and 220°C (Habschied et al., 2023) to stabilize the final grains and prepare them for the storage. This latter step contributes also to the development of sensory properties (flavours and colours) of the beer (Gupta et al., 2010).

For the second main step of the brewing process, malted grains are ground to crush the endosperm and mixed with water, gradually

increasing the temperature from 37 to 78°C. During heating, enzymatic activities that convert complex polymeric molecules, mainly starch and proteins, into oligo- and monomers are enhanced. Two essential enzymes, alfa and beta-amylases, are released and start breaking down amylopectin and amylose into simpler sugars (mainly glucose and maltose), which are required for the subsequent yeast fermentation. When the temperature reaches about 78°C the enzymes are denatured and their activities suppressed (Yin Tan et al., 2023). Subsequently, the resulting mixture is filtered (lautering step): the solid phase, known as spent grains (BSG), is discarded (first by-product from brewing), while the filtrate, called wort, is used for the production of beer.

The third main step consists in hop addition to the wort and boiling of the resulting mixture. The addition of hops plays a crucial role in the quality of the product. Hops are involved in the development of the bitter flavour largely due to the release of hop acids into the wort (Oladokun et al., 2017), as well as in the clarification and preservation of the wort, and in the stabilization of the foam (Kao, 2018). After boiling, the mixture is filtered again and the solid phase “spent hops” (BSH) is discarded (second by-product from brewing). Then, the liquid is cooled to temperatures ranging between 12 and 18°C and yeasts are added for the fermentation step (Jackowski, Niedzwiecki, et al., 2020).

For the fermentation stage, different yeasts can be used according to the sought-after flavour and aroma of the beer (Salaňă et al., 2023). Yeasts convert the fermentable sugars into ethanol and CO₂. The solution progressively changes colour to transparent amber and when the fermentation is over, the solid residues are filtered from the liquid. These residues, consisting in spent yeasts (BSY) and other insoluble materials, are discarded (third by-product from brewing) and the beer is ready to be bottled (Jackowski, Niedzwiecki, et al., 2020; Kao, 2018).

2.2 Brewing by-products



Figure 2.2-1 Brewing by-products generated during brewing: 1) brewer's spent grain (BSG); 2) brewer's spent hops (BSH) and 3) brewer's spent yeasts (BSY).

The higher demand and consumption of beer in the last decade led to a significant increase of beer production, and consequently of the waste generated. This has become a crucial challenge as regards the management of its disposal, since it is demanding not only on the economic side, but also on the ecological side (Rachwał et al., 2020). The three main organic residues generated from the brewery industry are spent grains, spent hops and spent yeasts (Figure 2.2-1: 1, 2, 3, respectively). Due to their content in bioactive compounds, these by-products can be recycled and used as sources for other applications instead of being disposed of. The chemical composition of these residues can vary depending on the varieties of grains, hops and yeasts used, as well as the different conditions applied during the brewing process. However, despite those differences, all these residues have a high content in health-promoting compounds (Jackowski, Niedzwiecki, et al., 2020; Mussatto, 2009; Rachwał et al., 2020).

BSG is the most abundant by-product from brewing, accounting for 85% of the total residues. It is estimated that for every 100 L of beer produced, 20 kg of BSG is generated (Agrawal et al., 2023). The high amount of this biomass, the costs for its stabilization and storage, and the low economic value make that this residue is mainly used as animal feed or otherwise sent to landfills (Rachwał et al., 2020). BSG is a lignocellulosic by-product consisting of the husks, pericarps, coats and residual endosperm (Codina-torrella & Roderó, 2021). Generally, it has a moisture content of 75-80% and its dry biomass is globally composed of 15-30% proteins, 20-70% fibers, 6-10% lipids and 0.5-2% phenolics. Furthermore, it also contains vitamins and minerals. Its

detailed chemical composition and potential re-uses and applications will be discussed more in detail in the following paragraphs.

The second by-product from beer production, filtered out after the wort boiling step, mainly consists of spent hops. The amount of hops used in the brewing process corresponds to 0.2-0.4% of the total wort, and for every hectolitre of beer produced, approximately 300 g of BSH are generated (Olivares-Galván et al., 2022). The principal components of BSH are proteins and fibers (Salaňă et al., 2023), this latter mainly composed of xylose and glucose, and in lower amount, of mannose and galactose (Kao, 2018). The most prevalent amino acids are leucine, lysine, valine, alanine, serine, glycine, tyrosine, and proline. Hops are also used as a source of phenolics, of which catechin, kaempferol, quercetin and proanthocyanidin C2 are the most prevalent (Olivares-Galván et al., 2022). BSH furthermore contains lipids, essential oils and minerals. Due to the presence of essential oils, the valorisation of this by-product is currently attracting more and more attention (Karlović et al., 2020). Since it is rich in nitrogen, BSH is used in agriculture as a soil fertilizer and compost. In addition, for the presence of organic acids, extracts from BSH are applied as an insect repellent and antibacterial agent (Salaňă et al., 2023). In contrast to the other two by-products generated during brewing, the bitter taste of BSH limits its use for animal feeding. However, this residue has been studied for medical and dietary applications as a nutraceutical or supplement with anticoagulant and antioxidant activity (Luzak et al., 2016). In addition, in the food sector, BSH has also been investigated for the production of food preservation films (Salaňă et al., 2023).

At the last stage of brewery process, the third by-product, BSY, is removed from the production chain, although it can be re-incorporated in the brewing process and re-used to produce beer up to 6 times (Rachwał et al., 2020). In terms of biomass quantity, it is the second by-product, corresponding to 10-15% of the total residue of the brewing process. Generally, the composition and amount of BSY vary according to the microorganism and inoculum concentration used in

the fermentation, as well as the wort composition and fermentation parameters (Kao, 2018). Besides the moisture content accounting for 85-90% of the weight, proteins and saccharides are highly present in BSY. The most abundant amino acids in BSY proteins are glutamic acid, histidine, alanine, and aspartic acid, which are known as flavour enhancer molecules (Olivares-Galván et al., 2022). This by-product is used to produce a bread spread and its typical taste of marmite is actually due to the presence of these amino acids (Kerby & Vriesekoop, 2017). Beta-glucans are the most important polysaccharides present in BSY, specifically located in the cell walls. These compounds contribute to the potential health benefits of BSY, due to their positive effects on human physiology given their immune-stimulant, anti-inflammatory, antimicrobial, and anti-tumoral properties, as well as their involvement in reducing cholesterol levels and in maintaining the gut microbiota balance (Tian et al., 2019).

3 Brewer's spent grain (BSG)



Figure 2.2-1 brewer's spent grain (BSG)

3.1 BSG composition and characterisation

BSG consists of the insoluble part of the grain including pericarp, husk and seed coat. The bulk of BSG consists in fibers, which range from 20 to 70% of the BSG dry weight basis (Olivares-Galván et al., 2022). This wide range is illustrative of the different factors that determine the composition of BSG (Agrawal et al., 2023; Almeida et al., 2017; Bonifácio-Lopes et al., 2020a; Jackowski, Łukasz, et al., 2020; Rachwał et al., 2020). The main fiber components are

hemicelluloses, cellulose and lignin, of which the relative percentage varies (Lynch et al., 2016; Naibaho et al., 2021; Olivares-Galván et al., 2022).

Hemicelluloses are a group of heterogeneous compounds with a complex chemical structure that includes pentoses, hexoses, and sugar acids. In BSG, this class is mainly composed of arabinoxylans (AX), which are β -(1,4)-linked xylose residues with arabinose units as side chains. In addition, this arabinose units can be esterified with phenolic acids such as ferulic and p-coumaric acids (Coelho et al., 2016). Together with a lower amount of beta-glucans (Bravi et al., 2021), AX are also the principal non-cellulose polysaccharides present in cereals and grasses (Ikram et al., 2017). Cellulose, a linear homopolymer of β -1,4 linked glucose residues, is the other abundant polysaccharide in BSG. With lignin, which is a polyphenolic three-dimensional macromolecule presenting a complex structure of coniferyl, sinapyl, and p-coumaryl alcohol monomers, cellulose plays a crucial role in maintaining the structure (rigidity and integrity) of plant cell walls (Gbenebor et al., 2023).

BSG is also considered a good source of proteins accounting from 15 to 30% of BSG dry weight (Agrawal et al., 2023). The main proteins in BSG are hordeins, which correspond to approximately 40% of the total protein present in BSG (Ikram et al., 2017). Hordeins, also known as prolamins due to their remarkable proline and glutamine content, are classified in four sub-categories (B, C, D and Y) according to their molecular weights (MW). The B and Y groups have a high content in S-containing amino acids and a lower MW (35-50 kDa), while C and D types are poor in S-containing amino acids and have a MW ranging from 55 to 100 kDa (Devnani et al., 2023). Other groups of abundant proteins in BSG are glutelins (21% of the total protein content), as well as albumins and globulins (accounting for about 7% of the total protein content) (Devnani et al., 2023). BSG proteins are rich in valine, alanine, serine, glycine, proline, glutamic acid, aspartic acid, and leucine (Ikram et al., 2017; Mussatto, 2009; Olivares-Galván et al., 2022). About 30% of the amino acids present

in BSG proteins are essential amino acids, among which leucine and lysine are the most abundant, followed by phenylalanine, isoleucine and threonine (de Paula et al., 2023) The essential amino acids (EAA) index of these protein is approximately 1.3 (Table 3.1-1). Among them, particular attention is given to lysine, whose concentration is significantly high in BSG compared to the other cereals, which are known to be poor in this essential amino acid.

Table 3.1-1 Amino acid composition of BSG protein isolate.

^a: references Connolly et al. 2013; da Fonseca et al. 2023; Hellebois, Gaiani, Paris, et al. 2021

^b: WHO/FAO/UN recommendations assuming a meal total protein requirement of 0.66 g/kg per day, the intakes are expressed as g/100 g protein for an adult (WHO report 2007)

Amino Acids	WHO/FAO/UN recommendations g/100g protein^b	EAA score	g/ 100 g of dw BSG protein from literature ^a
Histidine	1.5	1.5	2.3
Isoleucine	3	1.4	4.1
Leucine	5.9	1.2	7.1
Lysine	4.5	0.9	3.8
Methionine	2.4	0.5	1.3
Threonine	2.3	1.3	2.9
Phenylalanine + Tyrosine	3.8	2.2	8.2
Valine	3.9	1.4	5.6
EAA index		1.3	

Lipids are another class of compounds present in BSG. Among them, the most predominant are triglycerides and free fatty acids (Niemi et al., 2012). Mono- and diglycerides and phospholipids are also present in relevant amounts (del Río et al., 2013; Niemi et al., 2012). The main triglycerides identified are trilinolein, dilinoleoyl palmitin and dilinoleoyl olein. The most abundant free fatty acids are octadecadienoic (linoleic acid; 18:2), hexadecanoic (palmitic acid; 16:0), octadecenoic (oleic acid; 18:1) acids as well as, in smaller amounts, octadecatrienoic (linolenic acid; 18:3) and octadecanoic (stearic acid; 18:0) acids (del Río et al., 2013; Lordan et al., 2019;

Niemi et al., 2012). In grains, lipids are mainly located in the endosperm and embryo to provide energy and nutrients for plant development. Triglycerides, which make up 70-90% of total lipids in grains, are hydrolysed during the early stages of brewing, namely germination and mashing steps. This hydrolyzation results in higher presence of free fatty acids in the BSG fraction than in the grains prior to the treatments. These free fatty acids have a low solubility in the wort, which explains their abundance in the BSG (Kaukovirta-Norja et al., 1997; Lordan et al., 2019).

BSG largely consists of the outer layers of the grains (seed husk, pericarp, and seed coat), which are largely made up of cell walls. It has thus a high content in lignocellulose comprising the polyphenolic polymers of lignin, as well as hemicelluloses containing bound phenolic acids. As a consequence, BSG contains mainly HCA, namely ferulic, p-coumaric, sinapic and caffeic acids. In addition to HCA, BSG also contains HBA, albeit in lower concentrations, with syringic acid being the most prevalent (Guido & Moreira, 2017; Paula et al., 2023). Additionally, BSG contains free phenolic compounds, which are mainly made of flavonoids (catechins and oligomers, quercetin and myricetin (Birsan, 2022)). All these compounds, due to their bioactive properties and positive effects on human health, contribute to increase the value of BSG and make it a valuable source of bioactivities. It must be kept in mind that the content of phenolics, as that of the other BSG constituents, strongly depends on the type of grain used for beer production, agricultural practices, and the brewing parameters (Guido & Moreira, 2017).

BSG also contains vitamins and minerals. Folic acid, niacin, biotin, thiamine, choline, pantothenic acid, riboflavin, and pyridoxine are the most common vitamins found in BSG, although most attention is given to tocochromanols (tocotrienols and tocopherols) (Ikram et al., 2017). Among minerals, calcium, magnesium, phosphorus and sodium are most abundant. Even if in limited concentration, iron, copper and manganese are of interest as well (Mussatto, 2009).

3.2 *BSG applications*

Nowadays, BSG is still mainly used for animal feeding or, in areas with little cattle farming, disposed in landfills, thereby releasing about 513 kg of CO₂ equivalents for each ton (Assandri et al., 2021; Iñarra et al., 2022). BSG is considered a low cost biomass and considered one of the cheapest lignocellulosic biomasses (Buffington, 2014), currently valued in Europe between 35-40 €/ton. With a demand of spent grain to reach a value of USD 24 billion by 2033, the number of companies targeting the recycling of spent grain is increasing, especially in the food sector (Nyhan et al., 2023). In addition, the predictable generation and constant availability of this by-product and its chemical composition allow to consider alternative roads for its recycling with a high valorisation potential (Jackowski, Niedzwiecki, et al., 2020; Mussatto, 2009).

BSG can be re-integrated in the brewing process as a yeast carrier. It has been demonstrated that this biomass is suitable, since it has an adequate stability and high retention capacity for yeast cells. In this specific case of BSG re-integration in brewing process, no structural modification are needed, only sterilisation and washes are required (Brányik et al., 2001). Fresh BSG can also be subjected to pyrolysis and the resulting product, biochar, is used for soil improvement. Biochar produced from BSG has a high carbon and ash content, and contributes positively to the increase of nutrients in the soil. It has also been demonstrated that its application increases the soil water holding capacity (Jackowski, Niedzwiecki, et al., 2020). Another application of raw BSG is its thermal conversion into energy (biofuel) or its anaerobic fermentation to produce biogas (Pabbathi et al., 2022). However, choosing these processes as the first alternative for BSG recycling is not considered economically preferred. In its untreated form, this biomass can also be used as a sustainable building material or as filler and reinforcement matter. For example, it has been demonstrated that it is a good candidate to replace sawdust in bricks, even contributing to their porosity (Guido & Moreira, 2017), and it has been included in ground tire rubber, reinforcing polyurethane foam (Zedler et al., 2020). As well, because of its high fatty acid

content, BSG can be used as a cheap substrate to produce new biopolymers similar to plastic, like polyhydroxyalkanoate (PHA) (Lech & Labus, 2022). Besides these applications, BSG could also be exploited in biotechnological processes because of its rich and diverse chemical composition. For example, it can be used as a medium to produce enzymes or for the cultivation of microorganisms (Mussatto, 2009). It might also be a suitable and cheaper alternative substrate to other lignocellulosic material for microbial fermentation. For example, xylitol, lactic acid, and ethanol have been successfully produced via its fermentation (Mussatto et al., 2013). However, to make the lignocellulose more accessible for fermentation, thereby improving the efficiency of the process, BSG must undergo several pre-treatments (Ancuța Chetrariu & Dabija, 2020), such as enzymatic or alkaline and acidic hydrolyses, as well as microwave and ultrasound treatments (Mussatto, 2014). Another promising application is the use of BSG as a source of value-added products such as dietary fibers, prebiotics, and nutraceuticals, which can be re-integrated for example in the food sector (Lech & Labus, 2022).

3.3 BSG food industry application

Exploiting the composition in bioactive compounds may represent an alternative for BSG recycling (Lech & Labus, 2022). Accordingly, the use of BSG as source of functional ingredients in the food sector is currently under development.

For example, a market analysis has revealed that 19 food manufacturers in the US, Ireland, France, Germany, Denmark, Switzerland, the UK and Asia are integrating BSG and its extract components in their food products (Nyhan et al., 2023). For example, BSG can be milled to produce flour and be integrated into products such as pasta, bread, and cookies for their enrichment. Likewise, BSG flour has already been included in snacks or used as the main ingredient in food products because of its high fiber and protein content, bringing potential advantages into human diet (Jackowski, Niedzwiecki, et al., 2020; Mussatto, 2009; Rachwał et al., 2020).

In addition to integrating BSG into food products, companies are also taking the opportunity to produce functional BSG-derived ingredients by extracting its functional components (Nyhan et al., 2023). Generally, the focus in these valorisation efforts is on phenolics, arabinoxylans and proteins. Phenolics are mainly considered because of their health benefits, while arabinoxylan and protein extracts, in addition to potential beneficial effects on human health, are also used due to their techno-functional properties, which are of interest for the food sector. Phenolic acids such as ferulic, coumaric, caffeic, syringic, vanillic acids, and flavonoids such as catechins, are known for their antioxidant potential. Moreover, they are studied for their antimicrobial and anti-inflammatory properties and are considered as photo-protectant and food preservative agents (Ikram et al., 2017; Jackowski, Niedzwiecki, et al., 2020).

Arabinoxylans are the most abundant hemicelluloses in BSG and are known for their prebiotic and gut regulatory activities. Their intake is positively associated with an increased production of acetate and propionate from bifidobacteria, compounds shown to have anti-obesogenic, anti-inflammatory and neuroprotective bioactivities (Lynch et al., 2016, 2021). Moreover, arabinoxylans are also used as emulsifying, film-forming, gelling agents (Cervantes-Ramirez et al., 2022).

BSG protein extracts have emulsifying and gelling properties and are studied for their involvement in enhancing food flavour. Nevertheless, their application in food formulations remains difficult, mainly because of their low solubility, and further studies are required to optimize their valorisation (de Paula et al., 2023). BSG protein isolates, finally, are also being considered for their antioxidant activities and their potential anti-inflammatory, immunomodulatory and antihypertensive properties (Hellebois et al., 2021). In addition to phenolics, arabinoxylans and proteins, lipids are also present in BSG, but to which less attention is given (Niemi et al., 2012). BSG is a source of triglycerides, fatty acids and phospholipids, compounds with nutraceutical and cosmetic properties. For instance, BSG contains

linoleic acid, an essential dietary fatty acid, involved in different metabolic processes (Huang et al., 1999). Moreover, data of the few studies done on lipids specifically found in beer, provided preliminary results on the anti-thrombotic activity of beer polar lipids, but further studies are needed to assess the contribution of the different lipids (Lordan et al., 2019).

There is a growing focus on valorising food processing residues by re-integrating them in food industry as ingredients or by generating added-value products. The market for food products based on up-cycled residues knows a rapid growth with an expected annual increase of 5% for the next ten years. In addition, a factor not to be underestimated for the commercial success of BSG up-cycling in the food sector is consumer acceptance. As society is becoming more sensitive to environmental protection and recycling concepts, new opportunities are opening for future developments in this field (Nyhan et al., 2023).

4 Analysis and characterisation of compounds

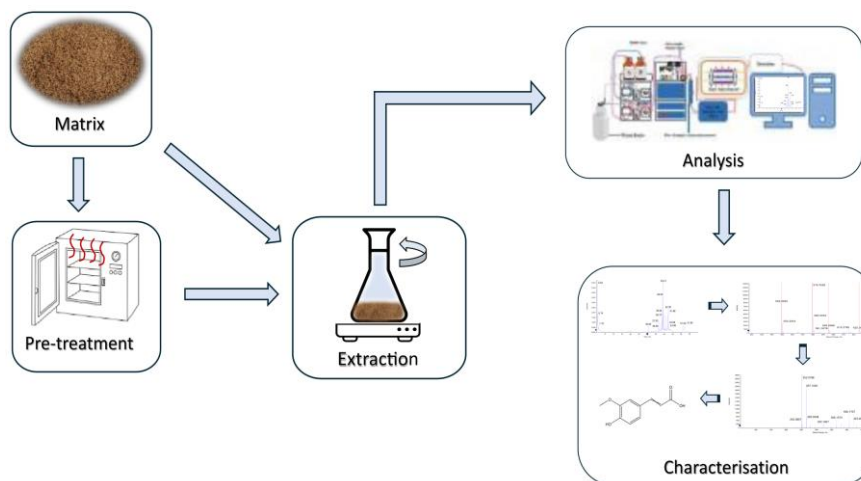


Figure 3.3-1 Workflow for the analysis and characterisation of bioactive compounds

Most of the natural compounds, currently used or studied to improve human life and health, originate from plants. For some of these natural compounds, different biological and functional activities such as antioxidant, anti-inflammatory, antidiabetic and anticancer effects have been identified. Once the bioactivity is experimentally confirmed, they are defined as bioactive compounds (Banwo et al., 2021). Plants, indeed, represent a valuable source of compounds, which can be used by humans as components of drugs for medical purpose, or in food as food additives or supplements. For this reason, these compounds are of interest and are at the basis of the application of plants and plant extracts all along human history. Unfortunately, the concentration of bioactive compounds in plants is often low and can be highly variable depending on their growth conditions. Therefore, applications often depend on the extraction of these active compounds from the plant material. Actually, the extraction process is a fundamental step in the use of active compounds from plant sources, and the selection of the most suitable extraction process represents a big challenge (Jha & Sit, 2022).

A general workflow of bioactive compound analysis (Figure 3.3-1) starts with the collection of the matrix, the natural source of these molecules, which is often followed by a pre-treatment stage. Subsequently, the workflow foresees the extraction of the targeted compounds and their characterisation. The form of the characterisation performed in this thesis is profiling of the chemical composition of the extracts. A subsequent characterisation would involve the assessment of the positive, but also potentially negative effects of the resulting products, ingredients, or compound enriched fractions. The final validation potential of the generated fractions will only be determined after subsequent activity analysis and the inclusion of economic and technical aspects.

4.1 Pre-treatment

Before extraction, the matrix can be pre-treated to facilitate the process and thus to improve the final extraction yield. Pre-treatments basically contribute to the de-structuration of the matrix, thereby facilitating the release of the targeted compounds. According to the compounds and plant matrices, several physical, biological, and chemical techniques can be applied. Among the many kinds of pre-treatments, the most common are hot air drying or freeze-drying, crushing and grinding (Arshadi et al., 2016), ultrasound, microwave and pulsed electric field techniques, fermentation (Cano y Postigo et al., 2021), as well as enzymatic, acidic or alkaline hydrolysis (Krakowska-Sieprawska et al., 2022).

Relevant for this thesis is drying of the matrix. This step is also used for decreasing the water content of the matrix, in order to limit the microbial degradation and to reduce the volume of the material. Therefore, it allows to store biomass for a longer period of time while reducing storage space and costs (Hassan & Muhamad, 2017; Krakowska-Sieprawska et al., 2022). Drying is either done at high (convection-drying) or very low temperatures (freeze-drying). The first method, which is considered the cheapest and easiest approach, is the most used, also because of its high feasibility at the industrial scale. However, it takes long times, and the temperatures applied

might lead to chemical, physical and physico-chemical modification of the matrix composition (Singh et al., 2020). The convection-drying approach will be discussed in detail in the chapter 3, where differences between dried and untreated BSG biomass will be described based on BSG extract chemical composition. As the material is dried by ice sublimation, freeze-drying has a much lower impact on the chemical composition of the matrix and it is therefore a suitable alternative to oven drying. However, freeze-drying also results in long processing times and in a high energy consumption. These factors are the main limitations for the application of freeze-drying at a larger scale, which ends up in a very high cost of execution (Krakowska-Sieprawska et al., 2022).

The biological strategy for pre-treating the plant matrix is also commonly used. It includes for instance the direct application of cell wall hydrolysing enzymes, which will make the desired compounds more accessible. Similarly, enzymatic activities of microorganisms can be exploited by inoculation bacteria, fungi, or yeasts, that are able to disintegrate plant tissues via fermentation. In addition to their hydrolytic activities, these microorganisms can also be used for the conversion of molecules present in the matrix into high-added value compounds (Zhao et al., 2014).

The use of acidic or alkaline solutions are very efficient and cheap techniques, although they require supplemental steps, like a solvent separation step, washes, an efficient chemical waste management and often specialized equipment for the treatment (Zhao et al., 2014). Alkaline and acidic methods, for example, are often applied to pre-treat cereal biomasses such as brewer's spent grain (Ancuța Chettrariu & Dabija, 2020).

4.2 Extraction

After the optional application of different pre-treatments, the selection of the most suitable extraction method is the next fundamental step to maximise the valorisation of the compounds of interest.

The extraction step represents one of the key procedures in the workflow of compound valorisation. It is influenced by factors such as the extraction methods applied, the raw materials and solvents used. The extraction techniques can be classified into conventional or unconventional (Figure 4.2-1) (Azmir et al., 2013). The conventional methods are mainly based on the dissolving power of solvents, the efficiency of which can be occasionally increased by heating and/or stirring. Among them, the most common techniques used for the extraction of compounds from plant material are Soxhlet, maceration and hydro-distillation (Jha & Sit, 2022).

For Soxhlet extraction a specific apparatus is required to extract in a continuous manner (Figure 4.2-1A). The solvent (located at the bottom of the apparatus) evaporates through heating and subsequently condenses at the top of the instrument. The drops are then collected in a sample thimble, which forms a siphon. When the volume reaches a certain height, the solvent passes back to the bottom of the apparatus and the cycle starts again (Martins et al., 2023). This operation is simple, and no filtration is required afterwards. High temperatures are applied during extraction with this relatively low-cost technique. However, it takes long times, it requires high volumes of solvent and it is not suitable for thermolabile molecules (Jha & Sit, 2022). Lipids are the most targeted class of compounds with Soxhlet extraction, mainly because of the properties (i.e. low boiling point, limited polarity, ...) of the solvents used for recovering lipids. Although it is also used for extracting more polar compounds such as phenolics, but generally with a prior de-fatting pre-treatment (López-Bascón-Bascon & Luque de Castro, 2019).

Maceration is another widely used and traditional extraction method (Figure 4.2-1B). This technique is usually performed at room temperature and under agitation. The material is mixed with a solvent (i.e. water, alcohol, etc...) in a closed vessel, and when the process is finished, the two phases (liquid and solid) are separated by centrifugation or filtration. Stirring is highly recommended, as it increases the exposure of the material to the solvent by facilitating its

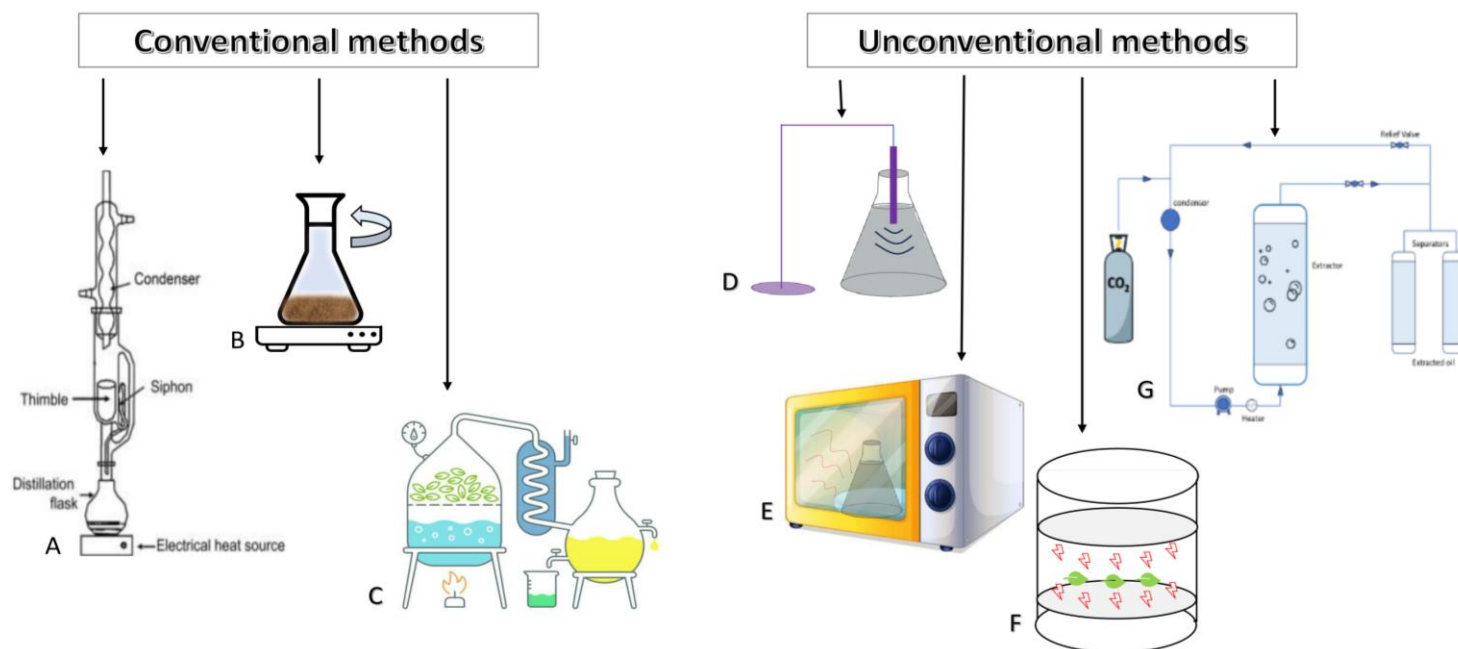


Figure 4.2-1 Some of the most common conventional and unconventional methods applied for the recovery of compounds: A) Soxhlet extraction; B) maceration extraction; C) hydro-distillation; D) ultrasound-assisted extraction; E) microwave-assisted extraction; F) pulsed electric field extraction and G) super critical fluid extraction

diffusion within the matrix. It is considered a simple technique, but on the other hand, it takes long time to be efficient. It is performed for numerous compounds, due to its flexibility in the execution (solvent, temperature, time, stirring can be adapted accordingly) and its feasibility at industrial scale. This technique is mostly suggested for thermolabile compounds, such as phenolics (Jha & Sit, 2022; Patra et al., 2022).

Hydro-distillation is the most common approach for the extraction of essential oils or volatile substances (Figure 4.2-1C). The material is mixed with water and heated up to the boiling point. For this method no organic solvents are required, and steam can be directly added in the system and applied into the material to enhance the extraction. The most important advantage of hydro-distillation is the lack of organic solvent; nevertheless the high temperatures used for evaporation of the solvent represent the biggest limitation for its application (Azmir et al., 2013; Jha & Sit, 2022).

The main issues of the traditional extraction methods are the long execution times, the high demand of solvents, the costs, and the environmental and operational risk related to their use. To overcome these problems, new extraction methods have been developed. These aim to combine high execution speed with limited environmental impact and high extraction yields (Lefebvre et al., 2021). The most studied unconventional techniques used for the extraction of compounds from plants are ultrasound assisted, microwave assisted, pulsed electric field, super critical fluid, and subcritical water extractions.

In ultrasound-assisted extraction (Figure 4.2-1D), ultrasound waves, with a frequency ranging from 20 to 100 kHz, are passed through the sample, promoting the rupture of the cell walls, thus leading to an increase of the permeability and diffusion capacity of the matrix. The main disadvantages of this technique are the several repetitions required to complete the extraction, and therefore resulting in high energy consumption and costs. In addition, when this technique is

applied in larger volumes, the exposure of the material to the solvent is not homogenous. Nevertheless, this method showed successful results in extracting several groups of compounds such as phenolics, carotenoids, and fibers (K. Kumar et al., 2021; Patra et al., 2022).

Microwave-assisted extraction uses electromagnetic waves to generate heat within the sample (Figure 4.2-1E). In this case, the heating is created from the inside to the outside of the sample, breaking bonds between tissues and molecules by internal water evaporation. The extraction is quick, highly reproducible with a low energy consumption (Usman et al., 2022). However, the yield can vary significantly according to the density and chemical composition of the matrix. As for the Soxhlet method, microwave-assisted extraction results in high temperatures during the extraction, damaging thermolabile compounds and affecting the composition of the final extract (Martins et al., 2023).

An innovative physical method is pulsed electric field extraction (Figure 4.2-1F). During this process, nano- and micro-pores are created in cell membranes by destabilizing the lipid bilayers and thus increasing permeability. One of the main advantage of this technique is the limited risk of thermal degradation, since no high temperatures are applied. In addition, this method can also be combined with other extraction techniques to improve the process yield or can even be exploited as a pre-treatment prior to subsequent extractions. Several compounds present in plant cells, such as pigments, carbohydrates, and phenolics have successfully been extracted with this approach (Puértolas et al., 2012). However, this technique has some limitations related to the presence of bubbles, the density of the solution and its integration in large scale processes, which still require further optimisation (Martins et al., 2023; Usman et al., 2022).

The use of super critical fluid extraction (Figure 4.2-1G) includes the application of super critical fluids, which are single-phase media with properties of both liquid and gaseous states. This phenomenon occurs when the solvent reaches its critical point, given by a specific

combination of temperature and pressure. At this point, the medium shows higher diffusion capacity than a liquid solvent and a higher solubilisation potential than a gaseous solvent. This method is highly recommended as is sustainable for the environment and considered safe for human consumption products. Super critical fluid extraction represents also a valid alternative to the use of organic solvent in the extraction of bioactive compounds (Uwineza & Waśkiewicz, 2020). The most commonly used solvent is CO₂, which is non- toxic, - flammable and -corrosive, chemically stable and nontoxic mL for humans (Gil-Martín et al., 2022). However, not all compounds can be extracted with CO₂ due to its low polarity, which makes the extraction of polar compounds such as phenolics and carbohydrates difficult. It is mainly efficient for the extraction of molecules with a low or medium polarity such as lipids. However, to increase the polarity and efficiency of this extraction method, a small concentration (i.e. from 1 to 10%) of a polar co-solvent can be combined with the CO₂. It is an innovative technique, simple and environment friendly by which high-quality extracts can be obtained in a short time. However, the up-scaling of this technique is complicated, due to the difficult optimisation of all parameters at larger scale. In addition to that, super critical fluid extraction equipment is expensive and not yet considered economically advantageous at an industrial scale, as compared to classical methods (Jha & Sit, 2022).

The extraction with subcritical water is considered safe for the environment and for human nutritional and medical applications. It is a cheap, fast and clean approach, wherein water, under a specific pressure and temperature above the boiling point, reaches a physical state below its critical point (Fărcaș et al., 2022). Due to its high dielectric constant, polarity and high boiling point, the extraction properties of water can be changed by adapting the temperature and the pressure. At room temperature, the dielectric constant and polarity of water is high; however, when the pressure and the temperature increase, the dielectric constant reduces reaching values typical for organic solvents, thus modifying its extraction potential. Due to water polarity, this technique is very efficient for polar compounds as

phenolics and carbohydrates (Daud et al., 2022). All these unconventional and modern methods are promising alternatives for the time-consuming and often not environmental-friendly traditional techniques. However, the difficult management of these systems and the very high costs of execution and maintenance still highly limit the application of these promising alternatives. As a consequence, the industrial extraction of compounds from plant matrices is still largely performed through maceration, hydro-distillation or with Soxhlet extraction.

An emerging alternative extraction method currently highly investigated is the use of deep eutectic solvents (DES). These solvents are formed by combining, in a specific ratio, a hydrogen bond donor (HBD) with a hydrogen bond acceptor (HBA) resulting in a liquid mixture that exhibits advantages compared to the traditional solvents (Ngasakul et al., 2023). For example, DES are easy to prepare, do not require further purification steps and are originated from low cost compounds (Cunha et Fernandes., 2018). The principle involves the formation of hydrogen bonds between the HBD and HBA resulting in a lower melting point than that of the individual components. Each DES exhibits distinct properties, which can be modulated based on the selected components (Cannavacciuolo et al., 2024). This innovative approach leads to the development of versatile solvents, which are currently considered more environmentally friendly than the traditional methods. While DES mainly involve synthetic or non-natural materials, the natural deep eutectic solvents (NADES), a new class of DES recently introduced, are specifically derived from natural sources, such as sugars, organic acids and amino acids. This enhances the sustainability and biodegradability properties of the DES (Benvenuti et al., 2019). Recent studies showed promising results on the extraction of phenolic compounds from BSG through the use of these solvents (Nath et al., 2023; Ngasakul et al., 2023). However, since DES/NADES extraction approach is still emerging, more research and analyses on their application need to be conducted (Benvenuti et al., 2019).

4.2.1 BSG general extraction methods

The extraction techniques mentioned above represent just a general overview of the methods applied on plant-based matrices, and foreseen for the most commonly targeted compounds. There are many other extraction approaches, pre-treatments, and combinations of those that can be applied. Each procedure is specific and must be adapted according to the molecules of interest and the starting material. Given the interest in BSG as source of added-value extracts, numerous studies have been conducted on BSG extractions. Although BSG is rich in fibers, most of these studies focus on the phenolic compounds and have antioxidant capacity as biological activity target. Despite the wide range of innovative extraction methods and their advantages, most of these studies apply traditional techniques for the extraction of these compounds. Prior to the extraction, BSG is often subjected to pre-treatments such as drying and grinding (Carciochi et al., 2018; Iadecola et al., 2022; Zago et al., 2022). Drying is mostly applied to reduce the high moisture content of BSG, thus increasing the storage time and reducing the volume of the biomass. Grinding is applied for homogenising the biomass and improving the subsequent extraction yield (Ancuța Chetrariu & Dabija, 2021). The maceration extraction procedure is one of the most often performed for the recovery of phenolic compounds; it uses different solvent mixtures (organic and non-organic), such as water, ethanol, methanol, acetone and ethyl acetate (Guido & Moreira, 2017). Few studies compared the use of non-traditional methods, like the ultrasound- and microwave-assisted extractions, to the traditional procedures (i.e. maceration) for extraction from BSG. These studies did not show a significant increase in the extraction yield (Birsan et al., 2019). The use of supercritical CO₂ showed even lower values compared to the classical methods (Spinelli et al., 2016). However, it must be considered that supercritical CO₂ extraction is not the optimal strategy to isolate phenolic compounds due to the low polarity of this solvent. Interestingly, combinations of microwave, ultrasound or enzymatic approaches (innovative techniques) with conventional extractions have shown positive results (Guido & Moreira, 2017).

Most studies on the extraction of fibers from BSG are based on differential precipitation obtained by using alkaline or acidic treatments. Recently, different strategies have been explored, for example the use of subcritical water for the extraction step (Alonso-Riaño et al., 2023), as well as the use of enzymatic pre-treatment of the biomass prior to alkaline extraction (Naibaho & Korzeniowska, 2021). These more innovative procedures resulted in an increase in process yield, thus opening interesting new alternatives for the isolation of BSG fibers. The exploitation of specific enzymes as a pre- or a combined treatment, proved to be a useful technique also for BSG protein extraction. Several studies were conducted for improving protein extraction from BSG, and the data reported are promising. For example, the combination of ultrasound and microwave pre-treatments followed by alkaline extraction exhibited high yields, as did enzymatic extraction in water with specific proteases (Devnani et al., 2023). Lipids have been less investigated, and little is known about the optimisation of their extraction from BSG. Lipids are mainly extracted using Soxhlet method with organic solvents such as hexane or acetone (del Río et al., 2013). However, some studies have shown similar results with supercritical CO₂ and Soxhlet extraction with hexane, demonstrating that innovative techniques may be applied for lipids (Ferrentino et al., 2019; Spinelli et al., 2016).

4.3 Identification and quantification of compounds in the extracts

Once an extract is obtained, the next step is the qualitative and quantitative analysis of the compounds of interest. Metabolite analyses are classified as targeted or untargeted; the first approach involves the identification and quantification of already known specific compounds or group of compounds, while the second method focuses on a comprehensive detection of as many compounds as possible, including known and unknown metabolites. The aim of the untargeted approach is to acquire a profile of the analysed extract without necessarily identifying or quantifying all detected compounds (Cevallos-Cevallos et al., 2009).

Metabolomics is an interdisciplinary field that deals with the identification and semi-quantification of known and unknown compounds with low molecular weight ($<\pm 2000\text{Da}$) (Segers et al., 2019). The main analytical methods used to obtain a metabolomic profile are spectroscopy and spectrometry, namely nuclear magnetic resonance (NMR) and mass spectrometry (MS) (David & Rostkowski, 2020). While NMR-based metabolomics can provide unbiased identification, the technique without fractionation of the extract generally only allows the unambiguous identification of only a few high-abundant compounds. However, the time that NMR requires to obtain good data is still considered one of the main drawbacks of this technique (Tampieri et al., 2021), together with the lack of an approved set of standard practices (Powers et al., 2024). NMR-based metabolomics is therefore most often used as screening tool to identify metabolite changes in large cohorts as is often done in medical studies or in quality control routines. Both analytical methods are often coupled with separation techniques such as high performance liquid-chromatography (HPLC), ultra-high performance liquid-chromatography (UHPLC) and gas-chromatography (GC) (Segers et al., 2019).

The direct injection of a biological sample, especially for MS, can provide a rapid analysis, although the high number of analytes can impact the performance of the machine (signal co-suppression or low ionisation efficiency) and make the data interpretation very complex (Zhang et al., 2012). To avoid these issues, a technique for the separation of compounds is often performed prior to MS analysis, and it is generally exploited as a sample-introduction system. By limiting ion-suppression and reducing the complexity of the generated data, coupling with analytical separation techniques increases the output and facilitates the subsequent analysis. The most common techniques are liquid- (LC) and gas-chromatography (GC) (Segers et al., 2019). According to the partitioning coefficient of the analytes between the mobile and the stationary phase, determined by the physicochemical properties of the analyte and the characteristics of the mobile and stationary phases, the analytes will preferentially interact with the

mobile or the stationary phases. Changing the characteristics of either phase will separate the compounds (Abdu Hussen, 2022). Based on the polarity of the stationary phase, different types of LC, such as reversed-phase (RPLC) and hydrophilic-interaction liquid chromatography (HILIC), can be used. In HILIC the stationary phase is polar, while in RP it is apolar (Žuvela et al., 2019). Another separation technique is gas chromatography. This method involves the use of a gas as a mobile phase, and a derivatisation step is often used to make the analytes more volatile (Cevallos-Cevallos et al., 2009). The system consists of a carrier gas, an injector, a column, and a detector. There are 2 types of injectors: the vaporisation and the on-column injectors. The first requires high temperatures (100-300°C) to quickly vaporise the sample, while the second injects the sample directly onto the column. There are different types of column, most used today are capillary columns, which are empty columns with the inner surface coated with the stationary phase; there are offering a high separation capacity and resolution (Forgács & Cserhádi, 2013). In a mass spectrometry-coupled system, the chromatographic technique is hyphenated to a mass spectrometer and an ion source. For LC-MS, liquid chromatography is directly coupled to an electrospray ionization (ESI) source, a soft ionisation method. The analytes in the liquid phase pass through a capillary tube with a strong electric voltage, thereby forming charged droplets. The solvent evaporates from these droplets until the force generated by repulsion between the charges surpasses the surface tension of the droplet (the Rayleigh limit) at which point the droplet will divide. This continues until all solvent is evaporated and the charged analyte remains isolated (Forcisi et al., 2013; Segers et al., 2019). The formed ions are thus injected into the mass spectrometer. Due to its high sensitivity and the wide range of covered metabolites, MS has become the technique of choice in metabolomics (Zhang et al., 2012). Mass spectrometers can be classified as low-resolution (LRMS) and high-resolution mass spectrophotometers (HRMS) according to the accuracy and the resolving power parameters. Accuracy refers to the difference between the theoretical m/z and the m/z measured of an ion (Tamara et al., 2022), while the resolving power indicates the capacity of the

instrument to separate the signals of two ions with a small m/z difference (Xian et al., 2012). These parameters are important for the type of analysis selected.

Low-resolution MS is mainly employed in targeted metabolomics, since this type of technique specifically focuses on the detection of a limited number of compounds with a known retention time and m/z -value. Targeted analyses are mainly applied in studies on specific biomarkers or bioactive compounds, enzymatic kinetics, metabolic pathways (Roberts et al., 2012), and effects of treatments or stress-induced responses on specific molecules (Katam et al., 2022). This approach is also widely used in BSG studies, for example for the quantification of phenolic compounds, such as p-coumaric acid, ferulic acid and catechin, whose presence is considered a critical parameter for the quality of BSG extracts (Bonifácio-Lopes et al., 2020b; Connolly et al., 2021; Guido & Moreira, 2017; Rachwał et al., 2020). In contrast, high-resolution MS analyses, which reach a higher level of accuracy and higher resolving power (Junot & Colsch, 2014), are mostly used for untargeted analyses. A high resolving power, for example, allowed to clearly discriminate vicine- and convicine-based compounds from fava bean extracts, in a study in which I collaborated, by allowing to distinguish a fragment unique to each of these molecules at respectively m/z 141.0423 and 141.02 (Sergeant et al., 2024).

Untargeted metabolomics is employed for numerous purposes, including obtaining a comprehensive metabolite profile of a sample and comparing various medical trials involving different treatments. Interestingly, untargeted metabolomics outputs can be combined with other omics datasets to acquire more accurate information. Furthermore, it is applied to discover biomarkers in response to various conditions (physiological, environmental, technical, etc...) and identify potential bioactive molecules. In this regards, plants have been intensively targeted due to their rich composition in bioactive compounds (Aloo et al., 2023; Jha & Sit, 2022). However, with the increasing attention to a more sustainable society and economy, agri-

and food-processed by-products have become of interest for their content in bioactive molecules. In that context, the application of untargeted metabolomics is crucial to reveal the potential of these by-products (such as spent ground coffee, spent cereals, fruits peels and husks (Campos et al., 2022; Di Matteo et al., 2023; Mir-Cerdà, Nuñez, et al., 2023; Zeng et al., 2022)) and to obtain a holistic screening of their metabolomic profile.

The output produced with MS, with compounds represented by retention time- m/z couples, generally known as LC-MS features, is analysed using specialised software. While the m/z value of a compound as obtained with MS allows to discern known metabolites, for instance used for targeted studies, it does not give sufficient information to identify unknown compounds, for example in untargeted studies. The capacity to identify unknown metabolites is increased thanks to the study of the fragmentation pattern of compounds in so-called tandem MS, MS/MS or MS^2 spectra. This fragmentation is induced by collisions between a neutral gas and the gas-phase ion. The generated fragments are subsequently analysed, generating a mass spectrum of fragments. With MS/MS analyses, the identification of known compounds is more accurate (Xiao et al., 2012), and the identification of previously unknown compounds is made possible.

In this study, a high-resolution Triple-TOF (Time-of-flight) mass spectrometer has been used. This instrument is a combination of two mass analysers: the quadrupole and the TOF. The quadrupole functions as a mass filter and allows to select ions with a specific m/z value for fragmentation, while in MS-mode it serves as ion-guide. The TOF, placed after the quadrupole, is a field-free flight tube wherein the ions fly at a constant speed inversely related to their m/z value. Measurement of the time taken by ions with different m/z values to reach the detector allows the accurate determination of the m/z of these ions (Haag, 2016; Mamyrin, 2001). The subsequent data treatment depends on whether a targeted or untargeted experiment is done.

In a targeted analysis the aim is to obtain an absolute quantification of known metabolites. The first step for this approach involves the generation of a reference database, containing the m/z , retention time, and, when possible, m/z values of fragments based on LC-MS runs with standards. A calibration curve of the standards is generated and the compounds can be quantified in the different samples (Gorrochategui et al., 2016; Schrimpe-Rutledge et al., 2016). Absolute quantification can be obtained after normalization of the data, avoiding biases created from experimental imprecisions, or analytical noise. Possible methods to normalise the data are, for example, the use of internal standards, quality control samples (QCs) or external calibration curves. Current methods for metabolite quantification generally use SRM or MRM, which respectively are Selected and Multiple Reaction Monitoring, wherein the MS will fragment all precursor ions with a specific selected m/z value and relative or absolute quantification is done based on the abundance of the specific fragments (Lv et al., 2022; Zheng et al., 2021). Since the compounds of interest are known, optimized methods for their analysis from extraction, sample preparation to chromatography and MS are generated, resulting in high-throughput procedures for the routine analysis of compounds.

While targeted metabolite analyses are used to confirm hypotheses or in routine settings, untargeted metabolomics is exploratory or used for the generation of new hypotheses. Contrary to targeted analyses, quantification is only relative and untargeted analyses are thus generally used for comparing samples. The amount of data generated in untargeted metabolomics is enormous and the use of a dedicated software is needed for the analysis (Allwood et al., 2021). Data treatment generally starts with alignment of the different runs, compensating for small differences in retention time and allocating a unique LC-MS feature (RT x m/z) to the same compound in the different runs. Further processing generally involves normalisation, scaling and transformation (Gorrochategui et al., 2016). The treated data are then used for statistical analysis (T-test, ANOVA, principal component analysis PCA, clustering, etc...) (Schrimpe-Rutledge et al.,

2016), highlighting those features that separate the different samples. Identification of unknown compounds is often a difficult task (Blaženović et al., 2018; Chaleckis et al., 2019), and an active field for research on the use of Artificial Intelligence (Asef et al., 2023; Palm & Krueve, 2022).

5 Conclusion

BSG is the most abundant solid by-product of the brewing process and its accumulation and management is becoming increasingly challenging. It is considered a low-cost biomass, which is currently poorly valorised, and is mainly used as cattle feed or sent to landfills. Interestingly, BSG has a chemical composition that includes valuable compounds with bioactive properties, opening new alternative roads for its up-cycling with a better valorisation potential. One of these is the generation of added-value products through the extraction and recovery of bioactive-enriched fractions. These compound-enriched fractions have a valorisation potential in food, pharmaceutical and cosmetic sectors, thereby being re-integrated in the industrial setting. The valorisation of agri-food biomasses by the extraction and characterisation of their valuable compounds represents a promising approach currently investigated for the development of a more sustainable and circular economy.

6 References

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CHAPTER 2.

SCOPE AND AIMS

In view of the increase in population and food demand, the agricultural and industrial sectors need to increase their production. This leads to an increase in the amount of biomass waste that is generated, thereby making the management of this waste more difficult and aggravating current societal issues related to the negative environmental impact of bio-waste. Spurred by the growing attention for environmental protection and the societal demand for a more sustainable economy, new possibilities to re-use waste are being explored.

As beer is the most consumed alcoholic beverage worldwide and its consumption is expected to increase, the brewing industry is expected to generate increasing amounts of biomass residues. Among these residues, with 6.8 million of tons generated in Europe in 2022, brewer's spent grain (BSG) represents the bulk of the solid waste produced. Currently, most of BSG is still poorly valorised and it is mainly used for animal feeding (70%) and for biogas production (10%). However, because the quantity of BSG produced exceeds the amount recycled, 20% of the generated BSG is sent to landfills. Such waste management practices do not exploit the potential of this biomass and consist of non-sustainable practices, which society attempts to mitigate.

The present PhD thesis is based on the hypothesis that developing alternative methods for recycling BSG can contribute to increase the sustainability of BSG management by generating new products that can be integrated in industrial activities. This hypothesis results more specifically in the following scientific question "Can part of the chemical composition of BSG be extracted and exploited for the generation of multiple products with a valorisation potential?" This

overarching question results in four more focused questions related to the development of a strategy to do so.

Can an untargeted metabolomic approach be used for the development and optimisation of a strategy to better valorise the complex composition of BSG?

Is it possible to generate a unique product from barley-based BSG, the most abundant type of BSG generated, based on the presence of specific molecules not found in other feed-stocks?

Is it feasible to fractionate BSG biomass and are the basic biochemical characteristics of the multiple fractions generated at the laboratory scale in line with those of extracts generated in studies that focused on the extraction of a single class of compounds?

Can the strategy for the better valorisation of BSG be applied at a pilot-scale and what are the critical parameters to optimize for industrial-scale application?

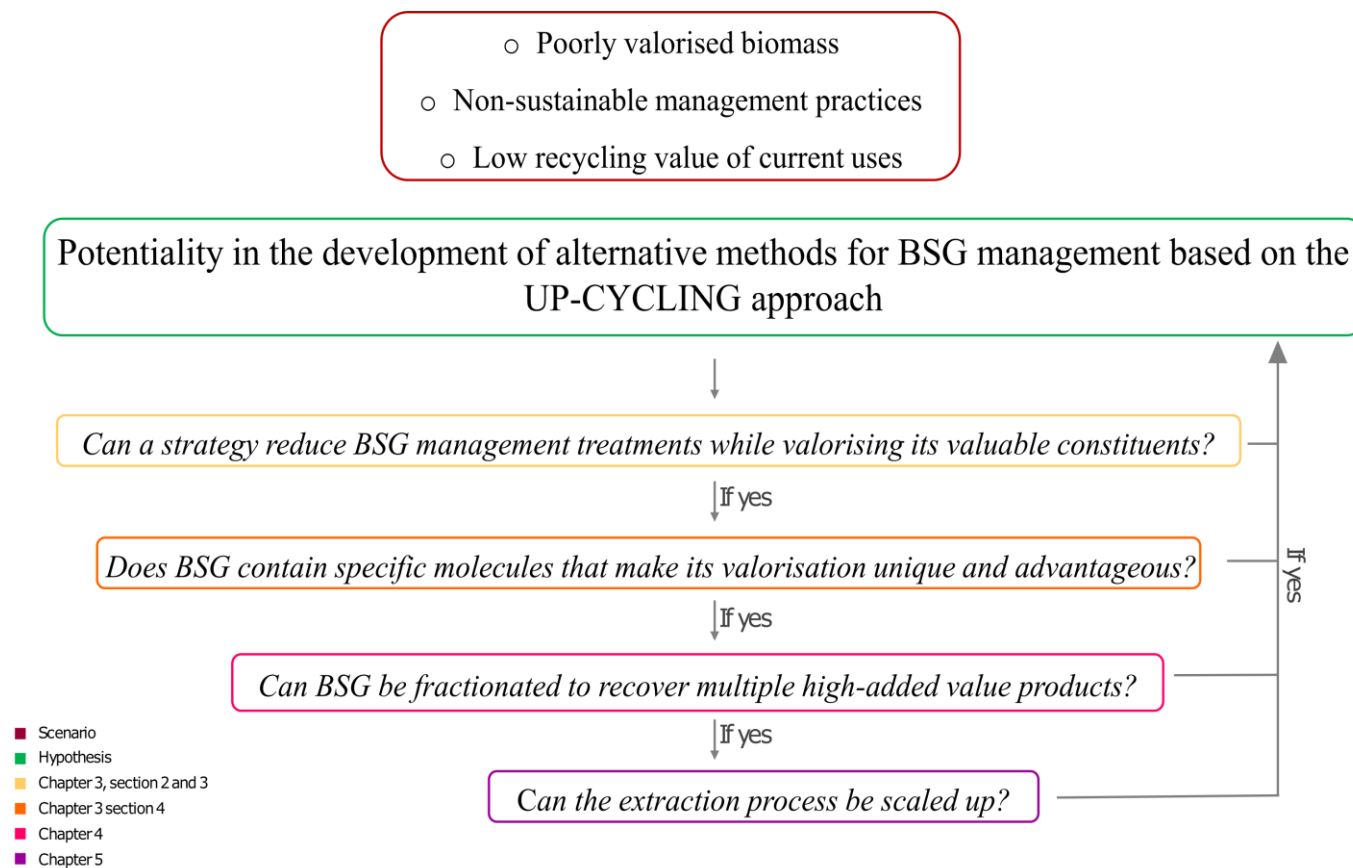


Figure 1 General scheme of the main objective

CHAPTER 3.

BSG CHARACTERISATION AND OPTIMIZATION OF THE HYDROETHANOLIC EXTRACTION

Foreword

The first experimental part of the present manuscript deals with the selection of a valid method for the extraction of bioactive compounds from BSG. The application that is aimed for it is as food supplement, with emphasis on the optimized extraction of phenolics and the antioxidant potential of the extract. Several extractions were performed and the approach that resulted in the best profile was selected based on metabolomic analyses and chemical assays. In addition, a detailed study on the impact of the most common method applied for BSG stabilisation (oven-drying) on the recovered extracts was conducted. A group of phenolic compounds specific of barley, which knowledge is still limited, was investigated and preliminary enrichment protocols assessed. The results from this chapter provide a more sustainable alternative to the most common BSG management and open new roads for the valorisation of barley-based BSG.

1 General introduction

As reported in the literature review (Chapter 1), BSG consists of the insoluble part of the grain including pericarps, husks, and seed coats and is the first and most abundant by-product from brewing. It accounts for 85% of the total solid brewery by-product and around 20 kg of wet spent grains are generated for each hectolitre of beer. Globally, BSG contains 15-30% proteins, 20-25% hemicelluloses, 12-25% cellulose, 12-28% lignin, 6-10% lipids and 0.5-2% phenolics. It furthermore contains vitamins and minerals (Lynch et al., 2016; Rachwał et al., 2020).

Phenolics are the main targeted compounds in extraction processes using BSG. Their health-promoting properties are mostly related to their antioxidant capacities. The total phenolic content (TPC) of BSG changes according to the malt type, and values reported in literature range from 1 to 3.3 mg gallic acid equivalents (GAE)/g dw BSG (Almeida et al., 2017; Socaci et al., 2018a). Among phenolics, a group of compounds highly present in BSG is the hordatine subclass, which is currently attracting the interest of the scientific community due to its potential bioactive properties like antifungal and antioxidant activities, and their possible involvement in stimulating gastrointestinal motility (Becker, Stegmüller, et al., 2022; Spreng & Hofmann, 2018; Stoessl & Unwin, 1970).

Many extraction methods have been explored on BSG, from conventional techniques to more innovative approaches to obtain health-promoting extracts (Guido & Moreira, 2017), but independent of the extraction approach followed, prior to that, plant-based materials are generally subjected to pre-treatments (Krakowska-Sieprawska et al., 2022). Due to its high moisture (approximately 75-80% w/w) (Ancuța Chetrariu & Dabija, 2020), as well as fermentable sugar and protein contents, fresh BSG is susceptible to degradation by microbial activity. Consequently, industrial valorisation of BSG generally requires drying as pre-treatment, thereby limiting microbial growth and increasing the storage time at room temperature (Mussatto et al., 2006). The most used method is oven-drying, that despite its

high energy demand and time consumption and its impact on the chemical composition of BSG (Lynch et al., 2016; Mussatto et al., 2006), is still the most cost-efficient technique for industrial valorisation of BSG.

BSG extracts have a heterogenous chemical composition, which may vary according to the barley variety employed for the brewing and all the parameters applied during the process. Therefore, a deep characterisation of the main compounds found in BSG extracts, and the addition of these identified compounds to our in-house database is a first step that was undertaken to facilitate the further investigation of BSG extracts.

This chapter is divided in 3 sections:

- *The first one mainly focuses on the characterisation of BSG extracts; at first stage as many compounds as possible were identified and secondly the antioxidant capacity of the extracts measured. Additionally, a new group of molecules will be described.*
- *The second part focuses on comparing different pre-treatments for BSG; extracts from untreated BSG (wet starting material) were compared with those from dried BSG (most frequently used pre-treatment), highlighting the impact of oven-drying on the chemical composition and the antioxidant properties of BSG extracts.*
- *The third section describes two different methods for the purification of hordatines: one through the application of a resin and the other one by performing a chromatographic fractionation.*

These three sections have a common goal, which is the first objective of this thesis: to obtain a valid extraction protocol, potentially applicable at larger scale, while focussing on the antioxidant capacity of the extracts.

2 Identification of Amadori products and oligosaccharide-glycated amino acids in brewer's spent grain extract

Highlights

- *A first characterisation of BSG extracts based on the most often used food grade extraction process has been performed. As many as possible compounds were identified to build a in-house database (enriched each time with new identifications as analyses occurred with new data). This database is developed in an internal project called DEREPE that aims to facilitate the identification process in further analyses. It is currently used and continuously implemented.*
- *A new class of products of the early stage Maillard cascade, known as Amadori products, have been identified and described in brewer's spent grain extracts for the first time.*
- *The highest antioxidant properties of BSG extracts resulted in 1.38 mg GAE/g dw BSG and 6.9 $\mu\text{mol FeSO}_4$ / g dw BSG, respectively measured through Folin-Ciocalteu and FRAP assays.*

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Abstract

Brewer's spent grain (BSG) is the most abundant by-product of the brewery industry. Its accessibility and chemical composition allow this material to be recycled for new applications mainly targeting the food industry, based on the health-promoting properties of phenolics in BSG extracts. This study investigates the major compounds identified in food-grade extracts from BSG by UHPLC-ESI-MS/MS analyses, with a focus on the presence of Amadori products. These fructosyl-derivatives are identified for most of the proteinogenic amino acids but for instance also for gamma-aminobutyric acid. While previously proposed to be formed, I show that Amadori products are formed by reaction of amino acids with oligosaccharides up to five hexoses. Moreover, the same glycation pattern was also observed for other nitrogen-containing compounds such as phosphatidylethanolamine lipids. The presence of these Maillard reaction products and glycated forms of amino-compounds, due to heat-treatment during brewing or stabilization of BSG prior to further processing, may impact the valorisation of this by-product and should thus be considered in future studies.

2.1 *Introduction*

In the context of circular economy, there is currently a focus on the upcycling of by-products from industrial processes. The food industry, in general, and the brewery sector, in particular, have a long tradition of re-use of their by-products, however upcycling remains often limited. The circular use of material aims at the efficient use of resources and, through that, at environmental protection. It is coupled with the increased economic development (Ancuța Chetrariu & Dabija, 2020) and is the focus of development in more and more countries. Keywords in this development are re-use, recycling and upcycling, and the term residue takes on another meaning: opportunity. As for all waste/residue streams, this also counts for residual biomass from forestry, agriculture, and industrial activities.

The global production of beer reached 1.9 billion hectoliters in 2022, with 343 million hectoliters being produced in Europe. For Europe, this results yearly in the production of 6.8 million tons of brewer's waste, that could be up/recycled into new commodities. Non-seasonality, nutritional value and low price of brewery by-products are facilitating their use as component of food, medical and biotechnological products (Karlović et al., 2020).

Barley, accounting for 19% of the European cereal production in 2020, is one of the most cultivated cereals after maize (23.7%) and wheat (41.6%). It is mainly used for animal feed and human consumption, with 21% of the cultivated barley destined for beer production (Mussatto et al., 2006). During brewing, barley is germinated and the germinated grains are subsequently dried at high temperature during the kilning, process to become malt. Malt is milled and again exposed to high temperatures during mashing, a process, during which starch is enzymatically degraded. The enzymatic degradation produces a liquid with carbohydrates, soluble proteins, polypeptides, and amino acids, called wort, and a solid fraction containing residues of the grains. This suspension is filtered (lautering step), hop is added before boiling and the resulting wort is used as a

fermentation base for yeast to produce beer. During the steps prior to lautering, the exposure to high temperatures induces the formation of compounds which, together with the addition of different types of hop, the formed by-products by yeast metabolism and the stability of flavour compounds during storage, determine the taste of the final product (Oladokun et al., 2017).

Today, the beer industry is looking for new types of products and increased productivity, which include the efficient reuse of its biomass waste (Rachwał et al., 2020). The first and most abundant biomass by-product originating from brewing is brewer's spent grain (BSG); it is discarded after the filtration step (lautering) and is a water-rich solid phase composed of the grain residues. Afterwards, a second waste is removed: spent hop, which represents the precipitated compounds, mostly proteins, after the boiling step. The third residue eliminated before bottling is spent yeast, a by-product reused in brewing (Anca Corina Fărcaș et al., 2017).

BSG represents about 85% of the total by-product: around 20 kg of wet spent grains are generated for each hectoliter of beer. BSG contains the husks, pericarp, and seed coat of the grains. It is rich in cellulose and non-cellulose polysaccharides, lignin, proteins, and lipids. The exact composition is influenced by the grain variety used, the agricultural methods and brewery's process conditions (Mussatto et al., 2006). Globally, spent grain contains 15-30% proteins, 20-25% hemicelluloses, 12-25% cellulose, 12-28% lignin and 6-10% lipids. It furthermore contains vitamins, phenolic compounds and minerals (Lynch et al., 2016; Rachwał et al., 2020). Due to its high moisture (approximately 75-80% w/w) (Ancuța Chetrariu & Dabija, 2020), fermentable sugar, and protein contents, fresh BSG is susceptible to degradation by microbial activity. In this context, industrial valorisation of BSG generally requires drying as pre-treatment, thereby limiting microbial growth and increasing the storage time at room temperature (Mussatto et al., 2006). Most used are oven- and freeze-drying. Although studies indicate that freeze-drying is likely the best technique to maintain the chemical properties of the starting

material, it is not economically sustainable on an industrial scale (Lynch et al., 2016). This makes oven-drying the most feasible technique for industrial valorisation of BSG, despite its high energy consumption and influence on the chemical composition (Lynch et al., 2021; Mussatto et al., 2006).

Because of the high amount of fibers and proteins and its low price, BSG is mainly used for animal feeding. It is known as a source of nitrogen and as a promoter of milk production in ruminants (Mussatto et al., 2006). However, environmental and economic factors make other uses more appropriate (Steiner et al., 2015). Indeed, due to the richness of its chemical composition, new potential uses of BSG have arisen in recent years, and BSG is now studied or used as an accessible and cheap base material in biorefinery, biotechnology, nutrition and medicine (Ancuța Chetrariu & Dabija, 2020). Many applications are being screened: biogas production through anaerobic degradation; bricks for building; substrate for fermentation processes or to grow microorganisms and as source of enzymes, molecules or prebiotics (Ancuța Chetrariu & Dabija, 2020). In nutrition, milled BSG can be used as flour or as component in the production of snacks (Lynch et al., 2016). Despite this wide range of uses, most research on BSG focusses on the health-promoting potential of BSG extracts due to the presence of antioxidants such as vitamin E and phenolic compounds (Bonifácio-Lopes et al., 2020), arabinoxylans, beta-glucans and fatty acids. Another alternative use of BSG is as source of proteins with interesting gelation properties (Hellebois et al., 2021).

In previous studies, BSG extracts have been analysed for the identification of health-promoting compounds, with absolute quantification of ferulic acid, p-coumaric acid and catechin often used as quality markers (Moreira et al., 2013a; Rachwał et al., 2020). Other parameters generally assessed are the TPC and the antioxidant capacity. The TPC of BSG changes according to the malt type, and values ranging from 0.4 to 3.3 mg GAE/g dw have been reported (Socaci et al., 2018a).

In addition to the above-mentioned, well-characterized molecules, reaction products between amino acids and reducing sugars, known as Amadori products, have been identified. Amadori compounds (Figure 2.1-1A, B and C), 1-amino-1-deoxyketoses, represent a class of intermediates in the Maillard reaction. More precisely, their formation takes place during the initial phase of this cascade (Davidek et al., 2005a; Xue et al., 2020). A reducing sugar reacts with a free amino group forming a glycosylamine, called a Schiff base. This unstable condensation product reversibly and spontaneously rearranges itself to generate Amadori compounds. Because the formation of Amadori products is faster than the reverse reaction and is promoted in acid conditions, these keto-amine structures are the predominant form found in equilibrium conditions (Troise et al., 2018). In a later stage of the Maillard reaction, degradation of Amadori products gives rise to oxoaldehydes, highly reactive dicarbonyl compounds with glyoxal, methylglyoxal and 3-deoxyglucosone being the most important. Further reaction of these oxoaldehydes results in advanced glycation end products (AGEs), generated by irreversible reactions between carbonyl and amino groups. As proposed by Hodge in 1955 (Hodge, 1955), a series of reactions starting from Amadori products and derivatives lead to the formation of volatile compounds and brown pigments, such as melanoidins, that give colour, aroma and flavour to processed food (Hodge, 1955; Hofmann & Schieberle, 2000). This mechanism occurs mainly during long-term storage and thermal processing of sugar-rich food (Del Castillo et al., 1999; Meitinger et al., 2014). The first objective of the work presented here was to identify as much as possible compounds found in BSG extracts obtained with the most frequently used solvent for food-grade extractions. With this effort, a database was built to facilitate the identification of compounds in further studies on BSG extracts. Therefore, yield optimization was not the purpose of the work presented here. Besides all the compounds generally found in BSG extracts, I identified several early-stage products of the Maillard cascade, known as Amadori products, which have not yet been described in BSG extracts so far. In addition, not only did I identify Amadori products for most proteinogenic amino acids, but Amadori

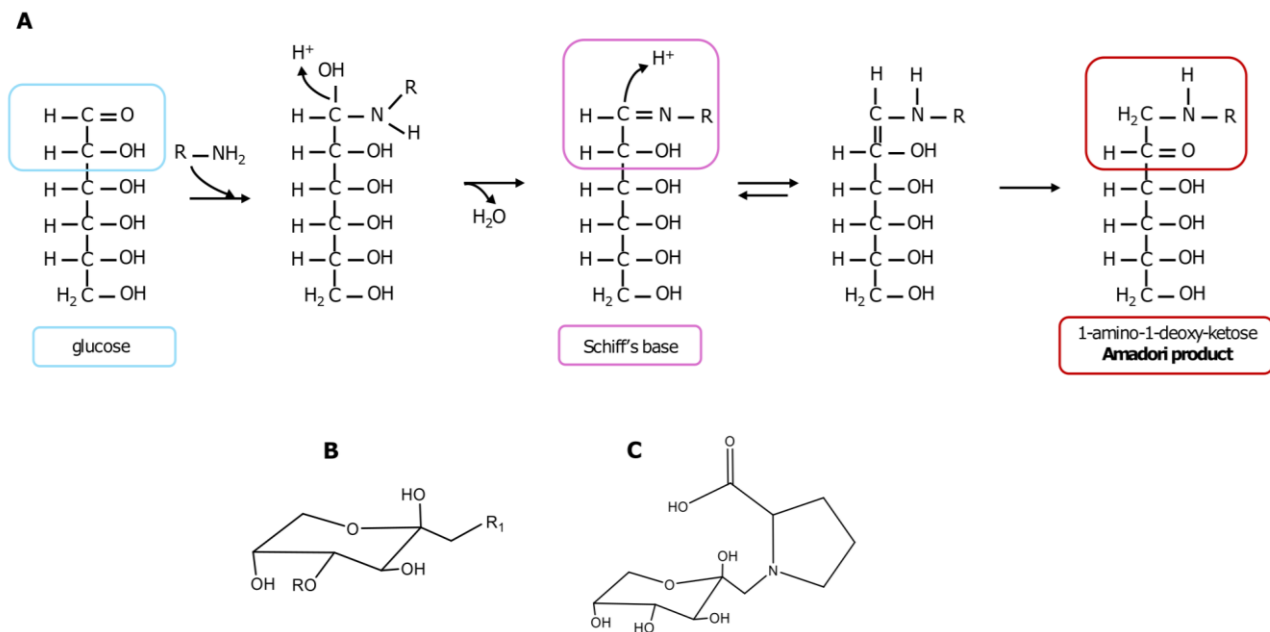


Figure 2.1-1 (A) Example of Amadori formation at early stage of Maillard reaction; (B) Typical chemical structure of Amadori products: $R = [Hex]_n$ with n from 0 to 4 in the compounds identified in this study; $R_1 =$ specific amino acid (Arg; Gly; Lys; Ala; GABA; Pro; Val; Met; pGlu; Tyr; Ile/Leu; Phe; Trp; His). (C) Structure of fructosyl-Pro.

reaction products between free amino acids and oligosaccharides of up to 5 monomers were also identified. In malt, disaccharide-derived glycated lysine (N- ϵ -maltulosyllysine) was previously identified and characterized and the presence of other disaccharide linked amino acids was hypothesized (Hellwig & Henle, 2020). Here, I confirm this hypothesis and extend it by identifying glycation products with oligosaccharides for most amino acids. Similar products were identified for non-proteinogenic amino acids and for phosphatidylethanolamine lipids. To our knowledge, these products have not been previously identified in BSG extracts and the formation of di- to pentasaccharide-glycated amino acids is not mentioned in literature.

2.2 *Materials and Methods*

2.2.1 Chemicals

The following chemicals were obtained commercially. Ethanol (WMR 20821.321); formic acid (LGC Standards-Promochem, SO-9679-B001); acetonitrile (Carl Roth, HN40.2); Folin-Ciocalteu reagent (Sigma Aldrich, F9252-100 ML); Na₂CO₃ (Sigma Aldrich, 223530-500G); gallic acid (Sigma Aldrich; 27645-250G-R); Hydrochloric acid (HCl) 0.1 M (ChemLab, 31955.373); Iron(III) chloride hexahydrated (FeCl₃ * 6 H₂O) (Merck VWR, 1.03943.250); Iron (II) sulfate heptahydrate (FeSO₄ * 7 H₂O) (VWR; 24244.232); Sodium acetate trihydrate (C₂H₃NaO₂ * 3 H₂O) (Sigma Aldrich, 331058-100G); Acetic acid, glacial (CH₃CO₂H) (J.T.Baker, 9524-33); Fluorescein (Sigma-Aldrich, 46955-100G-F); Trolox (Sigma-Aldrich, 238813-1G); AAPH (Sigma-Aldrich, 440914-25G); Potassium phosphate monobasic (KH₂PO₄) (Merck, 1.04873.0250); Potassium phosphate dibasic (K₂HPO₄) (Vel VWR, PROL26931.263)

2.2.2 Material

Fresh BSG was obtained from a local brewery (Brasserie Simon Sàrl, Wiltz, Luxembourg) and immediately frozen, vacuum-packed, and stored in polypropylene bags at -20°C. Subsequently BSG was dried

in an air convection oven at 50°C for 48 h as previously described (Bonifácio-Lopes et al., 2020), vacuum-packed and stored in polypropylene bags at room temperature.

2.2.3 Solvent extraction procedure

Three different extraction solvents were used: a) water b) 20% ethanol (v/v%) and c) 40% ethanol (v/v%). The three extractions were carried out at the ratio 30:1 (v:w; solvent:solid), based on literature and EU food-grade regulations (Meneses et al., 2013). For the extractions, dried BSG was ground with mortar and pestle and 30 mg of BSG were mixed with 1 mL of solvent (MilliQ water, ethanol 20%; ethanol 40%) and shaken at 1400 rpm for 4 hours at 21°C. Samples were centrifuged at 20 000 g at 15°C for 25 min. The supernatants were collected and dried with a vacuum concentrator (CentriVap; Labconco, Kansas City, MO, US). All extractions were performed in quadruplicate. Dried extracts were then dissolved in 300 µl MeOH:H₂O 5:95 and filtered on 0.22 µm PTFE syringe filters (Millex-LG; Merck KGaA, Darmstadt, Germany) prior to analysis.

2.2.4 Chemical assays

Total phenolic content (Folin-Ciocalteu)

The TPC has been measured following the method described by Singleton and Rossi (Singleton & Rossi Jr., 1965) with some modifications. Twenty-five µL of Folin-Ciocalteu reagent, 1N diluted twice from a 2N stock (Sigma Aldrich, F9252-100 ML), and 50 µL of extracted samples and standard dilutions were prepared and mixed in a 96-well plate and 125 µL 7,5% w/v Na₂CO₃ solution were added. The plate was covered with aluminium foil and incubated for 30 min at room temperature. The absorbance was detected with a spectrophotometric microplate reader (Spark, Tecan, Männedorf, Switzerland) at 765 nm. A standard solution of gallic acid was used to build the calibration curve (0.004; 0.008; 0.0125; 0.016; 0.025; 0.03; 0.05 mg/ mL) ($y = 4.0529x + 0.0407$; $R^2 = 0.9993$) and the results of TPC content were expressed as gallic acid equivalent (GAE). For each

extraction, four biological replicates were analysed in technical duplicates.

Ferric reducing/antioxidant power assay (FRAP)

The FRAP assay consists of the measurement of antioxidant capacity linked to the reducing power of molecules to convert Fe (III) into Fe (II) (Benzie & Strain, 1996). Ten μL of extracted samples were mixed with 190 μL of freshly prepared FRAP reagent (300mM acetate buffer; 10mM ferric-tripyridyltriazine; 20mM FeCl_3 ; pH 3.6) in a 96-well plate, covered with aluminium foil and incubated at room temperature for 20 min. A standard curve was constructed using FeSO_4 at different concentrations: 0; 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1 mM ($y = 0.6787x + 0.077$; $R^2 = 0.9981$) and the results were expressed as μM of ferrous equivalent. The absorbance was detected with a spectrophotometric microplate reader (Spark, Tecan) at 595 nm. For each extraction, four biological replicates were analyzed in technical duplicates.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay measures the free radical oxidation of fluorescein by the change in its fluorescence intensity. The ORAC values of the samples were measured using a 96-well fluorescence microplate reader (Spark, Tecan) adapting the method of Gillespie et al. (Gillespie et al., 2007). Samples were diluted (100x) and 25 μL diluted sample used for analysis. In each microplate well, 150 μL of fluorescein (0.08 μM of fluorescein in phosphate buffer 75mM) and 25 μL of sample or blank (phosphate buffer 75 mM, pH 7) or Trolox standards (1-20 μM) were added and mixed. The microplate was pre-incubated in the reader for 10 min at 37°C and AAPH (2,2'-Azobis(2-amidinopropane)) solution (40 mg/ mL) was added. The fluorescence was recorded every 5 min for a total of 12 cycles (60 min) and raw data were exported for further calculations. Trolox was used to build the standard curve ($y = 0.5745x$, $R^2 = 0.9799$) and results were expressed as μmol Trolox equivalents (TE)/g sample. For each

extraction, four biological replicates were analysed in technical duplicates.

2.2.5 UHPLC-ESI-MS/MS analysis

Five μL of each BSG extract were injected into an Acquity UPLC I-class ultra-high pressure liquid chromatography (UHPLC) system (Waters, Milford, MA, USA) coupled to an electrospray ionization high-resolution mass spectrometer (TripleTOF 6600+; SCIEX, Framingham, MA, USA) operating in both positive and negative ionisation modes. Formic acid 0.1% in water (A) and formic acid 0.1% in acetonitrile (B) were used in the following gradient: 0 min, 1% B; 4 min, 1% B; 16 min, 5% B; 35 min, 40% B; 45 min, 100% B; 50 min, 100% B; 54 min, 1% B; 60 min, 1% B. The flow was set at 0.5 mL/min and the temperature of the reverse-phase Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 μm particle size, Waters, Milford, MA, USA) was maintained at 50°C.

The electrospray ionisation parameters were set as follows: the source was heated at 650 °C, an ion spray voltage of 4.5 and -4.5 kV was applied for the positive and negative modes, respectively, with curtain gas (nitrogen) set at 30 psi; nebulizer gas (air) at 55 psi; and turbine gas (air) at 50 psi. The declustering potential was set at 60 and -60 V in positive and negative modes, respectively. Survey scans were acquired in 175 ms for information-dependent acquisition, and only the 10 most intense monocharged precursor ions were fragmented to produce MS^2 spectra in high sensitivity mode when a threshold of 100 counts per s was reached, with an accumulation time of 200 ms. The total cycle time was fixed at 2.2 s. A sweeping collision energy of 15 V below and above 15 V in positive and -15 V in negative mode was applied to all precursor ions. The dynamic exclusion was set for 2 s after three occurrences before the precursor could be fragmented again. Full high-resolution MS spectra were recorded between 100 and 2000 mass-to-charge ratio (m/z) for MS, whereas MS^2 scans were recorded between 50 and 2000 m/z , in profile mode.

2.2.6 Data analysis

For the identification of the compounds found in BSG extract, the raw positive and negative data-files were analysed in Progenesis QI (v2.3, Nonlinear Dynamics, Newcastle upon Tyne, UK). Since the aim was to build an as broad as possible database with compounds found in BSG, no adducts were considered in the analysis. In Progenesis, runs were aligned, peaks with the same molecular weight within a specified retention time frame grouped and the peak intensity of grouped peaks normalized and used for quantification. Peaks (retention time x m/z couples) without fragmentation spectrum were filtered out as were peaks corresponding to known or suspected contaminants; the remaining RT-m/z couples were used for identification. Initially, identifications within Progenesis were done based on MS/MS similarity with the NIST and MassBank databases. Compounds not found in these databases were identified through literature search and MS/MS comparison with the following databases: LipidMaps (<https://www.lipidmaps.org/>); HMBD (<https://hmdb.ca/spectra/ms/search>); GNPS (<https://gnps.ucsd.edu/ProteoSAFe/libraries.jsp>); Metlin (<https://metlin.scripps.edu>) and PubChem (<https://pubchem.ncbi.nlm.nih.gov>). For other compounds, comparison between experimental and theoretical MS/MS spectra was done using MetFrag (<https://ipb-halle.github.io/MetFrag/>) and CFM-ID (<https://cfmid.wishartlab.com/>). All identifications were used to create an in-house database of compounds identified in BSG. For this an msp-type file was created and used for dereplication. Close to all identifications reported here are level 2 based on m/z of the precursor and comparison of the experimental MS/MS with experimental or predicted MS/MS spectra from the sources cited above (Salek et al., 2013). Each identification was considered reliable with a MS² fragmentation score $\geq 75\%$ was attained and the retention time matches with that in the database.

2.3 Results

2.3.1 Compounds identified in BSG

BSG is mainly composed of the pericarp, coat, and husks of barley grains. It is rich in sugars, lignin and proteins, although its exact composition changes based on the used barley variety, harvesting, malting and mixing time (Ancuța Chetrariu & Dabija, 2020). The composition of BSG extracts likewise depends on these biological and process-related parameters (Steiner et al., 2015).

With the aim of identifying as many compounds as possible in BSG extracts and building an in-house database, 3 different extraction methods were performed using either milliQ water or milliQ water combined with 20% or 40% (v/v%) of ethanol as solvents at 21°C for 4 hours. Extracts were made and analysed with UHPLC-ESI-MS/MS, the data imported in Progenesis QI and the compounds corresponding to the most intense peaks identified. Each identification was considered reliable with a MS² fragmentation score $\geq 75\%$. The different extractions did not result in major qualitative changes in composition, but quantitative changes were observed. The aim of the current study is however not on these relative quantitative differences, but on the profiling of BSG extracts and the identification of reaction products between amino acids and reducing sugars not commonly identified in BSG. To build a database as comprehensively as possible, multiple adducts of the same compound were considered as individual database entries.

A total of 381 compounds were identified in the BSG extracts (Figure 2.3-1). Oligosaccharides were detected at retention times between 0 and 5 min. Oligo-hexoses containing 1 to 9 hexose residues (retention time (RT) 0.86 min m/z 1475.47, negative mode) were identified based on m/z and MS/MS, while ions corresponding to longer oligosaccharides were detected as doubly charged but not fragmented. Free proteinogenic amino acids, as well as Amadori compounds, were also identified within this retention time frame (Table 2.3-1). Most of these reaction products between amino acids and reducing sugars have

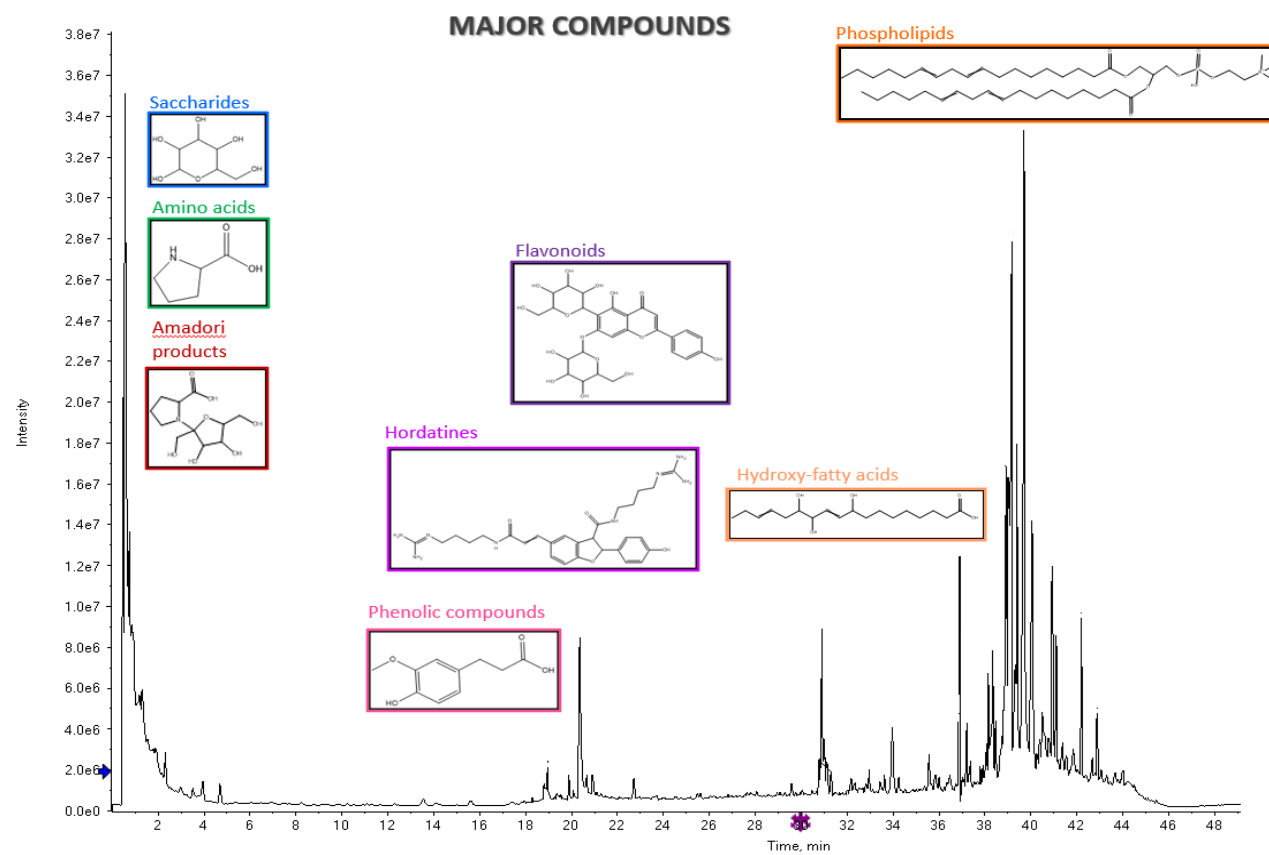


Figure 2.3-1 Main categories of compounds identified in brewer's spent grain (BSG) extracts with LC-MS/MS analysis. Following retention time: series of saccharides, amino acids, Amadori products, phenolic compounds, hordatines, flavonoids, hydroxy-fatty acids and lipids.

not been described in BSG extracts and represent a class of important intermediates in early stages of the Maillard reaction. These will be discussed separately. Cyclic dipeptides formed non-enzymatically under high-temperature conditions, known as diketopiperazines have been detected from 1 to 25 min. Twelve diketopiperazines containing mainly proline coupled with histidine, serine, lysine, arginine, alanine, tyrosine, leucine, phenylalanine, and tryptophan have been identified (supplemental file1).

Table 2.3-1 Free amino acids detected in brewer's spent grain extracts (BSG) with LC-MS/MS in positive and negative ionization mode. Retention time, molecular weight-to-charge ratio and ionization mode are shown.

RT min	m/z	Compound	Ion
0.55	148.0595	glutamic acid	[M+H] ⁺
0.94	182.0806	tyrosine	[M+H] ⁺
1.29	132.1013	isoleucine	[M+H] ⁺
2.34	166.0858	phenylalanine	[M+H] ⁺
4.7	205.0957	tryptophan	[M+H] ⁺
RT min	m/z	Compound	Ion
0.51	131.0450	asparagine	[M-H] ⁻
0.51	146.0448	glutamic acid	[M-H] ⁻
0.51	154.0611	histidine	[M-H] ⁻
0.9	128.0345	pyroglutamic acid	[M-H] ⁻
0.94	180.0655	tyrosine	[M-H] ⁻
2.31	164.0709	phenylalanine	[M-H] ⁻
4.68	203.0817	tryptophan	[M-H] ⁻

From 5 to 20 min, phenolics and hordatines were detected. According to Gorzolka et al. (Gorzolka et al., 2016), hordatines are highly abundant in BSG residue and present in BSG extracts (Steiner et al., 2015) made from barley based malt. Benzoxazinoids, typically found in BSG from rye or wheat based beers (Pieczonka, Paravicini, et al.,

2021; J. Pihlava & Kurtelius, 2016) were not detected, in agreement with the used BSG. Hordatines are agmatine derivatives of ferulic acid and p-coumaric acid, identified in barley as homo- or heterodimers. Homodimer of p-coumaroyl-agmatine corresponds to hordatine A; homodimer of feruloyl-agmatine to hordatine C and heterodimer of feruloyl-agmatine and p-coumaroyl agmatine to hordatine B. Hordatine D, only identified during MALDI analysis of our extracts (data not shown), is a heterodimer of sinapoyl-agmatine and feruloyl-agmatine. Glycosides of hordatine A and B, known as hordatine M, and diglycosides of these two hordatines were also identified as were hydroxy- and dihydroxylated hordatine A, B and C (Steiner et al., 2015). Furthermore, the hydroxycinnamoyl-agmatine precursors of these compounds were identified. Besides agmatine, other phenolamides, monomers and dimers of ferulic acid and p-coumaric acid conjugated to other polyamines such as putrescine and spermidine were also identified (supplemental file 1).

The phenolic compounds generally identified in BSG extracts were identified in the same time frame (5 to 20 min), namely: ferulic acid, p-coumaric acid and +/-catechin. Flavones such as saponarin, luteolin-6-glucoside and dihydrokaempferol and a flavin (riboflavin, vitamin B2), were identified at higher retention times (18 to 25 min). Among the BSG extracts analysed, the 40% ethanol extract had a higher TPC (1.38 mg GAE/g dw) than extracts obtained with milliQ water and 20% ethanol (0.74 mg GAE/g dw and 0.96 mg GAE/g dw respectively) (Figure 2.3-2). The results of the analysis of the antioxidant capacity measured with the FRAP assay are in accordance with the TPC measurement since 40% ethanol extracts showed the highest antioxidant activity (6.9 $\mu\text{mol FeSO}_4/\text{g DW}$ 40% ethanol > 3.78 $\mu\text{mol FeSO}_4/\text{g dw}$ 20% ethanol > 2.73 $\mu\text{mol FeSO}_4/\text{g dw}$ milliQ water). In addition, the ORAC assay showed the same trend, confirming that the extract with 40% ethanol had a higher antioxidant capacity (0.85 mmol TE/ g BSG) compared to the other 2 extractions (data not shown).

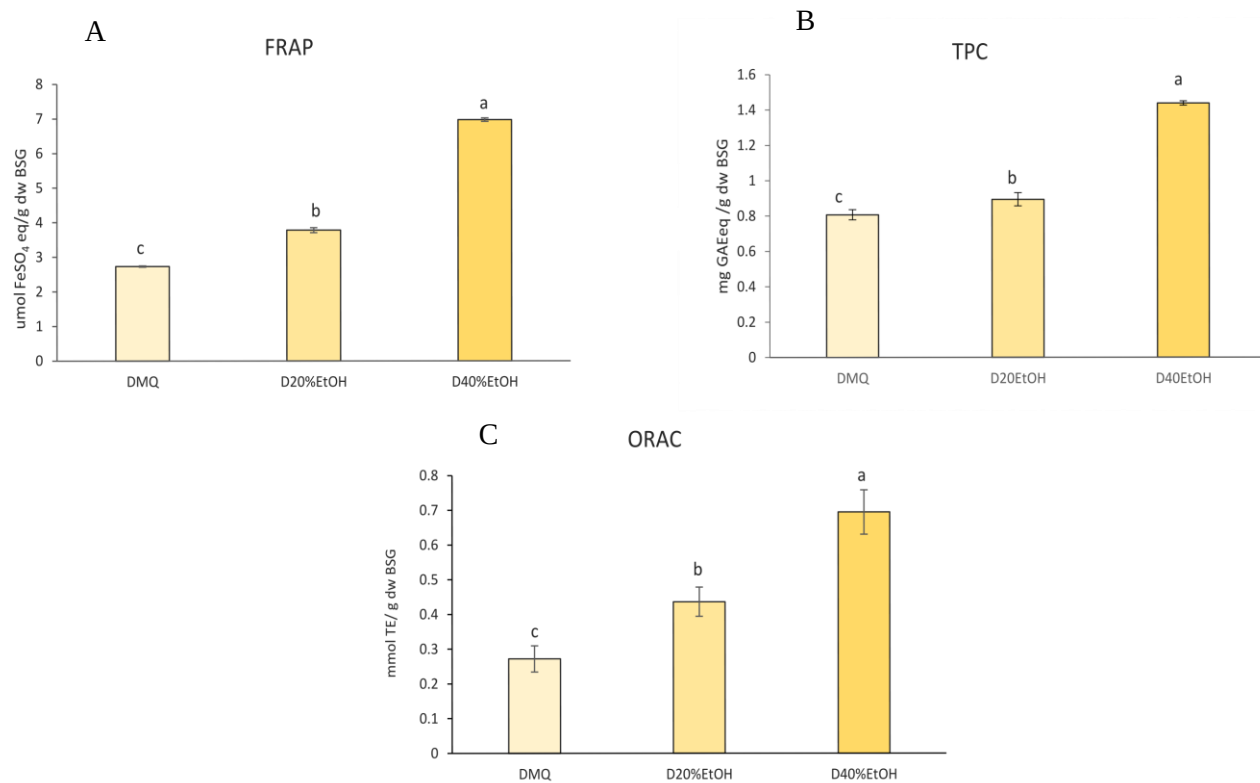


Figure 2.3-2 Antioxidant capacity of brewer's spent grain (BSG) measured by FRAP (A) and by ORAC (C), and the total phenolic content (TPC) (B) D indicates extracts from ground oven-treated BSG. The annotations MQ, 20 and 40 respectively indicate extracts obtained with milliQ water, 20% EtOH and 40% EtOH.

Tricin lignans and mono-, di- and trihydroxy fatty acids (18:1 to 18:3), were detected from 25 to 30 min. The highest peak in this range corresponds to hydroxylated C18:2 fatty acid, and multiple peaks correspond to di/trihydroxyoctadec-n-enoic acid (DiHOME, TriHOME) or di/trihydroxyoctadec-n,n-enoic acid (DiHODE, TriHODE) compounds. Numerous nitrogen-containing compounds are also detected. These currently unidentified compounds have a pattern of derivatisation with oligosaccharides comparable to that observed for amino acids, suggesting the presence of an amino group subject to the first stages of the Maillard reaction.

From 30 to 45 min, mono-acyl lipids were identified. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and monoacylglycerol (MG) are the main classes of lipids detected in the present study and in BSG extracts reported in literature (Anca C. Fărcaș et al., 2015; Niemi et al., 2012) and their acyl-composition is dominated by C18:2 and C16:0 fatty acids. Nonetheless, PC-containing C14; C16; C18; C18:1; C18:2, C18:3 fatty acids were identified in both positive and negative modes (supplemental file 1). Monoacyl-phosphatidylethanolamines were mainly identified as PE(18:2) and PE(16:0), both unglycated but also glycated with mono- and disaccharides as previously described in literature (Lin et al., 2018; Sookwong et al., 2011). MG and their galactosylated forms, respectively: digalactosylmono- and diacylglycerols (DGMG and DGDG) containing C16:0 and C18:2 fatty acids were also detected.

3.2.3.1 Amadori products

As mentioned above, and known from literature, free amino acids and hexose-oligomers were identified in our extracts. Knowing the potential coupling of these compounds through the first steps of the Maillard reactions, I systematically screened our data to identify Amadori products generated by condensation between a reducing sugar and compounds containing a -NH₂ group (Davidek et al., 2005b; Xue et al., 2020) (Figure 2.1-1A, B and C).

Since glucose is the most abundant sugar monomer in malt, glucose reaction products were observed, and I identified Amadori products with most of the proteinogenic amino acids and with the non-proteinogenic amino acid gamma-aminobutyric acid (GABA). The combination of positive and negative mode analyses resulted in the identification of products of such reaction for fifteen of the twenty proteinogenic amino acids: Gly, Lys, Ala, Val, Ile/leu, Met, Tyr, Phe, Trp, Pro, Arg, His and Glu/Gln (identified as pyro-Glu as discussed below) (Table 2.3-2 and Table 2.3-3).

Table 2.3-2 Fructosyl-amino acids detected in brewer's spent grain (BSG) extracts with LC-MS/MS in positive and negative ionization modes. Retention time, molecular weight-to-charge ratio and ionization mode are shown, amino acids are given in the three-letter code, pGlu: Pyroglutamate.

RT min	m/z	Compound	ion
0.51	337.1708	fructosyl-Arg	[M+H] ⁺
0.51	220.0808	fructosyl-Gly – H ₂ O	[M+H] ⁺
0.51	309.1649	fructosyl-Lys	[M+H] ⁺
0.55	234.0964	fructosyl-Ala - H ₂ O	[M+H] ⁺
0.55	266.1224	fructosyl-GABA	[M+H] ⁺
0.55	248.1104	fructosyl-GABA - H ₂ O	[M+H] ⁺
0.59	278.1227	fructosyl-Pro	[M+H] ⁺
0.78	280.1382	fructosyl-Val	[M+H] ⁺
0.78	262.1281	fructosyl-Val - H ₂ O	[M+H] ⁺
0.9	294.0999	fructosyl-Met - H ₂ O	[M+H] ⁺
0.9	290.0865	fructosyl-pGlu	[M-H] ⁻
0.94	272.0759	fructosyl-pGlu - H ₂ O	[M-H] ⁻
1.01	344.133	fructosyl-Tyr	[M+H] ⁺
1.01	326.1221	Fructosyl-Tyr – H ₂ O	[M+H] ⁺
1.56	292.13/294.15	fructosyl-Ile/Leu	[M-H] ⁻ / [M+H] ⁺
1.56	274.12/276.14	fructosyl-Ile/Leu - H ₂ O	[M-H] ⁻ / [M+H] ⁺
2.96	326.12 / 328.13	fructosyl-Phe	[M-H] ⁻ / [M+H] ⁺

RT min	m/z	Compound	ion
2.96	292.1174	fructosyl-Phe - 2 H ₂ O	[M+H] ⁺
2.96	308.11/310.12	fructosyl-Phe - H ₂ O	[M-H] ⁻ / [M+H] ⁺
5.36	365.13/367.14	fructosyl-Trp	[M-H] ⁻ / [M+H] ⁺
5.36	347.12/349.13	fructosyl-Trp - H ₂ O	[M-H] ⁻ / [M+H] ⁺

Table 2.3-3 [Hex]*n*- fructosyl-amino acids, detected in brewer's spent grain (BSG) extracts with LC-MS/MS in positive and negative ionization mode. Retention time, molecular weight-to-charge ratio and ionization mode are shown, amino acids are given in the three-letter code, pGlu: Pyroglutamate.

RT min	m/z	Compound	Ion
0.51	480.1809	fructosyl-His hex	[M+H] ⁺
0.51	469.2005	fructosyl-Lys hex	[M-H] ⁻
0.55	428.1751	fructosyl-GABA hex	[M+H] ⁺
0.59	440.1753	fructosyl-Pro hex	[M+H] ⁺
0.59	422.1646	fructosyl-Pro hex - H ₂ O	[M+H] ⁺
0.90	440.17/442.19	fructosyl-Val hex	[M-H] ⁻ / [M+H] ⁺
0.9	424.18.05	fructosyl-Val hex – H ₂ O	[M+H] ⁺
1.01	602.22/604.24	fructosyl-Val dihex	[M-H] ⁻ / [M+H] ⁺
1.06	472.14/474.16	fructosyl-Met hex	[M-H] ⁻ / [M+H] ⁺
0.98	452.1378	fructosyl-pGlu hex	[M-H] ⁻
1.02	614.1902	fructosyl-pGlu dihex	[M-H] ⁻
1.25	776.2428	fructosyl-pGlu trihex	[M-H] ⁻
1.49	938.2952	fructosyl-pGlu tetrahex	[M-H] ⁻
1.21	504.16/506.18	fructosyl-Tyr hex	[M-H] ⁻ / [M+H] ⁺
1.21	488.1742	fructosyl-Tyr hex - H ₂ O	[M+H] ⁺
1.32	668.2378	fructosyl-Tyr dihex	[M+H] ⁺
1.88	454.19/456.20	fructosyl-Ile/Leu hex	[M-H] ⁻ / [M+H] ⁺
1.91	438.1964	fructosyl-Ile/Leu hex - H ₂ O	[M+H] ⁺

RT min	m/z	Compound	Ion
2.22	600.2484	fructosyl-Ile/Leu dihex-H ₂ O	[M+H] ⁺
2.22	616.24/618.25	fructosyl-Ile/Leu dihex	[M-H] ⁻ /[M+H] ⁺
2.23	940.3482	fructosyl-Ile/Leu tetrahex	[M-H] ⁻
2.66	778.29/780.31	fructosyl-Ile/Leu trihex	[M-H] ⁻ /[M+H] ⁺
3.32	940.3481	fructosyl-Ile/Leu tetrahex	[M-H] ⁻
3.77	472.1799	fructosyl-Phe hex- H ₂ O	[M+H] ⁺
4.51	488.17/490.19	fructosyl-Phe hex	[M-H] ⁻ /[M+H] ⁺
4.55	634.2330	fructosyl-Phe dihex - H ₂ O	[M+H] ⁺
4.57	650.22/652.24	fructosyl-Phe dihex	[M-H] ⁻ /[M+H] ⁺
4.68	812.2801	fructosyl-Phe trihex	[M-H] ⁻
6.43	527.18/529.20	fructosyl-Trp hex	[M-H] ⁻ /[M+H] ⁺
6.44	511.1909	fructosyl-Trp hex - H ₂ O	[M+H] ⁺
6.44	689.2368	fructosyl-Trp dihex	[M-H] ⁻

In positive mode, the fragmented peaks correspond to dehydrated Amadori products for fructosyl-Gly, fructosyl-Ala, fructosyl-Met and fructosyl-Iso/Leu. In all these cases, an ion of lower intensity but identical elution profile is observed at the predicted molecular weight for the non-dehydrated compound. In MS, peaks corresponding to the neutral loss of 2 H₂O and (- CO - H₂O) from the non-hydrated compound are also often observed, corresponding to the typical fragmentation pattern presented in Figure 2.3-3 and resulting in spectra identical to published data (Davidek et al., 2005b). For fructosyl-Val, fructosyl-Phe, fructosyl-Trp and fructosyl-GABA, both the non-dehydrated and de-hydrated forms were fragmented and identified. Most of fructosyl-amino acids were identified in positive mode only, except for fructosyl-Ile/Leu; -Phe and -Trp, which were detected in both ionisation modes and fructosyl-pGlu that was only found in negative ionization mode.

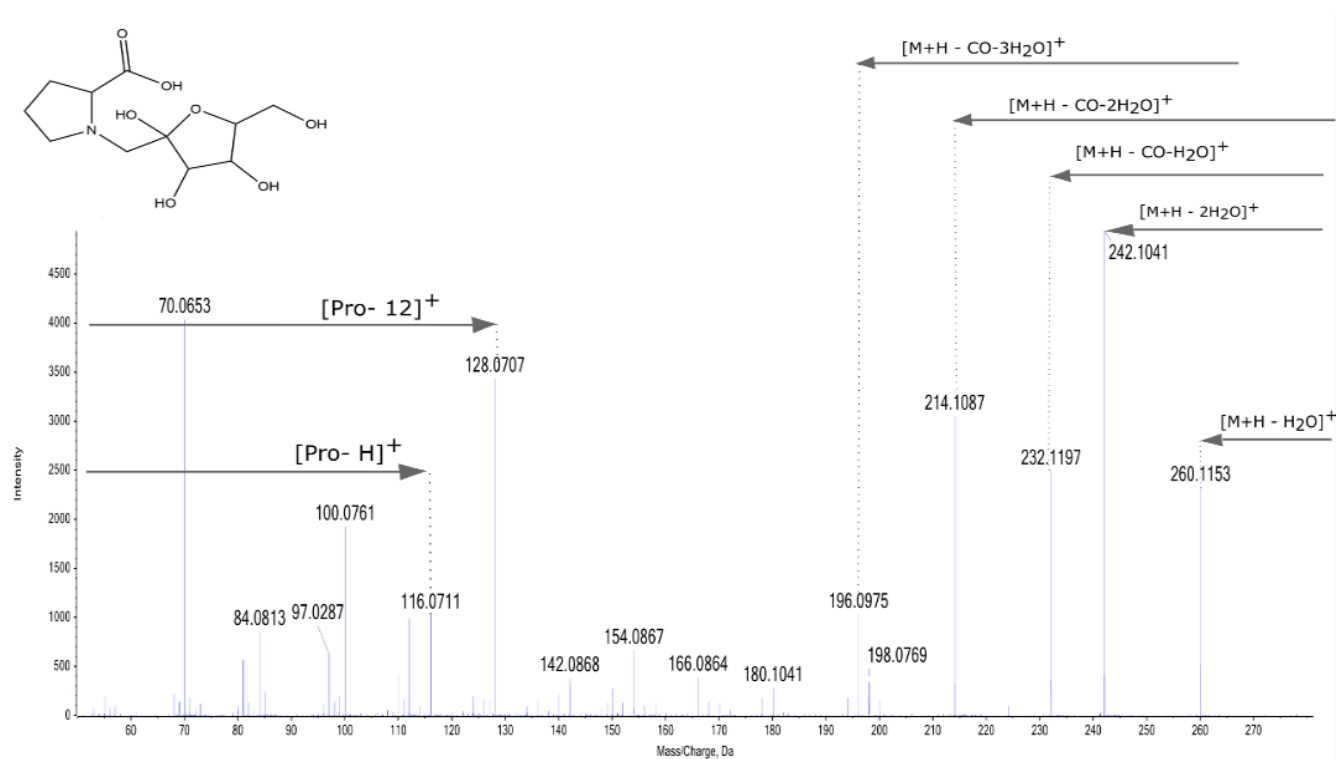


Figure 2.3-3 MS/MS spectrum of fructosyl-proline in positive ionization mode.

The MS/MS spectra of detected compounds show the same fragmentation pattern: as an example of the positive mode fragmentation, the MS/MS spectrum of m/z 278.123 RT 0.59 is given in Figure 2.3-3. This peak was identified as fructosyl-Pro based on a published fragmentation spectrum (Davidek et al., 2005b).

The pattern shows losses of 18 Da and 36 Da corresponding to the loss of one and two molecules of water. Afterwards, the loss of -CO combined with one, two or three water molecules results in losses of respectively 46 Da, 64 Da and 82 Da. Two more ions can be observed in the fragmentation profile, corresponding to the protonated amino acid and a peak at m/z of the amino acid +12 Da (Davidek et al., 2005b), corresponding to $[M+H - H_2O - C_5H_8O_4]^+$. Lower m/z peaks corresponding to the structure of the amino acid are also observed, for instance the peak at m/z 70.065 corresponding to the proline immonium ion in Figure 2.3-3.

In negative mode, the MS/MS fragmentation spectrum of these Amadori products with one monosaccharide has a base peak corresponding to the loss of a hexose (M-162.05). For instance, the fragmentation spectrum of fructosyl-Trp at m/z 365.13, has a base peak at m/z 203.08 $[M-H - C_6H_{10}O_5]^-$. Lower molecular weight product peaks correspond to the fragmentation pattern of the specific amino acid in negative mode. For cysteine, aspartic acid, asparagine, serine, and threonine, no Maillard reaction products were identified, while the presence of fructosyl-pGlu might account for Maillard reaction products of glutamic acid and glutamine as will be discussed below. In addition to these Amadori products resulting from the reaction between a reducing sugar monomer and the amino acid, I also identified reaction products between amino acids and longer oligosaccharides (Table 2.3-3).

Amadori products with oligosaccharides up to 5 monomers are detected, resulting in series of peaks at different retention times with an m/z shift of 162.05. Twenty-two compounds glycosylated with a saccharide of more than one sugar residue were detected in positive

mode: 15 amino acids coupled to a disaccharide, 6 products with a trisaccharide and Ile/Leu reacted with a tetra-saccharide. In negative mode, 18 amino acids glycosylated with oligosaccharides were identified: 8 Amadori products with a disaccharide, 5 with a trisaccharide, 3 with tetra-saccharide and Amadori products of Ile/Leu and pGlu with a penta-saccharide. For fructosyl-Gly, fructosyl-Ala, fructosyl-Arg and fructosyl-Lys, no reaction products with more than one sugar residue were identified.

In addition to Amadori products with free amino acids, fructosyl-derivatives of 7 dipeptides and 1 tripeptide were also identified. Similar to the MS/MS pattern of Amadori compounds with a single amino acid, the base peak of the fragmentation spectrum of these fructosyl-derivates corresponds to a loss of 162.05 Da ($[M-H - C_6H_{10}O_5]^-$) from the precursor ion, confirming the presence of a hexose (Figure 2.3-4B). These peptides are: pGlu-Pro; Ile/Leu-Pro; pGlu-Ile/Leu; Ile/Leu-Ile/Leu; Pro-Phe; pGlu-Glu; Leu-Pro for the dipeptides and pGlu-Pro-Gln for the tripeptide. The latter peptide sequence is abundant in grain proteins and was identified as tripeptide in wheat gluten hydrolysates (Gazme et al., 2019). The other dipeptides have previously been identified in fermented grain-based products. Concerning products of the second stage of the Maillard reactions, pyrroline (RT 5.11 m/z 253.1179) and perlopyrroline (RT 22.37 m/z 265.0963, positive mode) were identified (Chieri et al., 2019).

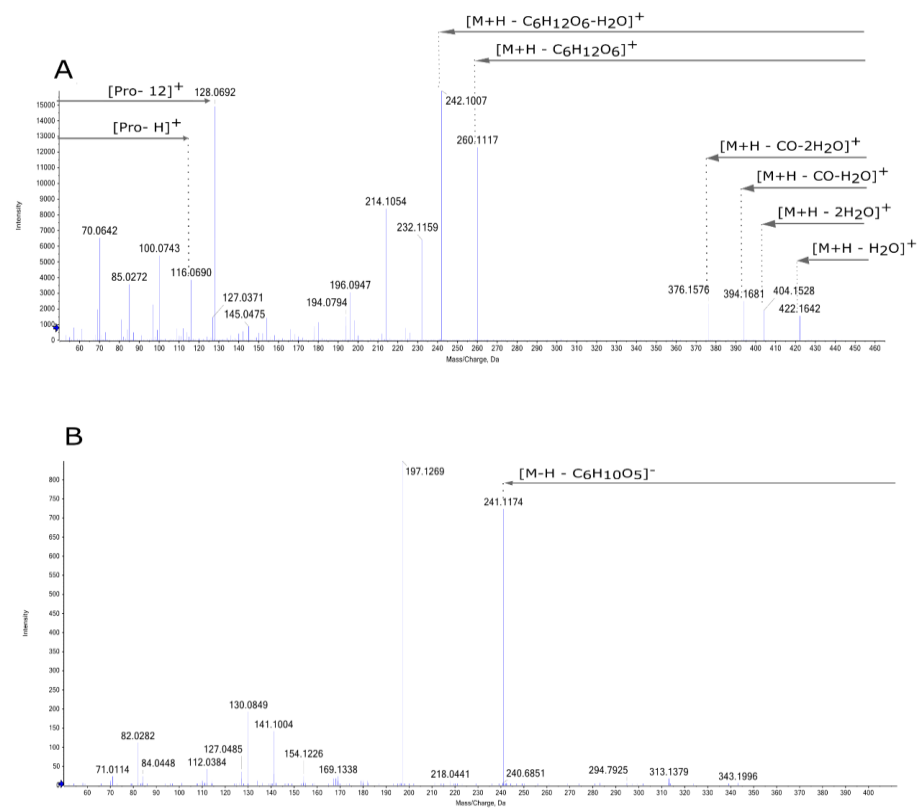


Figure 2.3-4 **(A)** MS/MS spectrum of fructosyl-proline hexoside in positive ionization mode. **(B)** MS/MS spectrum of fructosyl-pGlu-Ile/Leu in negative ionization mode.

2.4 Discussion

The worldwide increase in beer production results in high amount of biomass by-products that are currently poorly valorised as low-cost cattle feed or end up in landfills. The bulk of this residual biomass is BSG, the solid residue after filtration of mashed grains (Rachwał et al., 2020). In recent years, this by-product was intensively studied as source of bioactive molecules and a protein fraction with interesting properties (Hellebois et al., 2021).

In this study, BSG has been oven-dried, because it is the most frequently used stabilisation method in research and, based on previous study (Mussatto et al., 2006), the preferred pretreatment for industrial applications. Three solvents were used to make extracts from dried BSG: milliQ water and milliQ-ethanol mixtures (20% (v/v) and 40% (v/v)). The emphasis of the current study was on the identification of compounds and the generation of a database for use in subsequent optimisations. The global composition of the extracts is similar, and the quantitative differences observed in our dataset are those expected based on the polarity of the solvents used.

The chemical composition of the extracts is in line with previously reported analyses (Fărcaș et al., 2015; Gorzolka et al., 2016; Ikram et al., 2017). Phenolics are one of the most studied categories of compounds present in BSG extracts because of their antioxidant capacity and their benefit for human health. Ferulic acid and p-coumaric acid are the most frequently identified phenolics in BSG extracts (Connolly et al., 2021; Meneses et al., 2013). These were likewise found in our dataset both as free compound and conjugated to polyamines (putrescine, spermidine and agmatine in the identified hordatines) to form phenolamides.

The TPC and the antioxidant capacity measured in milliQ water, 20% ethanol and 40% ethanol extracts are in line with those reported in literature (Bonifácio-Lopes et al., 2020; Meneses et al., 2013; Socaci et al., 2018b). Here, the same trend is observed in the 3 chemical

assays (FRAP, ORAC, TPC), the highest values are obtained for extractions with 40% EtOH and these decrease further from 20% EtOH to water. The antioxidant capacity of the 40% EtOH extract, measured with the FRAP assay, showed higher values than those reported by Crowley et al. (Crowley et al., 2017). The highest value of TPC corresponds to 1.38 mg GAE/g dw of 40% EtOH extracts, which is higher than that of a 40% EtOH extract presented by Socaci et al. (Socaci et al., 2018b) (1.01 mg GAE/ g dw). Most studies agree that extractions with solvents mixtures (water:ethanol) result in extracts with higher TPC values than extractions with pure water (Bonifácio-Lopes et al., 2020; Meneses et al., 2013), as I obtain (Figure 2.3-2). Contrary to this, Almeida et al. (Almeida et al., 2017) presented data showing a decrease in TPC with increasing percentage of organic solvent in the extraction solvent. Beside ethanol, other solvent mixtures have been used to extract phenolics from BSG, some of them: 60:40 v/v acetone:water (Meneses et al., 2013); 1:80:10 v/v/v HCl:methanol:water; 0.75% NaOH:water (Moreira et al., 2013b), resulted in higher TPC values (respectively, 9.90 mg GAE/g dw (Meneses et al., 2013); 4.53 mg GAE/g dw (Guido & Moreira, 2017) and 19.5 mg GAE/g dw (Moreira et al., 2013b)) than those presented in this work. However, these solvents are considered not environmental friendly and limited by food-grade regulations (Ancuța Chetrariu & Dabija, 2020). An alternative, greener method for BSG extraction is the application of super critical CO₂ (Spinelli et al., 2016), however the TPC values reported are lower than those from solvent-based extractions (Macias-Garbett et al., 2021).

In this study, I focused on the identification of Amadori products extracted from BSG. Fructosyl-Lys, free and protein bound, is the most abundant Amadori product in malt and further processing of the malt results in flavour compounds originating from fructosyl-Lys in beer (Hellwig et al., 2016). However, the fructosyl-derivatives of the other amino acids are rarely identified. I show that these products are present in BSG extracts obtained according to current practice. Moreover, I confirm that Amadori products are formed between

different amino acids and oligo-saccharides, as was recently shown for N- ϵ -maltulosyllysine (Hellwig et al., 2016).

Amadori products were identified in processed food subjected to drying, roasting and fermentation. In dried vegetables (tomato, asparagus, carrot and celery), fructosyl-derivatives of Ile/Leu, Phe, Val, Gln, Asn, Thr, GABA, pyroglutamate, Ser, Arg, Glu and Asp were detected (Eichner et al., 1993). In dried pepper and tomato, fructosyl-Met, fructosyl-Tyr and fructosyl-His were identified (Meitinger et al., 2014) and Amadori products also were detected in dried fruit like jujubes, raisins (Yuan et al., 2017), apricots and plums (Davidek et al., 2005b). In roasted coffee and cocoa, fructosyl-Ile/Leu, fructosyl-Phe, fructosyl-Met, fructosyl-Val, fructosyl-Tyr and fructosyl-His were found (Meitinger et al., 2014); compounds also detected in barley, wheat malt and wheat beer (Meitinger et al., 2014). In a recent study, direct injection of extracts from brewery products and analysis with a Fourier transform mass spectrometer resulted in the generation of a Maillard reaction metabolome comprising 2800 small molecules (Pieczonka, Hem mLer, et al., 2021). However, the resulting compound list comprised of exact molecular weights and their associated molecular formulae and not of identified compounds. In a follow-up study, the same group compared the metabolite signatures of beers with different carbohydrate sources (wheat, barley, rice or corn) and grain-species specific compounds, but no Maillard reaction products, were identified (Pieczonka, Paravicini, et al., 2021).

As presented in Figure 2.3-3, the fragmentation pattern in positive mode is similar for all fructosyl-derivates. The base peaks are generated by de-hydration and the consecutive loss of -CO from the sugar molecule. The amino acid can be identified by the presence of an ion corresponding to the protonated amino acid, a peak at +12 Da and lower molecular weight peaks due to the fragmentation of the amino acid (Davidek et al., 2005b; Meitinger et al., 2014).

In addition to these well-known compounds, Amadori products with longer oligosaccharides of up to 5 hexoses were identified: 11 AA

coupled to a disaccharide; 5 to a trisaccharide, 3 to a tetrasaccharide and 2 to a pentasaccharide. In this count Ile and Leu are considered as one AA. In Figure 2.3-4A, the MS/MS spectrum of fructosyl-Pro hexoside, representative of the observed MS/MS pattern in positive mode, is shown. It is characterized by two losses of 180 and 324 from the precursor ion at m/z 440, respectively resulting in a product ions at 260.11 m/z $[M+H - C_6H_{12}O_6]^+$ and at 116.06 m/z $[M+H - 2 C_6H_{10}O_5]^+$, corresponding to proline. Lower molecular weight peaks correspond to those of the amino acid, observed as protonated ion (m/z 116.06 $[AA+H]^+$ in Figure 2.3-3) and with the addition of 12 Da (m/z 128.07 $[AA+12]^+$ in Figure 2.3-3) based on the annotation of Davidek et al. (Davidek et al., 2005b). In negative mode, MS/MS spectra of fructosyl-derivatives show fragment ions corresponding to the losses of hexoses from the precursor and the specific fragmentation of the amino acid. Amadori products of several dipeptides and one tripeptide were also detected, the fragmentation pattern of these compounds shows a base peak resulting from the loss of 162.05 Da from the precursor ion and further products characterizing the peptide, as shown in Figure 2.3-4B.

In our extracts, Amadori products of most proteinogenic amino acids were identified, except for glutamic acid, glutamine, aspartic acid, asparagine, threonine, serine, and cysteine. In the following, the absence of these compounds will be rationalized. Given that glutamic acid and glutamine are abundant in BSG extracts (Kumari et al., 2019) and that the fructosyl-Glu and fructosyl-Gln were absent in our analysis, I hypothesize that compounds identified as fructosyl-pGlu represent the Maillard reaction products of these two amino acids (Schlichtherle-Cerny & Amadò, 2002). The data presented by Eichner in 1993 (Eichner et al., 1993) are in line with this and it is proposed to use the abundance of fructosyl-pGlu as representing the sum of the content of fructosyl-Glu and fructosyl-Gln (Eichner et al., 1993). The low-level detection of fructosyl-Glu in other heat-exposed biomass containing relatively high amounts of free glutamic acid, such as sweet rice wine (Inoue et al., 2016), could likewise be due to the formation of pyroglutamic acid from glutamate. Similarly, while

fructosyl-Glu and nine other fructosyl-amino acids were identified in malt, fructosyl-Glu is the only one not found in the resulting beer (Wittmann & Eichner, 1989) after completion of the brewing process. Glutamine is known to be unstable: it degrades rapidly when exposed to high temperatures. At ambient temperature, Gln degrades slowly to pyroglutamate and ammonia, but after 30 min of incubation at 120°C of Gln-solutions a close to complete conversion of Gln to pyroglutamate is observed (Niquet & Tessier, 2007). Prolonged exposure to high temperatures, as during mashing, may thus explain the disappearance of fructosyl-Glu observed by Wittmann & Eichner (1989) (Wittmann & Eichner, 1989) and the fact that I do not observe fructosyl-Gln while I observe fructosyl-pGlu. Therefore, the observed pyroglutamate RT 0.90 (m/z 128.0345), fructosyl-pGlu and Amadori compounds with longer oligosaccharides are likely the sum of the contributions from Maillard reactions of both amino acids. However, specific studies on the thermal stability of fructosyl-Glu/Gln, that could confirm this hypothesis, are missing.

Why no Amadori product from asparagine is identified, although this amino acid is an important nitrogen source for yeast during the fermentation of wort (Hill & Stewart, 2019) and is present as free amino acid in BSG extracts (Table 2.3-1), is not clear. Because of its negative health effects, acrylamide, mainly formed through Maillard reactions with asparagine, is an important Maillard reaction product. However, in a recent study it was shown that no acrylamide was found in BSG extracts (Patrignani & González-Forte, 2021). Based on the latter study, the rapid formation of acrylamide from fructosyl-Asn cannot explain why I did not identify it.

No Amadori products of the two hydroxyl-containing amino acids, threonine and serine, were identified although molecular weights of known Maillard products derived from both (Noda et al., 2016) were systematically screened. In malt, fructosyl-Ser and fructosyl-Thr together with fructosyl-Gly showed the lowest concentrations compared to the other detected Amadori products (Wittmann & Eichner, 1989). Nevertheless, hydroxyl amino acids have been found

to be the most reactive in the formation of fructosyl-derivatives in several studies (Fry & Stegink, 1982; Piloty, 1979). The early elution of these compounds together with their low abundance in malt (Wittmann & Eichner, 1989) may explain why ions corresponding to these products were not observed/fragmented in this study.

Neither cysteine as free amino acid nor fructosyl-Cys were detected in our extracts. This is likely due to the high reactivity of the thiol-group with carbonyl or unsaturated carbonyl groups, inhibiting pigment formation through the Maillard cascade (Noda et al., 2016). Nonetheless, 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone (DHDMT), a product of the reaction between glucose and cysteine, was detected in processed food such as soy sauce, miso, beer, burnt onion, crust of bread and roasted wheat grains (Noda et al., 2016). DHDMT was not identified in the present study. Other known Maillard reaction products, used to assess quality for instance in honey (Sanz et al., 2003) and prepared food (J. Yu et al., 2018), were also looked for and none of these were identified.

In our extracts, oligo-hexoses containing 1 to 9 monomers were confirmed by MS/MS (supplemental file 2) and oligosaccharides of up to 20 monomers were observed based on m/z alone. With this mixture of oligo-hexoses in BSG and a lack of knowledge on the impact of oligosaccharide length on the reactivity of the reducing sugar towards amino acids, I postulate that Amadori products with even longer oligosaccharides are formed. The same reasoning was recently used to explain the higher abundance of N- ϵ -fructosyllysine over N- ϵ -maltulosyllysine by the predominance of glucose over maltose in malt (Hellwig & Henle, 2020). Detailed quantitative data on the content of oligosaccharides in BSG is limited, but more information is available for malt. Although the range of data reported for each compound is broad, most likely due to biological and process-related factors, glucose is, in general, more abundant than maltose. One study reported 19.5 g/kg for glucose and 11.8 g/kg for maltose in malt (Allosio-Ouarnier et al., 2000). Since these values are given in g/kg and maltose has a higher molecular weight than glucose but also only

one reducing sugar equivalent, the contribution of glucose to the Maillard reaction is expected to be several times higher than that of maltose. Furthermore, the quantity of longer oligo-hexoses was found to decrease significantly both in the previously cited study (Allosio-Ouarnier et al., 2000) and in other comparable analyses (Duke & Henson, 2008). The dominance of small saccharides over larger ones corresponds to the observed intensity of the fructosyl-AA-[Hex]_n products, with Amadori products with more than 3 hexoses falling below the detection limit for most amino acids.

The identification of PE lipids with oligo-hexoses attached to the ethanolamine group furthermore indicates that a similar reaction takes place between the PE head group and reducing sugars. The formation of these products is known to occur in heat-treated food and was recently studied in detail (Lin et al., 2018). High-temperature incubation of PE with glucose resulted in the fast formation and transient accumulation of Amadori-PE, an intermediate in the slower formation of carboxymethyl-PE (CM-PE) and carboxyethyl-PE (CE-PE). These products have also been reported to be formed *in vivo* in red blood cells (Fountain et al., 1999) and their monitoring is indicated as important in the assessment of ageing and age-related diseases (Sookwong et al., 2011). Furthermore, the quantification of these compounds was proposed as a quality criterion for processed food (Utzmann & Lederer, 2000).

Cyclic dipeptides, known as diketopiperazines, were identified in our extracts. Diketopiperazine formation is considered a side reaction of the Maillard cascade and, as other Maillard reaction products, contributes to the taste and colour of food. Furthermore, they display a broad spectrum of bioactivities including activities sought for in BSG extracts (Borthwick & Da Costa, 2017).

Amadori products are the precursors of AGEs. The latter can alter protein structure or conformation, leading to modification in tissues by changing intermolecular links, increasing the number of free radicals and inducing inflammatory responses. These biological activities are

prone to result in the development of cardiovascular diseases and diabetes (Delgado-Andrade, 2014), have a detrimental effect on cartilage and bones and result in a reduction of skin elasticity thereby promoting the ageing-process. All these physiological effects are the opposite of what food supplements are targeted at. On the other side, detailed studies of some of the compounds identified here, notably fructosyl-Arg and fructosyl-Tyr, show that they have a metal-chelating and antioxidant capacity (Ide et al., 1999; Mossine & Mawhinney, 2007). Other compounds such as fructosyl-Trp, -Tyr, -Gly, -Lys and -Leu have a measured antitumor and anti-inflammatory activity (Lee et al., 2016; Mossine & Mawhinney, 2007). Other beneficial biological activities (anti-bacterial and anti-fungal) are likewise attributed to some of these compounds or AGE products generated from these compounds. Considering these contradicting activities for the different compounds, more studies are needed on their metabolism during digestion and their bioavailability, as well as on the effects of their long-term ingestion.

The identifications reported here indicate that in the valorisation of BSG extracts as food supplement other compounds than those generally quantified should be considered. Although some of the compounds reported here only give a low-intensity signal during MS and thus are probably of relatively low abundance, their known biological activities may make their detection and quantification important. The data presented here show that BSG extracts, and most likely other processed food products, contain a larger diversity of Maillard reaction products than generally studied. Not only were monosaccharide Amadori products found for most proteinogenic amino acids, for dipeptides and for amino group-containing lipids, but reaction products with longer oligosaccharides were likewise identified. By these identifications in BSG, our data support the recent hypothesis that disaccharide-derived glycated amino acids other than lysine are present in malt (Hellwig & Henle, 2020).

During malting, the kilning step, high temperature drying, is the first step that may result in Amadori products (Wittmann & Eichner, 1989)

followed by the mashing phase. After mashing BSG is separated from the liquid phase, which continues the brewing process. Different kilning conditions and high temperatures during mashing are essential for taste development and, given the high temperatures, contribute to the formation of Amadori products found in beer (Inns et al., 2007), and thus BSG. Furthermore, the drying step prior to extraction, considered necessary to avoid microbial degradation in most studies on BSG valorisation, can affect the Amadori content and composition (Eichner et al., 1993). During mashing, free amino acids and reducing sugars are released enzymatically from malt proteins and oligosaccharides and solubilized in the wort. These free amino acids and reducing sugars are not only found in the wort but, as a comparative study on malt before mashing and on freeze-dried BSG showed (Jin et al., 2022; Robertson et al., 2010), also in BSG. While no exhaustive data on Amadori products is available on extracts from fresh, undried BSG it can thus be assumed that Amadori products identified in malt and after kilning are also present in BSG prior to drying. This however does not exclude that drying of BSG prior to the extractions done in this study contributes to the observation/abundance of these compounds. This is even highly likely knowing the presence of numerous Amadori products in dried food (Meitinger et al., 2014). Nonetheless, stabilization of BSG is considered essential due to its richness and its capacity to support microbial growth. Several methods have been used to stabilize BSG; besides oven drying, these include freeze drying or direct freezing. Although freeze-drying is considered a better technique, as the cold temperatures applied during the process maintain the BSG composition unaltered, it is not economically sustainable considering the amount of BSG biomass produced (Lynch et al., 2016; Mussatto et al., 2006). For this reason, oven drying is still the most suitable method for BSG stabilization due to its ease and lower execution costs (Krakowska-Sieprawska et al., 2022; Mussatto et al., 2006).

The biological activities of some of the Amadori products with sugar monomers identified here have been studied and often gave conflicting results from health-promoting to detrimental for health.

These may thus contribute or counteract the targeted effects of food supplements based on BSG extracts. Furthermore, the biological activities of Amadori products formed with sugar oligomers is not at all known. For this reason, the quantification of some of the compounds identified here, and in general detailed studies on the composition of food supplements, may be needed to optimize the use of BSG as source of food supplements.

3 The effect of stabilisation of brewer's spent grain prior to extraction; to dry or better not to dry?

Highlights

- *Extracts from untreated BSG, which were obtained with 40% EtOH mixture, showed the highest antioxidant potential among all extracts.*
- *The oven-drying pre-treatment applied on BSG biomass had an impact on the chemical composition of the obtained extracts. For sugars, Amadori products, amino acids, peptides, polyamines and phenolics classes the starting biomass was found to be the main determinant factor affecting the abundance of these metabolites, while the concentration of hordatines and lipids was mainly influenced by the extraction solvent used.*
- *The sugars, amino acids, polyamines and phenolic classes were relatively more abundant in extracts from untreated BSG, while Amadori products and peptides were found in higher relative concentrations in extracts from oven-treated BSG.*

Submitted for publication Minestrini, Simone Zorzan, Marcus Iken, Yvan Larondelle, Jenny Renaut, Jean-François Hausman, Kjell Sergeant* **The effect of stabilisation of brewer's spent grain prior to extraction; to dry or better not to dry?** submitted for publication in npj (Natural Partner Journals) Science of Food, second round of review process, resubmitted July 2024

Abstract

Extracts from untreated (fresh) and treated (oven-dried and ground) BSG, the most abundant residue from brewing industry, were investigated by comparing their antioxidant capacity and metabolomic profiles through UHPLC/-MS/MS analysis. Three extraction conditions were applied for each starting material, and the identified compounds were grouped in 8 classes based on their chemical structure. The accumulated MS-signal intensity of compounds in each class was compared among the starting materials and the extraction conditions. Extracts from untreated BSG with 40% hydroethanolic solvent showed the highest antioxidant capacity. Furthermore, for some groups of compounds (sugar, amino acids, phenolics, ...) drying was the main discriminating factor on the chemical composition, while for other groups (lipids, hordatines, ...) the determinant was the used extraction solvent. Our data showed that this energy- and time-consuming drying/stabilization pre-treatment can and perhaps should be avoided, which is possible since the availability of BSG is predictable in amount and time.

3.1 Introduction

Currently, there is a global focus on linking economic development with environmental protection. Therefore, sustainability and re- or upcycling are now considered essential factors in industrial development, and these three concepts must be considered as one entity. This emphasis is further necessitated by the growing population, which reached 8 billion in 2022, and concerns on whether, despite a 53% rise between 2000 and 2019, the global primary production can keep up with the growing demand (<https://www.fao.org/>). Coinciding with the context described above, the amount of generated organic by-products also increased, leading to a higher quantity of bio-waste to be transformed or disposed of (Bonato et al., 2022). The realisation that the so-called bio-waste can become resources rather than waste streams, is promoting scientific and societal interest to develop technologies for the circular use of biomass. The general aim is to use agro-industrial residues as renewable source for producing energy, materials, foodstuffs or to exploit them to extract useful compounds for pharmaceutical, medical and biotechnological purposes (Ancuța Chetrariu & Dabija, 2020).

With 1.82 billion hectolitres produced in 2020, beer is the most consumed alcoholic beverage, and brewing represents a highly productive and growing industrial activity. In 2022, European beer production reached 343 million hectolitres (<https://brewersofeurope.org/site/countries/key-facts-figures.php>) resulting in 20 kg of brewer's spent grain (BSG) produced for each 100 L of beer, making BSG the most abundant residue from the brewing process (Steiner et al., 2015). The low economical value of BSG, its still limited valorisation, together with the required costs for its storage, make that the two most common ways to recycle BSG are for animal nutrition (70%) and for energy production through anaerobic digestion (10%). Therefore, about 20% of the globally produced BSG is disposed of by landfilling (Kavalopoulos et al., 2021). On one hand, the current amount of BSG disposed of and its

expected increase, due to the rising of beer production, represent one of the biggest concerns of the brewery industry (Assandri et al., 2021; Bonato et al., 2022). On the other hand, since BSG is a residue from an industrial process and, contrary to other residual biomasses, its generation is non-seasonal, and its availability is constant and predictable both in amount and timing. These latter features open opportunities to find alternative uses to those mentioned above.

BSG is composed of non-soluble parts of the grain; husks, pericarp and seed layer-covering. It globally contains protein (15-30%), hemicelluloses (20-25%), cellulose (12-25%), lignin (12-28%), lipid (6-10%), phenolics, vitamins and minerals (Rachwał et al., 2020; Yu et al., 2019). Due to its chemical composition, other approaches for its upcycling, with higher valorisation potential, are being investigated among the scientific community, such as a biomass for biorefinery, a source of food ingredient or substrate for producing enzymes, bioactive molecules and prebiotics (Bonato et al., 2022; Ancuța Chetrariu & Dabija, 2020). However, it is important to note that according to the variety of the grain used as well as the type of brewery process parameters applied, its chemical composition can vary (Ancuța Chetrariu & Dabija, 2020; Inns et al., 2007).

The different phenolics found in BSG are the molecules most targeted in studies that aim to create new valorisation potential for BSG. BSG consists mainly of the outer layers of the barley grains, which is predominantly made up of cell walls wherein the highest accumulation of phenolic compounds is found. Phenolics can be found in their free or bound form. Free phenolics are in the case of BSG generally flavonoids (catechins and oligomers, quercetin and myceritin (Birsan, 2022)), while the bound phenolics are mostly hydroxy-cinnamic acids (HCA), such as ferulic, p-coumaric, sinapic, and caffeic acid. BSG as by-product of beer based on barley contains hordatines, condensation products of agmatine-HCA monomers, that are treated as a different group of compounds in this study. All these compounds are known to have a positive effect on human health, making that BSG is considered as a valuable source and the extraction

of these compounds a promising approach for BSG valorisation (Almeida et al., 2017; Andres et al., 2020; Socaci et al., 2018a).

Many studies focused on the optimisation of the extraction of phenolics from BSG. However, regardless of the exact extraction approach followed: from conventional techniques such as Soxhlet, maceration and hydro-distillation (Jha & Sit, 2022), to more innovative approaches, like ultrasound assisted, microwave assisted, or super critical fluid extractions (Guido & Moreira, 2017); prior to the extraction, plant-based materials are often subjected to pre-treatments (Krakowska-Sieprawska et al., 2022). Actually, most of the studies on BSG extraction do not use raw BSG as such, but apply drying and grinding as pre-treatments (Carciochi et al., 2018; Iadecola et al., 2022; Zago et al., 2022).

Drying is considered essential for BSG management: it avoids microbial growth and the related biomass degradation and thus increases the conservation time of the biomass. It also reduces the weight and volume, thereby decreasing transport and storage costs (Mussatto et al., 2006). Freeze-drying is considered the best drying technique to preserve the chemical properties of the used biomass as this treatment occurs at low temperatures (Krakowska-Sieprawska et al., 2022). However, the energy needed and cost and time-consumption for freeze-drying, make this method less suitable for industrial BSG valorisation (Hassan & Muhamad, 2017; Mussatto, 2014; Singh et al., 2020). Oven-drying is more cost-efficient but requires exposure to high temperatures and long drying times. It has a high energy consumption and, because of the high temperatures, influences the quality/composition of the extract (Krakowska-Sieprawska et al., 2022; Mussatto et al., 2006; Singh et al., 2020). Nonetheless, the detailed effects of oven-drying for industrial biomass valorisation are not known, and investment needs in technology and application hinders its use in BSG valorisation, especially for smaller breweries in less developed countries (Bonato et al., 2022; Krakowska-Sieprawska et al., 2022).

The pre-washing treatment (also referred to hydrothermal treatment based on liquid hot water) is mostly used on BSG for recovering high-molecular-weight fibers like cellulose, β -glucan, and arabinoxylan (Steiner et al., 2018). Furthermore, grinding is frequently used to homogenise the biomass and facilitate the extraction process (Ancuța Chetrariu & Dabija, 2021).

Exposure of biomass to high temperatures for several days, for instance during oven-drying, can affect the antioxidant potential of the extracts, negatively impacting the concentration of phenolic compounds. Lower concentrations of p-coumaric acid, catechins and procyanidins, considered quality parameters for BSG extracts (Aliyu & Bala, 2011; Krakowska-Sieprawaska et al., 2022; Mussatto et al., 2006), were obtained after drying. In addition, heat-treatment of organic material promotes heat-triggered reactions, like the Maillard cascade, thereby modifying the composition of the extracts (Yu et al., 2017). The first stable products formed during the early stage Maillard reaction are known as Amadori compounds. These products are formed by a condensation between a reducing sugar and a free amino group (Davidek et al., 2005b; Minestrini et al., 2022; Xue et al., 2020) altering the sugars and free amino acid content present in fresh material (Kumari et al., 2019; Meitinger et al., 2014; Ratsewo et al., 2019). Those compounds, indeed, have been identified in dried and processed food, mainly in vegetables and fruits (Meitinger et al., 2014; Yuan et al., 2017). Similarly, drying has been shown to have an impact on the polyamine and lipid content of extracts from plant matrices (Lehtinen et al., 2003; Palavan-Unsal et al., 2007).

In the most detailed study on the effect of drying of BSG so far (Santos et al., 2003), drying was shown to cause changes in the protein and lipid content of BSG extracts although only the impact on the lipids was significant. However, contrary to data on other matrices, freeze- nor oven-drying had a significant impact on the TPC (Santos et al., 2003).

Based on the hypothesis that drying of BSG prior to extraction will influence the composition of the extracts and thus the valorisation potential of BSG extracts, the detailed impact of the most used method for BSG stabilization (oven-drying at 50°C for 48h) on the main groups of metabolites detected in BSG extracts was studied. To attain this, the metabolomic profile of extracts generated from fresh and oven-treated BSG were compared. To avoid overfocussing on changes occurring for individual compounds, the identified compounds were classified in 8 groups (sugars, amino acids, Amadori products, peptides, hordatines, polyamines, phenolics and lipids) and, as previously done (Pinto et al., 2022; Salem et al., 2022), the relative accumulated MS-signal intensity of each group used to compare the treatments. I found that fresh BSG can be considered a better source of extracts than dried, stabilized BSG, resulting in a higher antioxidant capacity. Our data thus indicate that extracts destined for application in food can be realized using fresh BSG, an approach that avoids energy- and time-consumption for biomass stabilisation.

3.2 *Materials and Methods*

3.2.1 Chemicals

The chemicals mentioned below were purchased. Ethanol (WMR 20821.321); formic acid (Sigma-Aldrich, 56302); acetonitrile (Sigma Aldrich, 271004-100 ML); Folin-Ciocalteu reagent (Sigma Aldrich, F9252-100 ML); Na_2CO_3 (Sigma Aldrich, 223530-500G); gallic acid (Sigma Aldrich; 27645-250G-R); Hydrochloric acid (HCl) 0.1 M (ChemLab, 31955.373); Iron(III) chloride hexahydrated ($\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$) (Merck VWR, 1.03943.250); Iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$) (VWR; 24244.232); Sodium acetate trihydrate ($\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3 \text{H}_2\text{O}$) (Sigma Aldrich, 331058-100G); Glacial acetic acid (CH_3COOH) (J.T. Baker, 9524-33); Fluorescein (Sigma-Aldrich, 46955-100G-F); Trolox (Sigma-Aldrich, 238813-1G); 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (Sigma-Aldrich, 440914-25G); Potassium phosphate monobasic (KH_2PO_4) (Merck, 1.04873.0250); Potassium phosphate dibasic (K_2HPO_4) (Vel VWR, PROL26931.263)

3.2.2 Materials

A local brewery, Brasserie Simon Sarl, Wiltz, in Luxembourg, provided a single batch of wet barley-Pilsener BSG and it has been stored in two different conditions: dried and wet. The drying process consisted of placing BSG in an air convection oven at 50°C for 48 h, also mentioned by Bonifácio-Lopes et al., 2020. After drying, the BSG was milled (Waring Lab Blender) for 2 min at highest speed (18000) on ice and stored at 21°C under vacuum avoiding light sources. The wet BSG was stored as such under vacuum at -20°C. For conducting the extractions water and ethanol were used as solvents.

3.2.3 Solvent extraction procedure

Two preliminary experiments were performed for selecting the starting material and the solvent mixture to use. The first preliminary test was conducted on oven-treated, ground (48 h at 50°C) BSG and untreated, fresh BSG using water and different aqueous ethanolic mixtures (from 0% to 100% of ethanol) as solvent. Subsequently, according to the data (shown in result and discussion session 3.3.1), 40% of ethanol was selected as the highest percentage of the organic phase for the extraction solvent. Moreover, a second preliminary experiment was performed using untreated fresh BSG and ground fresh BSG using 0%, 20% and 40% of aqueous ethanolic solvent. This last test did not highlight remarkable differences between the two types of fresh BSG used. Since this article is part of a wider project aiming at the valorisation of BSG as such, according to the results from the trials mentioned above, the starting material used in the presented study was fresh, untreated, not ground (also referred to as wet) BSG and ground oven-treated (also called dried) BSG.

The experiment described in this study involved two batches of samples: untreated BSG and ground, oven-treated BSG. For each batch, 3 different extraction solvents (milliQ water, ethanol 20% and ethanol 40%) were used.

Table 3.2-1 Scheme of the experimental conditions and the sample annotation: W indicates extracts from wet, untreated BSG; D extracts from oven-treated and ground BSG. The annotations MQ, 20%EtOH and 40%EtOH respectively indicate extracts obtained with MilliQ water, 20% EtOH and 40% EtOH in a 1/20 BSG/solvent ratio.

SAMPLE ID	EXPERIMENTAL CONDITIONS
W_MQ	Fresh BSG → extraction with milliQ water (1:20 solid: volume)
W_20	Fresh BSG → extraction with 20% ethanol (1:20 solid: volume)
W_40	Fresh BSG → extraction with 40% ethanol (1:20 solid: volume)
D_MQ	Fresh BSG → oven-drying and grinding → extraction with milliQ water (1:20 solid: volume)
D_20	Fresh BSG → oven-drying and grinding → extraction with 20% ethanol (1:20 solid: volume)
D_40	Fresh BSG → oven-drying and grinding → extraction with 40% ethanol (1:20 solid: volume)

Extractions were carried out at the ratio 1:20 (solid:solvent) (Meneses et al., 2013) using 3 different solvents: a) milliQ water b) 20% of ethanol (v/v%) and c) 40% ethanol (v/v%) (Table 3.2-1) in 2 mL tubes under agitation (Bench-top Orbital shaker) at 1400 rpm for 4 hours at room temperature (21°C).

After extraction, samples were centrifuged for 25 min at 15°C at 20000 *g* and the upper phase was collected. For UHPLC-ESI-MS/MS analyses, 300 µL of supernatants were taken and dried under vacuum (CentriVap; Labconco, Kansas City, MO, USA). The dried residue was resuspended in 300 µL 5% methanolic aqueous solution and filtered with 0.22 µm PTFE syringe filters (Millex-LG; Merck KGaA, Darmstadt, Germany). The filtered supernatants of each sample were stored at -20°C for biochemical analyses. Four independent extractions were made for each condition.

3.2.4 UHPLC-ESI-MS/MS analysis

An Acquity UPLC I-class ultra-high pressure liquid chromatography (UHPLC) system (Waters, Milford, MA, USA) coupled to an electrospray ionization high-resolution mass spectrometer (TripleTOF 6600+; SCIEX, Framingham, MA, USA) working in positive and

negative modes was employed for injecting 10 μ L of each BSG extract. The solvents used were formic acid 0.1% in milliQ water (A) and formic acid 0.1% in acetonitrile (B). The applied gradient was: 1% B at 0 min; 1% B at 4 min; 5% B at 16 min; 40% B at 35 min; 100% B at 45 min; 100% B at 50 min; 1% B at 54 min; and finally, 1% B at 60 min. The column employed was a reverse-phase Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 μ m particle size, Waters, Milford, MA, USA) and the temperature set at 50°C, while the flow rate was maintained at 0.5 mL/min.

The ion spray voltage was set at - 4.5 and 4.5 kV respectively for the negative and positive mode, an application of 30 psi for curtain gas (nitrogen), of 55 psi for nebulizer gas (air), and for 50 psi for turbine gas (air), with a source temperature of 650°C. The de-clustering potential was fixed at -60 in negative and at 60 V in positive mode. For the generation of MS² spectra in high sensitivity mode, the fragmentation of the 10 most intense single-charged precursor ions were employed, to reach a threshold of 100 counts per s, with an accumulation time of 200 ms. The complete cycle lasted 2.2 s and for all precursor ion a sweeping collision energy of -15 V or 15 V in negative and positive mode was set respectively. Before the subsequent fragmentation, the dynamic exclusion was set at 2 s after three occurrences. For MS, the spectra were detected from 100 and 2000 mass-to-charge ratio (m/z), while for MS² in the profile mode spectra were visualised from 50 to 2000 m/z.

3.2.5 Chemical assays

Total phenolic content assay (Folin-Ciocalteu)

For TPC assay, reagents have been employed at the concentration mentioned hereafter: 1N Folin-Ciocalteu reagent (diluted from the stock solution, provided by Sigma Aldrich, F9252-100 ML) and 125 μ L 7,5% w/v Na₂CO₃ solution (Singleton & Rossi Jr., 1965). A specific volume of 125 μ L was mixed with 50 μ L of each sample and standard dilutions in a 96-well plate. The plate was incubated for 30 min in the dark at 21°C. The absorbance was measured at 765 nm with

a spectrophotometric microplate reader (Spark, Tecan, Männedorf, Switzerland). A calibration curve was built by using the standard solution of gallic acid at these concentration: 0.004; 0.008; 0.0125; 0.016; 0.025; 0.03; 0.05 mg/ mL, ($y = 4.9609x + 0.0373$; $R^2 = 0.9989$) and the results of TPC were expressed as gallic acid equivalents (GAE). Four replicates were analysed in technical triplicates, for each extraction condition.

Ferric reducing/antioxidant power assay (FRAP)

The ferric reducing power assay exploits the potential of molecules to reduce Fe (III) into Fe (II) (Benzie & Strain, 1996). This reducing power is proportional to the measurement of the antioxidant capacity. FRAP reagent were prepared fresh by mixing 300mM acetate buffer with 10 mM ferric-tripyridyltriazine and 20mM FeCl₃ at pH 3.6. A 96-well plate was filled with 190 µL of FRAP reagent and subsequently, 10 L of extracted samples were added and mixed directly in the plate. After an incubation in the dark at 21°C for 20 min, the absorbance was measured at 595 nm with a spectrophotometric microplate reader (Spark, Tecan, Männedorf, Switzerland). A standard calibration curve using FeSO₄ at different concentrations was built: 0; 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1mM ($y = 0.6853x + 0.0692$; $R^2 = 0.9998$) and the results were expressed as µmol of ferrous equivalent. Four replicates were analysed in technical triplicates, for each extraction condition.

Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay principle is to detect the decay of fluorescence intensity related to the measurement of the antioxidant potential to scavenge peroxy radicals. The experiment was performed in a 96-well fluorescence microplate reader (Spark, Tecan, Männedorf, Switzerland) by adapting the method of Gillespie et al., 2007. Hundred and fifty µL of fluorescein 0.08µM, previously prepared in phosphate buffer 75mM at pH 7, were mixed with diluted samples (100x), blank and standards. A pre-incubation step of the microplate lasting 10 min at 37°C was done in the microplate reader, and 25 µL

AAPH solution (40 mg/ mL) was added. The fluorescence was detected for 60 min during 12 cycles, each 5 min. A standard curve was made using Trolox dilutions (6.25; 12.5; 25; 50 μ M) ($y = 0.682x$; $R^2 = 0.9931$) and results were expressed as μ mol Trolox equivalents. Four replicates were analysed in technical triplicates, for each extraction condition.

Glucose assay

Glucose was quantified with the Megazyme Sucrose/D-Fructose/D-Glucose Assay Kit (K-SUFRG, Megazyme, Wicklow, Ireland). Ten μ L of extracts and standard dilutions were prepared and added in a flat-bottomed 96-well plate. Then, 10 μ L of Solution 1 (Buffer), and 10 μ L of Solution 2 (NADP⁺/ATP) and 5 μ L of Solution 3 (hexokinase and glucose-6-phosphate dehydrogenase) prediluted to 40% were added to each well. A pre-incubation of 30 min at 37°C was done before the detection. The absorbance was set at 340 nm (A1). Subsequently, 5 μ L of Solution 4 (phosphoglucose isomerase) prediluted to 40% were added. The absorbance was measured at 340 nm after 20 min of incubation at 37°C (A2). A standard curve was built for each measurement at several concentrations from 0 to 2000 mg/L of pure glucose. The equation ($y = 0.0006x + 0.065$ $R^2 = 0.995$) of the standard curves were used for quantification. All measurements were done in technical triplicates.

3.2.6 Data analysis

Compound identification

Compounds were identified analysing the raw data (positive and negative modes) in Progenesis QI (v2.3, Nonlinear Dynamics, Newcastle upon Tyne, UK) as previously described (Minestrini et al., 2022). Compounds (defined as RT x m/z couples) without MS/MS spectra and suspected and known contaminants were filtered out. Subsequently, MS/MS spectra were used for identification. Databases as NIST and MassBank were used for the first round of identification based on MS/MS similarity. Additional identifications were obtained

through comparison of MS/MS spectra with data available in literature or in others databases, such as: HMDB (<https://hmdb.ca/spectra/ms/search>); LipidMaps (www.lipidmaps.org/), GNPS (<https://gnps.ucsd.edu/ProteoSAFe/libraries.jsp>); MZCloud (www.mzcloud.org); PubChem (pubchem.ncbi.nlm.nih.gov) and Metlin (metlin.scripps.edu/index.php). For other compounds, theoretical spectra generated with MetFrag (<https://ipb-halle.github.io/MetFrag/>) and CFM-ID (<https://cfmid.wishartlab.com/>) were used for comparison. A newly identified compound was added in an in-house tool for dereplication and database-generation, that can be exported as msp-file, compatible with Progenesis. All compounds were identified at level 2 based on the m/z-value of the precursor and spectral matching of the fragmentation spectra (MS/MS) with experimental or theoretical MS/MS spectra (Rochat, 2017; Salek et al., 2013).

Data treatment

The moisture content of BSG was determined by weighting BSG before and after 48 h at 50°C in an air convection oven. A preliminary study showed that a constant weight, corresponding to a weight loss of 75%, was obtained after 48 h of drying in an air convection oven. Since all extractions were done on 75 mg of biomass, treated or untreated BSG, a factor four was used in all comparisons between the difference starting materials (Ancuța Chetrariu & Dabija, 2021). This factor was also used in the comparison of the signal intensity of UHPLC-ESI-MS/MS measurements.

Wet and dried BSG extracts were analysed by applying an untargeted metabolomic approach with UPLC-ESI-MS/MS, wherein the relative abundance of each compound based on its MS signal intensity was evaluated. Then, the resulting data were examined with Progenesis QI. Compounds were identified, and their relative abundances were calculated for all the 6 experimental conditions as previously described by Salem and colleagues (Salem et al., 2022). All identified

compounds were grouped in 8 classes (sugars, amino acids, Amadori products, peptides, hordatines, polyamines, phenolics and lipids) based on their chemical nature and functional properties. The intensity values of a compound were normalised based on sample weight and scaled with “Pareto scaling” (mean-centered and divided by the square root of the standard deviation of each variable) in Metaboanalyst 5.0 software (<https://www.metaboanalyst.ca>). To detect the significant discrimination factors among samples, the principal component analyses, heatmaps and ANOVA (p value < 0.05) were produced with Metaboanalyst 5.0.

3.3 Results and Discussion

The experiment, presented here, aimed to investigate the hypothesis that drying of BSG prior to extraction influences the composition of the extracts and thus the valorisation potential of BSG extracts. Through a detailed analysis of the composition of extracts from oven-treated and untreated BSG biomass, it is intended to show the potential of oven-drying on the chemical composition of BSG extracts. Study on BSG extraction are generally conducted on dried biomass. Among the few studies that used fresh BSG, Santos and co-workers focused on the impact of drying on the extraction of molecules from freeze-dried, oven-dried (60°C for 18h) and fresh BSG material. They measured 4 parameters: protein, phenolic, ash and lipid content, only showing a significant difference for the lipid content. A more recent study that aimed at the development of a biorefinery approach likewise used fresh untreated BSG but no comparison with dried biomass was made (Belardi et al., 2023).

Our data shows differences between extracts from fresh and oven-treated material for different groups of compounds. Furthermore, the antioxidant capacity, a biochemical parameter that is targeted in studies on BSG extracts, of extracts from fresh BSG was higher than those obtained from dried biomass. All samples were taken from the same BSG batch but treated differently: wet BSG was vacuum sealed and stored at -20°C, while the dried BSG used for extraction was

oven-dried at 50°C during 48 h, ground and kept in vacuum at 21°C in the dark.

A solid-liquid extraction was performed; the easy availability of infrastructure and the well-established production process at large scale, still make it competitive at industrial scale compared to new, alternative extraction methods (Didion et al., 2023). Ethanol was used as organic component of the solvent, it is non-toxic to human health and the environment, and is considered a green and GRAS (Generally Recognise as Safe) solvent (Lianza et al., 2022). Moreover, since the two of the main targets of BSG studies are the concentration of phenolic compounds and the antioxidant capacity of its extracts, these parameters were considered during preliminary tests.

3.3.1 Preliminary tests

Extractions with 0% to 100% of ethanol (v/v %, water/ethanol %) on oven-treated ground, and fresh BSG were conducted, and the antioxidant potential of these extracts measured. Among the extracts from oven-treated BSG, 60% EtOH resulted in the highest TPC and antioxidant potential, while 40% EtOH extracts from fresh samples gave the highest values. Comparing both batches, the latter resulted in a higher phenolics content and antioxidant capacity (supplemental file 3). According to these data, 40% EtOH was selected as the highest organic percentage of the solvent for the current study. In addition, with the second preliminary test, fresh untreated BSG was compared to fresh, ground BSG by using water, 20% and 40% of ethanolic mixtures. Both types of samples were investigated for their antioxidant capacities and their TPC. Since no significant differences were detected between their extracts, the starting materials selected for this research were oven-treated, ground BSG and fresh untreated BSG.

3.3.2 Analysis of MS intensity signals among the main categories of identified compounds

UHPLC-ESI-MS/MS analyses have been carried out on extracts from untreated and ground, oven-treated BSG. The main compounds were identified and grouped in 8 classes according to their chemical nature and properties. The relative MS signals of all compounds in a class have been used to compare the different experimental conditions. This approach does not provide data on the absolute quantity of every class or of specific compounds, however it provides information on the general impact of the oven treatment at 50°C for 48 h, combined with a grinding step on the global composition of the BSG extracts.

The highest intensity signals were detected at three retention time frames: from 0 to 5 min, from 15 to 25 min and from 30 minutes till the end of the runs. Signals at the earlier retention time correspond to the most hydrophilic compounds such as sugars, nucleotides, Amadori products and amino acids. Hordatines, polyamines and peptides and phenolics were detected in the time frame from 15 to 25 min, and several lipid classes were identified at later retention times. The MS-signal intensity of the identified sugars was higher in untreated BSG extracts compared to those from oven-treated BSG, this in agreement with studies conducted on malt (Jin et al., 2022; Wittmann & Eichner, 1989). Concomitantly, the relative intensities rose slightly with increasing EtOH concentration. The highest value was obtained with 40% EtOH extracts from fresh BSG. Looking at the corresponding PCA (Figure 3.3-1A), it is shown that the main segregating factor between those samples is the starting material (PC1 97.3%), and only little variance (PC2 1.5%) is explained by the solvent mixtures used for extraction (Figure 3.3-1A). Additionally, a glucose assay was performed confirming those data (supplemental file 4). The highest free glucose concentration was obtained for extracts with 40%EtOH from wet BSG samples. In detail, I measured from wet BSG, 13.6 mg glucose/ g dw BSG in 40% EtOH extracts, while only 11.2 mg glucose/ g dw BSG in water extracts and 10.9 mg glucose/ g dw BSG in 20% EtOH samples. Extracts from dried BSG had lower

concentrations but showed the same trend. A range from 0.5% to 4.2% w/w of free glucose was measured in 4 extracts from freeze-dried BSG (Jin et al., 2022), while a value of 1.3 mg glucose/ g was determined in oven-dried BSG (Gutiérrez-Barrutia et al., 2022). Moreover, Kumari and colleagues found in light BSG 3.33% glucose versus 0.5% in dark BSG (Kumari et al., 2019). These authors related the lower free glucose concentration in extracts from dark BSG with the increased occurrence of heat triggered reactions caused by a more intense kilning process for obtaining dark BSG. It was hypothesized that the decrease in free glucose concentration may be due to the generation of Amadori rearrangement products. These compounds are formed during the early stage of Maillard cascade from a condensation between a free amino group with a reducing sugar of which glucose is the most abundant in BSG (Kumari et al., 2019). As dark BSG, the dried biomass analysed in this study was subjected to a heat-treatment. Oven drying (at 50°C) may have contributed to the generation of Amadori products and explain the lower free glucose concentrations in extracts from dried BSG (Kumari et al., 2019; Meitinger et al., 2014).

I have recently shown that Amadori products are not only formed from glucose but also from oligo-saccharides (Minestrini et al., 2022), which could contribute to the lower relative MS-signal intensity for compounds here classified as sugars in extracts from oven-treated BSG. The Amadori rearrangement compounds identified in this study included fructosyl-glycation products with p-Glu (pyroglutamic acid), Met, Val, Tyr, Ile/Leu, Phe, Pro and Trp. This is in line with the most abundant Amadori products detected in barley malt which are fructosyl-Val, -Ile/Leu, -Phe and -Tyr (Luo et al., 2021). The significant increase of these compounds in dried BSG compared to fresh BSG extracts analysed in this study, may correlate with the heat treatment on dried BSG samples, since it was demonstrated that drying process boosts the formation of Amadori rearrangement products (De Brito et al., 2001; Inns et al., 2007; Kumari et al., 2019; Zhang et al., 2021). The PCA graph showed a separation between the two starting materials, again confirming that the pre-treatment

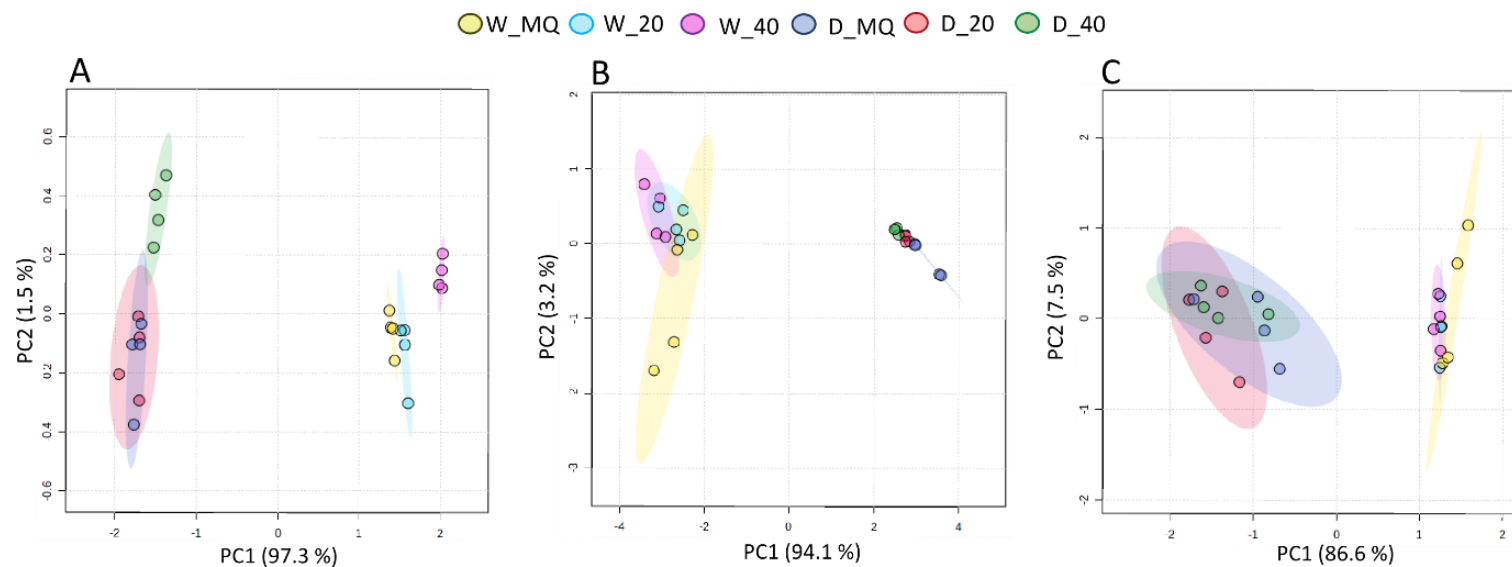


Figure 3.3-1 Principal component analysis (PCA) of: A sugars; B Amadori products; C amino acids. W indicates extracts from wet, untreated BSG; D indicates extracts from ground oven-treated BSG. The annotations MQ, 20 and 40 respectively indicate extracts obtained with milliQ water, 20% EtOH and 40% EtOH. In all the graphs, PC1 covers most of the variation among the samples showing a clear segregation between the two conditions: oven-treated and untreated. In the graph A, samples extracted with 40% ethanol in both oven-treated and untreated conditions resulted in a higher variation compared to the milliQ water and 20% ethanol extracts, while in the graphs B and C, the three solvent mixtures presented a similar trend.

had an important impact on the formation of these compounds (PC1 94.1%) (Figure 3.3-1B). Regardless of the starting material, the highest values for Amadori products were obtained in extracts with water, although the impact of the used extraction solvent is low. Fructosyl-Phe and fructosyl-Ile/Leu have already been reported to increase more than 13-fold after drying of BSG (Zhao et al., 2018). Amadori products, previously identified in wheat beer and malt, in barley (Hellwig & Henle, 2020; Meitinger et al., 2014) and in BSG extracts (Minestrini et al., 2022), are mainly generated during heating (drying, roasting, cooking, ...) of sugar-rich food such as vegetables, flour-based products and fruits (Davidek et al., 2005a; Eichner et al., 1993; Meitinger et al., 2014; Yuan et al., 2017). In addition to Amadori compounds, in the current study a Maillard reaction cascade product of lysine, called pyrraline (Liang et al., 2015), was identified and detected with higher intensity signal in oven-treated BSG samples. This molecule is used as an indicator of heating evaluation, as its abundance correlates with the intensity of heat treatment applied in food processing (Henle & Bachmann, 1996).

Similar to sugars and Amadori products, in the amino acids category, the PCA analysis shows that the highest variance is caused by the drying pre-treatment (PC1 86.6%) (Figure 3.3-1C). Using LC-MS, I only identified a limited number of amino acids: Tyr, Phe, Trp, p-Glu, Glu, Arg, Ile/Leu, Asp. As reported in the supplemental file 5, their relative abundances were higher in fresh BSG extracts than in those from dried BSG. This is similar to what is reported in a study on *Curcuma longa* L. wherein all the analysed amino acids were higher in the fresh material (Salem et al., 2022). Tyrosine was the amino acid with the highest MS-signal intensity in both treated and untreated BSG, followed by isoleucine/leucine. Instead, glutamic acid, which was observed in high relative concentration in fresh BSG extracts, showed an important decrease in extracts from dried BSG. Just as roasted and black malts contain less free amino acids than green, pale and lager malts, due to the higher temperature of kilning (Samaras et al., 2005), drying BSG reduces the free amino acid content (Salem et al., 2022). The generation of Amadori products may contribute to the

general decrease in the abundance of the amino acids as proposed previously (Kumari et al., 2019; Meitinger et al., 2014).

Di/tripeptides and derivatives of dipeptides such as diketopiperazines had a higher relative abundance in extracts from treated BSG. Confirmed by the corresponding PCA, even with this class of compounds, samples are highly segregated by PC1 (Figure 3.3-2A). In BSG extracts, the presence of these peptides is expected, since, during mashing the temperature gradually increases from 37 to 78°C and the enzymatic activity is enhanced resulting in the conversion of proteins into oligopeptides (Fontana & Buiatti, 2009; Yin Tan et al., 2023). However, the higher concentration in dried BSG sample may be related to the further heat treatment applied on wet BSG inducing further protein degradation (Gautschi et al., 1997; Melius & Yon-Ping Sheng, 1975; Poerschmann et al., 2016). During thermal treatments dipeptides can spontaneously form diketopiperazines by cyclisation of their chemical structures (Borthwick & Da Costa, 2017). In this study, thirteen diketopiperazine were identified in positive mode: 12 proline-based and 1 cyclo (Ile/Leu-Ile/Leu). Due to its ring configuration that stabilises the compounds during thermal treatment, proline is the main amino acid involved in diketopiperazine formation in beer and processed food (Gautschi et al., 1997; Ginz & Engelhardt, 2001; Poerschmann et al., 2016; Sakamura et al., 1978).

One group of highly present compounds in the BSG used here are hordatines. Hordatines are phenolamides and agmatine-hydroxycinnamic acid dimers, which due to their unique presence in barley-based BSG have been grouped in a single class separated from the phenolics and polyamines. Hordatines are formed by the dimerization of p-coumaroylagmatine and feruloyl- agmatine. In the present study, these molecules were detected in higher amounts in extracts with 40%EtOH in both dried and fresh sample. Although a slight increase in their concentrations was observed after the drying process, the parameter that affected the most was the solvent used for the extractions, described mainly by the PC1 (93%) variance (Figure 3.3-2B). Three different hordatines were identified in this study:

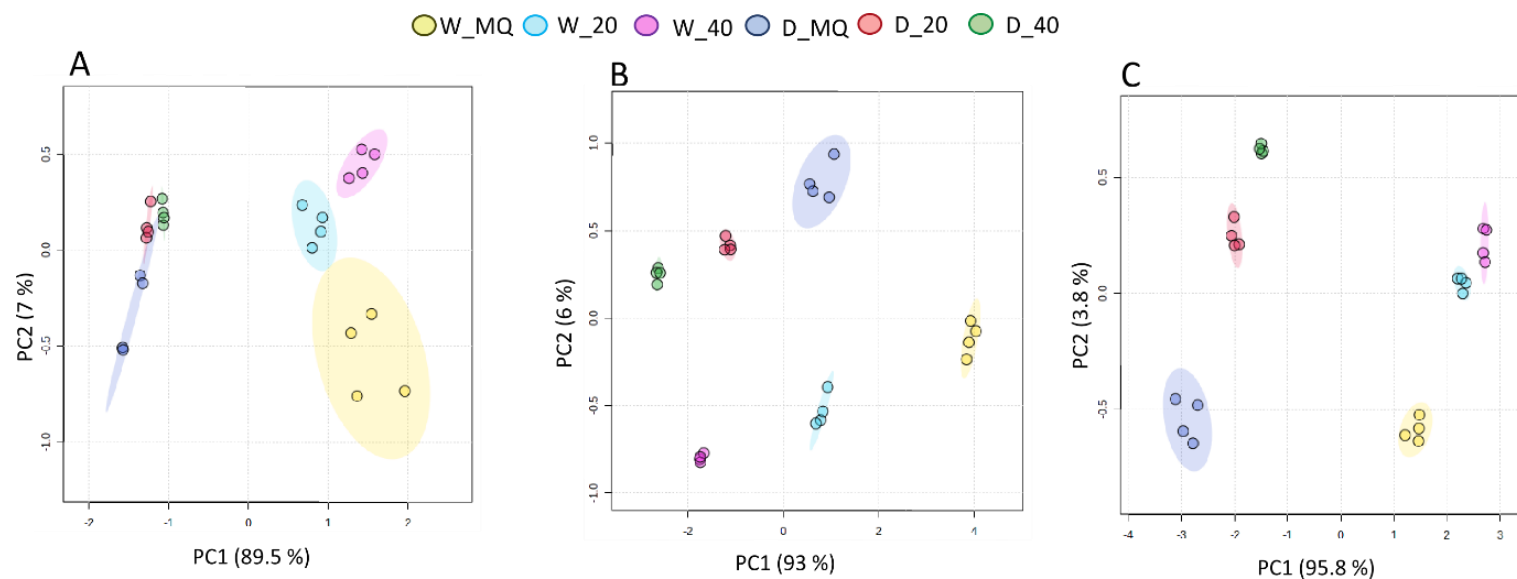


Figure 3.3-2 Principal component analysis (PCA) of: A peptides; B hordatines; C polyamines. W indicates extracts from wet, untreated BSG; D indicates extracts from ground oven-treated BSG. The annotations MQ, 20 and 40 respectively indicate extracts obtained with milliQ water, 20% EtOH and 40% EtOH.

A clear separation is shown in the graph A and C between oven-treated and untreated samples on the PC1, while on the PC2 the variability is mostly explained by the extraction solvent used. Conversely, the PC1 of graph B is mainly determined by the extraction solvent used while samples from the different starting biomasses are separated on PC2.

hordatine A is the homodimer of p-coumaroylagmatine; hordatine B is a p-coumaroylagmatine and feruloylagmatine heterodimer; hordatine C is formed by the dimerization of 2 molecules of feruloylagmatine. Hordatine A, B and C have also been detected as (di)hydroxylated compounds and glycated hordatine A and B (mono- and disaccharide) have also been identified.

The most abundant types of hordatine observed in 40% EtOH samples from both materials were hordatine A, which covered about 40% of the total MS signals of this group, followed by hordatine B, with 35% of the MS intensity. While being abundant, little data on direct health benefits of hordatines are available (Becker, Permann, et al., 2022; Wang et al., 2020), although they were recently found to have the highest antioxidant capacity of all individual beer constituents (Spreng & Hofmann, 2018). Furthermore, hordatines contain agmatine, a molecule involved in the arginine and polyamine metabolism and studied in relation to neuroprotection in clinical studies, and thus can potentially serve as source of this metabolite (Becker, Stegmüller, et al., 2022; Laube & Bernstein, 2017).

A group of polyamines including coumaroyl(hydroxy)agmatine, feruoyl(hydroxy)agmatine, dicoumaroylspermidine, p-coumaroylspermidine, feruloylspermidine and diferuoylputrescine showed lower MS signals in dried BSG extracts than in wet. Regardless of the type of BSG used, higher signal intensities were obtained from extracts with 40%EtOH compared to the use of lower concentrations of ethanol. The PCA analysis indicates that the treatment of the initial biomass is the main driver to separate the samples (Figure 3.3-2C). Spermidine, putrescine and agmatine conjugates are ubiquitous in the plant kingdom and have already been identified in barley and barley-based food and beverages, including beer (J. M. Pihlava et al., 2016; Roumani et al., 2020). Their content in food varies depending on the processing and storage conditions. Conflicting data on the effects of heat treatments on the abundance of polyamines have been reported in literature. Some studies report that high temperature treatments reduce the polyamine content (Muñoz-

esparza et al., 2019; Scoccianti et al., 1995), while other studies indicate that the polyamine content increases after heat treatment (Muñoz-esparza et al., 2019). These contradictory results may indicate that for some plant matrices heat treatments increase the extractability of polyamines while for other matrices thermal degradation may be dominant. Some polyamines have been reported to have beneficial activities on human health. A protective effect against oxidation has been demonstrated for spermidine (Muñoz-esparza et al., 2019) as well as for polyamines conjugated to HCA (Sang et al., 2007). Specifically, agmatine derivatives of HCA have anti-depressant and anxiolytic activity, and can be used for treating metabolic diseases (Akasaka & Fujiwara, 2020; Muñoz-esparza et al., 2019). Therefore, their higher relative abundance in extracts from wet BSG may lead to a higher valorisation potential of these extracts.

Considering the group of compounds referred to as phenolics, a comprehensive list of which is added in a heatmap in supplemental file 5. Among the extraction conditions used, extracts obtained with 40% EtOH gave the highest relative MS signal for both untreated and treated starting biomass. For most of these compounds, the MS intensities increased gradually from water to 40% EtOH samples (supplemental file 5). This agrees with the data from TPC, FRAP and ORAC assays, showing the global TPC and the antioxidant potential of the extracts (Figure 3.3-3). The TPC of untreated and oven-treated BSG extracted with 40% EtOH was 1.97 and 1.19 mg GAE eq./g dw BSG, respectively. For the other extraction solvents, lower values were obtained, e.g. 0.85 mg GAE eq./g dw BSG for wet and 0.67 mg GAE eq./g dw BSG for dried samples extracted with 20% EtOH. Although water extractions showed the lowest TPC, these values are still higher than those reported by Connolly, using fresh BSG (Connolly et al., 2021). The lower TPC in the study mentioned above may be justified by the different solid (biomass content)/solvent volume ratio and shorter extraction time applied. The TPC decrease from 40% EtOH to water was previously found for instance by Meneses et al. (Meneses et al., 2013). Our results for the 40% EtOH extracts are in the TPC value range (from 1.00 mg GAE eq./ g dw

BSG (Socaci et al., 2018a) to 3.3 mg GAE eq./g dw BSG (Almeida et al., 2017)) reported in literature.

The antioxidant capacity obtained with the FRAP and ORAC assays are in line with the trend of the phenolic content analysis: 40% EtOH extracts have a higher antioxidant capacity than 20%EtOH and water extracts (with respectively: 14 $\mu\text{mol FeSO}_4$ eq / g dw BSG for wet BSG, 5.37 $\mu\text{mol FeSO}_4$ eq / g dw BSG for dried BSG (FRAP) and 0.81 mmol TE/ g dw for wet BSG and 0.61 mmol TE/ g dw BSG dried (ORAC)) (Figure 3.3-3). These are higher than FRAP and ORAC values previously reported (Codina-torrella & Rodero, 2021; Crowley et al., 2017). Based on the results of the TPC, FRAP and ORAC assays, the TPC content and the antioxidant capacity are higher in extracts from untreated BSG compared to those obtained from oven-treated BSG. The differences between these extracts are significant, contrary to a previous study which found small, non-significant differences in TPC between extracts from wet, oven- and freeze-dried samples (Santos et al., 2003). While little data are available for extracts from fresh BSG, comparisons between the use of dried and fresh biomass have been made for other plant-based resources. As reported by Orphanides, drying pointedly decreases the antioxidant capacity and TPC of spearmint extracts (Orphanides et al., 2013). A similar observation was made for extracts from black grape (Çoklar & Akbulut, 2017). Furthermore, analysis of fruits such as apricots, cranberries, guava, mango and watermelon (Nunes et al., 2016; Shofian et al., 2011; Vinson et al., 2013), quinoa and cereals such as red rice (Jati et al., 2022; Miranda et al., 2010) resulted in higher TPC values in fresh compared to heat-treated biomass.

PCA analysis within this group of compounds indicates that the treatment of the BSG, projected on PC1 representing 66.2% of the variability, is the main determinant of the variation. Nonetheless and contrary to the previously discussed groups, the solvent mixtures used for the extraction also explain 24.8% of the variation on PC2. The impact of the used solvent is similar for untreated and oven-treated samples (Figure 3.3-4).

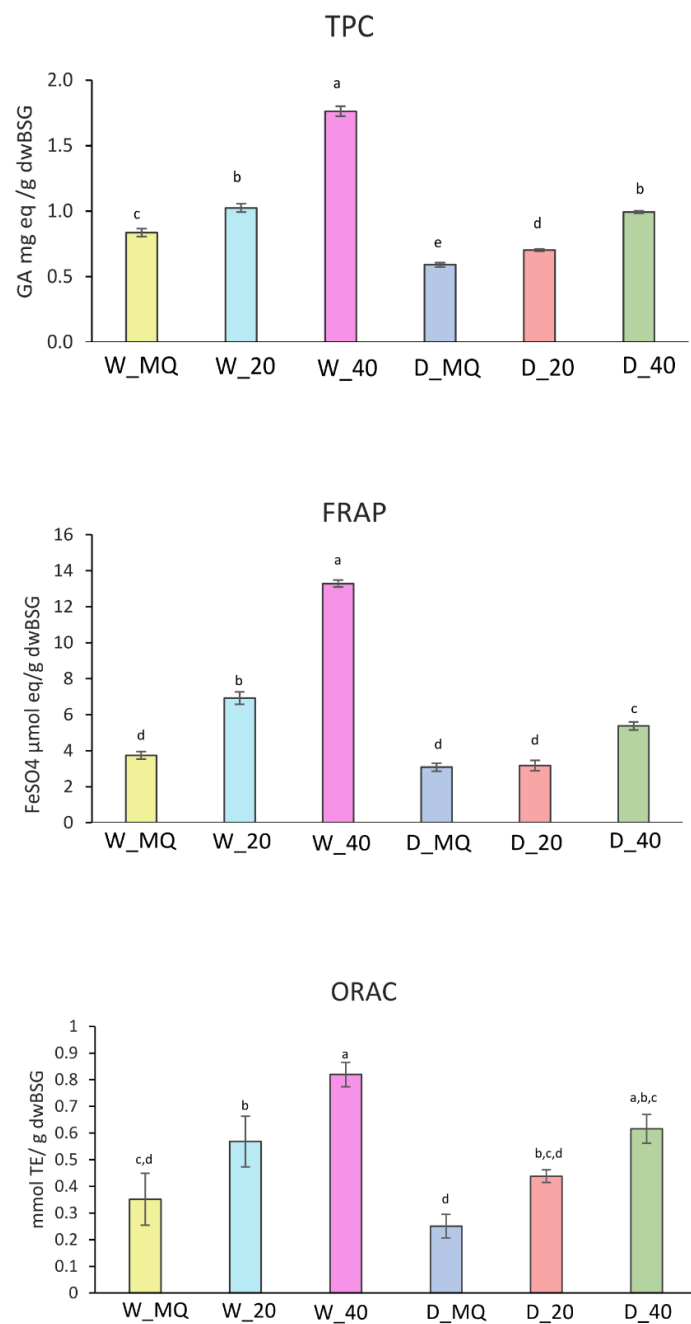
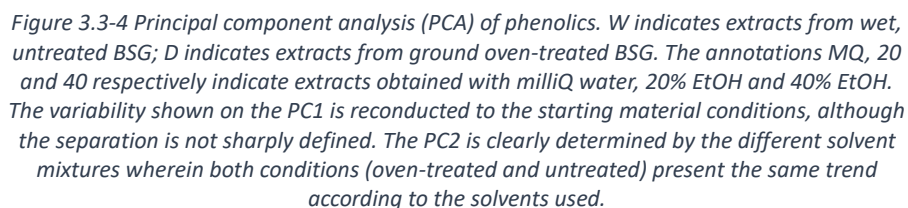


Figure 3.3-3 Antioxidant assay on BSG: total phenolic content (TPC) (Folin-Ciocalteu); antioxidant capacity (FRAP and ORAC). W indicates extracts from wet, untreated BSG; D indicates extracts from ground oven-treated BSG. The annotations MQ, 20 and 40 respectively indicate extracts obtained with milliQ water, 20% EtOH and 40% EtOH in a 1/20 mg BSG/ mL solvent ratio.

● W_MQ ● W_20 ● W_40 ● D_MQ ● D_20 ● D_40



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The first cluster contains among others riboflavin, dihydroxyferulic acid, p-coumaric acid and cinnamic acid, compounds that have a higher MS-signal intensity in extracts from untreated BSG. The lower relative abundance of p-coumaric and cinnamic acid in dried samples is in line with the known thermo-instability of HCA (Arruda et al., 2019), a downtrend also observed in temperature exposed broccoli, spinach and kale (Vargas et al., 2022).

In the second cluster, caffeic acid, hydroxybenzoic acid and hydroxybenzaldehyde were included, compounds that have higher signals in extracts from oven-treated samples. The increased abundance of these compounds were also found following the exposure of BSG to rising temperatures (Rahman et al., 2021). This suggests that the drying process influences their release from the plant matrix or that they are formed through thermal degradation of more complex phenolic compounds. In the third cluster, variations between extracts from wet and dried BSG are notable, but the different solvents also had a considerable impact on the clustering. Compounds as apigenin, scoparin, tricetin and oligomers thereof, (epi)gallocatechin, catechin, procyanidin dimer and trimer and saponarin gave a higher signal in 40% EtOH extracts compared to water and 20% EtOH extracts for both oven-treated and untreated BSG. The signal intensity of tricetin and its derivatives were not affected by the heat treatment, while catechins and oligomers thereof (procyanidin dimer and trimer and (epi)gallocatechin-(epi)catechin) showed a lower signal in extracts from dried BSG. The instability of these latter compounds to different conditions of temperature and pH are known (Khanal et al., 2010; Muhamad et al., 2015; Wu et al., 2019) and explains their lower signal intensity in dried BSG.

The different impact of the pre-treatment and extraction solvent on compounds classified as phenolics was highlighted by looking at which of the compounds contribute most to the accumulate signal for this group in extracts made with 40%EtOH (Figure 3.3-5). While in extracts from fresh BSG catechin and its oligomers represent 56% of the total signal intensity for the phenolic group, there relative

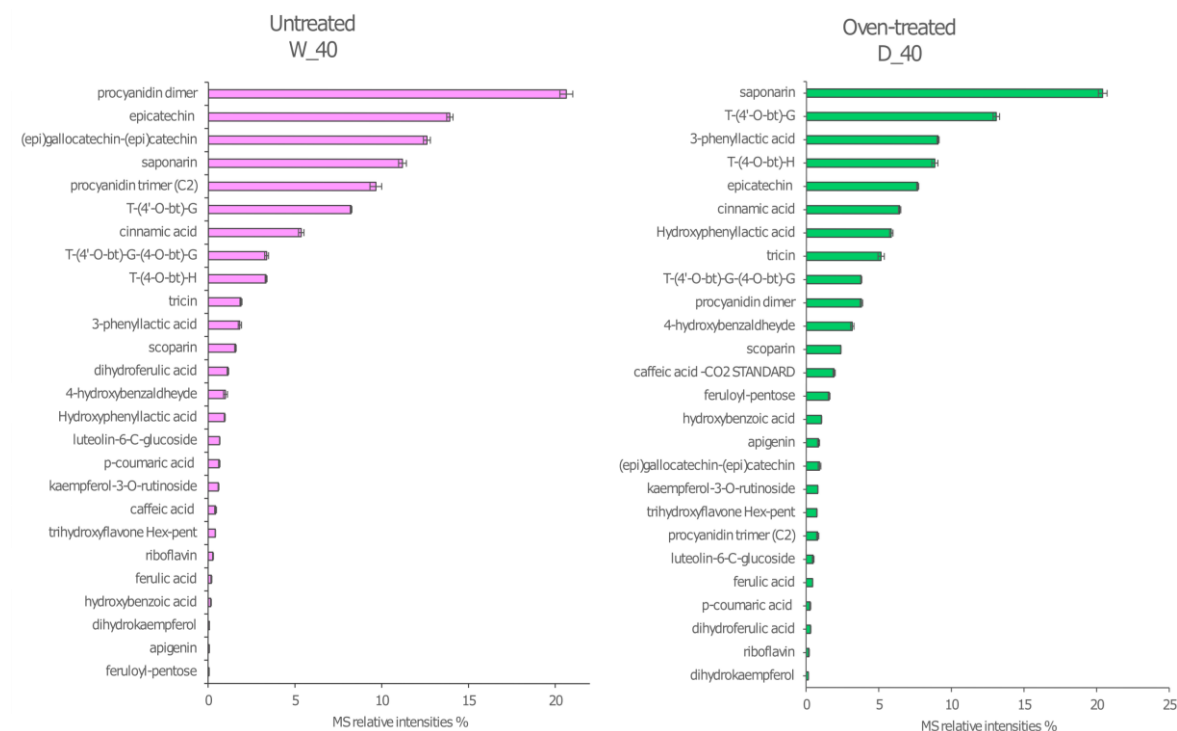


Figure 3.3-5 Graph of the phenolics in oven-treated and untreated BSG. D indicates extracts from ground oven-treated BSG; W indicates extracts from wet, untreated BSG. The annotations 40 indicates extracts obtained with 40% EtOH. The compounds are shown and represented as percentage of the sum of the signal for the entire group of phenolic

contribution decreases in extracts from dried BSG. This decrease which is attributed to the above mentioned known thermolability of these compounds, makes that saponarin and tricin derivatives make up for most of the accumulated signal intensity in extracts from dried BSG (51%). Lipids are the last group of compounds identified in this study. Lipids account for 6-10% of the dried BSG biomass (del Río et al., 2013), and include phospholipids, fatty acids, mono- and di- and triglycerides (Niemi et al., 2012). Accordingly, the principal lipids detected by the UHPLC-MS/MS analysis performed in this study were phospholipids, fatty acids, mono- and diglycerides and their galactosylated forms. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with linoleic and palmitic acid were the most frequently identified lipids, as previously reported (Gorzolka et al., 2016). Among the fatty acids present in BSG extracts (Anca C. Fărcaș et al., 2015; Lordan et al., 2019), linoleic acid was the most detected in this study. In addition, several hydroxylated fatty acids were also detected mainly based on C18 presenting one or two degrees of unsaturation (Hydroxyoctadec-n,n-enoic acids: HOME, DiHOME, TriHOME; HODE, DiHODE, TriHODE; HOTRE). As could be expected, extracts with 40%EtOH resulted in the highest MS intensity signals, and the extraction solvent is identified as dominant determinant in the PCA with PC1 representing 91.5% of the total variance found in the dataset (Figure 3.3-6). Comparing untreated and oven-treated BSG at 40% EtOH, glycated lipids were detected in higher amount in dried samples, while phospholipids were more present in extracts from untreated BSG. Hydroxy-fatty acids showed similar relative intensity signals between both types of starting materials (supplemental file 5). Owing to the high lipid content of BSG and the known disease-preventing and health promoting activities of some of the identified lipids, lipophilic extracts from BSG can be considered as good candidates for cosmetic, medical and nutraceutical applications (del Río et al., 2013).

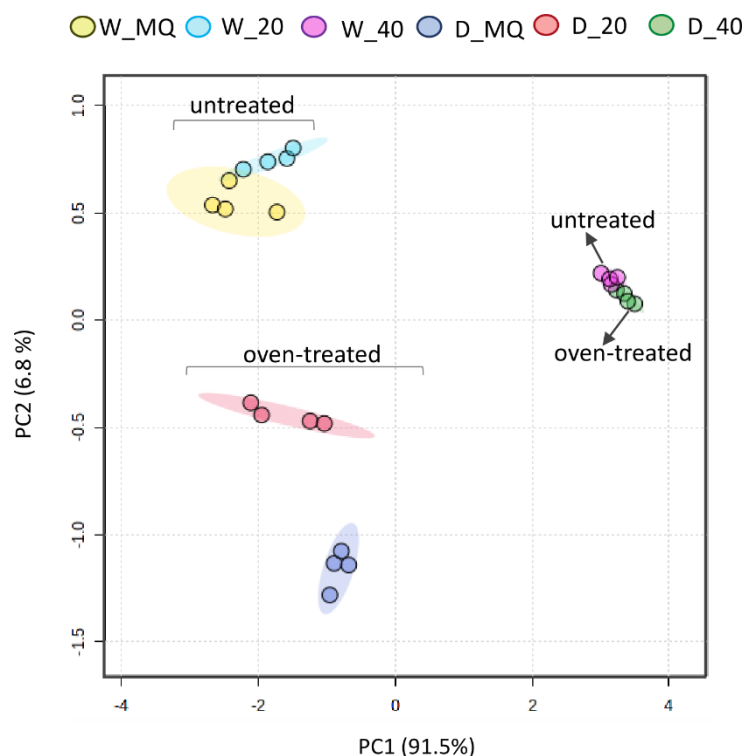


Figure 3.3-6 Principal component analysis (PCA) of lipids. W indicates extracts from wet, untreated BSG; D indicates extracts from ground oven-treated BSG. The annotations MQ, 20 and 40 respectively indicate extracts obtained with milliQ water, 20% EtOH and 40% EtOH. The PC1 covers more than 90% of the variabilities among all the samples. A complete separation is shown on the PC1 between the samples extracted with milliQ water and 20% EtOH from extracts with 40% EtOH. Samples are influenced to a lesser extent by the treatment of the starting material.

Considering the 8 groups of compounds, depending on the compounds the abundance is mainly determined by either the treatment of BSG prior to extraction or by the used extraction solvent. Only for the group of phenolics, as defined above, the interaction between these factors seems to play an important role. For sugars, Amadori products, amino acids, polyamines and peptides and peptide-derivatives the oven-drying pre-treatment has a substantial impact on the final composition of the extracts. Contrary to this, the relative abundance of hordatines and lipids was dominantly determined by the extraction solvent used. The phenolics undergo a subtler impact from both parameters, although the used solvent is the main determinant, the impact of the drying treatment is strongly dependent on the identity of

the compound. Our data indicate that fresh, unstabilised BSG forms an alternative for the generation of extracts destined for the food sector. Extracts from fresh BSG have higher biological activities and a higher or similar abundance of compounds that are currently targeted in studies on the valorisation of residual biomasses in the food sector. Furthermore, since BSG becomes available as the result of an industrial process, its availability is predictable in time and amount, thereby allowing to develop valorisation activities without requiring the investment of time and energy in the stabilisation of the biomass.

3.4 Conclusions

In the context of the necessity to increase the circularity of the economy and the efficient use of resources, the upcycling and/or reuse of by-products of the brewery industry is attracting a lot of attention. BSG makes up to 85% of the residues in the brewing industry and is mainly investigated as a source of compounds with health-promoting properties in the food industry. Most of the studies on the use of BSG include preliminary treatments of the biomass prior to extraction. BSG is mostly dried, which protects it from microbial degradation but also alters the composition of the biomass/extracts and increases process costs and energy consumption.

The described experiment aimed to test the hypothesis that drying of BSG prior to extraction influences the composition of the extracts and thus the valorisation potential of BSG extracts. The presented data give the most complete view on drying-induced changes in BSG extracts. Our data show that the antioxidant capacity of extracts from fresh BSG is higher than that of dried BSG. In the relative abundances of the different groups of compounds, different trends can be highlighted. Amadori products and peptide derivatives (diketopiperazines), are known to be formed during heat-exposure of biomass and are here indeed found to be more abundant in extracts from dried BSG. The abundance of hordatines and lipids is mainly determined by the extraction solvent used, independent of the input biomass. Although the results for the group here defined as phenolics

are nuanced, heat-exposure during drying has a negative impact on the relative abundance of phenolics, polyamines and amino acids and derivatives, the compounds most sought-after in the valorisation of BSG as food components. In conclusion, I show that the use of fresh BSG results in extracts with a specific composition, with a higher antioxidant capacity and a higher content in compounds generally targeted in plant extracts.

Although large-scale extractions and detailed analyses of the biochemical activities of the different extracts are needed, our data shows that fresh untreated BSG results in extracts with an at least equal quality than those obtained from dried BSG. Together with the lack of seasonality in beer production and thus the predictable availability of BSG in amount and time, this makes that the development of industrial utilization of non-stabilized BSG can be envisioned, thereby avoiding an energy-dense step to stabilize BSG prior to industrial use.

4 Isolation of hordatines, the most abundant class of phenolics in barley-based BSG

Highlights

- *Two different approaches including the use of an adsorbent resin in batch and a chromatography separation have been tested for the recovery of an hordatine-enriched fraction.*
- *Both techniques resulted in hordatine-enriched fractions.*
- *The resin approach is most likely feasible at larger scale for the ease of the execution and it resulted in a fraction more heterogeneous compared to the other technique.*
- *The chromatography approach showed a purer fraction, although its application, at larger scale, might be more complex and expensive*
- *The application of both techniques led to recover enrich-fractions with antioxidant capacity.*

Abstract

Among the phenolic compounds found in BSG extracts, there is a group of phenolamides, which is specific for barley-based malt, brewer's spent grain (BSG) and beer. These molecules are dimers of hydroxy-cinnamic acids (HCA) conjugated to agmatine and known as hordatines. Hordatines were recently identified in BSG and beer from barley malt. In the barley plant the antifungal activity of hordatines contributes to defence, but little is known about their other bioactive properties. Recently, they were identified as antioxidant agents in beer and as potent inhibitors of α -glucosidase. With this present study, I aimed to provide some preliminary data for the isolation and production of hordatine-enriched fractions from barley-based BSG and confirm their antioxidant properties. To attain this, two different approaches were developed: one by using an absorbent resin and the other by applying a chromatographic technique. While the first approach resulted in a more heterogenous fraction, the chromatographic approach resulted in a purer fraction and the antioxidant capacity of fractions obtained with both approaches was confirmed. The presented work is preliminary, and further optimisation and the analysis of the biological activities of hordatines is needed, but hordatine-enriched fractions could be a unique product in the upcycling of barley-based BSG.

4.1 Introduction

The extraction of phenolic compounds and the optimisation of the antioxidant potential of the extracts from BSG is the main focus of studies on the valorisation of this biomass. The most targeted compounds are the hydroxy-cinnamic acids (HCA) (ferulic, coumaric, sinapinic and caffeic acid with their dimers) and flavonoids such as catechin, tricin and quercetin. However, recently another group of phenolics, namely hordatines, of which much less is known, is attracting the interest of the scientific community.

Hordatines are secondary metabolites specifically biosynthesised in barley which, according to the scientific literature, are exploited by the plant for their antifungal properties (Stoessl & Unwin, 1970). Recently, these compounds have also been detected in beer and BSG. Hordatines are phenolamides, that are the condensation products of a phenolic acid, such as ferulic, coumaric or sinapic acids, and an amine. This latter compound, in the case of hordatines is represented by the molecule of agmatine. The phenolamide monomers (feruloylagmatine, coumaroylagmatine or sinapoylagmatine) react by generating a dimer, which results in different types of hordatines based on the phenolic acid included in the structure. Hordatine A (homodimer of p-coumaroylagmatine), hordatine B (heterodimer of p-coumaroylagmatine-feruloylagmatine), and hordatine C (homodimer of feruloylagmatine) are the most abundant hordatines detected (Figure 4.1-1). In contrast, hordatine D, which is formed by dimerization of sinapoylagmatine with feruloylagmatine, represents the least common hordatine found in BSG. In addition to these types, hordatines can occur in hydroxylated forms, which have up to 2 hydroxylations, and glycosylated forms, with one or more hexoses bound (Gorzolka et al., 2016; J. M. Pihlava, 2014).

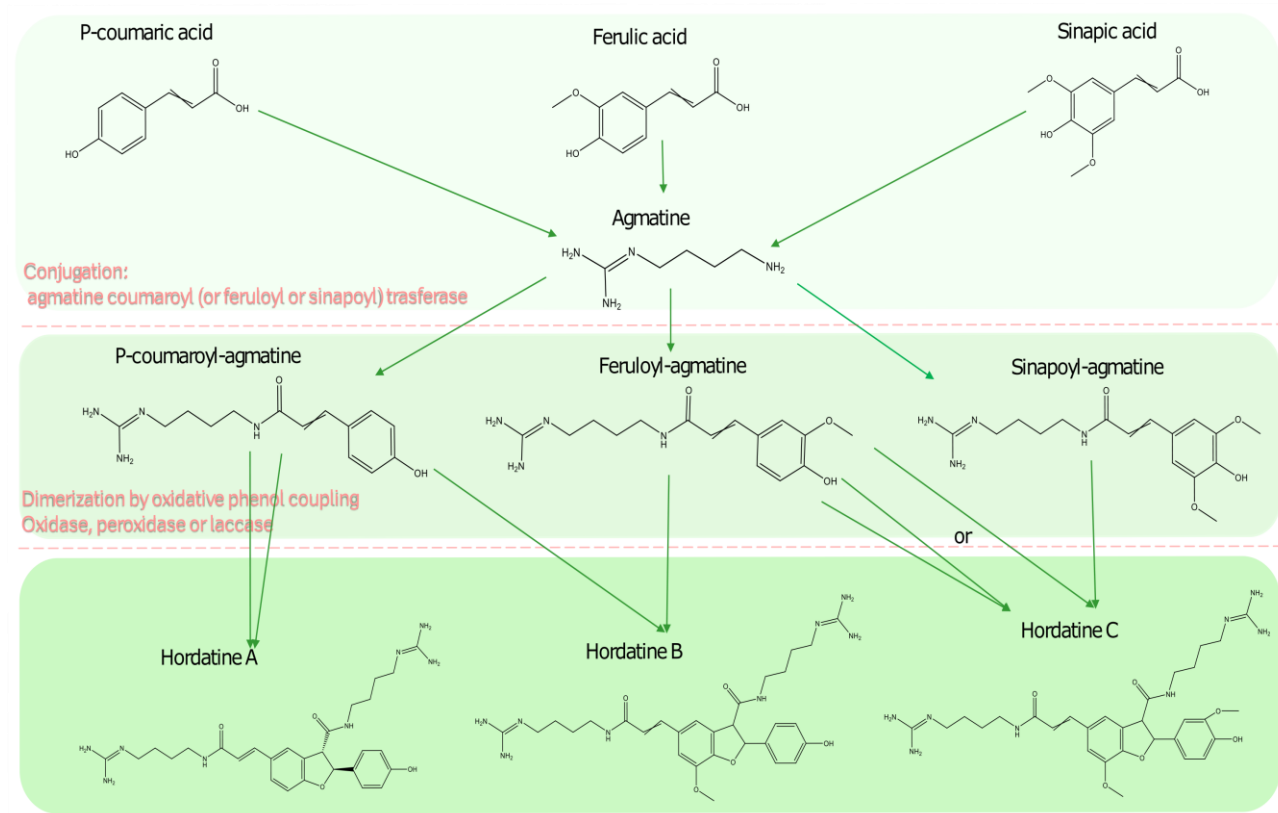


Figure 4.1-1 Scheme of hordatines biosynthesis

Hordatines were quantified throughout the different stages of barley germination, during which an increase in their biosynthesis was recorded, and their anti-fungal capacity was studied (Stoessl & Unwin, 1970). More recently, these molecules were also detected in beer and investigated for their astringent properties, which most likely contribute to the final taste of the beer (Pihlava, 2014), and for their possible involvement in stimulating gastrointestinal motility (Becker et al., 2022). However, as hordatines are phenolics, they might also exhibit interesting antioxidant capacity. In agreement with that, a recent study conducted on the antioxidants present in beer (Spreng & Hofmann, 2018) clearly showed their high contribution to the antioxidant capacity of beer.

In beer, the content of hordatines was estimated to range from 5 to 18 mg p-coumaric acid equivalent/L of beer (p-CA equivalent) (Pihlava & Kurtelius, 2016), meanwhile in BSG extracts their content ranged from 14.2 to 172.2 µg p-CA equivalent/mg extract (Becker et al., 2022). In these studies, hordatine quantification is done using p-coumaric acid as the reference standard, since no commercial references for hordatines were available and today their cost is high.

Since hordatines are detected in relatively high amounts in beer and BSG after undergoing several brewing steps, wherein high temperatures are reached (i.e. kilning), these molecules are considered stable and thermoresistant phenolics (Becker et al., 2022; Pihlava & Kurtelius, 2016). Not many studies were found in literature on the extraction of these molecules from BSG. The most recent study is a work conducted in 2022 by Becker and co-workers, in which they isolated the hordatines from BSG and tested the possible inhibitory activity of these compounds on α -glucosidase. Although this work gave interesting results, it is a preliminary study and further experiments on the isolation of hordatines and on the enzyme-inhibition assays are still required (Becker et al., 2022).

In line with the data mentioned in literature and based on the identification of high-intensity peaks in the BSG extracts indicated in chapter 3, sections 3.2 and 3.3, I aimed to investigate the feasibility of

two different methods to obtain hordatine-enriched fractions. Two protocols were optimised: one by using an adsorbent resin designed for food and biopharmaceutical processes, already used for the isolation of phenolic compounds (Buran et al., 2014) and the other one by applying a chromatography separation. These protocols were evaluated by the measurement of the antioxidant potential and the MS characterisation of the enriched fractions. Contrary to the main focus of most BSG studies on its content of HCA and flavonoids, which certainly contribute to the antioxidant potential of BSG extracts, I aimed to shift the attention to the hordatines. These compounds might exhibit several bioactive properties, which are still under study. Hordatines contribute largely to the antioxidant capacity of beer (Spreng & Hofmann, 2018), so I aim to further investigate the antioxidant capacity by their isolation. The development of feasible protocols to obtain hordatine-rich fractions might open new alternatives to the common sought-after compounds in food grade BSG extractions. Targeted hordatine extraction from barley-based BSG can thus lead to a specific and unique valorisation of this biomass compared to other agri-food residues.

4.2 *Materials and Methods*

4.2.1 Material and extraction parameters

The starting material used in this work, wet barley-Pilsener BSG, was provided by a local brewery, Brasserie Simon Sarl, Wiltz, in Luxembourg, and stored as such under vacuum at -20°C, as mentioned in the chapter 3. The extraction was performed following the approach used in chapter 3.3 with 40% EtOH (40:60 v:v ethanol:water). For the two experiments, 3 extractions were performed with the same parameters, but changing the amount of the starting material and adjusting the volume according to 1:20 s:l (solid:liquid) ratio; for the resin 50 g of wet BSG were used, while for the two trials with the chromatographic technique 10 and 50 g of wet BSG were utilised. Subsequently the extracts were dried and dissolved in different solvents according to which protocol was applied.

4.2.2 Isolation hordatine-enriched fractions

Resin in batch

AmberLite™ FPX66 (DuPont) was pre-treated and activated as previously described with some modifications (Buran et al., 2014). Briefly, the resin was transferred to a column and washed with 95% of ethanolic solution (v/v 95:5 ethanol: water) at ratio 1:10 (solid: liquid, resin: ethanolic solution) at flow rate of 400 mL/h. Maintaining the same ratio s: l (solid: liquid) and same flow rate, the resin was then washed with water and a solution of 4N HCl and 5N NaOH consequently. Afterwards, the resin was rinse with water until pH 7 was reached. The activated, hydrated resin was stored at 4°C prior to the experiment. Dried BSG extract, obtained by the 40% EtOH extraction, was dissolved in water to attain a concentration of 9.4 mg of dried extract/ mL. From this, 3 mL of the extract solution were used for the experiment. Different weights of dried resin, namely 1.7; 3.4; 6.8; 13.6; 20.5; 27.4; and 34 mg, were used to find the optimal resin/extract ratio to collect as much of the targeted molecules as possible. The static adsorption/desorption testing was performed following the protocol described by Buran with some modifications (Buran et al., 2014). The weighted amount of resin was added to the 3 mL sample solution and agitated at 100 rpm (Bench-top Orbital shaker) for 24 h at room temperature in 5 mL tubes. Then, the resin was allowed to sediment and the supernatants collected as the non-adsorbed fraction. The resin was transferred to 15 mL tubes and 6 mL of a 95% EtOH solution was added to the resin for the desorption step. Samples were again shaken for 24 h at room temperature at 100 rpm and finally the solution was collected and analysed as the hordatine-enriched fractions.

Chromatography

Dried BSG extracts were dissolved in 100% MeOH. For the first trial 256.3 mg of dried extract were dissolved in 6 mL of MeOH (42.72 mg BSG extract/ mL), while for the second trial 1.46 g of dried extract was dissolved in 10 mL of MeOH (146.7 mg BSG extract/ mL). The

two solutions were then separately used for the chromatography fractionation analysis.

For preparative HPLC fractionation, an NGC Discover Plus 100 system coupled with an NGC fraction collector (BIORAD LABORATORIE LTD) has been employed. The HPLC conditions were as follows: column C18-RP 12 g (column volume (CV) 17 mL, ratio of length and diameter 4.30, 9×2.1 cm; 180 psi max pressure, PHENOMENEX-CLARICEP Flash cartridge); solvent system A (formic acid/milliQ water (0.1:99.9; v/v), B (formic acid/acetonitrile (0.1:99.9; v/v); flow rate 15 mL/min; injection, through a 1 mL sample loop, volume 1.1 mL (4 times and 6 times respectively for the trial 1 and 2); gradient profile: equilibration of the column at 1% B for 3 CV, sample loading at 1% B and the elution step started at 1% B for 1 CV; gradient 1-10% over 1 CV; gradient 10-25% over 1 CV, gradient 25-40% over 2 CV and 40-100% over 1 CV. Then the column was washed at 100% B for 3 CV in isocratic mode. The wavelengths used for detection were 200; 235; 230; 280 nm (Figure 4.2-1). Fractions of 14 mL were collected and the ones showing an increased absorbance during the run were pooled and dried under vacuum in a CentriVap coupled with a Cold Trap (LABCONCO).

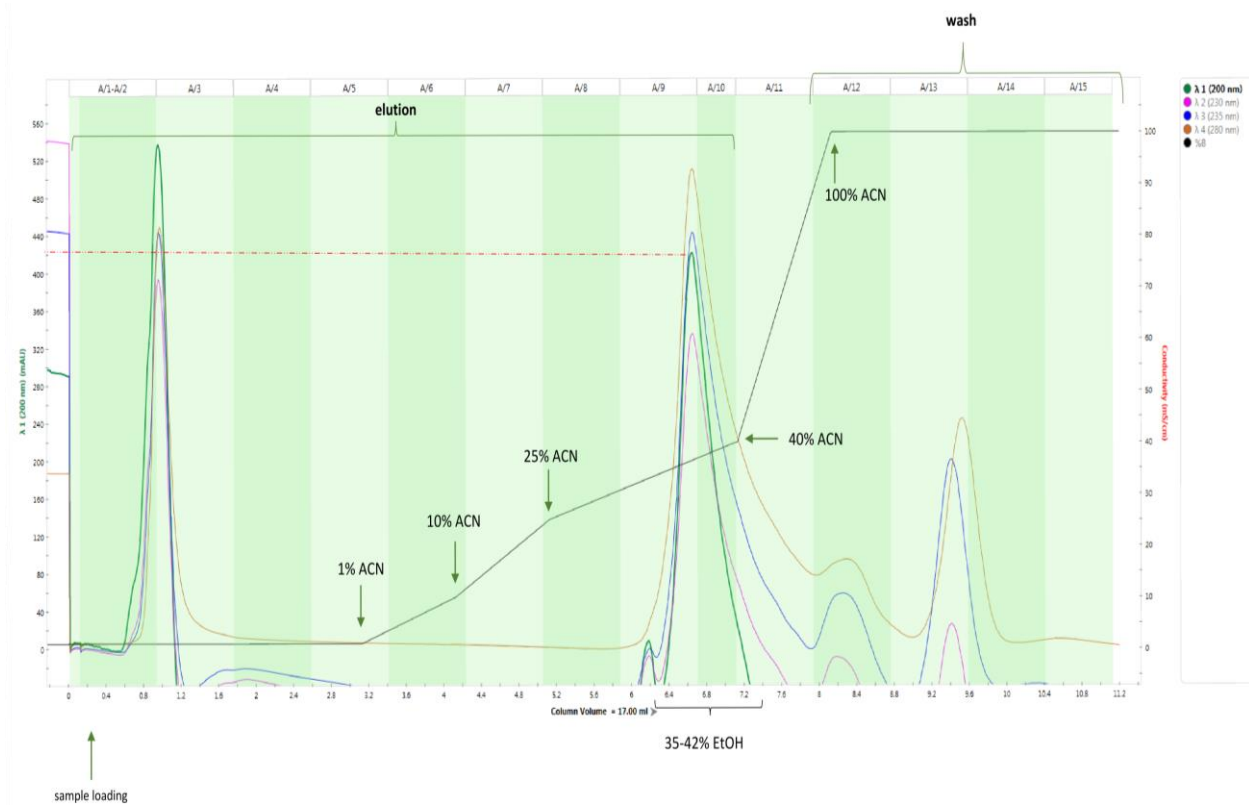


Figure 4.2-1 Example of one of the runs performed with the chromatographic protocol for hordatine-enrichment

4.2.3 Chemical assay

Ferric reducing/antioxidant power assay (FRAP)

All the collected samples from the resin approach, the non-absorbed and the desorbed fractions, and the two recovered fractions obtained from the two chromatographic trials were tested for their antioxidant potential by performing the FRAP assay, as described in the materials and methods of the section 2 and 3 of the chapter 3. FRAP reagent were freshly prepared by mixing 300mM acetate buffer with 10 mM ferric-tripyridyltriazine and 20mM FeCl₃ at pH 3.6. A 96-well plate was filled with 190 µL of FRAP reagent and subsequently, 10 µL of extracted samples were added and mixed directly in the plate. After an incubation in the dark at 21°C for 20 min, the absorbance was measured at 595 nm with a spectrophotometric microplate reader (Spark, Tecan, Männedorf, Switzerland). A standard calibration curve using FeSO₄ at different concentrations was built: 0; 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1mM ($y = 0.6521x + 0.0928$; $R^2 = 0.9994$) and the results were expressed as µmol of ferrous equivalent. Four replicates were analysed in technical triplicates, for each extraction condition.

4.2.4 UHPLC-ESI-MS/MS analysis

An Acquity UPLC I-class ultra-high pressure liquid chromatography (UHPLC) system (Waters, Milford, MA, USA) coupled to an electrospray ionization high-resolution mass spectrometer (TripleTOF 6600+; SCIEX, Framingham, MA, USA) operating in positive mode, since previous analyses showed that hordatines are best observed in positive mode analysis. The parameters have been described previously in paragraph 2.2 and 3.2 of the chapter 3. For the resin approach, samples treated with 6.8; 13.6; 20.5 and 27.4 mg of resin were selected for the UHPLC-ESI-MS/MS analysis, while for the chromatographic method, the fractions obtained from both trails were analysed. In addition, on the second trial, the hordatine content was evaluated by using p-CA (p-coumaric acid) as external standard, using an approach adapted from Pihlava et al, due to the lack of

commercially available hordatine references (J. Pihlava & Kurtelius, 2016). Quantification was done based on the absorbance at 280 and 330 nm, and an external calibration curve from 5 to 200 $\mu\text{g}/\text{mL}$ p-CA was constructed. Due to the different UV absorption between pCA and hordatines, a factor 5 was considered for the calculation, as reported by Becker et al. (Becker et al., 2022).

4.3 Results and Discussion

Hordatines represent a subclass of phenolic compounds classified as phenolamides. These compounds are found in barley and barley-derived products. As shown under the heading 2 and 3 of Chapter 3, independently of which extraction method has been used to treat BSG, hordatines were detected among the main compounds present in BSG extracts. According to the data shown so far, fresh untreated BSG was used as the starting material and 40%EtOH as the extraction solvent to generate extracts for this study. The two main purposes of this study on the recovery of an hordatine enriched fraction were to get information on the composition of the obtained fractions with both techniques, and to evaluate the antioxidant potential related to the presence of hordatines in these fractions.

The non-absorbed fractions and the fraction desorbed from the resin were analysed. Firstly, after 24h of incubation with the resin, the liquid phase (non-absorbed compounds) of all samples was collected, and the antioxidant capacity of these recovered phases measured. As expected, the antioxidant capacity decreased in the non-absorbed fractions when more resin was added. In the samples with the lowest amount of resin, 1.7 mg of resin added to 3 mL of resuspended extract, an antioxidant capacity of 0.44 $\mu\text{mol FeSO}_4 \text{ eq/g dw BSG}$ was measured. When 34 mg of resin was added this value decreased to 0.10 $\mu\text{mol FeSO}_4 \text{ eq/g dw BSG}$. However, from the addition of 13.6 mg of resin a further increase in added resin resulted in a gradually lower decrease in antioxidant capacity at each point until a plateau was reached. Afterwards, the resin was washed with 95% EtOH to release the phenolics and the desorbed fraction collected and

analysed. Here, the antioxidant capacity increased when more resin was added, with 0.47 to 0.80 $\mu\text{mol FeSO}_4$ equivalent/g dw BSG for 1.7 and 34 mg, respectively, confirming the hypothesis on the antioxidant capacity of hordatines (Figure 4.3-1).

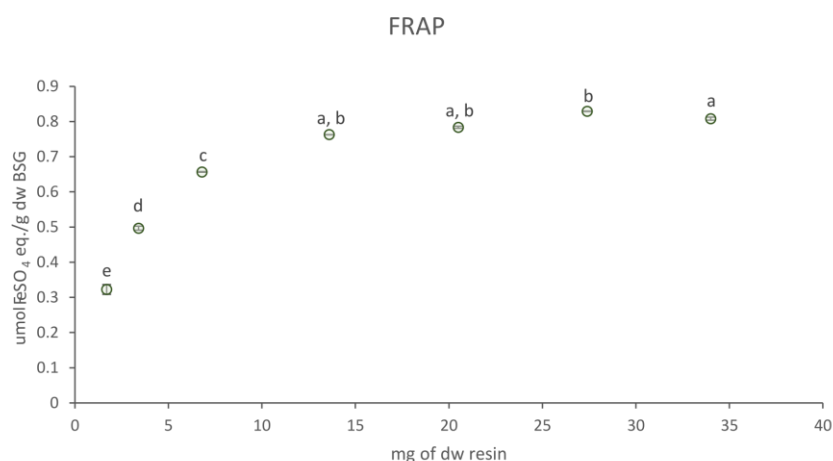


Figure 4.3-1 Antioxidant capacity (FRAP) results on the hordatine-enriched fraction (desorbed fraction) obtained by the resin approach. The amount of dw resin used: 1.7; 3.4.; 6.8; 13.6; 20.5; 27.4 and 34 mg.

In addition, as mentioned above, a plateau was reached when more than 13.6 mg of resin was added to 3 mL of solubilized extract. The desorbed fractions treated with 6.8; 13.6; 20.5 and 27.4 mg resin were then selected for the subsequent MS characterisation. In the analysed fractions, the intensity of the MS signal of the peaks corresponding to hordatines gradually increased when more resin was employed. However, another group of compounds, which is highly present in BSG extracts, namely lipids, was also detected. Among lipids, the highest MS-signal intensities were measured for PC containing C16 and C18:2 fatty acids. However, contrary to the proportional increase in hordatines and the antioxidant capacity (Figure 4.3-1), the relative MS intensities of the lipids were not rising depending on the amount of resin, showing already maximum signal intensities in samples treated with the lowest amount of resin. This demonstrates that there is no apparent correlation between the amount of lipids and the

antioxidant potential of the enriched fractions. Conversely, the cumulative MS signal intensity of hordatines is positively correlated with increased antioxidant capacity. Therefore, this showed that hordatines are the main factor influencing the antioxidant capacity of the enriched fractions obtained in this study, thus confirming the Spreng and Hofmann's study on the contribution of hordatines as an antioxidant agent in beer (Spreng & Hofmann, 2018). The presence of lipids could be attributed to the hydrophobic properties of the resin, which may retain lipids via hydrophobic interactions. The fraction treated with 27.4 mg resulted with the highest relative MS signal intensity of hordatines, it was then selected as candidate for the qualitative comparison (Figure 4.3-2A) with the chromatographic approach. Different types of hordatines were identified: hordatines A, B and C and their glycosylated (up to 3 glycation) and hydroxylated (up to 2 hydroxylation) forms. Hordatine A has the highest MS-signal intensity followed by hordatine B and C (Figure 4.3-2A).

The chromatographic approach was tested in two different trials in order to confirm the feasibility and reproducibility of the developed protocol (Figure 4.2-1). For the first trial, 10 g of untreated BSG were used for the extraction and 0.26 g of dried extract were obtained. For the second trial, 50 g of untreated BSG were used and 1.46 g of dried extract were collected. Samples were dissolved in 100%MeOH for the chromatography analysis. For the first trial the injected concentration was 42.6 mg/ mL. Four runs were performed and fractions covering the main peaks (an example is shown in Figure 4.2-1) were collected and pooled. The pooled sample was evaluated for its antioxidant capacity corresponding to 0.21 $\mu\text{mol FeSO}_4$ eq/g dw BSG. Finally, the fraction was analysed with UHPLC-ESI-MS/MS. The LC-MS data showed that 39% of the signal corresponds to hordatine A, 30% to hordatine B and 4% to hordatine C. The glycated and hydroxylated forms of the main hordatines were also observed but with low intensities, in line with the results from the resin experiment (Figure 4.3-2).

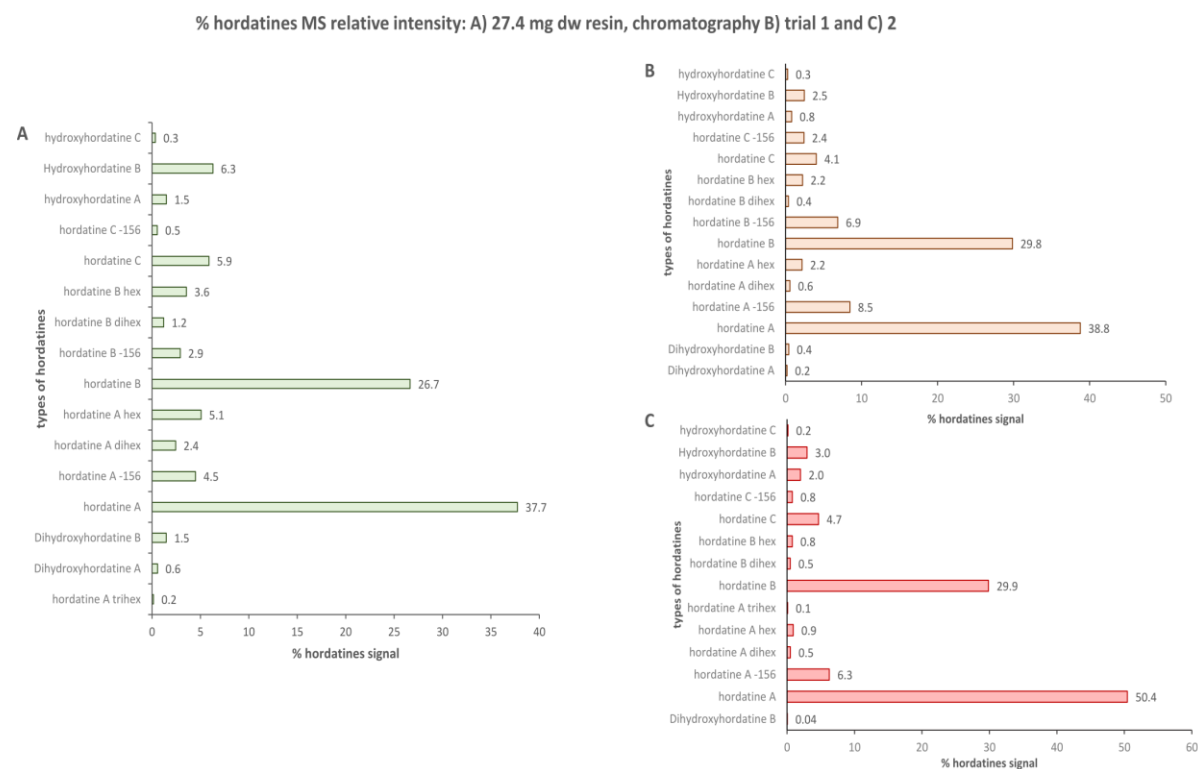


Figure 4.3-2 Percentage of MS relative intensities of the identified hordatines: A) from 27.4 mg of dw resin; B) and C) from chromatography, respectively, trial 1 and 2. The obtained enriched fraction from the resin approach corresponds to the extract treated with 27.4 mg of dw resin. Trial 1 and trial 2 referred to the two extracts obtained from 10 and 50 g of untreated, wet brewer's spent grain (BSG).

For the second trial, based on the previous data and with the aim of increasing the concentration of hordatines obtained, a higher sample concentration of 146 mg/ mL was used. Six runs were then carried out and, again, the targeted fractions were collected and pooled. The antioxidant capacity measured from this latter corresponded to 0.96 $\mu\text{mol FeSO}_4$ eq/g dw BSG (Figure 4.3-3) and, as in the first trial, the UHPLC-MS/MS analysis was performed to identify the composition of the obtained fraction. Similar to the first trial, hordatine A had the highest MS-signal intensity (Figure 4.3-2B and C). The sample treated with 27.4 mg of resin was selected for the comparison of the hordatine distribution in the different approaches. The data from the resin experiment with 27.4 mg of resin added are in agreement with those obtained with the chromatographic methods: hordatine A has the highest relative MS signal with 37% of the total intensity, followed by the hordatine B accounting for 27% and the hordatine C for 4% (Figure 4.3-2A).

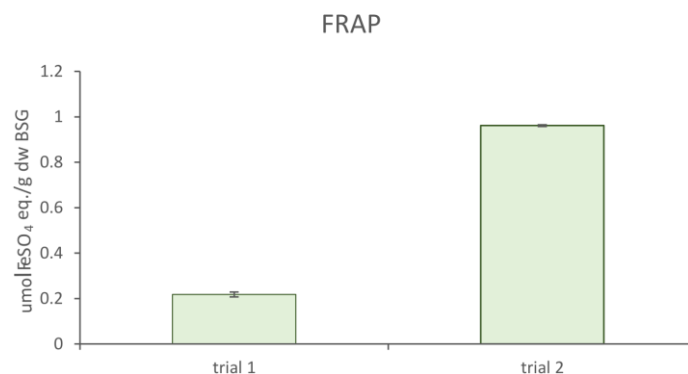


Figure 4.3-3 Antioxidant capacity (FRAP) results of the hordatine-enriched fractions obtained by the chromatographic approach. Trial 1 and trial 2 referred to the two extractions performed from 10 and 50 g of untreated, wet brewer's spent grain (BSG).

In contrast to enrichment with the resin, the chromatographic fractionation resulted in fractions containing the hordatines as main compounds. In addition, the content of hordatines from the second trial was quantified, and it amounted to 175 μg p-CA equivalent/mg of dried fraction and 242 μg p-CA equivalent /g of dw BSG. These are

preliminary data, but the obtained values are slightly higher than those reported previously (Becker et al., 2022).

To conclude, the resin approach yielded a heterogeneous fraction containing a mixture of lipids and hordatines, contrary to the chromatographic method, with which a purer hordatine fraction with a slightly higher antioxidant capacity calculated on BSG dry weight basis was obtained. Depending on the application that is envisioned both approaches for obtaining hordatine-enriched fractions may be used. The choice between approaches will furthermore be determined by factors such as ease and cost of execution. Both approaches use resources that can be re-used, and the amount of resin can be optimized due to the plateau that forms when super-optimal amounts are added.

This small size study aimed to provide preliminary data on the generation of hordatine-enriched fractions, compounds that are highly abundant in BSG extracts and for which no specific valorisation is currently known. However, recently these compounds started attracting the attention of the scientific community for their potential bioactivities. Among others this resulted in studies on how to synthesise these compounds either to produce standards for in-depth studies or as semi-chemical production platform for industrial commodities (van Zadelhoff et al., 2024; Van Zadelhoff et al., 2022). In the past, hordatines were investigated for their antifungal activity in the plant world, today they are mostly studied for their potential benefits for human health. I confirmed their potential as antioxidant agents (Spreng & Hofmann, 2018).

Today, BSG valorisation mainly targets the optimization of the extraction of HCA and flavonoids. However, an economically sound approach to obtain hordatine-enriched products may provide a new and unique valorisation potential for barley-based BSG. Here, two different approaches were investigated, each giving interesting results in obtaining very different hordatine enriched-fractions. Further

optimisation is needed; the repeatability must be assessed and the valorisation potential of hordatine enriched-fractions explored.

5 References

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CHAPTER 4.

BSG VALORISATION: A NOVEL BIOREFINERY APPROACH

Foreword

In the previous chapter, a hydroethanolic extraction from BSG was optimised. Therefore, this extraction was selected as the first step of the biorefinery model developed in this Chapter 4. This approach involves the application of a sequential extraction cascade aiming to maximally valorise the recycling potential of BSG. It resulted in four outputs namely, hydroethanolic, ethanolic, protein and arabinoxylan fractions, which were then characterised.

1 BSG: a novel biorefinery approach

Highlights

- *A biorefinery approach was developed through sequential extraction from untreated BSG.*
- *Four fractions with potentially valorizable properties, namely hydroethanolic, ethanolic (lipid), protein and arabinoxylan fractions, were collected.*
- *On a weight balance base, 49% of the starting BSG biomass has been extracted and is potentially valorizable with this process.*

Patent application initiated, filling foreseen November 2024

Abstract

Brewer's spent grain (BSG) is the most abundant solid residue from the brewing process and its production is foreseen to increase in the near future. Currently, BSG is poorly valorised: it is mainly used as cattle feed or disposed of in landfills, and furthermore, its management is becoming increasingly challenging. To comply with socio-economic demands to increase the circularity of the economy, new up-cycling strategies are needed. The scientific focus in this context is on the upcycling of agri-food residues into added-value products. Although BSG is a heterogenous material, studies on BSG generally focus on the optimisation of a single extraction thus limiting its valorisation potential to a specific class of compounds. To maximally valorise BSG, a biorefinery model was developed through sequential extraction that results in four fractions, which were previously targeted in single-extraction studies. The fractions were characterised, compared to single extraction studies and the specific parameters for each fraction were found to be in line with values reported in literature. The shown data demonstrate that four potentially valorisable outputs can be recovered using the described extraction cascade. However, further research is needed to assess the detailed activities of these fractions and to perform the extractions at a scale compatible with industrial activities.

1.1 Introduction

With the expansion of the industrial activities and the population increase, the generation of agricultural and food processing waste is increasingly a crucial societal issue. Over the past years, the amount of food waste has risen and is expected to rise as a consequence of the higher population's demand for food, leading to an increased attention and concern about its management and environmental and economic impact. Accordingly, the management of its disposal has become more challenging, due to the reduction of the physical space for landfilling, which causes an increase in the costs associated with the handling of biomass residues. Furthermore, the society is more and more sensitive to these issues and sustainable plans for a more ecological economic development are currently part of the global progress strategy (i.e. Sustainable Development Goals, UNEP, 2015; Bioeconomy Strategy, European Commission, 2018; and the Circular Economy Plan, European Commission, 2015) (Caldeira et al., 2020).

A large amount of bio-waste is generated all along the food supply chain with food processing as a step that highly contributes to the generation of organic residues. Some of this waste can be prevented by applying proper management strategies to reduce food loss and residue generation, while another part cannot be avoided (Morone et al., 2019). For these latter organic residues, new alternatives to disposal need to be developed and potential solutions can be found in the recycling concept by promoting the valorisation of these residues through their re-use and their re-integration in industrial cycles (Teigiserova et al., 2019).

Recycling opens new roads to waste exploitation, thereby changing the concept of waste, which is no longer considered only as residue to be disposed of, but becomes a source to be valorised. More and more models of waste valorisation are currently explored by the industrial sector and the scientific community. One promising strategy is the application of the biorefinery approach (Arias et al., 2023). Until

recently, the general concept of biorefinery involved the fractionation of lignocellulosic biomass into valuable products mostly including bioenergy (Mohanty et al., 2021; Yaashikaa et al., 2022). Currently, however, the biorefinery model also envision the production of high added-value products like essential oil, organic acids (lactic and citric acids), phenolic compounds, pectin, proteins, carotenoids, dietary fibres etc (Sarangi et al., 2023), which can find their re-integration in the food, pharmaceutical and cosmetic sectors (Carmona-Cabello et al., 2018). Agro-industrial waste represents a valid matrix for the development of this system. Fruits, vegetables, cereals, meat and dairy products, such as orange and banana peels, spent coffee and cocoa grounds, tomato and potato pulps, bran and coat from corn, barley or wheat are just some of the potential biomasses suitable for a biorefinery model application (Caldeira et al., 2020; Yaashikaa et al., 2022).

One of the globally developed food manufacturing sectors that contributes to the generation of organic residues is the brewing industry. Beer is the most consumed alcoholic beverage worldwide and its production increased in the last years and is foreseen to rise in the near future. The most abundant by-product generated from beer production is BSG accounting for 85% of the total residues from the brewing process (each 100 L of produced beer results in about 20 kg of wet BSG) (Agrawal et al., 2023; Mussatto et al., 2013). BSG is a lignocellulosic biomass consisting of the insoluble part of the grain: husks, seed coats and pericarps. It is a heterogenous material generally composed of fibres (20-70%), proteins (15-30%), lipids (6-10%), phenolics (0.5-2%), vitamins and minerals (Bonifácio-Lopes et al., 2020). However, its chemical composition varies depending on the grains used, the agricultural practises and the parameters used during the brewing process (Jackowski et al., 2020). Today, this biomass is considered a low-cost residue (35-40 euro/fresh ton), and it is mostly used as cattle feed. However, because BSG contains health promoting components, it is also investigated as a source of bioactive compounds and functional ingredients for the food, pharmaceutical and cosmetic sectors (Rachwał et al., 2020).

BSG can be milled to flour and, mixed with other types of flour, and integrated into products such as pasta, bread, and cookies for their enrichment. Another option consists of using BSG for the extraction and isolation of bioactive compounds. The focus in these valorisation efforts is mainly on phenolics, arabinoxylans and proteins. The first two are mainly studied for their health benefits, while the techno-functional properties of BSG protein extracts can be exploited for applications in the food sector (Hellebois et al., 2021). Phenolic acids such as ferulic, coumaric, caffeic, syringic or vanillic acid, and flavonoids such as catechins, are known for their antioxidant potential. Arabinoxylans are the most abundant hemicellulose type in BSG and known for their prebiotic and gut regulatory activities (Lynch et al., 2016, 2021). BSG protein extracts showed emulsifying and gelling properties, and they are also studied for their involvement in enhancing food flavour. Moreover, BSG protein isolates are considered for their antioxidant activities and their potential anti-inflammatory, immunomodulatory and antihypertensive properties (Hellebois et al., 2021). In addition, BSG is a source of triglycerides, phospholipids and fatty acids. Some of the lipids identified in BSG extracts are known for their nutraceutical, pharmaceutical and cosmetic properties. Linoleic acid, an omega-6 unsaturated fatty acid, is highly present in BSG and is an essential dietary fatty acid. Moreover, the potential contribution of BSG lipids in increasing cardiovascular health has already been described and it is under study for further developments (Lordan et al., 2019). Despite their potential BSG-extracted lipids are less studied compared to the other BSG constituents.

Although there are numerous studies on the valorisation of BSG for its bioactive compounds, most studies focus on the optimized extraction of a single class of molecules (specifically phenolics, protein and arabinoxylans), thereby only targeting a part of the valorisation potential of BSG (Lisci et al., 2024). Being an heterogenous biomass, BSG is a rich source of different groups of compounds, which can be extracted separately to obtain different high added-value fractions. The application of a biorefinery approach might thus represent an

innovative alternative for a holistic exploitation and valorisation of BSG (Pabbathi et al., 2022). Current biorefinery studies on BSG mainly focus on producing bioethanol, biobutanol, biodiesel and biogas generally applying acidic or enzymatic pre-treatments prior to solid phase fermentation (Aggelopoulos et al., 2014; Pabbathi et al., 2022). To our knowledge, few studies used a biorefinery approach for a multi-product valorisation of BSG. Among them, the main targeted bioactive molecules in a sequential system are the arabinoxylans with a consecutive conversion of the residual biomass in bioethanol or biobutanol (González-García et al., 2018; López-Linares et al., 2020). Likewise, a biorefinery approach was described wherein a first bioactive fraction is obtained and evaluated for its antioxidant capacity, while the second and last step of the process focused on bioethanol production (Lisci et al., 2024). In these three studies, only the first fraction involved the recovery of bioactive compounds with the production of energy-carriers on the residue. In contrast, some studies on BSG valorisation proposed a sequential approach for the recovery of several potential bioactive fractions including proteins, hemicelluloses and lignin (Belardi et al., 2023; Vieira et al., 2014). While Vieira et al. focused on the extraction of two fractions, namely proteins and arabinoxylans (Vieira et al., 2014), Belardi and colleagues modified the process by also recovering cellulose and lignin fractions (Belardi et al., 2023).

In the present work, I developed a laboratory-scale biorefinery model resulting in multiple outputs. In contrast to published work, our model starts with the sequential recovery of a hydroethanolic fraction containing compounds of interest for the food industry, followed by the recovery of a lipid-enriched extract. Only afterwards, the proteins and arabinoxylans fractions are collected. Finally, a four-step sequential extraction system has been designed, each fraction corresponding to a specific class of compounds. All the fractions have been evaluated for their extraction yield, which was comparable to yields obtained in studies focussing on one specific extraction.

1.2 *Material and Methods*

1.2.1 **Material**

A batch of wet barley-Pilsener BSG was provided by a local brewery in Luxembourg (Brasserie Simon Sarl, Wiltz). The freshly collected BSG was immediately put under vacuum and stored at -20°C and thawed (referred to as fresh) as previously described (Minestrini et al., 2022).

1.2.2 **Preliminary extractions**

To assess the biorefinery approach including four sequential extractions hydroethanolic (first extraction), ethanolic (second extraction) and alkaline (third and fourth extractions) extractions, a preliminary test was performed to determine the most suitable solvent for the lipophilic extraction. An extraction was performed with 100% hexane, ethanol, or ethyl acetate, as well as with the Folch mixture, namely 2/1 v/v chloroform/methanol mixture. The test was performed on dried BSG residue collected after the first step. The extractions were carried out under nitrogen to avoid oxidation as follows: 500 mg of dried BSG residue (from the first step) were mixed with 5 mL of solvent (500 mg/5 mL, 1:10 w/v ratio). An ultrasound pre-treatment was performed for 10 min at 37 KHz at 20°C and then the extraction was carried out at room temperature for 2 hours under agitation (100 rpm, Bench-top Orbital shaker). After extraction, samples were filtered with glass fibre filters (Carl Roth GmbH, type CR259, 47 mm diameter, $\geq 0,6 \mu\text{m}$), and the supernatants were collected and characterized by UHPLC-ESI-MS/MS analysis, as previously described (Minestrini et al., 2022).

1.2.3 **Biorefinery approach: sequential extraction procedure**

The designed biorefinery approach involves four sequential extraction steps (Figure 1.2-1): hydroethanolic (first extraction), ethanolic

(second extraction) and alkaline (third and fourth extractions) extractions.

- 1- Hydroethanolic extraction: 50 g of fresh BSG were extracted with 40% ethanol solvent mixture (40:60 ethanol: water v/v %) at the ratio 1:20 (solid: solvent, w/v) at room temperature for 4 h under agitation (100 rpm, Bench-top Orbital shaker). Afterwards, the solution was filtered with a glass fibre filter (Carl Roth GmbH, type CR259, 150 mm diameter, $\geq 0,6 \mu\text{m}$). The extract, representing the first bioactive fraction, was collected for analysis. The extraction efficiency (weight of extract/weight of dw BSG) was measured. The antioxidant capacity (FRAP assay) and the total phenolic content (TPC, Folin-Ciocalteu) of the extracts were determined, and a metabolomic analysis was performed. The solid residue after filtration was collected and dried under a ventilated hood for 48 h at room temperature to reach a final moisture content of below 8%.
- 2- The dried solid residue after step 1 was used for a second, lipophilic extraction. The extractions were carried out under nitrogen to avoid oxidation as follows: 50 g of dried BSG residue (from the first step) were mixed with 0.5 L of solvent (50 g/0.5 L, 1:10 (w/v) ratio). An ultrasound pre-treatment was performed for 10 min at 37 KHz at 20°C and then the extraction was carried out at room temperature for 2 hours under agitation (100 rpm, Bench-top Orbital shaker) After extraction, samples were filtered with glass fibre filters (Carl Roth GmbH, type CR259, 150 mm diameter, $\geq 0,6 \mu\text{m}$), and the extracts, representing the second fraction, were collected and characterized by UHPLC-ESI-MS/MS analysis, as previously described (Minestrini et al., 2022).

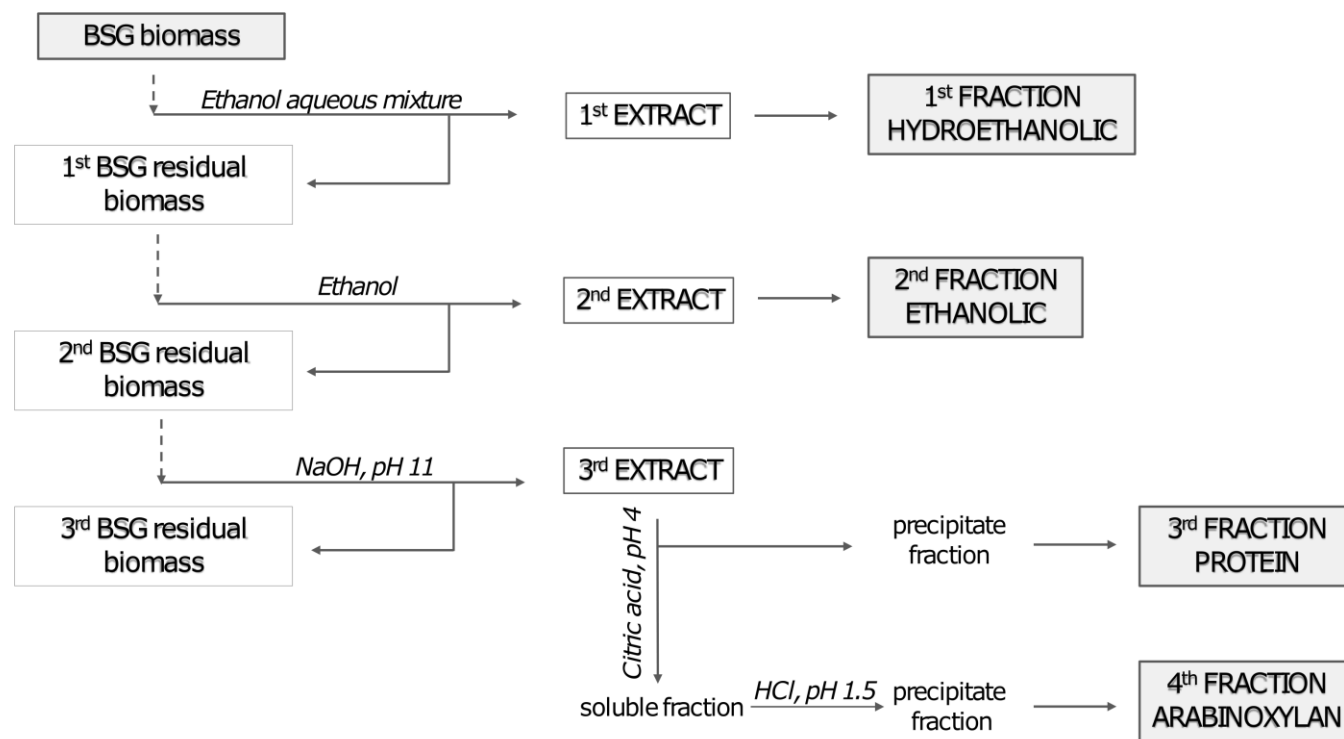


Figure 1.2-1 Scheme of the biorefinery approach used in the present study for brewer's spent grain (BSG): the four sequential extractions and the related collected fractions are illustrated

The solid residue was again collected and dried under a ventilated hood for 24 h at room temperature to a final water content of less than 6.2 %.

- 3- The dried residue collected from the ethanolic extraction, was ground and used for the extraction of proteins and arabinoxylans. These two fractions were obtained in one step following the protocol described by Vieira et al. with some changes (Vieira et al., 2014). With the aim of selecting the most suitable pH to obtain the highest extraction efficiency, two different pH values, 10 and 11 were tested. The dried residue of a sample, treated as described above in steps 1 & 2, was suspended in water in a 1:20 (w: v) ratio and the pH adjusted to 10 or 11 with 5M NaOH. The solution was agitated at 300 rpm at 50-55°C for 4 h after which it was centrifuged for 10 min at 3250g. The pellet was discarded and the supernatant was collected and acidified to pH 3.5-4 (pH range corresponding to the isoelectric point of the dominant BSG proteins) using a saturated citric acid solution and maintained under constant agitation for 1 h at room temperature to allow proteins to aggregate and precipitate. Afterwards, the solution was centrifuged for 10 min at 3250g and the supernatant and pellet separated. The pellet, composed of precipitated proteins, was washed 3 times with water and the pH was adjusted to pH 7 with 0.1M NaOH and dialysed (Benzoylated dialysis tube, Sigma-Aldrich, cut-off 2 kDa) for 48 h. Finally, the retentate was freeze-dried and kept at room temperature in the desiccator. Since the best results were obtained with a pH of 11, the supernatant separated from the protein pellet collected after acidification at pH 3.5-4 was used for subsequent experiments.
- 4- The pH of the supernatant separated from the protein pellet was then adjusted to 1.5 with 1M HCl. To precipitate the arabinoxylans, ethanol was added till a final concentration of 70%. The solution was incubated at 4°C over-night, and the arabinoxylan precipitate was collected through centrifugation

for 10 min at 3250g at room temperature. The dried pellet represents the 4th and last fraction containing arabinoxylans. All the experiments were conducted in quadruplicate.

1.2.4 Raw BSG composition analysis

The moisture content of the raw BSG used as starting material was established by weighing the BSG sample before and after oven-drying at 105°C for 24 h. The protein content was obtained by elemental analysis (Elementar, Variocube, Germany) with a conversion factor of 6.25 x N%. The lipid content was determined through hexane extraction and the ash fraction was assessed by weighting after 6h in a furnace at 550°C. The fibre and carbohydrate content of raw BSG was considered as the remaining part and calculated by difference (Hellebois et al., 2021).

1.2.5 Analysis of the hydroethanolic fraction

Total phenolic content assay (Folin-Ciocalteu)

The reagents used were as follows: 7.5% w/v Na₂CO₃ solution and 1N Folin-Ciocalteu reagent (diluted from the stock solution, supplied by Sigma Aldrich, F9252-100 ML) (Singleton & Rossi Jr., 1965). The 25 µl of Folin-Ciocalteu reagent were combined with 50 µL of each sample and standard dilutions and then a volume of 125 µL of Na₂CO₃ solution was added to each mixture in a 96-well plate. The plate was covered with aluminium paper to avoid degradation of the reagents by light and incubated at room temperature for 30 minutes. Using a spectrophotometric microplate reader (Spark, Tecan, Männedorf, Switzerland), the absorbance at 765 nm was determined. Gallic acid was used to generate a calibration curve ($y = 6.6173x + 0.0524$; $R^2 = 0.9971$), and the TPC results expressed as gallic acid equivalents (GAE). Four replicates were analysed in technical triplicates.

Ferric reducing/antioxidant power assay (FRAP)

The ability of molecules to convert Fe (III) into Fe (II) is utilized by the ferric reducing power assay (Benzie & Strain, 1996). The reduction of the iron, namely reducing power, is directly correlated

with the antioxidants present in the sample. The greater the amount of reduced iron, the more intense the blue colour of the solution, and the higher the antioxidant capacity of the sample. Fresh FRAP reagent was made by combining 20mM FeCl₃, 10mM ferric-tripyridyltriazine, and 300mM acetate buffer at pH.3.6 Hundred-ninety µL of FRAP reagent was added to a 96-well plate, to which 10 µL of the extracts were added. After a static incubation of 20 minutes in the dark at room temperature, the absorbance was measured at 595 nm using a spectrophotometric microplate reader (Spark, Tecan). FeSO₄ at various concentrations (0-1 mM) was used to build a standard calibration curve ($y = 0.7132x + 0.0813$; $R^2 = 0.9994$) and the results were expressed as µmol of ferrous equivalents. Four replicates were analysed in technical triplicates.

1.2.6 Analysis of the preliminary and optimised (ethanolic) lipophilic extract

UHPLC-ESI-MS/MS analysis

Five µL of each extract were analysed using an Acquity UPLC I-class ultra-high pressure liquid chromatography (UPLC) system (Waters, Milford, MA, USA) connected to an electrospray ionization high-resolution mass spectrometer (TripleTOF 6600+; SCIEX, Framingham, MA, USA) operating in both positive and negative modes. Formic acid 0.1% in milliQ water (A) and formic acid 0.1% in acetonitrile (B) were the solvents used. The gradient applied was as follows: 1% B at 0 min, 1% B at 4 min, 5% B at 16 min, 40% B at 35 min, 100% B at 45 min, 100% B at 50 min, 1% B at 54 min, and 1% B at 60 min. A reverse-phase Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size) was used with a flow rate at 0.5 mL/min at 50°C.

The analysis was performed with the following parameters: the source was heated up to 650°C; for the negative mode the voltage of the ion spray was fixed at -4.5 and for the positive at 4.5 kV; the curtain gas (nitrogen) was at 30 psi; the nebulizer gas (air) was at 55 psi and the

turbine gas (air) 50 psi. Declustering potential was set at 60 V and -60 V, respectively in positive and negative modes. The 10 most intense single-charged precursor ions were selected for MS² analysis in high sensitivity mode, with an accumulation time of 200 ms and a threshold of 100 counts per second. A sweeping collision energy of -15 V or 15 V in the negative and positive modes was applied for each precursor ion during the 2-second cycle. The dynamic exclusion was set at 2 s after three occurrences prior to the subsequent fragmentation. In the profile mode, spectra for MS² were acquired from 50 to 2000 mass-to-charge ratios (m/z), whereas spectra for MS were detected between 100 and 2000 m/z.

UHPLC-ESI-MS/MS identification

Progenesis QI (v2.3, Nonlinear Dynamics, Newcastle upon Tyne, UK) was used to analyse the UHPLC-ESI-MS/MS data. Firstly, a filtration step was performed to remove those LC-MS features, defined as the specific RT (retention time) and m/z (mass/charge) couple, without MS/MS spectra and known contaminants. The MS/MS spectra were used for compound identification. Initially, these were searched against the MassBank and NIST databases. Further identifications were obtained by doing spectral matching with other databases: HMDB (<https://hmdb.ca/spectra/ms/search>), LipidMaps (www.lipidmaps.org/), GNPS (<https://gnps.ucsd.edu/ProteoSAFe/libraries.jsp>); MZCloud (www.mzcloud.org), PubChem (pubchem.ncbi.nlm.nih.gov) and Metlin (metlin.scripps.edu/index.php) and by comparing with data present in literature. Compounds not found were checked using de novo MS/MS tools such as MetFrag (<https://ipb-halle.github.io/MetFrag/>) and CFM-ID (<https://cfmid.wishartlab.com/>). When a compound was identified, it was added in an in-house database, thus contributing to the expansion of this database. Data files were then generated in msp-file format which is compatible with Progenesis. All the reported identifications are based on correct m/z of the precursor (mass error <10 ppm) and MS² similarity with experimental or theoretical MS/MS spectra,

according to the level 2 identification (Rochat, 2017; Salek et al., 2013). A compound was considered tentatively identified when a score of ≥ 75 on a scale of 100 was attained and the retention time matched with that in the database. Subsequently, to generate a general view on the composition of the extracts, the identified compounds were classified in 5 categories based on their chemical structure. The sum of the MS-signal intensity of the compounds in each category was calculated (Chapter 3.2) and the extraction solvent offering the highest accumulated MS-signal intensities was selected.

1.2.7 Analysis of the protein and arabinoxylan fractions

Hydrolysis and amino acid composition

Sample preparation and derivatization

Five mg of the lyophilized protein fractions in specific tubes for hydrolysis were hydrolyzed by incubation in 1 mL of 6M HCl at 110°C for 24h under nitrogen to prevent amino acid oxidation as previously described (Hellebois et al., 2021). Two hundred μL of each hydrolysate were collected and mixed with 20 μL of the internal standard (10mM of D-4 Alanine) and dried under vacuum in a CentriVap (LABCONCO, Kansas City, USA). Calibration curves were built with a standard mix (Sigma-Aldrich) at concentrations from 0.53 to 2.22mM. This mix contains Alanine, Arginine, Aspartic acid, Cysteine, Glutamic acid, Leucine, Isoleucine, Lysine, Serine, Threonine, Tyrosine, Valine, Histidine, Methionine, Phenylalanine, Proline and Glycine. For each standard point, 200 μL were collected, mixed with 20 μL of the internal standard mentioned above and dried. Subsequently, samples were derivatized according to a previously described method (Smart et al., 2010) and then analyzed with the GC-MS. Briefly, dried samples were derivatized as follows: the samples were solubilized with 200 μL of 1M NaOH and transferred to silanized glass tubes. To the solubilised samples, 167 μL methanol and 34 μL pyridine were added, followed by the addition of twice 20 μL of methylchloroformate. The mixture was vortexed for 30 s after each addition of methylchloroformate. Afterwards 400 μL of

chloroform were added and vortexed for 10 sec. By adding chloroform two layers were formed in the solution: the upper, aqueous phase was discarded while the lower phase was dried with sodium sulphate (the amount of sodium sulphate added was dependent to the volume of water left in each sample). Finally, the remaining liquid, corresponding to the chloroform-solution, was transferred into amber GC vials for GC analysis.

GC-MS and data analysis

The GC-MS (GC (7890B)-MS (5977A), Agilent Technologies, Santa Clara, US) coupled with an autosampler (MPS, GERSTEL, Muhlheim, Germany) was set following a previously defined method (Smart et al., 2010). Briefly, 1 μ L of each sample was injected with the autosampler in splitless mode at 280°C. The flow through the column (Agilent 19091S-433UI, 30 m x 250 μ m x 0.25 μ m) was set at 1 mL He/min. The oven temperature was elevated from 45°C (hold 2 min), to 180° (hold 5 min) at a speed of 9°C/min; to 220°C (hold 5 min) at 40°C/min; to 240°C (hold 11.5 min) at 40°C/min; to 280°C (hold 2 min) at 40°C/min, finally, to 300°C (hold 1 min)) at 40°C/min. Prior to each run, the column was equilibrated for 5 min, each run lasted 40 min. Data analysis was performed by using the free Automated Mass Spectral Deconvolution and Identification tool (AMDIS, <https://www.amdis.net>, V2.71) and a library that was previously described (Smart et al., 2010). Amino acids were identified and subsequently quantified with the Metab package (version 1.32.0) in R studio (R 4.2.3).

1.2.8 Hydrolysis and quantification of arabinoxylans

Sample preparation and derivatization

The arabinoxylan hydrolysis protocol was adapted from Lynch et al. (Lynch et al., 2021). Briefly, 10 mg of dried arabinoxylans isolate were mixed with 2 mL of 4N trifluoroacetic acid and incubated for 2 hours at 121°C in Pyrex tubes. The hydrolysed arabinoxylan fractions were then analyzed and the monomers quantified by GC-MS. First,

samples were derivatized based on a previously described protocol (Villas-bôas et al., 2006), with some modifications. Twenty μL of hydrolysate were mixed with 20 μL of internal standard (10 mM of D-4 Alanine) and dried under vacuum in a Centri Vap. In the same way, standard curves for arabinose, xylose, glucose, mannose, and galactose were prepared with concentrations ranging from 1.5 to 20 mM. All samples were resuspended in 80 μL methoxyamine hydrochloride in pyridine (2g/100 mL) and incubated for 90 min at 30°C at 750 rpm (Thermomixer Eppendorf, Fisher scientific). Subsequently, 80 μL of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were added and the samples incubated for 30 min at 37°C at 750 rpm. Finally, the samples were transferred into amber GC vials and analysed.

GC-MS and data analysis

One μL of sample was injected into the GC-MS through a splitless mode at 280°C. The flow through the column (Agilent 19091S-433UI, 30 m x 250 μm x 0.25 μm) was at 1.2 mL He/min. The oven temperature was initially set at 60°C and then increased gradually at a speed of 3°C/min up to 150°C and then at 5°C/min up to 280 until the end of the run. The column was equilibrated for 6 min before each run, which lasted 44 min.

For the data analysis, the identification of the MS spectra was performed on the basis of an in-house MS database and the quantification of the compounds was made by using the height of the GC peaks as described before (Villas-bôas et al., 2006). Data were normalised using the internal standard.

1.2.9 Estimation of the process costs

An initial estimation of the cost of the process was performed. This estimation includes the direct manufacturing costs (DMC), the fixed manufacturing cost (FMC) and the general expense (GE). The DMC comprises the cost of raw material, utilities, waste, labour,

supervision, maintenance, operating supplies and the laboratory charge. The raw material refers to the cost of the biomass used in the process. The utilities include electricity, solvents and chemicals, and operating labour costs. The direct supervision and clerical labour are covering the costs of engineering, administrative and support personnel and account for 10% of the labour cost (Turton et al. 2012). The maintenance and repair costs correspond to the cost of labour related to the maintenance of the system and are calculated using a factor 0.001 on the CAPEX (investment of the infrastructure: mainly equipment and building) (Turton et al., 2012). The operating supplies and laboratory charges encompass the supplies that support daily operations that do not involve raw materials, such as paper, filters, protective clothing etc., as well as the costs for process monitoring and quality control analysis. The FMC are costs that do not vary with the production rate of the process and involve the depreciation, the local taxes and the overhead costs. Depreciation is calculated over a 10-year period using a classic straight-line depreciation model and reflects the reduction in value of the system for a specific period due to the usage, obsolescence and deterioration of technologies. The local taxes are estimated by using a factor 0.01 of the CAPEX (Turon et al., 2012), while overhead costs are equivalent to 50% of the total labour cost.

1.3 Results and Discussion

The composition of the wet barley-Pilsener BSG used in this work is shown in Table 1.3-1. The reported data are in line with the chemical composition of raw BSG previously described in literature (Chin et al., 2024; Connolly et al., 2013).

Table 1.3-1 Proximate composition of the raw BSG used as starting material.

BSG components	g/100 g dw (dry weight)
Protein	28.3
Lipids	7.3
Ash	2.3
Fibres + carbohydrates	62.1
Moisture content in fresh BSG	76.50%

1.3.1 First fraction: hydroalcoholic compounds

One of the most investigated potential uses of BSG is as source of compounds with antioxidant properties for food industry applications. The first step of this sequential approach therefore aimed to obtain an extract with antioxidant potential. This fraction was obtained with a 40% EtOH aqueous maceration extraction, which consists mainly of hydrophilic compounds such as oligosaccharides, amino acids, phenolics, fatty acids and phospholipids. The parameters of this first extraction were selected in order to obtain an efficient (in terms of antioxidant capacity and extraction yield) and simple method with a low environmental impact and with the potential to scale up the approach (Putra et al., 2022).

This first extraction led to a TPC of 1.6 mg GAE/g dw BSG and an antioxidant capacity corresponding to 6.8 $\mu\text{mol FeSO}_4$ eq./g dw BSG. These results are in line with values reported in literature for TPC

(from 1.00 mg GAE eq./ g dw BSG to 3.3 mg GAE eq./g dw BSG (Macias-Garbett et al., 2021; Meneses et al., 2013; Guido & Moreira, 2017, Socaci et al., 2018)), as well as for the antioxidant capacity values obtained with the FRAP assay (Codina-torrella & Rodero, 2021; Crowley et al., 2017). Alternative methods to maximise the recovery of these compounds from BSG are being investigated. Among them, extraction with super critical CO₂ is promising. However, the TPC reported in literature of extracts with super critical CO₂ is lower (0.35 mg GAE/g dw BSG (Spinelli et al., 2016)) compared to those obtained in our study and in other studies using conventional solid:liquid extractions (Macias-Garbett et al., 2021). It is worth noting that in addition to the extraction method applied, the TPC and antioxidant activity values are also influenced by the grain variety used and the agricultural and the brewery process conditions applied (Mussatto et al., 2006). Although different studies have tested methods to obtain high-antioxidant BSG extracts, the approach that is most suited for industrial scale application is not established. Factors such as process feasibility, process costs and logistics are essential factors to consider to make this assessment (Jha & Sit, 2022). Additionally, although the TPC of BSG extracts is relatively low compared to those found for other plant-based biomass residues and product extracts (Table 1.3-2), the amount of BSG that is produced, its low-cost (approximately 35-40 euros/ton in Europe) and the non-seasonality of its production could make BSG a viable option for the recovery of phenolic compounds. This then further emphasizes the need to optimise larger scale extraction of these metabolites.

Most of the studies that aim to obtain a comparable extract from BSG use dried, stabilized biomass. This stabilisation is considered essential to increase the storage time and to facilitate the management of BSG, which presents a high moisture content and a rich composition. However, this pre-treatment can impact the composition of BSG, as described in chapter 3.3. Starting with fresh BSG not only results in a higher antioxidant capacity, but avoiding the drying step furthermore reduces the energy needed. When applied on a larger/industrial scale, it will lead to a lower environmental and economic impact (Belardi et

al., 2023). Consequently, the starting material used in this study was fresh BSG.

Table 1.3-2 Total phenolic content (TPC) of some plant-based biomass residues and products

Matrices	Analysed parts	TPC mg GAE/g dw biomass	References
Apple	pomace	0.89	(Gumul et al., 2021)
Olive	pomace	23	(Böhmer-Maas et al., 2020)
Grape	pomace	97.9	(Silva et al., 2024)
Grape	pomace	41.5	(Zhang et al., 2023)
Orange	peel	5.8	(Suleria et al., 2020)
Pomegranate	peel	3.8	(Suleria et al., 2020)
Papaya	peel	3.1	(Suleria et al., 2020)
red rice	bran	2.3	(Min et al., 2014)
purple rice	bran	1.8	(Min et al., 2014)
soybean (green)	seeds	12.4	(Deng et al., 2013)
Tomato	fruit	7.94	(Deng et al., 2013)
Onion	bulb	6.2	(Deng et al., 2013)
Broccoli	flower	9.84	(Deng et al., 2013)

1.3.2 Second fraction: hydrophobic compounds

As preliminary experiment, I tested 4 different solvents, namely hexane, ethyl acetate, chloroform/methanol (Folch method) and ethanol, to select the most suitable extraction solvent. The efficiency of the solvents was judged based on an untargeted metabolomic approach aiming initially to detect and identify the main compounds present in the extracts. The identified compounds were classified in 5 groups based on their chemical structures (hydroxy fatty acids (HFAs), phosphatidylcholine (PC), phosphatidylethanolamine (PE), mono/di-galactosyldiacylglycerol (MGDG/DGDG) and glucuronosyl-diacyl/monoacylglycerols (GlcA-DAG) and the extracts made with the four solvents were compared based on the cumulative MS signal intensities of these five groups.

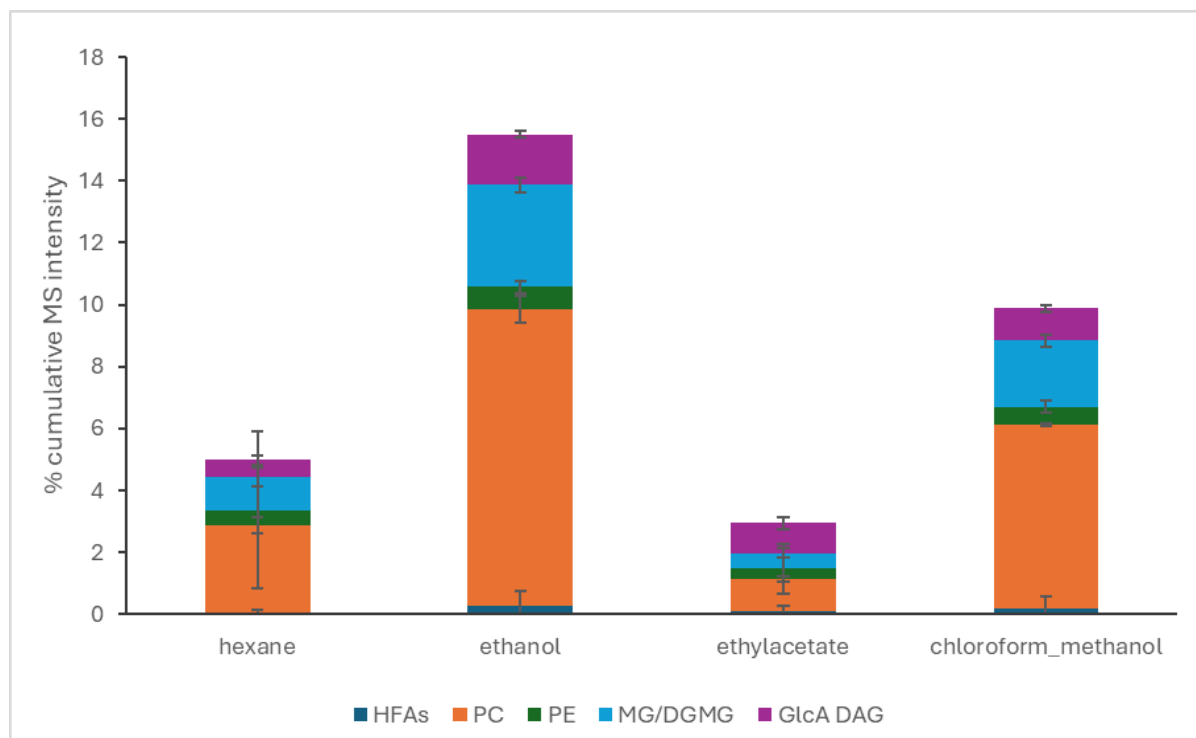


Figure 1.3-1 Percentage of the relative cumulative MS signal intensity of the 5 main categories of lipids identified in this study, 100% being the total ion signal intensity, hydroxy fatty acids (HFAs), phosphatidylcholine (PC), phosphatidylethanolamine (PE), mono/di-galactosyldiacylglycerol (MGDG/DGDG) and glucuronosyl-diacyl/monoacylglycerols (GlcA-DAG), extracted with 4 different solvents: hexane, ethanol, ethyl acetate and chloroform/methanol. The percentage of the relative MS cumulative intensity, based on the total ion signal intensity was used to compared 4 used solvents. The relative intensity of each category within each solvent it is also represented

Extraction with ethanol came out as best in this comparison (Figure 1.3-1). Extracts with hexane and ethyl acetate, the most used solvents for lipid extractions, were similar and had the lowest content for all lipid groups. The mixture of chloroform/methanol resulted, as expected based on their polarity (Saini et al., 2019), in extracts similar to those obtained with ethanol (Figure 1.3-1). The reason for these results is that most of the identified lipids are amphipathic, therefore more extractable with the less apolar solvents such as ethanol. The ethanolic lipophilic fraction produced 3.5 g extract/100 g dw BSG, which is consistent with the range reported for lipid extractions from cereals (wheat bran 4.5 g extract/100 g dw biomass, sorghum 3.5 g extract/100 g dw biomass (Yadav et al., 2021; Leguizamón et al., 2009)). The contribution of each of the 5 groups of identified lipids to the cumulative MS-signal intensity is shown in Figure 1.3-2. PC-lipids, containing C14, C:16, and C:18 with up to 3 unsaturations and C:20 with up to 2 unsaturations, contribute most to the total signal intensity. In this category, the highest MS signals were observed for PC (16:0/0:0) and PC (18:2/0:0). The dominant fatty acids identified in our extracts in the free and bond forms are C18:2 and C16:0, as previously found for BSG (del Río et al., 2013; Niemi et al., 2012). These two fatty acids, and to a lesser degree C18:1, represent the predominant fatty acids detected in BSG extracts (Ferrentino et al., 2019; Niemi et al., 2012).

In addition to PC-lipids, PE and HFAs have the highest accumulated MS-signal intensity. This is in line with what was described by Niemi et al. (Niemi et al., 2012), with free fatty acids and phospholipids as predominant compounds, after the triglycerides. The main free fatty acids that were detected are unsaturated C18-fatty acids, mostly with 2 unsaturations and hydroxyl groups. Hydroxyoctadecanoic acids with up to 3 hydroxylations and up to 3 unsaturations were detected. The highest MS-signal intensity was recorded for hydroxyoctadecadienoic acid (HODE) which had one of the highest MS intensities of all detected hydrophobic compounds. Moreover, to our knowledge, the HFAs have not yet been described in lipophilic BSG extracts before.

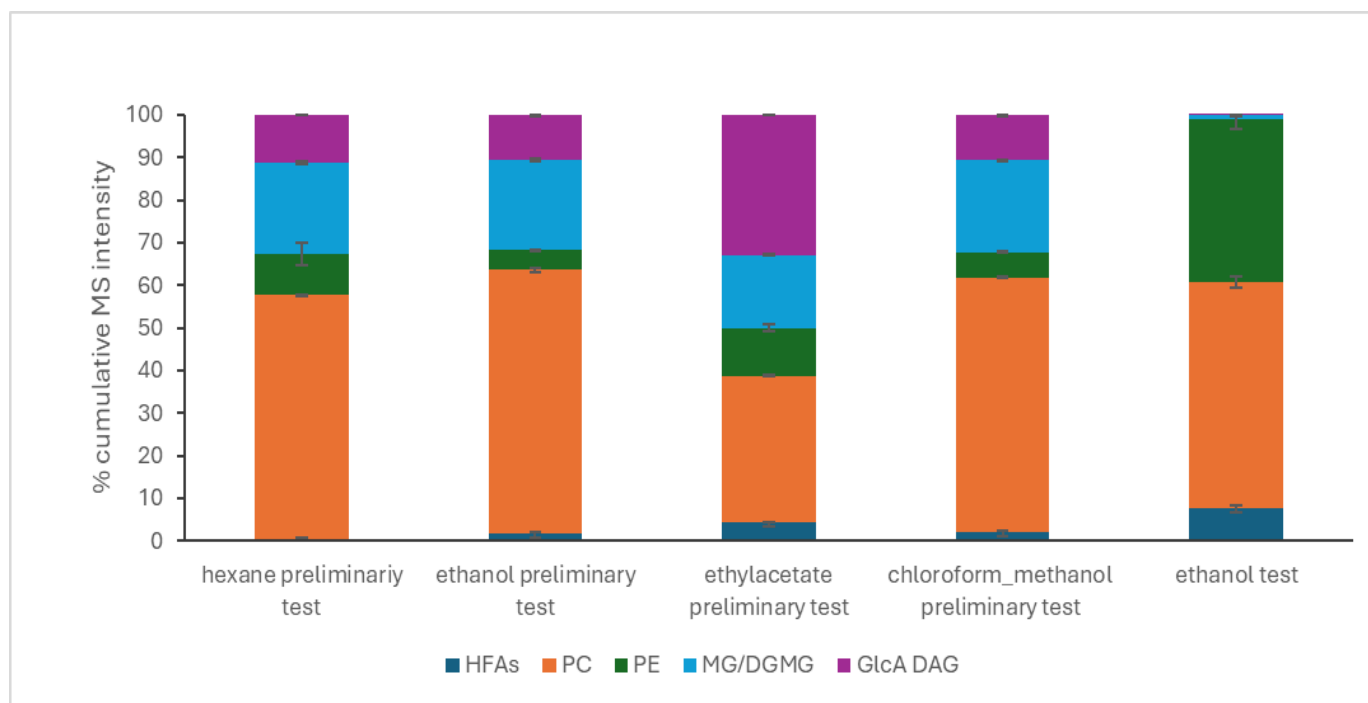


Figure 1.3-2 Percentage of the relative cumulative MS signal intensity of the five main categories of lipids identified in the ethanolic extracts of BSG, 100% being the total ion signal intensity attributed to lipids for each analysis: hydroxy fatty acids (HFAs), phosphatidylcholine (PC), phosphatidylethanolamine (PE), mono/di-galactosyldiacylglycerol (MGDG/DGDG) and glucuronosyl-diacyl/monoacylglycerols (GlcA-DAG). Ethanol preliminary test represents the results from the preliminary experiment reported in the figure 1.3-2 and the ethanol test represents the test described in the biorefinery approach. .

The one remaining lipid class, MG/DGMG had a low MS-signal intensity, confirming the lipid profile of BSG extracts reported by del Río (del Río et al., 2013). Despite the known beneficial effects of lipids in pharmaceutical, food and cosmetic applications (del Río et al., 2013), there are only few studies that focussed on the extraction of lipids from BSG (del Río et al., 2013; Ferrentino et al., 2019; Niemi et al., 2012). The most used approach for lipid extraction is Soxhlet-based extraction, a technique known to be time consuming. For example, the extraction performed by del Río and colleagues (2013) lasted for 8 hours in a Soxhlet apparatus. In addition, this approach includes the use of toxic solvents such as hexane and heptane (del Río et al., 2013; Niemi et al., 2012). Only Ferrentino and co-workers dedicated a study to the use of super critical CO₂ extraction as alternative method for extracting lipids from BSG. They tested different extraction parameters to optimise the process and targeted the antioxidant capacity as potential bioactivity of these hydrophobic extracts. Moreover, a targeted characterisation of the fatty acid profile was performed (Ferrentino et al., 2019). In contrast, in the present study the extraction was performed through a maceration approach by using ethanol as solvent at room temperature for 2 hours.

The most abundant lipids detected in this work are phospholipids and, within these compounds, PCs represent the highest cumulative MS intensity signal. PCs have been studied for their bioactivities on humans notably for their anti-inflammatory properties in the treatment of chronic diseases such as ulcerative colitis (Treede et al., 2007), and their hepatoprotective effects by supporting the integrity of cellular membrane and reducing the oxidative stress at the liver level (Chen et al., 2019). It has also been demonstrated that PCs are involved in alleviating high-fat diet induced-obesity and related problems (Lee et al., 2014). In addition, being the precursor of the neurotransmitter acetylcholine, PCs are investigated for their implication in brain function and cognition (Kapoor et al., 2023). Furthermore, PCs are also under study for their potential role as a delivery system (Tree-Udom et al., 2023). Some of the identified fatty acids, in particular linoleic acid (18:2), the most abundant free fatty acid, an essential

unsaturated omega-6 fatty acid for which dietary uptake is required, are also known to have health-promoting bioactivities.

1.3.3 Third fraction: proteins

The protein content of the BSG used in the present study was determined for each step of the biorefinery cascade. An elemental analysis was performed on the raw BSG, on the residues after the hydrophilic, hydrophobic and on the recovered protein fraction. The raw BSG used contains 28.3% (w/w on dry basis) protein, in line with the values reported in literature (Bonifácio-Lopes et al., 2020; Connolly et al., 2021; Vieira et al., 2014). After the first extraction, the protein content in the residue decreased to 25.6% and applying the second, ethanolic extraction decreased it with a further 2.2%. In total almost 5% of the protein present in the raw BSG was extracted in the first two steps of the cascade, most likely through the extraction of proteins such as albumins and hordeins which are soluble in water and alcohol mixtures (Celus et al., 2006; Wen et al., 2019).

In the protein fraction, the protein content was determined to be 44.4% and 63.5 % w/w (respectively for the alkaline extractions performed at pH 10 and 11) compared to the protein content of alkaline extracts reported in literature that are going from 46 % (Connolly et al., 2013; Devnani et al., 2023; Junttila, 2022) to 75% (Hellebois et al., 2021). The weight of protein isolate obtained from the extraction amounted to 45 mg protein isolate/g dw BSG at pH 10 and 90 mg protein isolate/g dw BSG at pH 11. These results are in agreement with the previously published data ranging between 20 and 90 mg of protein isolate/g dw BSG (Connolly et al., 2013; Junttila, 2022; Vieira et al., 2014). The total protein matter in the protein isolate calculated for the two tests was found to be 20 mg protein matter /g dw BSG at pH 10 and 56 mg of protein matter/g dw BSG at pH 11.

Different methods for protein extraction have been proposed (for example alkaline, acids, reducing agents, salt, and deep eutectic solvents). Of these, alkaline extraction is the most widely applied

since it results in the highest extraction yields (Wen et al., 2019). Alkaline extraction of proteins consists of two steps; protein solubilization at alkaline pH followed by recovery of the solubilized proteins by precipitation, centrifugation or microfiltration (Devnani et al., 2023). Here, alkalization with NaOH at pH 10 and 11 was used to solubilize the proteins from the residue of the hydrophobic extraction followed by precipitation with citric acid as was previously applied on BSG (Vieira et al., 2014). Citric acid is an organic acid which is accepted by European legislation as a food grade agent and additive. It has a relatively high acidity among the organic acids which makes this acid suitable for precipitating BSG proteins at pH 3.5-4, corresponding to their isoelectric point (Wen et al., 2019). Since temperatures higher than 60°C may induce too much denaturation (Jaeger et al., 2021), the temperature was stabilized between 50 and 55°C (Connolly et al., 2013) and an extraction ratio of 1:20 (solid: solvent) was used. Temperatures higher than 60°C were not considered because of their potential impact on protein denaturation. As different studies found different optimal pH values for the solubilisation of BSG protein: respectively 10 and 11 (Connolly et al., 2013; Hellebois et al., 2021), these pH values were tested. Since the results were higher at pH 11, this parameter was selected and the protein isolates obtained at this pH value were tested for their amino acid content. The amino acid content was determined by GC-MS (Table 1.3-3) and expressed as g amino acid/100g of BSG protein isolate. The most abundant amino acids were glutamic acid and glutamine, the sum of which reached 21.4 g/100g of BSG protein fraction. Other highly abundant amino acids were lysine and aspartic acid with respectively 6.5 and 6.4 g/100g of BSG protein fraction and proline, leucine, isoleucine, tyrosine, and valine with values above 4 g/100g of BSG protein fraction (Table 1.3-3). These amino acids are the most abundant amino acids in BSG and protein isolates from BSG (Connolly et al., 2013; da Fonseca et al., 2023; Hellebois et al., 2021; Qin et al., 2018).

Most of the published studies on BSG protein extracts used pre-treated starting material, subjected to hot drying (Connolly et al., 2013),

defatting or alkaline and acid treatments (da Fonseca et al., 2023; Hellebois et al., 2021; Qin et al., 2018).

Table 1.3-3 Amino acid composition of BSG protein isolate. The data are based on four replicates.

^a: WHO/FAO/UN recommendations assuming a meal total protein requirement of 0.66 g/kg per day, the intakes are expressed as g/100 g protein for an adult (WHO report 2007);

^B: Connolly et al. 2013; da Fonseca et al. 2023; Hellebois et al., 2021 references based on g/100g of dw BSG protein isolate;

^c: the value of phenylalanine and tyrosine are expressed as the sum of their concentration in the WHO/FAO/UN recommendations, accordingly for the calculation of EAA score g amino acid x in sample/ g amino acid x (in WHO/FAO/UN recommendations) were expressed as the sum of their concentration.

Amino Acids	g/ 100 g of dw BSG protein	WHO/ FAO/ UN ^a (g/100g protein/day)	EAA score	Literature references ^b (g/ 100 g of dw BSG protein)
Histidine	2.7 ± 0.02	1.5	1.8	1 - 3.63
Isoleucine	4.6 ± 0.08	3	1.5	2.5 - 5.7
Leucine	4.8 ± 0.06	5.9	0.8	4.8 - 9.5
Lysine	6.5 ± 0.1	4.5	1.4	1.8 - 5.9
Methionine	1.6 ± 0.05	2.4	0.7	1.3
Phenylalanine	3.7 ± 0.06	3.8 ^c	2.3 ^c	4.6 - 6.7
Tyrosine	4.9 ± 0.05			1.6 - 3.5
Threonine	2.9 ± 0.07	2.3	1.3	1.8 - 4.1
Tryptophan	nd	0.6		
Valine	4.6 ± 0.06	3.9	1.2	3.5 - 7.8
Essential	36.4	27.9		
EAA index			1.4	
Alanine	4.1 ± 0.06			3 - 4.3
Aspartic acid	6.4 ± 0.06			5.2 – 9
Glutamic acid + glutamine	21.4 ± 0.06			17.2 – 24
Glycine	2.9 ± 0.06			2.9 – 5.1
Proline	4.8 ± 0.06			8.4 – 9.7
Serine	2.2 ± 0.01			3.2 – 4.3
Non-essential	41.8			

The initial extractions to which BSG was exposed to in this study result in a protein fraction similar to those obtained after the pre-treatments used in other studies. Therefore, despite the unavoidable loss of proteins in the first steps of the sequential approach, the amino acid composition of the protein fractions are in line with those reported in literature (Connolly et al., 2013; da Fonseca et al., 2023). Only the value for proline is at 60% of the lowest value obtained in the reference studies.

The high content of lysine is of interest since this amino acid is low in the flours and grains of the most consumed cereals, like wheat, maize and rice (Matthews, 2020; Shewry, 2024; Ummels et al., 2023). Besides the specific concentration of amino acids, BSG protein isolates have been shown to have sought-after bioactivities, such as antioxidant and anti-inflammatory, immunomodulatory and antihypertensive properties (Hellebois et al., 2021). Therefore, BSG protein isolates can be exploited for food fortification. Furthermore, BSG protein isolates also have a high water and oil holding capacity, techno-functional properties that can be valorised in the production of viscous and gelled food like mayonnaise, yogurt or plant-based meat (Devnani et al., 2023). Additional valorisation can be based on the stabilising of emulsions and foams by BSG protein isolates (Connolly et al., 2013; da Fonseca et al., 2023). While these functional attributes have been established for BSG protein isolates, further studies on the isolates obtained in our extraction are needed to confirm that these are conserved.

1.3.4 Fourth fraction: arabinoxylans

AX-enriched fractions were obtained by further acidifying the supernatant after precipitation of the proteins with citric acid. After precipitation of the proteins at a pH between 3 and 4, the supernatant was further acidified to pH 2 with hydrochloric acid.

The AX extraction resulted in 5,6 g AX extract/100 g dw BSG. This value is within the broad range of values previously reported; from 0.3 to 6,4 (Coelho et al., 2016) and from 1 to 13.5 g AX extract/100 g dw

BSG (Vieira et al., 2014). This wide range might be due to various factors, including the complexity of the extractions consisting of 6 or 4 steps, respectively for Coelho (2016) and Viera (2014) groups. In contrast, here the AX fraction was obtained in one-solubilisation step.

Based on GC-MS quantification of arabinose and xylose after hydrolysis of the AX fraction, the total amount of AX recovered in this fourth extract accounted for 2.63 g AX/ 100 g dw BSG. In the two studies previously mentioned, the values vary from 3 to 8 g AX/100 g of dw BSG (Vieira et al., 2014) and from 0.36 to 5.1 g AX/100 g dw of BSG (Coelho et al., 2016). These differences can again be explained by the exact conditions used. In both previous studies the highest amount of AX was obtained when using the highest concentration of alkaline reagent, in our study the pH value was set at 11 as it allowed to obtain both a protein and an AX fraction.

An important factor for the valorisation potential of AX fractions is the ratio between arabinose and xylose, reflecting the AX branch degree and giving information about the structure of the fibres. The arabinose/xylose (Ara/Xyl) ratio of our AX-enriched fraction was 0.65, similar to data shown by Li et al., who reported Ara/Xyl values between 0.68 and 0.72 for AX extracted from barley malt beers. While this Ara/Xyl ratio gives a first impression on the valorisation potential of our fraction, more research, among others on the length and the polymerisation degree of the AX is needed.

Generally, BSG is a biomass rich in fibres, wherein the hemicelluloses, specifically AX, are the most abundant. These compounds are known for their pre-biotic, immune and gut regulatory activities (Zannini et al., 2022). In addition, AX are involved in the intestinal protection against pathogenic bacteria through the enhanced production of short-chain fatty acids (acetic, propionic, and butyric acids) (Weng et al., 2023). Their impact on lowering cholesterol levels, reducing cardiovascular diseases (McRae, 2017), obesity (Tucker & Thomas, 2009) and gastrointestinal disorders make dietary fibres sought-after phytochemicals to be valorised in the food,

pharmaceutical and medical sectors (Hussain et al., 2020; Zannini et al., 2022).

With the generation of the four fractions described here more than one fourth of the total BSG biomass was fractionated. The total residual biomass of the process accounted for 51 g dw BSG residual biomass/100 g dw BSG starting biomass (Figure 1.3-3). This amount of BSG can be further exploited for example as a source of energy, in form of biofuel and biogas or as biomaterials for packing and building (Jackowski et al., 2020; Pabbathi et al., 2022). More than one fourth of the starting BSG biomass was extracted in 4 fractions that each individually have been targeted in the different studies that have been used for comparison.

The individual steps in the described extraction cascade can be modified depending on specific needs or the availability or experience with other extraction techniques. Steps can be modified by including enzymatic or chemical hydrolysis for instance to release cell wall bound phenolics (Mussatto et al., 2007). The final step can be expanded to include the isolation of other fibres using a recently described approach (Belardi et al., 2023). With all these alternatives available, choices were made. At each step approaches were selected that are today used in large-scale extractions from vegetal biomass, and these selections used studies on the industrial feasibility, accessibility, and cost-effectiveness of alternatives as guide. Here only data obtained at laboratory-scale is presented and validation/confirmation that the proposed extraction cascade can have a significant role in the valorisation of BSG as source of chemicals, material and energy (Carmona-Cabello et al., 2018) at a larger scale is needed.

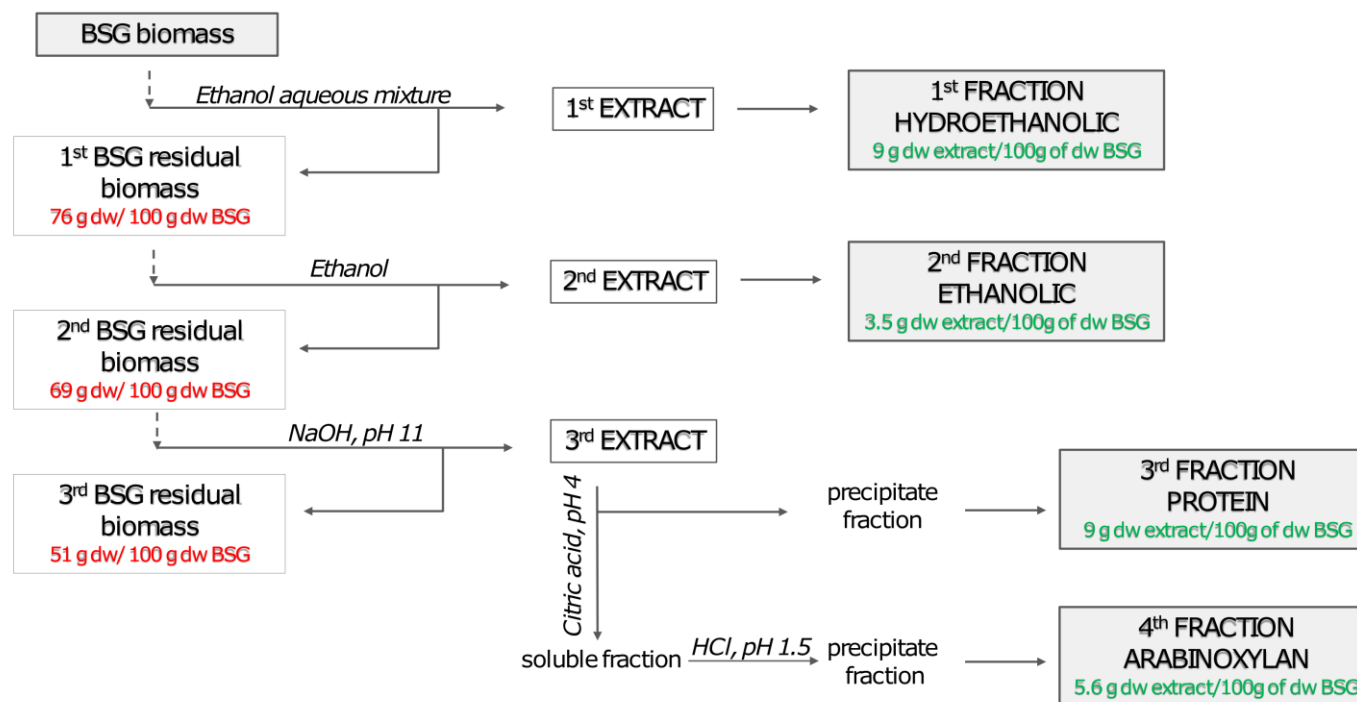


Figure 1.3-3 Scheme of the biorefinery approach used in the present study for brewer's spent grain (BSG): the four sequential extractions, the related collected fractions and the mass balance of the residual BSG produced and the recovered fractions are illustrated

1.3.5 Estimation of the process cost

The potential and approximate cost of the entire biorefinery process, normalized to 1 ton of dw BSG, is presented in Table 1.3-4.

Table 1.3-4 Estimated cost of the main variables including in the proposed process normalised for the time of instrument usage for 1 ton of BSG

DMC (DIRECT MANUFACTURING COST)	Comment	Total cost* (€/ton dw BSG)
Raw material		134
Utilities		3117.52
Waste		800
Labour		3596
Direct supervisory and clerical labour		359.6
Maintenance and repair	0.01 total CAPEX	225
Operating supplies	0.003 total CAPEX	67.5
Laboratory charge	0.1 labour cost	359.6
FMC (FIXED MANUFACTURING COST)		
Depreciation	Over 10 years	2250
Local taxes and insurance	0.01 total CAPEX	225
Overhead cost	0.5 total labour	1798
GE (GENERAL EXPENSE)		
Administration cost	0.15 total labour	539.4
TOTAL COST		13471.62
TOTAL COST/KG EXTRACT FOR 1 TON SCALE OF BSG		64.4
*Normalised for the total time of usage		

The total cost of this process according to this estimation is 13471 euros for the extraction from a ton of dw BSG. The total CAPEX was estimated at approximately 1.4 million euros. This covers instrument cost as well as all associated dependent installations, such as piping,

painting, data processing tools, sensors, light, etc. In table 1.3-4, this is found among others in calculated cost of depreciation, and used as a base to calculate maintenance and repair, operating supplies and local taxes and insurance costs. The main costs are labour, utilities (Table 1.3-5) and overhead.

*Table 1.3-5 General market prices of inputs used in this biorefinery approach proposed. *Artz et al., 2021; Boulamanti and Moya., 2017, Eurostat 2023*

Inputs	price/unit*
Utilities	
Electricity	0.04 €/kWh
Water	0.03 €/m ³
Ethanol	1 €/L
NaOH	1.5 €/kg
Citric acid	1.5 €/kg
HCl	1 €/L
Operating labour	
Average hourly wage	29 €/hours

The cost of the utilities has a relevant impact on the total cost estimation, specifically the cost of ethanol, although recycled for up to 80%. Nonetheless, it is also important to consider that new ethanol is required after a certain number of extraction cycles. In the calculated example given in the table above (Table 1.3-4), the market price for dw BSG was used. A value of 134 euros was obtained from a previous study (Buffington, 2014), which compares it to the market price of fresh BSG, being approximately 35-40 euros. The value of dw BSG was used since, apart from costs associated with drying, this also includes transport and storage. The same estimation using the market price for fresh BSG resulted in a slightly lower cost. Therefore, the overall cost to produce 1 kg of extract by applying the parameters of this estimation amounts to 64.4 euro/kg extract.

The above mentioned estimation provides a general idea on the potential cost of this process, although up-scaled data and more detailed analyses, such as life cycle analysis (LCA) and techno

economic assessment (TEA), are recommended to evaluate the development of this biorefinery approach at larger scale. In addition to estimate the process costs, it is important to define the targeted market, specific activity and associated usage dose to further estimate to optimal production scale to reach profitability. The values highly depend on the application in sectors like food fortification, cosmetics or pharmaceuticals. A recent study showed that integrating BSG fractions as a food ingredient has lower environmental impact compared to standard sources, such as soybean, whey, pea, wheat (Nyhan et al., 2023). Especially, it results in reduced global warming (measured in kg CO₂ equivalent), freshwater eutrophication (expressed as kg P equivalent) and land use (expressed as m² a crop equivalent) (Nyhan et al., 2023).

Upcycling BSG provides significant cost benefits by reducing food waste and greenhouse gas emissions, which is increasingly important in the context of rising global food insecurity and waste. It is estimated that the upcycled BSG market is expected to reach USD 24 billion by 2033 (Nyhan et al., 2023).

1.4 Conclusion

Due to its chemical composition, its non-seasonal nature, and the sheer amount available, BSG is a good candidate feed-stock for industrial development. Here, I present data from a preliminary, laboratory-scale application of a biorefinery strategy that aimed at maximally valorising this biomass. To our knowledge, the sequential extraction of four different fractions from BSG has not been yet tested. Starting from wet BSG, hydrophilic, hydrophobic, protein and arabinoxylan fractions were generated in a cascading approach. With this process, 27 g/100 g of dw BSG was recovered in the four fractions. The compositional data of these four fractions is in line with data obtained in studies that optimise the extraction of one of these fractions. Furthermore, more research is also needed to deepen the investigation of the potential bioactivities of these fractions in real-life applications. Through this process, 51 g/ 100 g dw BSG represents the

final residual biomass, whose use can be further explored. In addition, while this is shown here at the laboratory scale, further research is needed on the economic and technologic point of view for the application of this approach at larger scale. An initial estimation of the cost of the process at 1ton scale was done, but more accurate economic assessment is required.

2 References

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CHAPTER 5.

UP-SCALING

Foreword

This chapter describes a study on the scalability of the hydroalcoholic extraction performed in chapter 3 is described, as it represents the initial step in the biorefinery approach (described in chapter 4) intended for future industrial-scale application. Extractions were performed on a range from mg- to almost kg- of BSG biomass and were evaluated based on the yield and the antioxidant properties of the extracts.

1 Up-scaling of BSG extraction

Highlights

- *The up-scaling approach was performed using 0.075 g; 0.5 g; 10 g; 25 g; 50 g and 850 g of wet BSG.*
- *The extraction efficiency expressed as the amount of extract per the quantity of biomass used for the extraction was constant at 8 mg dw BSG extract/100 g dw BSG for all the conditions except for the extractions performed with the highest amount of biomass.*
- *The antioxidant capacity measurements show that the largest extraction scale resulted in an extract with the lowest antioxidant capacity.*
- *For the largest scale extraction, a pilot scale extractor was used and the stirring type and an additional step of volume reduction was added, most likely negatively affecting the extraction parameters.*

Abstract

The valorisation of agri-food residues by their conversion into added-value products is a major focus in the development of a more sustainable society. Brewer's spent grain (BSG) is the most abundant solid residue from brewing with a low market value, and currently poorly valorised. Recently, BSG has gained interest for the extraction of its valorisable compounds. While many extraction methods have been optimized in a laboratory scale, few have focused on larger-scale extractions. With the aim of providing data on the up-scaling of extractions from BSG, a hydroethanolic extraction was up-scaled from the mg scale to 850 g of BSG in several steps. Up to the extraction using 50 g of fresh BSG, an extraction efficiency of 8 mg dw BSG extract/100 g dw BSG was obtained. In contrast, for the extraction with 850 g the extraction efficiency and the antioxidant capacity of the extracts showed the lowest values. These lower values may be attributed to a change in equipment and the necessity to introduce an extra step to manage the volumes of the extracts. These data show that up-scaling is challenging and that, in addition to laboratory scale optimisation, the development of a larger scale approach and its optimisation is necessary.

1.1 Introduction

Bioactive compounds isolated from plants are highly sought-after for the realisation of the ambition to increase the sustainability of society. This is not new, since ancient times plants have been cultivated and/or exploited for the extraction of compounds that give health benefits or can be used for manufacturing purposes (Tsiaka et al., 2017). What is, however, new is that the interest shifted from the use of plants cultivated for that purpose to the use of alternative sources. For high-added value compounds for medical use for instance plant cell cultures are being developed, while the focus for the production of compounds that are needed in higher quantities and at lower prices is on the valorisation of residual biomass streams (Caldeira et al., 2020).

As described in the previous chapters, the disposal of plant based-agri-food by-products is becoming less accepted by society and new strategies are currently being investigated to reduce their accumulation. One of the used strategies is upcycling, which involves the generation of valuable products from low-value residues. While valorisation of biomass as source of energy (heat, biogas, bio-ethanol) is attractive (Yaashikaa et al., 2022), the complex chemical composition of biomass makes that other uses can be envisioned such as the biorefinery concept, wherein the biomass is fractionated to obtain different products (Zeko-Pivač et al., 2022). The recovery of bioactive compounds from plant-based matrices involves a series of steps, the exact parameters of which depending on the starting materials used, the targeted compounds, and the extraction methods applied. Many extraction protocols have been optimized according to the chemical content of the starting materials and the selected metabolites (Jha & Sit, 2022).

Different methods have been developed for the extraction of metabolites from plants biomass. At the laboratory scale recent innovations led to the replacement of traditional extraction methods (maceration, Soxhlet, and hydro-distillation) with more innovative and modern methods (ultrasound-assisted extraction, microwave-assisted

extraction, and subcritical fluid extraction) (Azmir et al., 2013). The traditional techniques suffer from long execution times, high solvent volumes and often high temperatures leading to a high energy consumption (Belwal et al., 2020), issues that the new methods attempt to solve (Cannavacciuolo et al., 2024). While these new extraction techniques perform better at the laboratory scale and comply with current sustainability demands, at the industrial scale the new methods are not necessarily more efficient (Escobar-Avello et al., 2019). Actually, as for the traditional methods, each of the new methods has its limitations: microwave-assisted extraction is not recommended for thermolabile compounds; ultrasound-assisted extraction potentially contributes to the formation of radicals and super critical fluid extraction comes with high equipment costs. These issues make that today the application of these techniques at industrial scale is still limited (Gavahian et al., 2019), and, furthermore, validated large-scale designs are still missing (Gori et al., 2021). Consequently, large scale extractions are currently largely done with the traditional methods (Gori et al., 2021).

Several criteria need to be considered for reproducing the result of laboratory scale extractions at a larger scale. The temperature, and the pressure are considered primary criteria, while other factors, such as the stirring, are considered secondary parameters, and are directly dependent to the dimension of the extraction vessel (Martinez & Sales Silva, 2013). For example, agitation of the solution in small scale extractions can be done with shakers, mixers, or magnetic stirring devices, while at larger scale, agitation is likely obtained with an overhead stirrer immersed in the solution. The optimisation of the parameters at each scale to achieve repeatable, accurate, and reproducible results, represent the main challenge of upscaling. So far, the scale-up potential of an extraction method by following these criteria is still limited to the traditional extraction methods, which are recognised as consistent and reliable techniques and, therefore, suitable for larger scale application (Tsiaka et al., 2017).

Among the bioactive compounds from plant-based-by-products, phenolics are highly targeted for larger scale extractions because of their beneficial effects on human health (Belwal et al., 2020). The extraction of phenolic compounds from BSG has been intensively studied, and various extraction methods have been developed, including conventional and unconventional approaches. However, most of these studies focused on optimising methods at the laboratory scale, and few produced data on upscaling or pilot scale extractions (Sganzerla et al., 2021; Swart et al., 2022).

One recent study focused on the upscaling of subcritical water extraction from BSG and characterized the obtained extracts, not only for carbohydrate content, but also for protein, amino acid and phenolic compounds (Alonso-Riaño et al., 2023). However, upscaling studies on BSG using the traditional maceration technique have not been published so far. Conversely, Putra and colleagues proposed a study on the extraction of phenolics from fruit bunches of oil palms with maceration, and considered it the simplest and economically preferred approach in terms of feasibility, reproducibility and extraction efficiency (Putra et al., 2022).

In line with the study by Putra et al (2022), we explored the potential for scaling up a hydroethanolic extraction applying a traditional maceration method. Given the limited research on the scalability of the extractions on BSG, we aimed at investigating the potential and limitations of this extraction approach throughout a preliminary up-scaling study. Since the antioxidant capacity and the total phenolic content are the main parameters to assess the quality of BSG extracts, we aimed to determine whether how these factors might be affected at different scales.

1.2 *Materials and Methods*

1.2.1 Material and extraction parameters

Wet barley-Pilsener BSG provided by a local brewery, Brasserie Simon Sarl, Wiltz, in Luxembourg was used as the starting material

for this work, as mentioned in the previous chapters (3 and 4). Two batches of barley-Pilsener BSG generated from two independent brews under the same process conditions were used for this study.

Freshly received, the BSG was vacuum sealed and stored at -20°C and thawed (referred to as fresh, wet and untreated BSG) prior to use. With the first batch of BSG, extractions with 0.075 g, 0.5 g, 10 g, 25 g, 50 g were performed, while with the second batch extractions with 10 g, 25 g, 50 g and 850 g of BSG were carried out. Based on the different amounts of fresh BSG, the solvent volume was adapted according to the extraction ratio previously set at 1:20 g/ mL (solid: liquid). All extractions were performed in four replicates. The extraction was carried out with 40% EtOH mixture (40:60, v:v, ethanol: water) for 4 hours at room temperature under agitation. Due to the different quantities of BSG used for the upscaling, the size and the types of the extraction vessel, the agitation mode and speed, the filtration and the management of the collected extracts were adapted as follows (Table 1.2-1). For 0.075 g and 0.5 g samples, 2 mL Eppendorf tubes and 15 mL falcon tubes were used, while glass flasks of 0.5, 1 and 2 l were used for extractions from 10, 25 and 50 g of BSG, respectively. The largest scale extraction starting with 850 g of BSG was done in a 20 L pilot plant (Pignat) system (Figure 1.2-1).



Figure 1.2-1 The 20 L Pignat pilot plant extractor used for the extraction of 850 g of untreated BSG

The agitation system and the speed of the mixers were adapted according to the availability of shakers to accommodate the extraction recipients as follows: for 0.075 g samples agitation was set at 300 rpm, for extractions from 0.5, 10 and 25 g BSG the agitation was decrease at 180 rpm, while for 50 g samples the agitation decreased to 100 rpm (Bench-top Orbital shaker). For the 850 g BSG samples, the mixtures were agitated by an overhead stirrer with a paddle impeller integrated in the pilot system. After extraction, the extracts were separated from the solid residues by centrifugation for the 0.075, 0.5 g samples, while extracts from 10, 25 and 50 g were filtered with glass fibre filters (Carl Roth GmbH, type CR259, 150 mm diameter, $\geq 0.6 \mu\text{m}$). The pilot scale extractor (Pignat) used in this work is equipped with a filtration and distillation system. Therefore, after 4 hours of maceration, the mixtures were directly filtered into the pilot extractor to separate the solvent from the solid residues. The remaining primary filtrate, approximately 17 L, was concentrated to 2 L at 50°C for 8 h at 150 mbar and filtered again with the glass fibre filters used for 10 to 50 g samples mentioned above. All the filtered extracts were finally stored at -20°C for subsequent analyses.

Table 1.2-1 Scheme of the experimental materials used for each scale of the upscaling process. All the extractions were performed with 40% EtOH in a 1/20 BSG/solvent ratio.

biomass g	vessel	agitation	filtration	volume reduction
0.075	plastic tube 2 mL	Orbital 300 rpm	centrifugation	/
0.5	plastic tube 15 mL	Orbital 180 rpm	centrifugation	/
10	glass flask 0.5 L	Orbital 180 rpm	glass filter (0.6 μm)	/
25	glass flask 1 L	Orbital 180 rpm	glass filter (0.6 μm)	/
50	glass flask 2 L	Orbital 100 rpm	glass filter (0.6 μm)	/
850	aluminium 20 L	Overhead stirring	glass filter (0.6 μm)	50°C at 150 mbar

1.2.2 Chemical assays

Total phenolic content assay (Folin-Ciocalteu)

The reagents used are as follows: 7,5% w/v Na₂CO₃ solution and 1N Folin-Ciocalteu reagent (diluted from the stock solution, supplied by Sigma Aldrich, F9252-100 ML) (Singleton & Rossi Jr., 1965). Twenty-five µL of Folin-Ciocalteu reagent were combined with 50 µL of each sample and standard dilutions and then a volume of 125 µL of Na₂CO₃ solution was added in a 96-well plate. The plate was covered with aluminium paper to avoid light degradation of the reagents and incubated at room temperature for 30 minutes. Using a spectrophotometric microplate reader (Spark, Tecan, Männedorf, Switzerland), the absorbance at 765 nm was determined. Gallic acid was used to generate a calibration curve ($y = 4.8007 + 0.0461x$; $R^2 = 0.9968$), and the TPC results expressed as gallic acid equivalents (GAE). Four replicates were analysed in technical triplicates.

Ferric reducing/antioxidant power assay (FRAP)

The ability of molecules to convert Fe (III) into Fe (II) is utilized by the ferric reducing power assay (Benzie & Strain, 1996). The reduction of the iron, namely, reducing power, is directly correlated with the antioxidants present in the sample. The greater the amount of reduced iron, the more intense the blue colour of the solution, and the higher the antioxidant capacity of the sample. Fresh FRAP reagent was made by combining 20mM FeCl₃, 10mM ferric-tripyridyltriazine, and 300mM acetate buffer at 3.6 pH. Hundred-ninety µL of FRAP reagent was added to a 96-well plate, to which 10 µL of the extracts were added. After an incubation of 20 minutes in the dark at room temperature, the absorbance was measured at 595 nm using a spectrophotometric microplate reader (Spark, Tecan). FeSO₄ at various concentrations (0-1mM) was used to build a standard calibration curve: ($y = 0.6921x + 0.0866$ $R^2 = 0.9999$) and the results were expressed as µM of ferrous equivalent. Four replicates were analysed in technical triplicates.

1.3 Results and discussion

The TPC and the antioxidant capacity of the extracts obtained from the first batch of BSG were measured respectively with the TPC and FRAP assays. The TPC of the samples from the first batch ranges from 1.64 to 1.74 mg GAE/g dw BSG (Figure 1.3-1) and the antioxidant capacity from 3.7 to 6.4 $\mu\text{mol FeSO}_4$ eq/g dw BSG (Figure 1.3-1). The scale-up from 0.075 g to 50 g with the first batch of BSG resulted in similar TPC values and no significant differences were observed. The antioxidant capacity, measured with the FRAP assay, increased with increasing amounts of start material, with the antioxidant capacity of the 0.075 g and 0.5 g extracts significantly lower than that of the 10 to 50 g extracts. To test the reproducibility of these first steps in the upscaling extractions, the extractions were repeated with 10, 25 and 50 g of BSG from the second batch of BSG. When comparing the three extractions performed with both batches, while the antioxidant capacity measured with the FRAP assay is the same independent of the batch used, the TPC of extracts from the second batch is significantly lower. When making the step to 850 g of BSG as starting biomass, and the associated change in the used equipment, both the TPC and the antioxidant capacity decreases compared to the 10, 25 and 50 g samples. Actually, the extracts from the pilot scale presented a TPC of 0.28 mg GAE/g dw BSG and an antioxidant capacity of 2.5 $\mu\text{mol FeSO}_4$ eq/g dw BSG. The currently preferred valorisation of BSG focusses on the use of phenolics, which can be used in the food industry for their beneficial effects on human health, mainly associated with their antioxidant capacity. Consequently, the total phenolic content and the antioxidant activity are the quality parameters generally used to compare different BSG extractions. However, data on the effect of larger scale extraction are still scarce.

In this experiment, the solid to solvent ratio and the temperature were kept constant, while the size and the type of extractor and the stirring changed. These data illustrate the impact of changing equipment and approach, as is required to make extracts from higher amounts of

biomass. The lower antioxidant values for the 0.075 and 0.5g samples compared to those of 10 to 50 g might be explained by the different extraction vessels used. Extractions from the lowest amount of BSG were done in plastic tubes, while those from 10 to 50 g were performed in glass flasks. This change also required to modify the agitation technique and the method for separating the extract from the remaining solid phase. These parameters had to be adapted again for the 850 g pilot-scale extraction. For example, the agitation/stirring speed was previously reported to affect the extraction efficiency. Specifically, Belwal et al. (2020) tested stirring from 300 to 600 rpm showing an increase in the extraction efficiency at 600 rpm attributed to the enhancement of the circulation flow and the greater exposure of the biomass to the solvent. Moreover, the Pignat pilot-scale extractor is equipped with a filtration and distillation system, therefore primary filtration and distillation at 50°C to reduce the volume were performed; steps not performed in the other extractions. This extra step at higher temperatures may, similarly to the drying of the BSG as pre-treatment (chapter 3.3), have a negative impact on the TPC of the extract and therefore result in a lower antioxidant capacity.

The extraction yields of the second batch of BSG were calculated and confirm that the Pignat-system performs less good than the approaches used for the small-scale extractions. In addition to the lower values in the biochemical assays, the extraction yield of the pilot extraction is lower than those of the other extractions. There are no comparisons available in literature wherein up-scaling was performed with maceration, and the extraction yields given in Table 1.3-1 can only be compared to those obtained during laboratory scale extractions. A study conducted by Herbst et al. resulted in extraction efficiency, expressed as the amount of the extract per the quantity of the starting biomass, between 6.6 and 10 g extract/100 g dw BSG depending on the solvent used during Soxhlet extraction with different solvents (Herbst et al., 2021). Moreover, they also studied a pressurized liquid extraction approach, resulting in higher extraction yields than Soxhlet (Herbst et al., 2021). A recent study performed extractions from BSG with supercritical CO₂, resulting in yields

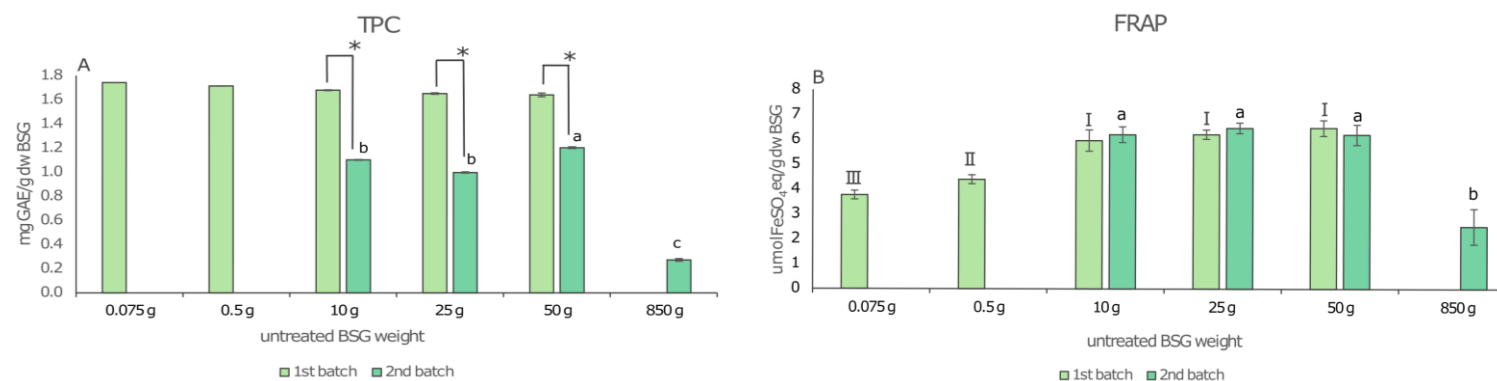


Figure 1.3-1 Biochemical assays on BSG during upscaling: A) total phenolic content (TPC) (Folin-Ciocalteu); B) antioxidant capacity (FRAP). The annotation 0.075 g; 0.5 g; 10 g; 25 g; 50 g and 850 g indicate the wet weight of the BSG used for the extraction. A conversion factor 4.2 (calculated based on the weight difference of dried and fresh BSG of the analysed samples) was applied for expressing the results based on dw BSG. All the extractions were performed for 4 h at room temperature with 40% EtOH in a 1/20 BSG/solvent ratio. The data are based on four replicates. In the TPC graph (A), the “*” indicates significant differences between the two batches of BSG analysed; the letters “a, b and c” indicate the significant differences between the extracts within the 2nd batch. No significant differences were observed in the 1st batch. In the FRAP graph (B), the “I” indicates the significant differences within the 1st batch. The letters “a and b” indicate the significant differences within the 2nd batch. No significant differences were observed between the two batches of BSG analysed

between 5.7 to 13.6 g extract/100 g dw BSG (Lisci et al., 2024).

Table 1.3-1 extraction efficiency expressed as the amount of extract per the quantity of biomass used for the extraction, the data are based on four replicates.

g fresh BSG	g extract/100 g dw BSG
10	8.5 ± 0.19
25	8.5 ± 0.4
50	8.8 ± 0.3
850	6 ± 0.6

Here, only data on the first step of the pilot-scale extraction cascade described in chapter 4 are presented. With the lower values in the biochemical assays obtained for extracts of this first step, the other fractions were not further characterized. Further up-scaling experiments need to be conducted to improve the extraction yield and to increase the antioxidant capacity and the TPC, bridging the gap between results from laboratory-scale with the pilot seen in Figure 1.3-1. For example, optimisation of the preliminary pilot scale extraction might be achieved by testing different stirring types (such as saw tooth impeller, Rushton turbine, anchor or counter flow impeller) and speeds optimising them according to the matrix and the extraction parameters applied (Torotwa et al., 2018). Another important factor to consider for improving this extraction process at larger scale is the application of alternative methods to reduce the volume of the extract thereby avoiding exposure of the extracted substances to high temperatures. In the Pignat system used in this study for the larger scale extraction, the distillation step was conducted at 50°C for several hours. As discussed in Chapter 3.3, heat treatment can negatively impact the antioxidant capacity of the collected fraction. Therefore, exploring methods that minimize or avoid the heating exposure, such as spray drying, could potentially enhance the extraction process and preserve the quality of the extract. The optimisation of process parameters is indeed considered as the

key point to bring good results to industrial applications (Martinez & Sales Silva, 2013).

Although there are numerous studies that primarily aimed to maximize the phenolic content and antioxidant capacity of BSG extracts, most of these studies are at laboratory scale. Literature on upscaling and pilot scale extractions is limited and more resources should be allocated to make upcycling of BSG part of the international efforts to increase the circularity of the economy. The data presented here illustrate that with the used equipment for the extraction from 850 g of BSG the biochemical activities of the extracts are less than those obtained at laboratory scale, and optimisation of the process parameters at pilot scale is needed.

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CHAPTER 6.

GENERAL DISCUSSION AND CONCLUSION

1 General discussion

BSG is the main solid residue generated by the brewing industry and its management is becoming increasingly challenging. The volume of BSG produced is exceeding the amount that is currently recycled. Consequently, around 20% of BSG ends up in landfills. Despite the current recycling methods, the full potential of this biomass remains underexploited.

In this study, we investigate the hypothesis that developing alternative recycling methods for BSG management can effectively reduce its accumulation while enhancing its value. Specifically, this research examines whether a method can be designed to better valorise the chemical composition of this biomass by extracting its compounds. It explores the possible development and optimisation of an extraction process through an untargeted metabolomic analysis. Additionally, we investigated whether the application of a biorefinery approach aiming at obtaining multiple exploitable products could be considered a viable strategy for a better recycling of BSG and its potential up-scaling.

1.1 Untargeted metabolomics as a driving approach for the development and optimisation of a BSG extraction method

To address the previous hypotheses, the first phase of this study was dedicated to the metabolomic characterisation of BSG extracts aiming at identifying as much compounds as possible to obtain a comprehensive metabolomic profile. The resulting UHPLC chromatogram showed within the first timeframe ranging from 0 to 5

minutes, the more hydrophilic molecules, such as oligosaccharides, free amino acids, diketopiperazines, and Amadori products (Chapter 3).

Amadori products are important intermediates of the Maillard reaction cascade and are a group of compounds that spontaneously form by a condensation reaction between molecules containing an amino function and reducing sugars. I could identify Amadori products formed from sugar oligomers, thereby confirming the presence of these compounds in BSG extracts (Minestrini et al., 2022). While the formation of Amadori products through the reaction of sugar monomers is known and found in numerous matrices, the condensation with sugar oligomers was just hypothesized (Hellwig & Henle, 2020). In the BSG extracts from this study, Amadori products derived from most proteinogenic amino acids were identified. These compounds had been mainly detected in processed food subjected to heat-drying and roasting. They were found in dried vegetables such as dried tomatoes and peppers, as well as in dried fruits like raisins, apricots plums, roasted coffee and cocoa (Davidek et al., 2005; Meitinger et al., 2014; Yuan et al., 2017). While on one side, Amadori products are precursors of AGEs, which are molecules known for their detrimental effects on human health, on the other side, some of Amadori products are being investigated for their beneficial properties such as metal chelating, and antitumor activities (Lee et al., 2016; Mossine & Mawhinney, 2007). Considering their controversial effects on human health, it is then important to identify these compounds and further investigate their bioactivities, since they were detected in several food matrices.

Along the chromatogram, from 5 to 20 minutes, phenolic compounds, including hordatines a group of dimeric phenolamides unique for barley, were detected, while the more hydrophobic compounds were identified in the last time frame. In total, 381 compounds were identified in positive and/or negative mode, constituting the metabolite profile used in the subsequent experiments.

The untargeted approach used in the initial phase of the study provided a detailed metabolomic profile of the main metabolites detected in the recovered extracts. This method aided to identify compounds that may affect the potential health-promoting properties of the final product and therefore to address the further steps of the study. As previously mentioned, Amadori products are early-stage compounds formed during the Maillard reaction cascade, which is triggered by heating. In brewing, high temperature treatments are applied both during the malting phase, specifically kilning, and the mashing step, and this is crucial for the formation of Amadori products (Inns et al., 2007). In addition, BSG, once generated, is often subjected to an additional drying treatment for its stabilisation by lowering its high moisture content (70-80%). This helps to prevent microbial activity and reduces the volume and weight of the biomass. This drying treatment involves heating and is the most commonly used method for stabilising BSG, especially oven-drying. Therefore, most of the studies conducted on BSG valorisation used heat-dried BSG as starting material. Accordingly, the first batch of extractions used in the current study was performed using oven-dried BSG.

Oven-drying for BSG stabilisation involves long-time exposure of the biomass to high temperatures and leads to a high energy consumption and a high investment in cost and time (Krakowska-Sieprawska et al., 2022; Mussatto et al., 2006; Singh et al., 2020). The exposure to high temperatures may impact the chemical composition of the biomass and thus the resulting extracts. Moreover, drying may also trigger the formation of new compounds, such as Amadori products and diketopiperazines, and affect the bioactive properties of the extracts. Other options for BSG preservation include the application of preservatives such as organic or inorganic acids, and other commercial additives, mainly suppressing microbial growth rather than reducing the water content and volume of the biomass. In addition, these products most likely affect the chemical composition of the biomass and may modify the extracts (Terefe, 2022). Alternatives to oven-drying to reduce the water content are available, however, their application is still limited at industrial level and the improvement and

implementation of these alternatives are currently being studied. Nonetheless, these alternatives are considered promising as they are estimated less economically, energetically, and environmentally impactful compared to oven-drying (Terefe, 2022). One of such alternatives is the use of a membrane filter press, although a further pre-treatment with hot water is necessary prior to the mere action of pressing (Arnaud, 2024; El-Shafey et al., 2004). Another recent approach, that is considered more sustainable, is the use of a superheated steam. This technology is currently investigated for application in the food industry, for example, for improving the structural and rheological properties of cereal-based foodstuffs (Ma et al. 2022). While the rheological properties are improved, treatment with superheated steam involves very strong heating, which can negatively impact the quality of the matrices. Nonetheless superheated steam treatment is considered promising, and further study on the influence of this stabilisation method on the chemical composition of matrices and the potential application at larger scale must be conducted (Sehrawat et al., 2016). Despite the advancements made, the industrial-scale application of such alternatives is still rare and the requirement to develop these technologies currently limits the potential of BSG recycling, especially for smaller breweries in less developed countries (Bonato et al., 2022; Krakowska-Sieprawaska et al., 2022). Consequently, oven-drying is still the most widely used approach and even considered essential for BSG valorisation. However, the detailed effects of oven-drying on BSG and its extracts is still unknown.

Based on the identification of Amadori products and diketopiperazines, which are compounds formed as a result of heat exposure, and the hypothesis that oven-drying, as pre-treatment for BSG may affect the chemical composition of the extracts, a detailed study was conducted to investigate the impact of oven-drying on the main groups of metabolites detected in BSG extracts. Therefore, an untargeted metabolomic approach was applied for comparing extracts generated from untreated, fresh and oven-treated BSG. The identified compounds were classified in 8 groups (sugars, amino acids, Amadori

products, peptides- and -derivatives, hordatines, polyamines, phenolics and lipids) according to their chemical structures and their properties. The cumulative relative MS intensities of all compounds in each group were then used to compare the extractions. While this approach results in the loss of detail on the impact of heat on individual compounds, it generates a global view on the impact of experimental conditions, and it was previously used as such (Pinto et al., 2022; Salem et al., 2022). In the same experiment, different extraction solvents were tested. Ethanol and methanol are the most common solvents used for the recovery of phenolic compounds from biomass (Ngasakul et al., 2023) and this group of metabolites is one of the most targeted in BSG studies. We selected ethanol because it is the safer solvent for the human health and the environment, and it is considered a GRAS-solvent by the Food and Drug Administration (FDA) (Lianza et al., 2022).

Preliminary data indicated that mixtures of ethanol and water with more than 40% of ethanol resulted in extracts with a lower antioxidant capacity and total phenolic content. Therefore, the tested extraction solvents were water and 20 and 40% ethanol in water (Chapter 3). The extracts from untreated BSG had a higher antioxidant capacity than those from the oven-dried biomass and the 40% EtOH mixture resulted in the highest values for the targeted biochemical parameters. Oven-drying was the main determinant in the cumulated signal intensity for sugars, Amadori products, amino acids, polyamines, and peptides and peptide-derivatives. On the contrary, the cumulative MS-intensity of metabolites classified as hordatines and lipids were most affected by the solvent mixture used to make the extract. The group of phenolic compounds had a subtler impact from both parameters (starting material and solvent used). Although the used solvent is the main determinant, the effect of the drying treatment is strongly dependent on the identity of the compounds.

This study indicates that fresh, untreated BSG can be considered as an alternative to dried, oven-treated BSG for the generation of extracts for application in the food sector, thereby confirming the hypothesis.

Untreated BSG showed higher antioxidant activities and presented a similar or higher abundance of compounds that are currently targeted by most studies on BSG valorisation. BSG is a by-product of an industrial process. Therefore, its availability is dependent on the brewing process and it is predictable in time and amount. This makes that both the size of the extraction process and the productivity of industrial BSG valorisation can be engineered, and as the data presented in chapter 3.3 shows, this can and should be done without consuming energy and time for BSG stabilisation.

The economic impact of BSG stabilisation is huge. A study showed that while the prize of fresh BSG is approximately \$ 40 per ton the actual cost that an industrial actor needs to consider when valorising BSG is three times higher. This sur-cost corresponds to the investment in equipment, energy and time needed to stabilize the biomass (Buffington, 2014). Based on this economic evaluation and on the results described in this work, the use of fresh, untreated BSG is a promising alternative to oven-dried BSG for the valorisation of this biomass by avoiding energy and costs.

The selection of the method and the solvent mixtures used for this experiment (chapter 3.3) was made considering the feasibility and the ease of the approach on one side, and the potential economic and environmental impact on the other. However, a lot of new extraction approaches have been proposed in the last years and are generally considered as more sustainable and effective. One example is the use of deep eutectic or natural deep eutectic solvents (respectively DESs and NADESs) for extraction. These solvents are a mixture of a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) which, when mixed in an optimal ratio, form an eutectic system with high extraction potential. Recently, studies on DES and NADES extraction from BSG showed promising results, especially for the extraction of phenolic compounds (Nath et al., 2023; Ngasakul et al., 2023). Nonetheless DES/NADES extraction comes with some limitations, and the environmental safety, the effectiveness and the potential for upscaling of these emerging solvents needs to be

assessed. However, DES/NADES is considered the extraction approach of the future, spurring the scientific community to further explore this road (Benvenuti et al., 2019).

1.2 A promising group of unique phenolic compounds for barley-based BSG valorisation

Independently to the extract that was studied, hordatines were among the phenolic compounds with the highest intensity peaks. While studies on BSG extracts have mainly been focussed on the hydroxycinnamic acids (HCA) and flavonoids, hordatines are only recently attracting scientific interest. Nonetheless, some bioactivities are being studied for these compounds (antifungal and antioxidant activities) (Becker, Stegmüller, et al., 2022; Spreng & Hofmann, 2018; Stoessl & Unwin, 1970). Hordatines, formed by the dimerization of phenolamides consisting of a phenolic acid and an agmatine, are specific metabolites from barley. Therefore, the presence of hordatines in my extracts, identified through untargeted metabolomic analysis, along with their specificity to barley matrices, may offer a unique and advantageous approach to the valorisation of barley-based BSG biomass. Given their higher concentration compared to other phenolic compounds in BSG and their potential bioactivities, the development of a hordatine-enrichment protocol was considered of interest. In addition, the potential health benefits and the use of these molecules in functional foods is currently under investigation (Hamany Djande & Dubery, 2024).

Two methods were tested to generate hordatine-enriched fractions from BSG extracts (Chapter 3). The first one is based on adsorption of phenolics, hordatines included, on an adsorbent resin, used in batch, designed for food and biopharmaceutical processes. In the second method, the extracts were injected on a preparative C18-column and the hordatine-containing fraction collected. Each method has its strengths and its weaknesses. While the chromatographic method is more selective, it is time consuming and, for bigger volumes, requires

expensive equipment, the resin-based approach is most likely easier to scale up although less selective.

The protocols were optimized and evaluated based on the antioxidant potential of these fractions since the antioxidant capacity is the main biochemical activity targeted in BSG extracts and one of the parameters followed throughout this thesis. Furthermore, a UHPLC-MS/MS characterisation of the enriched fractions was performed. Depending on the method used, different metabolite profiles were found. The enriched fractions obtained with the resin method were heterogenous, containing a mixture of lipids, hordatines and other phenolics. In contrast, the chromatographic method resulted in fractions containing nearly exclusively hordatines. The relative abundance of the hordatines, the ratios between the intensities of the different hordatines (A, B, C and the hydroxy and glycated forms), were the same independent of the used method. Both enrichments resulted in fractions with a high antioxidant potential, confirming the data presented by Spreng and Hoffman (Spreng & Hofmann, 2018).

The growing interest in hordatines is among others illustrated by efforts to synthesize these compounds *in-vitro* for the production of standards, with a high market value. Furthermore, *in-vitro* synthesis for the application of these molecules in industrial products, for example as food preservatives, is attempted (van Zadelhoff et al., 2021, 2024). In addition, recent studies on the biological activities of hordatines (e.g. inhibitory activity of α -glucosidase, as COVID-19 treatment, regulation of gastro intestinal smooth muscles (Becker, Permann, et al., 2022; Dahab et al., 2020; Hamany Djande & Dubery, 2024; Spreng & Hofmann, 2018)), make the recovery of hordatines-enriched fractions promising, providing a unique product associated to the valorisation of barley-based BSG.

1.3 BSG fractionation: a novel biorefinery approach

The optimisation of the hydroethanolic extraction from untreated BSG described in chapter 3, is the first step towards the main objective of

this research project that is to provide data for maximizing the valorisation of BSG. Although there are numerous studies conducted on the valorisation of BSG for its bioactive components, such as phenolics, lipids, fibers or proteins, most focus on the optimization of the extraction of a single class of compounds. In this way, only a limited part of the potential of BSG is exploited (Lisci et al., 2024). Therefore, considering the heterogeneity of its constituents, a viable strategy could involve the fractionation of BSG to extract multiple enriched products, thereby increasing its up-cycling value. Although such biorefinery approach can be a promising strategy, so far it is only applied for the production of biofuel and biogas (Aggelopoulos et al., 2014; Pabbathi et al., 2022), leaving the complex composition of BSG un-valorised. The use of a biorefinery approach on this biomass with the aim of generating a multi-product output with a focus on the activities of these products, is scarcely explored. Closest to this is the combination of the extraction of fibers and proteins (Belardi et al., 2023; Vieira et al., 2014), but this was done without further exploring the possibility to generate other, more diverse fractions. In Chapter 4, a preliminary model was established by applying a sequential extraction cascade based on fresh, untreated BSG, resulting in, four potential valuable enriched fractions.

The extraction cascade starts with the recovery of a hydroethanolic-phenolic fraction followed by the recovery of a lipid-enriched fraction. As last step, the protein and the arabinoxylan fractions were collected. By applying a sequential extraction scheme, four potential valuable fractions were obtained in one process. Each step of the cascade was optimised, and the chemical profile of these fractions characterised. Results were compared to data obtained in studies focused on the recovery of one specific fraction.

Starting from untreated, fresh BSG, the first, hydroethanolic, fraction was collected and characterized as described in chapter 3. Although the BSG used in this study contains 7.3% lipids on a dry weight base (table 1.3 in chapter 4), and that lipids have a known valorisation potential in different economic sectors, only few studies were

conducted on BSG lipids (del Río et al., 2013; Ferrentino et al., 2019; Niemi et al., 2012). In a preliminary experiment different solvents commonly used for lipid extraction were tested, and ethanol was selected for the extraction cascade. The main lipids detected were phospholipids, specifically phosphatidylcholines (PC) and phosphatidylethanolamines (PE), and hydroxy-fatty acids (HFAs). The lipid class with the highest MS-signal intensity were PC, which are investigated for their benefits for human health (Chen et al., 2019; Treede et al., 2007) and the main fatty acid identified is linoleic acid (18:2), an essential unsaturated omega-6 fatty acid.

The solid residue after lipid extraction was used for the extraction of proteins. Different technologies have been proposed for the extraction of proteins from residual biomass, the most widely used is the alkaline method resulting in the highest extraction yields (Wen et al., 2019). The extraction performed in this study included two steps: protein solubilisation in alkaline conditions (pH 11) followed by their precipitation at a pH-value corresponding to their isoelectric point (pH 3.5-4) (Devnani et al., 2023). Both the extraction yield and the amino acid composition of the obtained extracts were in line with values reported in literature. The amino acids with highest concentrations correspond to those previously described in BSG biomass and in protein isolates from BSG. Given that protein extracts from BSG are generally obtained after different pretreatments such as oven-drying (Connolly et al., 2013), defatting or alkaline and acid treatments (da Fonseca et al., 2023; Hellebois et al., 2021; Qin et al., 2018), this was an expected result. In the biorefinery approach described here, the initial extractions (namely hydroethanolic and ethanolic extractions) allow simultaneously to collect two potential bioactive fractions, while removing molecules that could interfere with protein recovery, thereby facilitating the subsequent extraction.

Food-related applications of the protein-enriched BSG fraction have been previously demonstrated. Studies found that BSG protein isolates exhibit antioxidant, anti-inflammatory, immunomodulatory and antihypertensive activities (Hellebois et al., 2021). Furthermore, this

fraction also shows high water and oil holding capacity and gelation, foaming and viscosity increasing capacities (Connolly et al., 2013; da Fonseca et al., 2023; Devnani et al., 2023), techno-functional properties of interest for the food-industry.

An arabinoxylan enriched-fraction is the fourth and last BSG fraction collected in this biorefinery process. Arabinoxylans (AX) are the most abundant hemicelluloses present in BSG. The extraction yield and the absolute AX content in the extract generated in the cascade are in the range reported in literature (Coelho et al., 2016; Reis et al., 2015; Vieira et al., 2014). The range reported in literature is very broad since these parameters depend on numerous factors including the selection of pre-treatments, the different extraction conditions and used solvents. These parameters, together with the differences in the used BSG, make the yield and quality of AX-enriched fractions variable. The AX-branch degree, calculated as the ratio between arabinose/xylose, is an important parameter that describes the AX-structure and represents a first indication on the valorisation potential of AX-extracts. The AX-branch degree of the fraction obtained here is close to that from analyses only targeting AX-extraction from barley malt beers and BSG. A study conducted by Lynch et al. concluded that a A/X ratio equal or higher than 0.6 indicates a highly branched AX structure. This high level of arabinose substitution acts positively on the stimulation of the probiotic activity of bacteria of the genus *Lactobacillus* (Lynch et al., 2021). A similar AX ratio was obtained by Calvete-Torre et al. in a study on the microbiome modulatory activity of AX extracted from BSG. They analysed the microbiota of 2 populations of healthy or Crohn's disease (CD) patients. Although the authors mentioned certain limitations, mainly related to the inter-individual variability in both groups of people, they observed a positive impact on microbiome modulation through the administration of the BSG AX-enriched fraction (Calvete-Torre et al., 2023). Although this study thus demonstrates that AX-fractions extracted from BSG have an application potential in the medical sector, this was only a first test and further research needs to be done. More analyses need to be performed on the AX-fraction described in chapter 4. To

obtain a complete characterisation of the fraction, the length and the polymerisation degree of the AX need to be determined. In addition to the pharmaceutical and medical sectors, which are interested in AX isolates due to the pre-biotic, immune and gut regulatory properties that these compounds show (Zannini et al., 2022), the food industry is also looking for these valuable dietary fibers for food fortification (Liu et al., 2023; Zhang et al., 2019).

The application of the biorefinery approach allows to valorise more than a fourth of the starting BSG biomass. It resulted in four fractions which have an added-value potential. The remaining solid residue from this process can be further exploited for the production of bioenergy, biofuel or as packaging and building materials (Jackowski et al., 2020; Pabbathi et al., 2022). Today about 6.8 million of tons BSG are annually produced in Europe, which, based on a fresh BGS price of 35 euro/ton, represents a relevant value. Significant research and R&D efforts are being done to increase the value of this biomass. In this frame, increasing BSG value is under investigation by the scientific community. However, this research effort mainly focuses on recycling BSG by generating only one output. In contrast, the application of a biorefinery approach is rare (Zeko-Pivač et al., 2022). With the model proposed in chapter 4, the output-value of BSG is estimated to increase since with one process four valuable fractions with potential applications in different industrial sectors are collected. As proposed by Zeko-Pivač et al. the enhancement of the value of BSG and its integration in a circular economy model requires that a feasible and simple cascade approach for obtaining several added-value products is developed (Zeko-Pivač et al., 2022).

The presented approach and the data show that it is possible to extract four valorisable fractions from BSG, this however is only a first form, and each step can be modified. For example, if triglycerides are the targeted molecules for the second step of the cascading, the lipophilic extraction can be performed with more apolar solvents (del Río et al., 2013; Ferrentino et al., 2019; Niemi et al., 2012). More fibre-rich fractions can be obtained by implementing additional steps after the

extraction of the proteins. This was for instance done in a recent publication and granted patent (WO2023012841 (A1) Date of Issue: 9 February 2023) (Belardi et al., 2023). In the model described by Belardi et al., the sequential extraction starts with the recovery of proteins, and the solid residue of this step is furthermore fractionated for the recovery of cellulose, functionalised AX and lignin products.

The objective of the work described in chapter 4 was to build a process that is easy enough to be scaled up to industrial level while resulting in multiple fractions from BSG. The same extraction cascade can be applied on other biomasses. Initially this approach may be easily applied on residues from other industrial activities based on grains such as wheat, oat or rice bran, presenting a similar chemical composition. However, there are no *a priori* reasons why it would not be applicable for the upcycling of unrelated biomasses. The feasibility, the ease, the cost, and time-effectiveness of the process, but also knowledge reported in scientific literature were considered to build this model. However, as mentioned above, decisions were taken, and alternative extraction methods can replace the ones used in chapter 4. Furthermore, further research and analyses are needed to ascertain that the generated fractions show the expected biological activities, which for the moment are attributed based on data from previous studies. This was not feasible within the time-frame of my thesis but is essential for the development of an industrial activity based on this extraction cascade.

1.4 Potential up-scalability of the extraction model and the limitations of the approach

Currently, the research community is increasingly focused on the extraction of valuable compounds from BSG, although most of the current approaches mainly occurred at laboratory scale. Given the large quantity of BSG produced and the necessity to find effective strategies to turn this biomass from waste into a resource, the feasibility of scaling up the method developed in this study was investigated.

An up-scaling plan from laboratory to pilot-scale was established for the first extraction (chapter 3.3) of the biorefinery model described in the chapter 4. Different amounts, from 75 mg to 850 g of untreated BSG were used for performing the hydroethanolic extraction. The antioxidant capacity and the extraction yield of the obtained extracts were evaluated. Besides the selection of an extraction method, other parameters need to be considered as in all scale up efforts. The solid to solvent ratio, the temperature, and the pressure are considered primary criteria and should be kept constant across the different scales. Other parameters are considered secondary factors, which often depend on technical limits and/or possibilities related to the volume of the vessels used must be adapted to each scale level (Martinez & Sales Silva, 2013).

In this first experiment the primary factors were kept constant, while the method of agitation and filtration depended on the volume of the extraction. The major change in the measured parameters was from the extraction of 50 g to that of 850 g. Both the extraction yield and the antioxidant capacity, calculated on g dw BSG base, were similar up to the extract from 50 g of BSG, but decreased significantly for the largest sample size. This variation is associated with a change from agitation with a shaker for the smaller sample sizes to the stirring of the extraction mixture with an overhead propeller. In the Pignat pilot-scale extractor, used for the 850 g sample, the volume of the initial extract is furthermore reduced by distillation at 50°C for 8 hours, an extra step not needed for the extracts of the smaller sample sizes. In chapter 3 the negative impact of exposure to high temperatures, *in casus* oven-drying, on the antioxidant capacity of a hydroethanolic BSG extract is described, a similar decrease may be caused by the distillation of the primary extract. These data clearly show that the up-scaling is not straightforward and the application of different approaches to reduce the volume of the extract is needed, spray drying may be an alternative (Spinelli et al., 2016).

This represents only the beginning of an up-scaling study, and further efforts must be put on the adaptation of the secondary extraction

parameters, since their optimisation is considered the key point for the industrial scale application (Martinez & Sales Silva, 2013).

2 Conclusion and perspectives

The present study investigated an alternative approach to enhance the valorisation of BSG biomass converting the re-use of this biomass from recycling to up-cycling.

Three main objectives were addressed to make progress towards the maximal valorisation of BSG: the development and selection of an optimised extraction considered suitable for food-grade applications, the development of a biorefinery model resulting in multiple fractions with valorisation potential, and a preliminary up-scaling.

For the development and optimisation of a food grade extraction (hydroethanolic extraction), an untargeted metabolomic approach was applied and a metabolomic profile was generated. Based on these data, an extraction protocol was developed. This resulted in a method that bypasses the commonly used oven-drying step for stabilizing BSG biomass. However, while this optimisation study provides an overview on the metabolomic profile of BSG extracts and gives insight into the effect of oven-drying on BSG, it also raises new questions. To further optimise the BSG pre-treatments in the valorisation process, extending the experiment to compare freshly produced BSG, without storage, with BSG that has been frozen at -20°C and defrosted (as used in this study), and with freeze-dried BSG, should be also considered.

In addition, since the phenolic compounds are among the most targeted molecules in BSG studies, a more detailed quantification of these compounds may be of interest for future investigations. In particular, further research is needed on the isolation and bioactivities of hordatines, which, despite the increasing attention from the scientific community, are still not well investigated. Considering that hordatines are produced by barley as a defence mechanism against

phytopathogenic fungi, it is worth exploring their antifungal and antimicrobial activities by targeting both plant and human pathogens. Furthermore, as hordatine isolates showed antioxidant capacity, optimizing methods to increase their concentration may open new possibilities for studying their use also as human health-promoting products.

Based on the first hydroethanolic extraction, a biorefinery approach was developed to maximise the recycling potential of BSG. This approach resulted in the recovery of four valorisable fractions. While this approach offers additional perspective on the recycling of BSG, more studies need to be conducted to evaluate the effectiveness of the process. Here, a preliminary cost analysis of the whole process was examined, although more comprehensive and detailed economic evaluations, such as a life cycle analysis and techno-economic assessment are required (Nyhan et al., 2023). Additionally, the environmental impact of the process, and the management of potential waste streams generated need also to be considered.

From this process, the recovered fractions are deemed high-value products for the food, pharmaceutical and cosmetic sectors (Calvete-Torre et al., 2023; Devnani et al., 2023; Hellebois et al., 2021; Lynch et al., 2021). However, in this study their effective bioactivities were not measured, except for the antioxidant capacity tested for the first hydroethanolic fraction. Therefore, studies on the techno-functional properties and bioactivities of these fractions are needed.

The feasibility and flexibility of the entire process present opportunities for further implementation. For example, the fractionation steps may be expanded to include the recovery of other components like lignin and cellulose, as proposed by Belardi and colleagues (Belardi et al., 2023). Additionally, investigating new alternative and promising techniques, such as the application of (natural) deep eutectic solvents, could represent a further opportunity for the improvement of the extraction.

This study also aimed to investigate the potential scalability of the process. However, given the challenges faced during the up-scaling of the hydroethanolic extraction, the other steps of the biorefinery approach have not yet been explored. While hydroethanolic extraction maintained consistent results at different laboratory scales, a significant decrease was observed when doing pilot-scale extractions. These results are not surprising and coincide with technical changes associated with the scaling up, most likely related to stirring and distillation. To determine the main critical factor, further experiments should be performed. For example, comparing the antioxidant capacity of the extract before and after the distillation, could give indication on the impact of this additional step. Furthermore, applying different stirring speeds or tools during extraction may also give insight for the optimisation of the process. The presented results stem from a first trial and before testing the scalability of all steps included in the biorefinery approach, this first stage should be optimised to successfully scaling up the extraction.

In conclusion, the data presented here contribute to knowledge that is needed for maximising the valorisation of BSG as low-cost biomass. BSG thus no longer represents a residue to be disposed of but can rather be considered as a promising resource for future applications.

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