Enrichment of brewer's spent grain polyphenols and assessment of their role in inhibition of cholinesterases, amylase and glucosidase

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A thesis submitted for the degree of Doctor of Philosophy (Ph.D) to the University of East Anglia, School of Biology, for research conducted at the Quadram Institute Bioscience UK and Teagasc Food Research Centre IE.

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Preface

The research presented in this PhD thesis was undertaken between October 2017 and April 2021, as a 3-year Walsh fellow studentship co-funded by Teagasc IE postgraduate development programme (grant number 2014027) and Quadram Institute Norwich UK (grant number 42880-000-Q), with a 4-year academic registration at University of East Anglia Norwich UK. On top of the 3-year period funding, due to COVID-19 pandemic, a 6-month extension was approved to be able to finalize the overall experimental work. This Ph.D. studentship was a collaboration between two Food and Health Research Institutes, Quadram Institute Norwich UK (Pete Wilde's group) and Teagasc Food Research Centre Dublin IE (Dilip Rai's group).

Abstract

Polyphenols are important components of the human diet and are studied for their antioxidant properties and health benefits. Brewer's spent grain (BSG) is a valuable source of polyphenols, such as phenolic acids and flavonols, and is readily available. To use these polyphenol-rich BSG reservoirs as health promoters, efficient extraction and enrichment methodologies must be developed, followed by *in vitro* testing and chromatographic analysis.

Here, several classic and novel solid-liquid extraction technologies were explored to extract polyphenols from three types of BSG. This was followed by liquid-liquid partitioning and flash chromatography to obtain polyphenol rich fractions, which were then identified and quantified using LC-MS/MS. The ability of these BSG extracts and fractions to inhibit the enzymatic activities of acetylcholinesterase, butyrylcholinesterase, α-amylase and α-glucosidase, associated with Alzheimer's disease and diabetes, respectively was assessed in vitro.

The results showed that saponification with 0.75% sodium hydroxide is the best method to extract bound phenolic acids from BSG, and 60% aqueous-acetone solution to extract free phenolics. Among the organic solvents tested to obtain rich-phenolic extracts, diethyl ether and ethyl acetate showed the highest recovery. LC-MS/MS analysis showed that BSG bound phenolic extracts are abundant in hydroxycinnamic acids, i.e. ferulic acid, *p*-coumaric acid, including dimers and trimers of ferulic acid, and 4-hydroxibenzoic acid. On contrary, BSG free phenolic extracts were abundant in the flava-3-ol catechin. BSG Dark polyphenol rich extracts, its flash chromatography fractions presented a higher inhibitory capacity for acetyl- and butyrylcholinesterase activities compared to the other tested samples, as well as for α-amylase and α-glucosidase. BSG diethyl ether fractions showed that the decarboxylated di-ferulic acid exhibited a significant contribution towards anticholinesterase activities.

This work highlighted the potentials of using BSG polyphenols for these degenerative diseases and pave way for further research towards their nutraceutical and phytopharmaceutical benefits.

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'This thesis is dedicated to my beloved wife, ***Ioana-Raluca****, for her unwavering support and unconditional love throughout the years.'*

Outputs from this Project

Publications:

1. **Birsan, R.I**.; Wilde, P.; Waldron, K.W.; Rai, D.K.; Recovery of Polyphenols from Brewer's Spent Grains. Antioxidants 2019, 8, 380. https://doi.org/10.3390/antiox8090380

2. **Birsan, R.I**.; Wilde, P.; Waldron, K.W.; Rai, D.K. Anticholinesterase Activities of Different Solvent Extracts of Brewer's Spent Grain. Foods 2021, 10, 930. https://doi.org/10.3390/ foods10050930

3. Rico, D., Peñas, E., García, M. D. C., Martínez-Villaluenga, C., Rai, D. K., **Birsan, R. I**., ... & Martín-Diana, A. B. (2020). Sprouted barley flour as a nutritious and functional ingredient. *Foods*, *9*(3), 296.

4. Hossain, M.B.; Lebelle, J.; **Birsan, R.I;** Rai, D.K. Enrichment and Assessment of the Contributions of the Major Polyphenols to the Total Antioxidant Activity of Onion Extracts: A Fractionation by Flash Chromatography Approach. *Antioxidants* 2018, *7*, 175. https://doi.org/10.3390/antiox7120175

Oral presentations:

1. **Birsan, R.I.**; Polyphenols from brewer's spent grains & their anti-cholinesterase activities. Teagasc Walsh fellowship Student Seminar, 2019 and Teagasc Walsh Scholars of the Year Competition, Moorpark Fermoy, IE, 2020.

Poster presentations:

1. **Birsan, R.I.;** Alka Choudhary; Ramon-Aznar, Roca; Wilde, P; Rai, D.K. Impact of solvent selection for the extraction of bioactive molecules from brewer's spent grains; 46th Annual Food Science and Technology Conference (IFSTI), Dublin, Ireland, 6th December 2017

2. **Birsan, R.I.;** Alka Choudhary; Brijesh Tiwari; Wilde, P.; KW Waldron; Rai, D.K.; Anticholinesterase and antidiabetic activities of enriched polyphenolic extracts from brewer's spent grains; 2nd Food Bioactives and Health Conference, NOVA, Lisbon (Portugal) – 26-28th September 2018

3. **Birsan, R.I**.; Wilde, P.; KW Waldron; Rai, D.K.; Recovery of polyphenols from light, dark and mix brewer's spent grains; 3rd World Congress on Polyphenols Applications, Malta Sep-Oct 2019

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Abbreviations

AC	Acid hydrolysis
Ace	Acetone
AChE	Acetylcholinesterase
ACN	Acetonitrile
AD	Alzheimer's Disease
AL	Alkali hydrolysis
ANOVA	Analysis of variance
AX	Arabinoxylan
BChE	Butyrylcholinesterase
BSG L or D	Brewer's spent grain light or dark
BuOH	*n*-Butanol
C18	Carbon 18
DE	Diethyl ether
DNS	3,5-Dinitrosalicylicacid
DPPH	2,2-diphenyl-1- picrylhydrazyl
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
dw	Dry weight
EA or EtOAc	Ethyl acetate
EEC	European Economic Community
EFSA	European Food Safety Authority
ESI	Electrospray ionisation
EtOH	Ethanol
FCR	Folin-Ciocalteu reagent
FRAP	Ferric ion reducing antioxidant power
g	Gram
GAE	Gallic acid equivalent
GC	Gas chromatography
h	Hours
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
Hex	Hexane
HPLC	High-Performance Liquid Chromatography
IC50	Half-maximal inhibitory concentration
kDa	kilo-Dalton
m/z	Mass to charge ratio

MAE	Microwave assisted extraction
MeOH	Methanol
mg	Milligram
Min	Minutes
mL	Millilitre
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MS/MS	Tandem Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut-off
NaOH	Sodium hydroxide
ORAC	Oxygen radical absorbance capacity
PTFE	Polytetrafluoroethylene
Q-TOF	Quadrupole Time of Flight
RP	Reverse phase
SET	Single electron transfer
TDF	Total dietary fibre
TPC	Total phenolic content
TQD	Tandem Quadrupole detector
TRAP	Radical-trapping antioxidant parameter
UAE	Ultrasound assisted extraction
UPLC	Ultra-Performance Liquid Chromatography
US-FDA	United States Food and Drug Administration
v/v	volume/volume
w/v	weight/volume
WR	Water Residue

Chapter 1

1. Introduction and literature review

This chapter sets the background for the conducted research work, underlines its goals and objectives, and includes a comprehensive review of the related literature in the areas of antioxidants from brewer's spent grain, phenolic compounds extraction and characterization techniques, as well as the *in-vitro* assays and statistical methods used.

1.1 Introduction

The importance of food and nutrition for human health and welfare has been widely recognised. Dietary guidelines from government bodies recommend a sufficient intake of fruits and vegetables because of their high concentrations of dietary fibre, essential nutrients and phytochemicals to improve worldwide health (Bazzano et al., 2003). Phytochemicals, such as flavonoids, pectins, anthocyanidins and phenolic compounds are abundant in the peels or husk, compared to the edible sections of the fruit, vegetables or grains, which have a lower concentration of these bioactive components (Ferarsa et al., 2018, Medina-Torres et al., 2017, Zuorro et al., 2019, López-Perea et al., 2019). Attributed to the prevalence of high value-added compounds, the vital natural antioxidants found in the (poly)phenolic compounds of fruit and vegetable peels may improve human health (Lynch et al., 2016, Masibo and He, 2008). A diet rich in fruits, vegetables and legumes containing bioactive components are necessary for immunity-related responses and the prevention of stress-related illnesses such as melanoma, cardiovascular disease, diabetes, erythrogenic and neurological diseases, and many other malignancies (Socaci et al., 2018, Ayala-Zavala et al., 2011, Kalpna and Mital, 2011, Prakash et al., 2007, Group et al., 2011). Over the past few years, several research studies have shown the beneficial health effects and biological properties of dietary phytochemicals, with respect to prevention and reduced risk of these chronic diseases (Dauchet et al., 2009, D'Onofrio et al., 2017, Key, 2011, A Stravodimos et al., 2017, Hamer and Chida, 2007).

The downside on the consumption of fruits and vegetables is that it generates a huge quantity of waste and residue, from both domestic and industrial use, including peel, seed, and unwanted or inedible plant tissue. Some researchers believe that commercial processing of these wastes might provide high-value products including taste and colour additives, dietary supplements,

cosmetics, and medications owing to their abundance of bioactive phytochemicals (Galanakis, 2012, Renard, 2018). Because of consumer preferences and culinary trends, there has been a resurgence of interest in fruits and vegetables peel as a source of bioactive and functional components. Alongside, functional food or food ingredients have led to global interest (annual growth rate of <10%) and are being examined intensively for their potential role to maintain a good health or otherwise optimize health (Hasler, 2002, Bogue et al., 2017).

The brewing industry generates millions of tonnes of waste by-products, which poses a management challenge from the perspectives of both the environment and the economy. Every year, massive volumes of this biomass accumulate, causing pollution problems and environmental deterioration, and especially reducing the quantities of a valuable resource that might otherwise be used for food, a wide range of additives and fuel. For the majority of food waste produced in the EU today, landfilling, composting, and incineration are the most common options to manage these wastes, and with limitations to be used in the food sector as animal feed or as food ingredients due to disease concerns (Salemdeeb et al., 2017). It is possible to extract high-value components from brewing by-products, such as proteins and polysaccharides, as well as fibres, flavour compounds, and phytochemicals, specifically phenolic compounds, all of which may be employed as nutritional and pharmacologically effective constituents (Baiano, 2014, Mussatto, 2014, Fărcaş et al., 2017, Amoriello and Ciccoritti, 2021). Brewing industry generates a variety of by-products, mainly wastewater, spent grains, spent hops, spent yeast, and germ/rootlets (Mussatto, 2006b, Karlović et al., 2020). The most abundant by-product produced by the brewing industry, representing around 85% of the total brewing waste, is brewer's spent grains (BSGs), which is the leftover residue of barley (Mussatto, 2006b).

1.2 Brewing process: from barley to spent grains

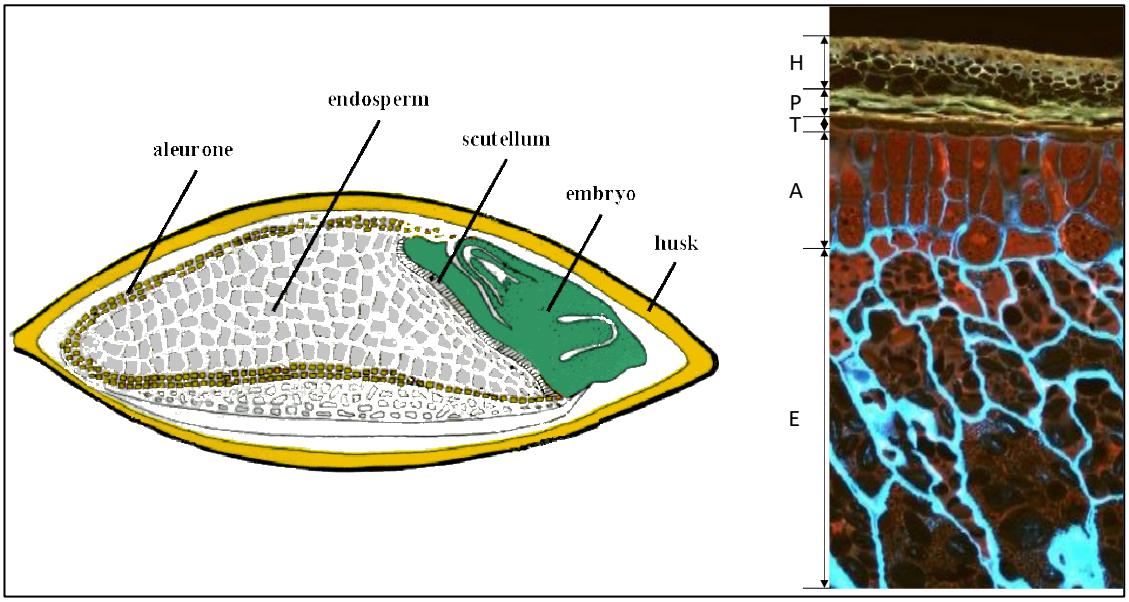
Brewing is a centuries-old practise and technology, as well as one of the world's oldest food technologies, along with baking. It is believed that barley malt-based brewing most likely originates in Middle East, shortly after the beginning of agriculture around 6 000 BC (Boulton and Quain, 2008). First-generation

fermentations may have been unplanned processes, whereby an accidental contamination of a natural sugar with yeast in the presence of good amount of water, triggered these spontaneous fermentation reactions. The malting process is also believed to have been accidental, and the brewing process was later refined in Europe by Christian abbey-dwelling monks (Abbey beers type) as it was part of daily life, which probably was related to the alcohol's physiological effects (Brewer'sofeurope, 2022, Boulton and Quain, 2008). Moreover, when illnesses like cholera spread via polluted drinking water, drinking beer rather than water had its advantages. Beside beer, brewing also creates residues, such as spent grains, which are the malts' insoluble parts that are rich in polymers, such as proteins, dietary fibres, carbohydrates and phenolic compounds (Gupta et al., 2010).

1.2.1 Barley

Barley grain is an important food source in many parts of the world due to the soluble and insoluble dietary fibre and micronutrients such as vitamins (E or B-complex), minerals and health-promoting phenolic compounds (Gupta et al., 2010). Barley grain comprises of the embryo, endosperm and several surrounding layers with diverse functions, such as store of carbohydrate (mostly starch) and proteins, enzyme secretion (aleurone) and protection (husk, pericarp and testa). A schematic representation of barley grain, with the different structural components, is presented in Figure 1-1.

Figure 1-1 Structure of a barley grain (left) (Group, Bach-Faig et al. 2011); Microscopic image of a cross-section of a barley grain (right) (H=husk, P=pericarp, T=testa, A=aleurone, E=endosperm) (Niemi 2016).



The husk and pericarp are represented in yellow (Figure 1-1) and are rich in phenolic components, whilst nearly all the cellulose (96%) in barley is located in the husk (Duffus and Cochrane, 1993). Below the pericarp, the testa is rich in hydrophobic cutin, whereas the aleurone contains most of the storage proteins in the grain and all together are referred to as bran (rich in dietary fibre). The largest part of the barley grain is represented by the endosperm, with cell walls composed of β-glucan and arabinoxylan (70-20%), vice-versa respective aleurone layer (26-67%) (Duffus and Cochrane, 1993). Overall, the endosperm stores energy in the form of starch, whereas the embryo stores lipids and proteins to initiate the plant germination and growth. Barley is primarily used for malt and further for beer production, giving beer its colour and malty sweet flavour and most important, is a source of fermentable starch for yeast to ferment to alcohol.

1.2.2 Malting and brewhouse operations

The brewing process is divided into eight distinct stages: malting, milling, mashing, brewing, cooling, fermentation, rack, and finishing (Briggs et al., 1981). The first step in the malting stage is steeping where the grains are mixed with cold water until they reach a moisture content of 42 to 48%. During this step, the grains will begin to germinate after approximately 24-hours, and the germination will be allowed to continue for a period of over 5 days. After this period, the germination process is stopped by kiln-drying (up to 80°C) or roasting (110-

250°C) in a forced flow of dry air, thus obtaining barley malt (Figure 1-2). Depending on the cooking temperatures, the lightly-roasted malt will produce a pale beer, whereas deeply roasted malts will produce dark or black beer (232°C Guinness malt) (Guinness, 2018). The overall process described above is called malting of barley. The most important physiological changes of malting, which also determine the quality of malt, include fast and uniform germination, synthesis of hydrolytic enzymes in the scutellum and aleurone layer to degrade the endosperm cell walls thus releasing the starch granules from the matrix and making it more accessible to saccharolytic enzymes expressed in the barley during germination (Group, 1999).

The main brewhouse operations consist of mashing, filtration and wort boiling. The malt is milled and mixed with water in a mash tun, at specific ratios and with increasing temperature to promote the activity of malt-derived enzymes. The mashing duration is around two hours and after this period the soluble substances (wort) and the residual solid (Brewer's spent grain) are separated by filtration (draining). The mashing process is usually performed at pH 5.5, with gradual temperature increase (37 - 78°C) to favour the enzyme activity, ultimately degrading the cell walls, proteins and starch.

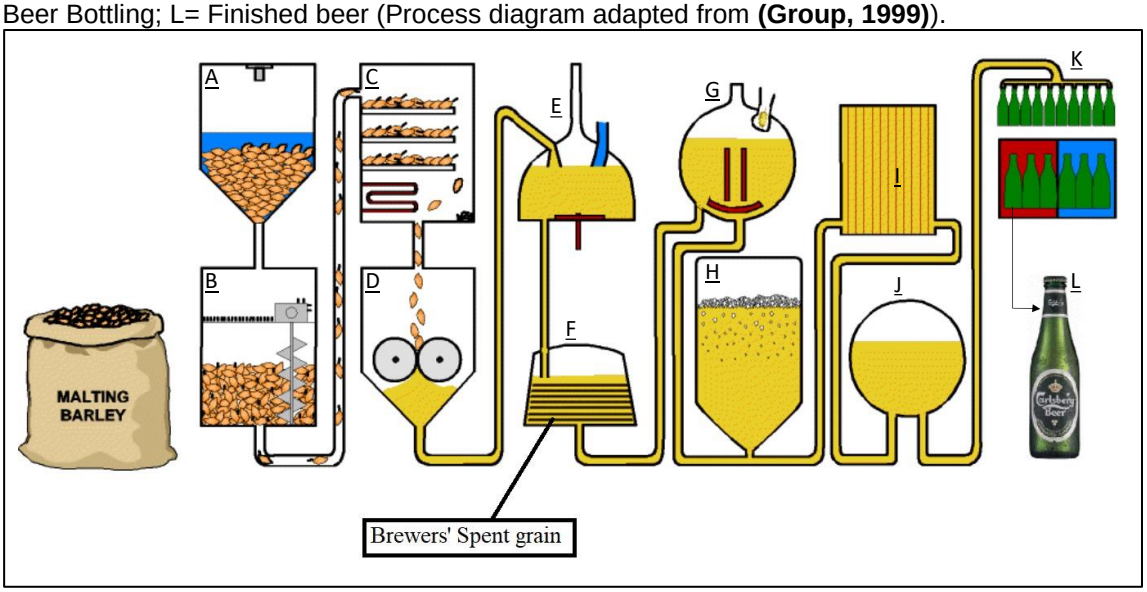
Figure 1-2 Malt Barley Spectrum of Colours after drying and roasting (from top left to bottom right: light (50°C) to caramel (148°C) to dark roast (232°C), to obtain gold, copper and black colour beer (Routson 2012).

The cell walls are first broken down by (1-3,1-4)-ß-glucanase and xylanase, proteins are degraded into peptides and amino acids by endo-peptidases and carboxypeptidases, and lastly starch is broken down to maltose, maltotriose and dextrins by amylases, dextrinases and α-glucosidases. After filtration, the wort is mixed with hops and boiled for around one hour. At this step, the bitterness and the aromatic components from hops are transferred to wort, from which the beer gets its bitter taste, flavour and foam stability. Following the brewhouse operations, fermentation, the mixture is then cooled and filtered, and the hopped wort is fermented by yeast to obtain the final product, beer (Mussatto, 2009, Group, 1999). The overall brewing process is illustrated in Figure 1-3.

In addition to normal industrial materials like packaging, breweries create a wide variety of brewing-specific waste materials. During the brewing process, several by-products are generated from the raw materials (barley), such as brewer's spent grain (BSG) from barley following wort separation, spent hops from hops following wort boiling and surplus yeast from yeast following

fermentation. Thomas et al., 2006 showed that per 100L of beer produced, the following amount of waste is generated: water 300 – 1 000L, 14 kg dry weight of BSG, 0.16 kg dry weight of spent hops, 0.35 kg dry weight trub, and 3 kg dry weight of yeast (Thomas and Rahman, 2006). For every 5L of beer manufactured, approximately 1kg of BSG is produced (Mussatto, 2006b). Considering this ratio and the production of beer in Ireland of 7.1 million and UK of 32.2 million hectolitres for the year 2020, an estimate of 142 kilotons of BSG was generated in Ireland and 644 kilotons in UK, respectively (Europe, 2020). As it can be observed, BSG is the main low-value solid by-product from beer production (after mashing and lautering process), representing 85% of total by-products generated and 31% of the original barley weight (Gupta et al., 2010).

Figure 1-3 Production of beer A= Steeping (barley, water); B= Germination (barley); C= drying and kilning (malt); D= Milling (malt); E= Mashing (malt, water); F= Filtration (wort, Brewer's spent grain); G= Wort boiling (wort, hops); H= Fermentation (wort, yeasts); I, J= Maturation (beer); K= Beer Bottling; L= Finished beer (Process diagram adapted from **(Group, 1999)**).

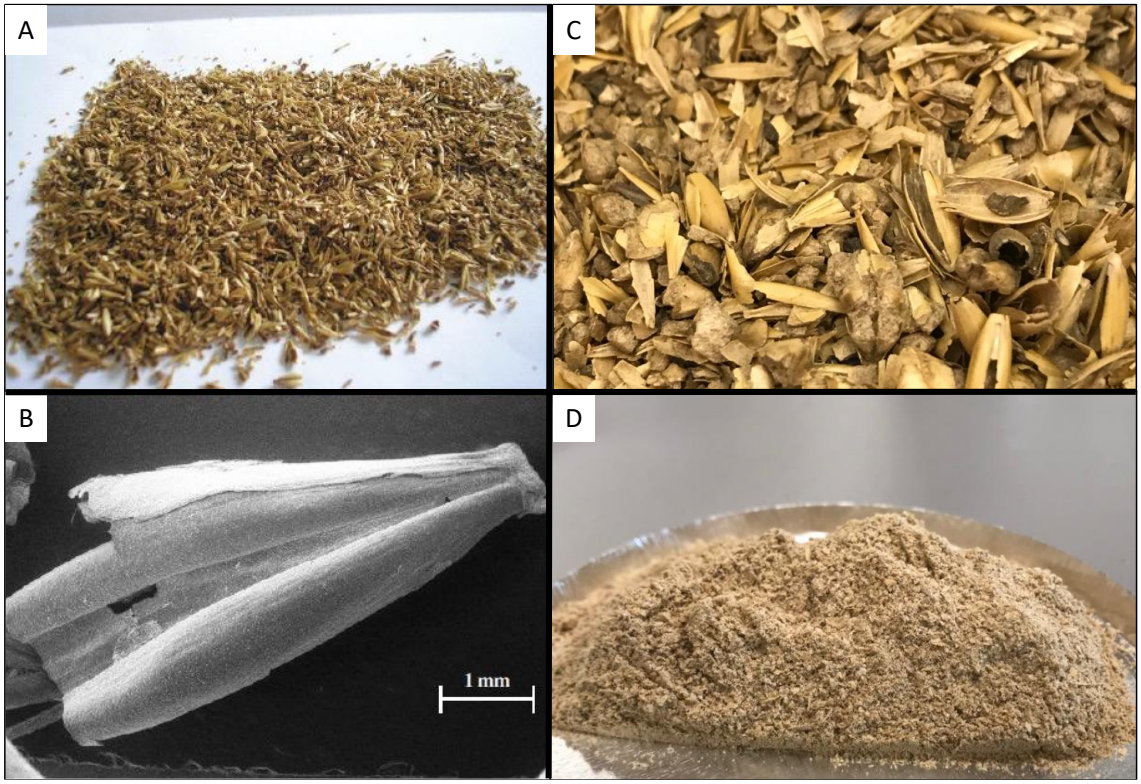


1.2.3 Brewer's spent grain:

Figure 1-4 shows the appearance of BSG recovered from the brewing process, including a microscopic image of BSG particles and the final milled version used for the experiential approach. BSG is mainly used as cattle feed or disposed of to landfills. Unfortunately, due to high moisture content (75-80%) and some remaining fermentable sugars, BSG is difficult to preserve under environmental conditions (perishable around 7 days after production at +4 °C, and 2 days at room temperature) (Robertson J.A., 2010). Due to this reason and the low economic value of BSG, most of the brewing companies consider it as

waste rather than a by-product (El-Shafey et al., 2004). Nevertheless, BSG is rich in dietary fibre, bioactive proteins, phenolic acids and lignin, thus BSG might be a potential source of food grade material with high value applications if proper processing methods are developed (McCarthy, 2013, Niemi, 2016, da Rosa Almeida et al., 2017). Extraction of these high-value bioactive components may add value to the BSG, by using them as functional ingredients in nutrition and pharmacology (Mussatto, 2006b, Socaci et al., 2017).

Figure 1-4 BSG sample dried (A, C), milled (D), and micrograph of BSG particle by scanning electron microscopy (B) (Mussatto 2006).



1.2.4 Chemical Composition of BSG

According to Mussatto *et al.* 2006, the chemical composition of BSG varies due to barley variety, harvesting time, brewing process (malting, mashing) and the quality and type of adjuncts used. Almost half of the BSG dry mass comprises of carbohydrates, followed by a variation in protein (10-27%) and lignin (11-28%) content. Lipids are present in a lower content, varying from 4-10% and the phenolic compounds between 0.2 to 2% (Bartolomé et al., 2002, Santos et al., 2003). The high variation in BSG characteristics may be due to factors such as genetic diversity in crops, brewery production specificity, treatment, and pretreatment after beer production. In addition to being used in the manufacture

of breakfast cereals, bread and other baked goods, it can also be used for culturing microbes, preparing compost, and producing biogas. As BSG is made from raw materials approved for human consumption, it may be an important ingredient for developing new foods thus increasing its nutritional value. It has been demonstrated that the inclusion of BSG in bakery products (bread, extrudate, and snacks) not only improves digestibility, but it may also present health promoting effects, such as reducing the risk of cardiovascular diseases, constipation, colon cancer, obesity, and diabetes (Yitayew et al., 2022).

The approximate chemical composition of BSG is presented in Table 1-1, together with barley and barley pale malt.

Table 1-1 Chemical composition of BSG

Reference	Cellulose	Hemicellulose	Lignin	Proteins	Ash	Extractives
Barley grain *Robert, 1988*	1.4-5	4.4-7.8^{AX} 3.6-6$^{\beta G}$	2-2.9	8-11	3.1	51-71^{S} 2-3^{L}
Barley pale malt *Cook, 2013*	5	6-8^{AX} 6.9 $^{\beta G}$		8-11	3	58-60^{S} 2-3^{L}
Barley spent grain						
Mussatto et al. 2006	16.8	28.4^{AX}	27.8	15.3	4.6	5.8
Kanauchi et al. 2001	25.4	21.8^{AX}	11.9	24.0	2.4	nr
Carvalheiro et al. 2004	21.9	29.6^{AX}	21.7	24.6	1.2	10.6^{L}
Silva et al. 2004	25.3	41.9^{AX}	16.9	nr	4.6	9.5
Forsell et al. 2011	14.4	23.9^{AX}	18.9	26.9	3.4	6.8^{L}
Faulds et al. 2008	19.4	26.5^{AX}	20.1	17.6	nr	5.2^{L}
Robertson et al. 2010	14-24	22-29^{AX}	13-17	10-24	nr	0.7-0.9^{PA}
Xiros et al. 2008	12	40^{AX}	11.5	14.2	3.3	2.0^{PA}

Extractives= a fraction consisting of either of waxes, lipids^{L}, gums, starches^{S}, resin, tannins, essential oils, phenolic acids^{PA}, other cytoplasmatic constituents; nr=not reported; β-Glucan$^{\beta G}$; AX – arabinoxylan;

1.2.4.1 Carbohydrates

BSG is mainly composed of barley grain husk, thus most of the carbohydrates present originate from in the cell wall such as cellulose and hemicellulose (almost 50%) as illustrated in Figure 1-5. Cellulose is a polysaccharide consisting of β (1-4)-linked D-glucose units, which are linearly arranged and held together by hydrogen bonds, whereas hemicellulose is mostly arabinoxylan, a branched polymer consisting of β (1-4)-linked xylose residues with single units of arabinose as side chains (Mussatto, 2006a). The hydrogen

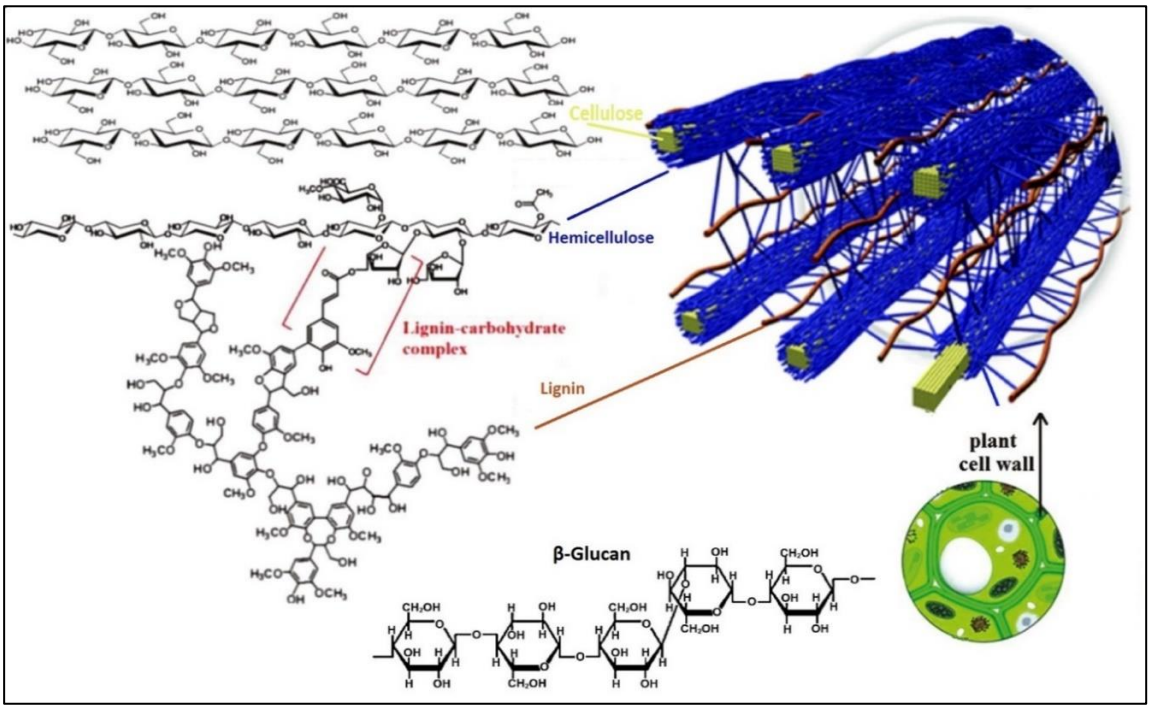
bonds in cellulose make this polysaccharide insoluble in water and resistant to depolymerisation, whilst arabinoxylan is less rigid and susceptible to enzymatic action. In aleuronic tissue, the arabinose side chains can be substituted with ferulic or *p*-coumaric acid via ester-linkage, which cross-link arabinoxylan chains together, strengthening the cell wall (Ishii, 1997, Bunzel et al., 2001). A similar substitution can happen in pericarp and husk, where ferulic acid can cross-link arabinoxylan to lignin (Bunzel et al., 2004).

Starch is the most abundant polysaccharide in barley grain and may reach up to 70% of the dry weight (Zhu, 2017). During mashing, almost all the starch is solubilised into wort and around 2-13% remains in BSG, along with residual quantities of mixed linked β-glucan (0.5 to 1.1%) (Robertson J.A., 2010).

1.2.4.2 Lignin

Lignin is the second most abundant constituent of the plant cell wall, and together with cellulose and hemicellulose form a framework that strengthens the plant cell wall. It is a polyphenolic macromolecule that has an important role in protection of the plant against microorganisms and due to its hydrophobicity prevents the absorption of water by the polysaccharides, thus improving the water transportation in the vascular tissues (Figure 1-5) (Sarkanen and Ludwig, 1971, Volynets et al., 2017). Lignin is present in the outer layers of the barley grain, husk and pericarp, representing about 12-28% of BSG (Table 1-1). Lignin is formed from three phenolic units, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol linked together in a branched network. The lignin units are derived from ferulic and sinapic acids, via the phenylpropanoid pathway (Aura et al., 2013). Beside the two phenolic acids, other phenolic compounds were identified in BSG, such as ferulic, *p*-coumaric, syringic, vanilic, *p*-hydroxybenzoic acid (Mussatto et al., 2007a).

Figure 1-5 Cellulose, hemicellulose and lignin in lignocellulosic biomasses, with related chemical structure. Adapted from Volynets et al., (2017).



1.2.4.3 Proteins

The protein content in barley grain varies between 8-11%, whereas in BSG the proportion is increased and varies between 15-27% (Table 1-1). This is due the fact that during the malting (barley germination step) and mashing process (extensive solubilisation of the endosperm in barley malt), only a quarter of the total proteins are degraded to amino acids and polypeptides by endogenous peptidases (Jones and Budde, 2005), the rest of insoluble proteins remaining in BSG, reaching around 27%. The most abundant type of proteins in BSG are hordeins and glutelins. Hordeins are storage proteins, whereas glutelins are structural proteins. Other types of proteins are albumins and globulins, representing only 10% of the total BSG proteins (Celus et al., 2006). Among the amino acids, glutamic acid/glutamine and proline are the most abundant, representing 30% of the total amino acids in BSG. This is because hordeins consist mainly of these two amino acids (Treimo et al., 2008). Other amino acids have been reported in BSG, like leucine, valine, alanine, serine, glycine, and aspartic acid (Mussatto, 2009).

1.2.4.4 Lipids

The main physiological role of lipids in the grain is to provide energy and nutrients to the seedling during germination, hence their location in endosperm and embryo. The lipid content of whole barley grain is less than 5%, with linoleic acid being the main fatty acid (55%), followed by palmitic (22%) and oleic acids (13%) (Kaukovirta-Norja et al., 1993). The mashing process of malt increases the lipid content in BSG due to solubilisation of other compounds (such as polar lipids), varying between 5-11%. (Table 1-1) Triglycerides (69%) are the main lipid class present in barley and malt, but during the mashing process they are partly de-esterified by lipase releasing free fatty acids (Kaukovirta-Norja et al., 1993). Lipids in wort are important during fermentation due to their beneficial effect on yeast metabolism, as well as negative effect in beer, affecting shelf life, taste and foam stability, therefore the brewer's try and retain lipids in the BSG (Bravi et al., 2014).

1.2.5 Bioactive components from BSG

The main constituents in BSG of importance, due to their potential health benefits, are the dietary fibre (e.g. arabinoxylan, lignin, β-glucan), peptides and phenolic compounds (e.g. hydroxycinnamic acids).

Arabinoxylan (AX) is the predominant component of hemicellulose, which can account up to 42% of BSG (see Table 1-1). In contrast, the content in wheat and barley is between 4-10% and 4-8%, respectively (Steiner et al., 2015b). Solubility is of paramount importance in promoting the health effects of AX. Water-extractable AX can act as prebiotic in the large intestine due to the fermentation capability of the colonic microflora, especially bifidobacteria and lactobacilli. Bifidobacteria, during fermentation of AX, produce short chain fatty acids. The short chain fatty acids have been associated with protection against pathogenic bacteria, induce immune responses, reduce cholesterol synthesis, stimulate colonic blood flow, enhance muscular contractions and may reduce the development of colon cancer (Grootaert et al., 2009). Besides, during breakdown of AX, xylooligosaccharides are released, which are known to have prebiotic activity (Wang et al., 2010). Unfortunately, only in the large intestine that certain microbial species possess specific enzymes, like α-L-arabinofuranosidase,

necessary for AX breakdown (Grootaert et al., 2009). The size of xylooligosaccharides varies depending on the degree of polymerisation. It has been shown that selective bacterial species prefer low molecular weight fractions of AX (66 kDa), shown by increased short chain fatty acids production, especially butyrate($C_4H_8O_2$) (Hughes et al., 2007). Propionate ($C_3H_5O_2$) has also been detected with a significant increase of bifidobacterial population (Lynch et al., 2016). In an *in-vitro* colonic model, xylooligosaccharides fermentation did not have a significant effect on the microbial community composition; instead, it shifted the bacterial fermentation site towards the distal colon. This could be of great importance as colon cancer usually occurs towards the distal parts of the large intestine, and by extending the sugar fermentation towards these parts would increase the production of short chain fatty acids, that would reduce the risk of developing colon cancer (Grootaert et al., 2009). In another study, it has been shown that ingestion of AX may help control post-prandial glycaemic responses (Lu et al., 2000).

Lignin is the second major component in BSG accounting up to 28% of BSG. Since 2008, European Commission has considered lignin as dietary fibre, but only when it remains intact with other original plant polysaccharides (CotE, 2008). Lignin was considered a robust polymer that gut microbiota could not break down, but Niemi et al., 2013 demonstrated that lignin rich fractions prolonged the survival of bifidobacteria against glucose as substrate. This was confirmed by the presence of lignin-derived phenolic metabolites during fermentation suggesting the partial breakdown of lignin (Aura et al., 2013). Prolonged formation and increase / protection of the gut microbiota may help in release and the formation of new metabolites that may originate from the degradation of lignin or from phenolic precursors, such as ferulic acid. These newly formed metabolites may present several health-promoting properties (Niemi et al., 2013).

β-glucan constitutes ~1% (w/w) in BSG and has been strongly associated with reducing the risk of coronary heart diseases (Steiner et al., 2015b). The mechanism proposed is based on its soluble nature and the ability to form a gel-like network, and thereby increasing gastro-intestinal viscosity (Truswell, 2002). The viscous β-glucan polymer has a cholesterol lowering effect by reducing the

reabsorption of bile acids and promoting the synthesis of bile acids from cholesterol (Steiner et al., 2015b). In fact, EFSA has approved the health-claims of β-glucans from oats and/or barley in reducing post-prandial blood glucose responses when present in ≥ 5.2% of total carbohydrates in the food (EFSA Panel on Nutrition et al., 2021)

In general, **dietary fibre** is also considered to have a role in modulation of the immune system. It has been proven that increased intake of dietary fibres decreases the level of pro-inflammatory effectors (pro-inflammatory IL-6 and TNF-α cytokines, C-reactive protein), thus minimizing the risk of colorectal cancer (Ma et al., 2008). Also, the M-cells in the Peyer's patches can uptake and transport dietary fibre to the immune cells and increase the production of pro- or anti-inflammatory cytokines (Volman et al., 2008).

Besides dietary fibre, **proteins** are also of interest, predominantly as ingredients in form of protein hydrolysates or concentrate, with bio- or techno-functional applications (e.g. solubility, emulsification, elasticity, foaming, immunomodulatory effects, and antimicrobial activity)(Martin, 2013). Dairy products have been the main source of bioactive peptides so far, but owing to their high carbon footprint, attention is now turned to plant-based proteins (Lynch et al., 2016, Samtiya et al., 2021, Pradhan et al., 2022). Bioactive peptides isolated from cereals, like barley, wheat soy, rapeseed, presented *in-vitro* antioxidant and antihypertensive effects (Balgir, 2016). BSG protein rich isolates (containing approx. 50% protein dry weight) and associated hydrolysates showed slight antioxidant activity, and demonstrated the selective inhibition on the generation of the pro-inflammatory cytokine INF-γ, *in-vitro* (McCarthy, 2013). Connolly *et al.*2014 showed the potential of protein hydrolysates from BSG to exert functionality for regulation of diabetes and hypertension, by increasing α-glucosidase, dipeptidyl peptidase IV and angiotensin converting enzyme (ACE) inhibition (Connolly et al., 2014). With the help of *in-silico* protein sequencing methods, these were identified as parts of the storage proteins from oats and barley that show various biological activities such as ACE-I inhibitors, prolyl endopeptidase (PEP) inhibitors, renin inhibitors (Cavazos and Mejia, 2013).

The disequilibrium between prooxidants and antioxidants in the body (oxidative stress) is thought to be the main cause in development of several

degenerative diseases (Shahidi and Chandrasekara, 2010, Mussatto et al., 2008). The generation of reactive oxygen species, such as free radicals and peroxides, damage most of the cell components, including proteins, lipids, lipoproteins and DNA (Valko et al., 2007). To guard against this oxidative stress, the antioxidant defences present within the cell as well as those accessible extracellularly must be effective. Unfortunately, the excessive creation of free radicals or an improper intake of nutrients, may overrun the antioxidant defence systems leading to a disruption in the redox balance. On this aspect, several studies have shown that good nutrition, a diet rich in fruits and vegetables, is the most important environmental component, and a possible strategy in managing these chronic ailments. Antioxidants that include vitamins, α-tocopherol, and phenolic compounds, and are found in high amounts in fruits and vegetables, seem to be responsible for the protective effects and minimizing the oxidative stress (Jin and Russell, 2010, Balasundram et al., 2006, Ikram et al., 2020). Therefore, BSG's phenolic acids, including other phenolic compounds, may help to reduce the damage caused by the reactive oxygen species, either directly or via boosting the endogenous antioxidant defence systems (Shahidi and Chandrasekara, 2010).

BSG has been considered a potential adjunct for human food products due to its composition and nutritional values. The incorporation of BSG in food products, has shown to increase the main content of the compounds with the role as functional and bioactive ingredients. As such, the baking and extrusion processes have utilized BSG's dietary fibre-rich and protein-rich flours (Ainsworth et al., 2007); incorporation of approximately 10% in the production of classic bread doubled the content of crude fibre (D'Appolonia and Prentice, 1977), whereas in cookies, the addition of 15% BSG resulted in an increase of 27% of protein content, and three times the total dietary content, and seven times when BSG incorporation was increased to 25% (Öztürk et al., 2002); a 30% incorporation of BSG in extruded snacks resulted in a significant increase in nutritional and physical values, but as well in dietary fibre and protein (Ainsworth et al., 2007). Even thought, the main concern remains and is the acceptance of the final products for human consumption due appearance, texture and flavour, as some studies showed that the addition of a maximum of 10% BSG in crispy slices was accepted by consumers (Ktenioudaki et al., 2013).

1.2.5.1 Polyphenols from BSG and their health benefits

Polyphenols are naturally occurring compounds widely accepted as benefiting health due to their antioxidant properties. They are secondary metabolites in plants derived from phenylalanine and tyrosine (to a lesser extent) and produced via the shikimic acid pathway, that are typically engaged in plant response to environmental stress conditions, with an important role in defence against parasites, pathogens and predators, and some also contribute as attractants (colour) to pollinators and UV-protection (Mazid et al., 2011, Shahidi and Naczk, 2003). Chemically, phenolic compounds are a group of small molecules that have an aromatic ring that contains one (phenol) or multiple hydroxyl (polyphenol) moieties, including their functional derivatives (Shahidi and Naczk, 2003). More than 8000 phenolic structures have been identified in plants, ranging from simple molecules, like phenolic acids, anthocyanins, to highly polymerized substances, like tannins, in different quantities (Jin and Russell, 2010). Naturally, polyphenols are commonly found in conjugated form, with one or more sugar residues (i.e. glucose) linked to the hydroxyl group, however direct connections different type of monosaccharides, polysaccharides or oligosaccharides to the aromatic carbon may also occur (Pandey and Rizvi, 2009). Moreover, it is also common for polyphenols to interact with other type of compounds, such as carboxylic and organic acids, amines, lipids but as well forming linkages with other phenols (Kondratyuk and Pezzuto, 2004). They may be classified into at least ten distinct groups based on the number of phenol rings they contain and the structural elements that link these rings together, with flavonoids and phenolic acids being the most prevalent in plants (Kondratyuk and Pezzuto, 2004).

In grain cereals, phenolic acids are the most common sub-classes of polyphenols and can be present in both free soluble form (on the outer layer of the pericarp, conjugated with sugars or organic acids) and bound form ester- or ether-linked to plant cell wall components, such as lignin (Sosulski et al., 1982, Forssell et al., 2008, Mussatto, 2014). Also, phenolic acids, with their carboxyl and hydroxyl groups, may create bridges or cross-links with starch and other polysaccharides through hydrogen bonding, chelation, or covalent bonds (Yu et al., 2001) (Figure 1-6-A). Phenolic acids, which are a subset of polyphenols, are

further divided into two categories according to their hydroxylated derivates, benzoic acid and cinnamic acid derivatives based on C_1–C_6 and C_3–C_6 backbones, namely hydroxybenzoic acids and hydroxycinnamic acids (Tsao, 2010). Hydroxybenzoic acids are derived directly from benzoic acid, and variations in their structure due to hydroxylation and methylation lead to the formation of other acids, such as vanillic acid, protocatechuic acid, syringic acid, and *p*-hydroxybenzoic acid. The most common hydroxycinnamic acids consist of *p*-coumaric, ferulic, caffeic, and sinapic acids, whereas hydroxybenzoic acids consist of hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acids (Figure 1-6-B, C). The highest levels of phenolics are found in the husk, pericarp, testa, and aleurone cells of cereal grains, whereas only trace amounts are found in the starchy endosperm (Yu et al., 2001). There is a vast variation among the reported concentrations of phenolic compounds in BSG, especially for the most abundant, ferulic and *p*-coumaric acid (Table 1-2), whereas sinapic, caffeic and syringic acid were reported at much lower concentrations (0.4 to 42 mg/100g BSG) (Hernanz et al., 2001, McCarthy et al., 2013b). These vast variations in the phenolic content of BSG may be attributed to breweries' malting and mashing processing conditions, variety, location, growth conditions and harvesting period of barley, and lastly the type and quantities of cereal grains, and adjuncts added during the brewing process (Mussatto, 2006b).

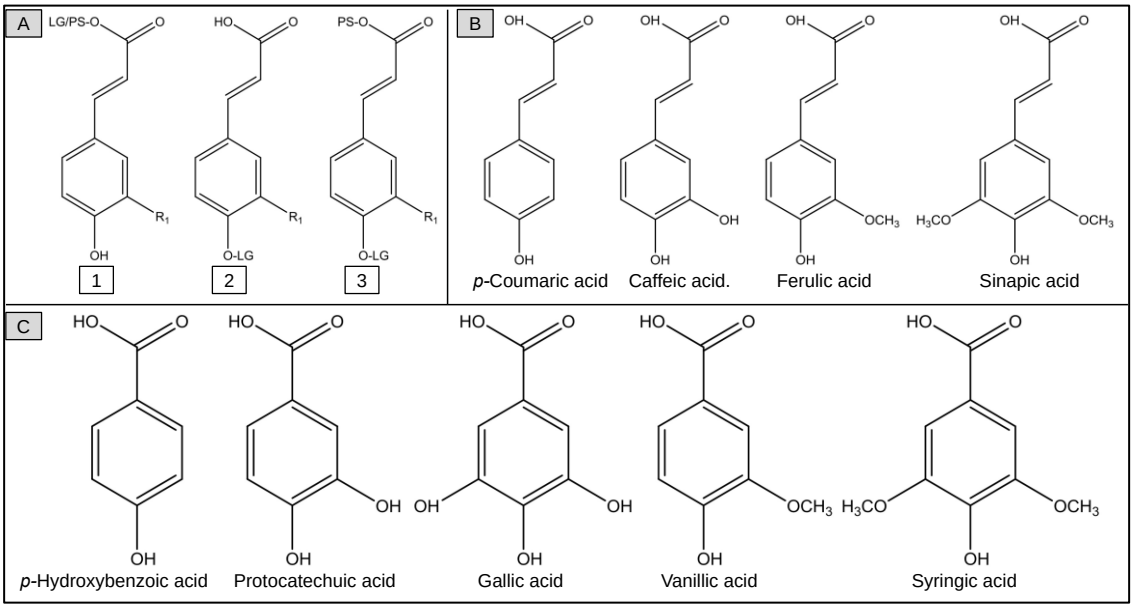
The beer brewing process differs from one brewery to another, and it has been shown that not only the barley variety used in the brewing process showed variation in the phenolic content and antioxidant activity, but as well the malting process significantly changed their content in the final product, being either the waste such as BSG, or beer (Lu et al., 2007). If there is an interest in obtaining a high yield of phenolics from BSG residue, this information is of importance, as a high yield of phenolics in beer would mean a low phenolic content in BSG residue, and vice-versa, as approximately 80% of phenolics in beer come from barley.

Table 1-2 The approximate concentration of ferulic acid and p-coumaric in BSG (mg/g BSG dw) as reported in the literature, modified from McCarthy et al.,2013.

Reference	Ferulic acid	*p*-coumaric acid
Hernanz et al., 2001	1.9	0.7
Bartolome et al., 2002	1.7-2.4	0.7-1.2
Mandalari et al., 2005	6	3
Mussatto et al., 2007	2.9	2.8
Athanasios et al., 2007	0.2-0.3	0.1-0.2
Forssell et al., 2008	0.5	0.5
Szwajgier et al., 2010	3.3	0.1
Moreira et al., 2012	0.8-1.5	0.2-0.5
McCarthy et al., 2013	0.2	0.1
Sibhatu et al.,2021	0.5	nr
Ideia et al., 2020	4.7	nr

nr = not reported

Figure 1-6 (A) Schematic representation of linkages of ferulic acid and p-coumaric acid to lignin (LG) and/or polysaccharides (PS): (1) ester bonds, (2) ether bonds, and (3) ester–ether bridges, R_1 = H for p-CA and R_1 = OCH_3 for FA **(Max et al., 2009)**; (B, C) Chemical structure of phenolic acids: (B) – hydroxycinnamic acids (C) – hydroxybenzoic acids



Phenolic compounds from barley are characterised as *in-vitro* antioxidants exhibiting antioxidant and antiradical characteristics, as well as presenting other biological properties (Gorinstein et al., 2007, Rice-Evans et al., 1997). As aforementioned, BSG is mostly comprised of the husk-pericarp-seed coat and is primarily composed of cell walls. Thus, BSG has the potential to be an important source of phenolic acids because the husk contains the majority of the barley

grain's phenolics, and hydroxycinnamic acids accumulate (esterified) to cell walls (Mussatto, 2006b).

Regarding their biological activity, hydroxycinnamic acids act as antioxidants by scavenging DPPH (radical) in the order of caffeic acid > sinapic acid = ferulic acid > ferulic acid esters > *p*-coumaric acid (Lynch et al., 2016). Interestingly the lesser antioxidant phenolics, i.e. ferulic and coumaric acids, have shown as potential agents in Alzheimer's disease therapy (Szwajgier and Borowiec, 2012). Moreover, ferulic acid has a broad range of biological activities including anti-inflammatory, anti-allergy and hepatoprotective properties. It also has an anti-carcinogenic, anti-thrombotic and antiviral effect, as well as metal chelation and enzyme regulation (Kumar and Pruthi, 2014). The antioxidant effect of ferulic acid is complex, including both the suppression of reactive oxygen species (ROS) and free radical production. This acid also chelates protonated metal ions like Cu (II) and Fe (II). Besides being a free radical scavenger, ferulic acid is an inhibitor of free radical-producing enzymes and an amplifier of scavenging enzyme activity (Zduńska et al., 2018).

Furthermore, fruits and vegetables, which are high in caffeic acid and ferulic acid, may aid the body's defence against carcinogenesis by reducing the creation of N-nitroso compounds (Kuenzig et al., 1984). Aside from its antioxidant properties, caffeic acid has several additional pharmacological effects ranging from anti-inflammatory to anticancer properties, which is due to its diorthohydroxyl aromatic (catecholic) moiety. Moreover, there have been several recent studies demonstrating the protective effects of caffeic acid in animal models of Alzheimer's disease as well as other forms of neurotoxin exposure (Habtemariam, 2017). Sinapic acid is another hydroxycinnamic acid present in a variety of food plants, including spices, berry fruits, vegetables, cereals, and oilseed crops. Infections, oxidative stress, inflammation, cancer, diabetes, neurodegeneration, are among some of the pathological disorders for which sinapic acid has been tested and reported to have beneficial role (Chen, 2016). Syringic acid is another naturally occurring phenolic acid (hydroxybenzoic acid in fruits and vegetables, and has antioxidant, antibacterial, anti-inflammatory, and antiendotoxic properties, making it a good therapeutic agent for a variety of disorders (diabetes, cardiovascular diseases, cancer, cerebral ischemia, neuro-

and liver damage). Moreover, it can influence the dynamics of various biological targets implicated in disease development, including proteins, transcriptional factors, growth factors, and signalling molecules, and this therapeutic effect may be due to the presence of methoxy groups at positions 3 and 5 on the aromatic ring (Srinivasulu et al., 2018). Even though catechin has been reported in many dietary products and fruits, with high concentrations in green tea leaves, apples, cacao, cherries etc, it has been found also in cereal grains, but at lower concentrations (Jadeja and Devkar, 2014, Quinde-Axtell and Baik, 2006b). Catechin's antioxidant activity has been thoroughly proven via a variety of *in vitro*, *in vivo,* and physical approaches. Catechin alters the molecular pathways behind angiogenesis, extracellular matrix breakdown, cell lysis control, and multidrug resistance in malignancies and associated illnesses. Based on epidemiological and experimental investigations, a favourable link between green tea intake and cardiovascular health has been shown owing to many attributes such as antioxidative, anti-hypertensive, anti-inflammatory, anti-proliferative, anti-thrombogenic, and anti-hyperlipidemic properties (Zanwar et al., 2014). The number and position of the hydroxyl (-OH) groups linked to the aromatic ring are one of the various factors that affect the antioxidant and antiradical efficacy of these phenolic compounds. The health benefits exerted by these phenolic compounds are related to their chemical structure and the number of -OH groups on the phenolic ring, connection that has been shown to correlate positively with their antioxidant capabilities (Kalinowska et al., 2021, Zanwar et al., 2014).

Phenolic compounds have the potential to interact with a wide range of food matrix constituents, macronutrients such as carbohydrates, lipids, proteins, or micronutrients such as vitamins, minerals, and even similar phenolic compounds. Due to these interactions, the bioactivity of specific phenolic compounds may be altered resulting in synergistic, additive, or antagonistic effects among these compounds, depending on various circumstances (i.e., roasting process). For example, the interaction between *p*-coumaric and ferulic acid in respect to antioxidant capacity is additive, but when caffeic acid is present, the type of interaction changes to antagonistic (Salazar-López et al., 2017). These interactions have been demonstrated in several food matrixes, such as fruits (oranges, apple), vegetables (broccoli, tomato) and legumes (black beans, soybeans) (Wang et al., 2011). On the other hand, the positive health-effects of

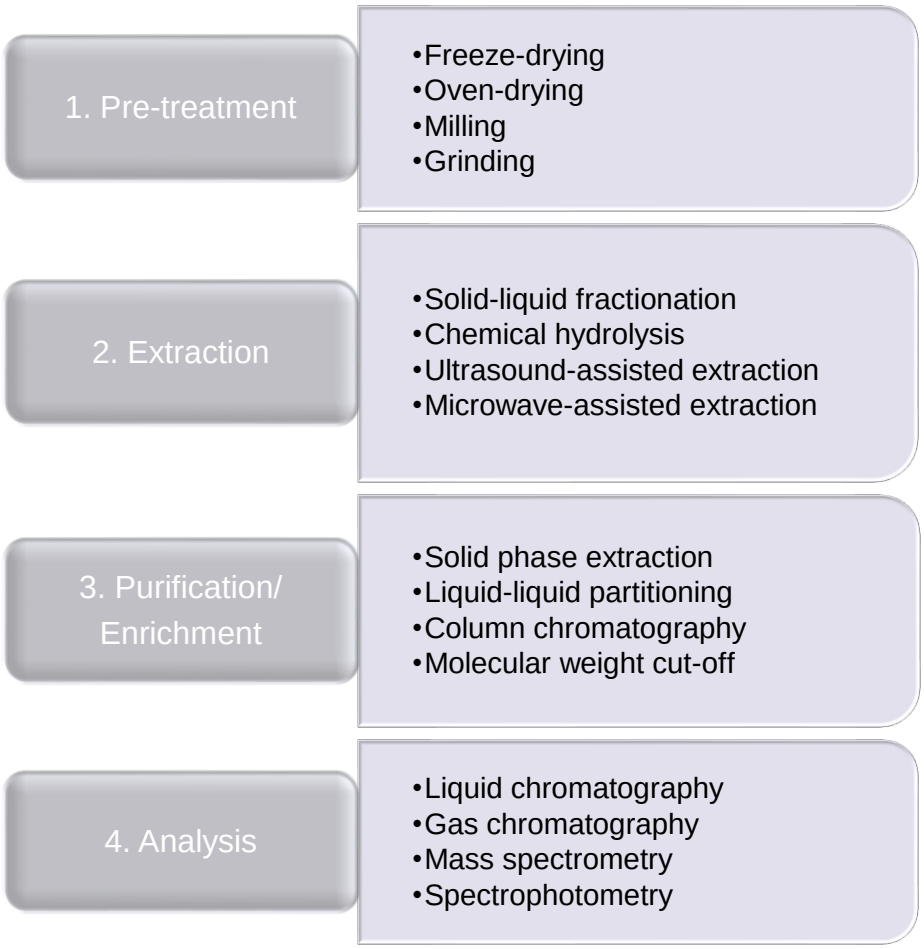
coffee phenolics have been extremely variable and depended on a variety of factors, including the roasting process parameters and the presence of various phenolic compounds (Moreira et al., 2017).

Thus, it is important when designing novel physiologically active substances, such as functional food additives, nutritional supplements, food antioxidants, preservatives, or medications, to have an understanding about the relationship between molecular structure and biological activity. Physicochemical characteristics of polyphenols vary widely, even though they all have a common phenolic feature. Extracting polyphenols remains a difficult task despite recent advancements in modern technology because of their chemical complexity and the frequency with which they appear in plants. Glycosylation and polymerization patterns, as well as varied dietary matrices, all add to the complexity. BSG is a lignocellulosic material composed of several polysaccharides, which can be degraded into their corresponding components and used as nutrients in food or pharmaceutical sectors. Bioactives like dietary fibre, proteins and phenolic compounds would increase the value of BSG as a by-product if proper extraction and processing technologies were to be used. Due to the diversity of target compounds, their location and interaction within the plant matrix, it is important to develop a suitable extraction technique. Due to the type and variation of the compounds, extraction and separation are not always easy and straightforward.

1.3 Extraction and separation of polyphenols

The initial stage in the study of medicinal plants is extraction, as the preparation of plant extracts for experimental purposes is the first and most important stage in generating a high-quality study result. An overall description process from pre-treatment to analysis is shown in Figure 1-7.

Figure 1-7 Strategies for preparation and characterization of extracted bioactives from plant materials. Adapted from (Jin and Russell 2010)



The procedures involved in analysing phytochemicals in plant materials include several steps, sample pretreatment, extraction, separation, and purification (Stalikas, 2007, Routray and Orsat, 2012). Depending on the type of plant material (water content), the initial steps may be maceration, milling, crushing, or homogenization prior to air– or freeze-drying, or vice-versa. Drying presents several advantages, such as reducing the amount of space required for storage, increasing shelf-life and the yield per mass of the raw material (results expressed in general per dry weight basis) (Routray and Orsat, 2012). However, some drying procedures, such as freeze-drying, might have an adverse impact on the sample component profiles; consequently, the desired pretreatment of the samples should be chosen with caution (Dai and Mumper, 2010). Moreover, the maceration, milling, crushing, or homogenization may enhance the interaction surface area between the solvent (extractant) and the solute-containing material. These initial pretreatment procedures cause cellular disruption in the plant

material, thus increasing the extraction yield of desired bioactive compounds. Even so, several studies showed inconsistency among the chosen pretreatments (Mussatto, 2006b, Bartolomé et al., 2002, Tang et al., 2005, Huige, 2006), and some factors, such as, acidity, polarity, chemical structure of the bioactive compounds (arrangement of hydroxyl groups and aromatic rings) and the concentration levels, including the recalcitrance of the plant matrix, impact the efficiency of the pretreatment process (Stalikas, 2007, Routray and Orsat, 2012). The efficiency of the extraction approach is determined not only by the extraction yield, but as well by the minimal impact on the extracted component quality.

With the above considerations in mind, the most crucial stage in the recovery and separation of bioactive compounds from plant sources is clearly selecting the appropriate extraction procedure. Moreover, the extractability is affected by a number of variables, including the plant source and type of compounds to be extracted, the solvent composition, particle size of the raw substrate, solid-to-solvent ratio, extraction time and temperature, among others (Zhang et al., 2018, Chaves et al., 2020). Furthermore, it may be necessary to clean-up (i.e., filtration) the obtained plant extracts as to remove any unwanted compounds, such as sugars, terpenes lipids, resulting in an enriched phenolic extract, which sometimes may also require concentration prior analysis (i.e., vacuum concentration) (Stalikas, 2007, Vichapong et al., 2010).

There are several comparable research publications describing the progress on using various methods for the extraction of bioactive compounds from cereals, by using the classic extraction approach, such as acid-base extractions, but as well using novel extraction technologies such as microwave- and ultrasound-assisted extraction(Camel, 2001, Athanasios et al., 2007b, Moreira et al., 2012b, Wang et al., 2008, Hernanz et al., 2001, Mussatto et al., 2007a). The primary extraction procedures for phenolic compounds from BSG, will be reviewed in this section.

1.3.1 Solid-Liquid extraction

One of the most often utilised procedures to extract phytochemical from plant materials are wet extractions (solid-liquid extraction), like hydrothermal, enzymatic, acid-base procedures or with organic solvents, and this is because of

their simplicity to use, broad applicability, versatility and efficiency to extract phenolic compounds from plant materials (Socaci et al., 2018, McCarthy et al., 2012, Zuorro et al., 2019, Dorta et al., 2012, Meneses, 2013, Gupta et al., 2010). When using solid-liquid extraction (SLE) several conditions needs to be taken in consideration, in terms of temperature, time, pressure, particle size, agitation, solid/solvent mass ratio, solvent concentration etc., that affects the extraction performance including yield and rate of extraction, and the quality of the extracted product (Pronyk and Mazza, 2009, Pereira et al., 2016, Berk, 2018). For example, Mussatto et al., (2007) studied the influence of several extraction conditions, such as different NaOH concentrations (1, 1.5 and 2% w/v), temperatures (80, 100 and 120°C) and reaction time (30, 60, 90 minutes) on the alkaline hydrolysis of BSG to release FA and *p*-CA. The highest phenolic yield was obtained by using the longest extraction period, at higher temperatures and NaOH concentrations (90 minutes, 120°C and 2%NaOH, respectively) (Mussatto et al., 2007a). Elevated temperatures have shown to increase the diffusivity of the solutes, whilst lowering the energy barrier of the extraction process (Chan et al., 2014). For example, it has been shown that the optimal extractions conditions of 200°C and 3.5 minutes are sufficient to obtain the highest amount of FA from wheat bran, as beyond these conditions, the thermal degradation occurs (Pazo-Cepeda et al., 2021).

1.3.1.1 Conventional extraction technologies

It is generally known that phenolic compounds occur in plant cells in both free and bound forms, with the free phenolic compounds being solvent extractable. The bound phenolics, on the other hand, cannot be extracted into aqueous/organic solvent combinations because they are covalently attached to a range of components within the plant matrix (Pérez-Jiménez and Torres, 2011).

1.3.1.1.1 Organic solvents

Traditionally, phytochemicals, such as free phenolic compounds, are in general extracted with organic solvents from plant materials. Thermal assisted extraction is one of the most prevalent methods for this purpose. This approach was used by Meneses et al., 2013 where they evaluated the efficiency of several organic solvents, such as ethanol, methanol, acetone, hexane, ethyl acetate, water and mixtures of this organic solvents with water at specific ratios, under

stirring, to extract polyphenols from BSG. Not only they found that all the obtained extracts exhibited antioxidant activity based on the Folin-Ciocalteu assay, but as well by using organic solvent: water mixtures (60%acetone v/v), they managed to obtain the highest total phenolic content recorded, to our knowledge, of 9.9±0.4 mg of gallic acid equivalent per g BSG dry weight (Meneses, 2013).

The majority of studies on the quantities and compositions of dietary polyphenols focus on extractable polyphenols assessed in aqueous organic extracts, whereas large amounts of bioactive polyphenols remaining in the extraction residues (bound phenolics) are ignored (Arranz et al., 2009).

1.3.1.1.2 Acid and alkali hydrolysis (saponification)

Acidic and alkaline hydrolysis are also used to extract phenolic compounds from plants and processed products of plant origin, and they are essential for the phenolics' stability in the extract (Khoddami et al., 2013). Alkaline hydrolysis is preferred for releasing esterified phenolic acids (linked to cell wall polysaccharides by ester bonds), whereas acid hydrolysis is preferred for releasing glycosylated phenolic acids (related to solubilising sugars by ether bonds) (Khoddami et al., 2013).

In general**, Acid hydrolysis** is used with dilute or concentrated acids, such as sulphuric or hydrochloric acid to breakdown cellulose and hemicellulose in the lignocellulosic biomasses to release sugar molecules. Thus, acidic hydrolysis might be an efficient way for releasing phenolic compounds that have been trapped in the cores of plants and are bonded to the cell wall matrix (Mitchell et al., 2014). The application of acid hydrolysis would enable the identification of greater concentrations of bound phenolic compounds and other types of compounds, such as hydrolysable tannins. Several phenolic compounds, such as ferulic acid, *p*-hydroxybenzoic acid, catechin, have been identified as the primary bound phenolic compounds released by the acidic hydrolysis from various types of fruits and vegetables (Arranz et al., 2009, Su et al., 2014, Sani et al., 2012, Verardo et al., 2011, Yu et al., 2001). Because acidic hydrolysis may degrade hydroxycinnamic and benzoic acids (Krygier et al., 1982), alkali hydrolysis is the most common solid-liquid extraction for ester bond cleavage in plant materials**.**

Alkaline hydrolysis (saponification) is a common solid-liquid extraction method for recovering bound phenolic compounds from BSG residues. Following saponification with 2M NaOH, for 16h at 20°C, Hernanz et al., 2001 managed to recover the most abundant phenolic compounds, FA and *p*-CA, including dehydrodimers of FA, at concentrations raging between 1.86 to 1.95 mg/g BSG dw for FA, and between 0.57 and 0.8 mg/g BSG dw for p-CA, respectively. Using this approach, these levels were at least 5 times higher compered to unprocessed BSG (Hernanz et al., 2001). Bartolome et al., 2002 used a similar extraction and analytical approach as Hernanz et al., 2001, where eight batches of BSG, that were preserved using various procedures (freeze-drying, oven drying and freezing), were tested for the release of FA and *p*-CA by using saponification with 1M NaOH. The obtained results were comparable with those of Hernanz et al., where FA concentration varied between 1.7 to 2.4 mg/g BSG dw, and *p*-CA between 0.7 and 1.2 mg/g BSG dw, respectively (Bartolomé et al., 2002). Using a solid-liquid ratio of 1:20 w/v, Mussatto et al., 2007, studied the saponification of BSG in the presence of varying concentration of NaOH (1.0, 1.5 and 2.0% w/v), and various other conditions, such as temperature (80, 100 and 120°C) and time (30, 60 and 120 min). The highest FA yield of 2.86 mg/g BSG dw was achieved by using saponification with 2% NaOH, at 120°C with an extraction period of 90min. Even though the extracted FA yield was approx. 43% higher compared with the above presented data, a pretreatment with dilute sulphuric acid is necessary, thus making this process more labour-intensive and time consuming (Mussatto et al., 2007a). In a later study by McCarthy et al., 2012, phenolic rich fractions were obtained from BSG pale and dark using saponification with 1M NaOH at room temperature for 16h. These generated fractions presented the highest FA yield of 113 μg/mL and 27 μg/mL, and total phenolic content of 0.64 mg GAE/mL and 0.73 mgGAE/mL, respectively, among the other generated fractions. Moreover, these fractions were examined for their ability to protect against genotoxic effects of antioxidants in human myeloid leukaemia U937 cell lines and showed the greatest protection among other tested fractions (McCarthy et al., 2012). The observed variations in the concentration of FA, *p*-CA and total phenolic content between the applied saponification procedures may be explained not only by the overall conditions applied in the extraction procedures but also the by barley varieties, harvesting period and growing conditions.

1.3.1.2 Novel extraction technologies

Novel extraction technologies have been actively studied in food industry applications as sustainable and less-hazardous techniques of extraction due to increased customer demand for ecologically acceptable alternatives to harmful chemicals (Wen et al., 2020). Several innovative extraction strategies for recovering phenolics from various fruit and vegetables wastes have been developed recently. In terms of efficiency, solvent volumes, extraction time and temperature, most of them are thought to be superior compared to classic extraction methodologies. Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are some of the innovative extraction methods accessible (Moraes et al., 2013, Conidi et al., 2018, Handa et al., 2008, Sadeghi et al., 2017). Ultrasound is commonly employed to enhance traditional solvent extraction, although microwaves are recognised for their capacity to remove components without the need of solvents (J Mason et al., 2011). These technologies may provide an increased extraction efficiency and a high extraction yield of specific compounds for the creation of nutraceuticals or functional food components (Sadeghi et al., 2017). Moreover, uses of carcinogenic chemical solvents are eliminated or drastically reduced, and the extracted compound quality is improved by these new procedures.

1.3.1.2.1 Microwave-assisted extraction

MAE is a unique microwave-based technology for extracting soluble compounds from a variety of plant materials into a fluid (Paré et al., 1994). MAE has gained a lot of attention in recent years because of its capacity to minimise extraction time, cost, and sustainability, as well as the possibility of automation extraction, including on-line quality evaluation by connecting to analytical instruments (Conidi et al., 2018, Ekezie et al., 2017). MAE has several advantages, including boosting extract yield, reducing thermal degradation, and controlled heating of plant material. Because it uses less organic solvent, MAE is classified as a green technology (Zhang et al., 2018). The overall extraction principle is described in Chapter 2, section 2.4.2.1.

MAE is in general used with aqueous solution of certain organic solvent to increase solvent penetration and hence heating efficiency (Chan et al., 2011).

For example, MAE in combination with methanol were used to extract phenolic compounds from grape skins and seeds, citrus mandarin peels, or with ethanol to extract phenolic compounds from peanut skins, or with acetone to extract phenolic compounds from plant roots, etc (Chan et al., 2011). Stefanello et al., 2018, used both maceration and MAE with 50% methanol, 50% acetone, and 0.75% NaOH aqueous solution to extract phenolic compounds from five different type of lignocellulosic materials, corn silage, rice bran, corn bran, wheat bran, and BSG, respectively. Based on the total measurement of polyphenols and flavonoids, the most effective solvent was the aqueous solution of NaOH (0.75% v/v), in combination with maceration for BSG (approx. 17 mg GAE/g sample) and corn silage, and with MAE for corn bran and rice bran, whereas for wheat bran the results were not significantly different. Among the organic solvents used, maceration with 50% acetone was the most efficient solvent in all the substrates compared to MAE (Stefanello et al., 2018b). Even though MAE in combination with aqueous NaOH solution showed a lower phenolic content (17% lower mg GAE/g in BSG) compared to maceration, Moreira et al., 2012 managed to generate a 5-fold increase in FA (approx. 13mg/g BSG dw) compared with the classic solid liquid extraction technique. In their study, the efficiency of using MAE in the extraction of phenolic compounds from BSG was performed in 15 min, at 100°C and 20 mL of 0.75%NaOH extraction solution (Moreira et al., 2012b).

1.3.1.2.2 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is one of the most basic and easy to perform new extraction technologies that uses acoustic cavitation power to create mechanical vibrations to release soluble compounds from a variety of plant materials into the surrounding medium (Rostagno et al., 2003, Gallo et al., 2018, Moraes et al., 2013). When compared to traditional extraction procedures, UAE has grown more attractive owing to various factors such as lower energy consumption, shorter extraction times, less active component destruction, and higher extraction yields. In laboratory studies, UAE has been used to extract a wide range of therapeutic chemicals from plants, including alkaloids, flavonoids, glycosides, phenolic compounds, and polysaccharides (Chemat et al., 2017, Zhang et al., 2018). The first record of UAE usage was in 1956 by Head et al. to isolate alkaloids from *Cinchona succirubra* and has been used since to extract

therapeutic chemicals from a variety of plants (Head Jr et al., 1956, Khadhraoui et al., 2021).

Ultrasound has been investigated for its impact on polyphenol extraction in a number of studies, and has been showed to improve the extraction yield of polyphenols from a diverse range of plant residues (Virot et al., 2010, Vilkhu et al., 2008). Although several aspects on the stability of the bioactive extracts have not been thoroughly addressed, subsequent investigations have showed that UAE results in phenolic compounds which are less degraded than when using other extraction procedures (Pingret et al., 2012). An optimised UAE was used to extracts polyphenols from barley grain, resulting in an enhanced total phenolic yield of approx. 20 mg GAE/g dw. The extraction method was optimised by using response surface methodology, adapting several extraction parameters, such as temperature, ethanol concentration, time, solid:solvent ratio, which showed the best conditions of 50°C, 100% ethanol, 18 min. and 60 mg/L (Wang et al., 2013). Wang et al., 2008 used a similar approach to extract phenolic compounds from wheat bran, and showed the optimum extraction conditions to be 64% ethanol, 60°C, and an extraction time of 25 min., which resulted in a yield of total phenolic content of 3.12 mg GAE/g dw (Wang et al., 2008). The recent work by Alonso-Riao et al., 2020, has shown that extractable compounds from BSG may be extensively valorised utilising UAE. This study showed that 30 min. of treatment using water as the solvent, and 5s of 20 kHz waves at 47°C, the overall polyphenol extraction yield was improved, resulting in a total phenolic content of 3.3 mg GAE/g BSG dw and FA yield of 10.7 µg/g BGS dw, respectively (Alonso-Riaño et al., 2020).

Extraction methods and solvents must be chosen carefully to obtain the most effective and potent extract possible, and it is not always that a high yield of extract does not always ensure a high yield of bioactive components. Consequently, extracts contain these compounds should be treated with caution to avoid oxidation and thermal degradation. As a result, when choosing an extraction process, considerations on the quantity and quality of bioactive components should be made.

A summary of the most common extraction techniques for plant material, with associated properties are summarized in Table 1-3.

Table 1-3 Various extraction techniques with their main characteristics, advantages and disadvantages, mechanism of work

Characteristic	Modern Extraction Technology				Conventional Methods		
	Ultrasound Assisted	**Microwave Assisted**	**Supercritical Fluids**	**Accelerated by Solvents**	**Mechanical Agitation**	**Soxhlet**	**Chemical hydrolysis**
Principle/ mechanism	Acoustic cavitation /cavitational dislodgement, microjetting and microstreaming effects, disintegration of solid materials and disruption of cell walls	Conversion of electromagnetic waves into thermal energy, microwave heating without thermal gradient, evaporation of moisture creating high pressure on the cell wall, cell wall and organelles disruption	Increased density and reduced viscosity of extraction fluid at temperature and pressure above critical points, altered diffusivity, surface tension, heat capacity and thermal conductivity, increased penetration and mass transfer	Increased solubility and diffusion rate at elevated temperature (above boiling point) under pressurised condition, reduction in viscosity and surface tension of solvents, increased mass transfer	An impeller rotates in a tank to give enhanced rates of mixing and mass transfer (3)	The analyte is concentrated from the matrix as a whole or separated from particular interfering substances (1)	Alkali solution break ester bonds compounds from cell wall; Acid-disrupting biomass network of intra- and interchain hydrogen bonds, decrystallize cellulose (2)
Control parameters	Frequency, amplitude, power, pressure, temperature, and viscosity of media	Magnetic field strength of magnetron, type of microwave device, microwave power, frequency and time, dielectric properties of sample and solvent, number of extraction cycles	Type of supercritical fluid (most commonly CO_2), used modifier (co-solvent), temperature, pressure, fluid flow rate and pressure control	Temperature (in the range of 50-200˚C), Pressure (3.5-20 MPa), type of extraction solvent, temperature, static time, and number of cycles	Speed, time, power, viscosity, and density of the liquid (3)	Pressure, temperature, solvent type, time (1)	Alkali/Acid concentration, time, temperature, combination with other processes or solvents (2)
Advantage	Easy to handle, safe (atmospheric pressure and ambient temperature), moderate use of solvent, reproducible	Fast, easy to handle, moderate use of solvent	Fast, safe, no filtering required, high selectivity	Fast, safe, no filtering required	Not use of sophisticated equipment	Not use of sophisticated equipment	
Disadvantages	Required filtration step, possible degradation of compounds at high frequencies	Risk of explosion (solvent must absorb microwave power), expensive, required filtration step	Many parameters to optimize	Possible degradation of thermo-labile compounds	Risk of spills and exposure to organic vapours, degradation of	Exposure risk to organic vapours, degradation of	Long time, high temperatures, large amount of chemicals,

					thermos-labile compounds, required filtration step	thermos-labile compounds	environmentally unfriendly
Driving force	Acoustic cavitation	Microwave power	Pressure in conjunction with supercritical fluid	Heat in conjunction with the solvent under pressure	Solvent contact	Heat	Chemical contact
Extraction time	10–60 min	3–30 min	10–60 min	10–20 min	Several hours	6–24 h	1-24 h
Sample size	1–30 g	1–10 g	1–5 g	1–30 g	1–30 g	1–30 g	High
Solvent amount	50–200 mL	10–40 mL	30–60 mL	15–60 mL	Large volume	150–500 mL	Ratio 1:10
Power	Moderate	High	Moderate	Moderate	High	High	Low

Note: The overall principle and mode of action is described in Chapter 2. Table adapted from (Medina-Torres et al., 2017) and (Kumari et al., 2018); (1) (de Castro and Ayuso, 2000); (2) (Binder and Raines, 2010); (3) (Scargiali and Brucato, 2007).

1.3.2 Separation and purification of polyphenols

It is necessary to further separate and purify the components from a extract obtained via the preceding procedures since they are complex and comprise of a wide range of natural products that must be separated and purified in order to attain the active fraction or pure bioactive compounds. Following the crude extraction, solid-liquid extraction is often preceded by a filtration or separation phase, such as liquid-liquid extraction (LLE), chromatography or ultrafiltration, for either clean-up of the extracts, or to selectively isolate and purify bioactive compounds, such as polyphenols.

1.3.2.1 Liquid-liquid extraction (LLE)

LLE is most often used to selectively separate and purify bioactive components, such as polyphenols and simple phenolics, from crude liquid extracts from a diverse range of solid plant materials. Ethyl acetate is a widely used organic solvent to recover phenolic compounds and is also recognised as less toxic and of lower risk to human health by US-FDA and EFSA . For instance, ethyl acetate (EtOAc) has been used to extract and separate a diverse range of phenolics, i.e. *p*-coumaric acid, vanillic acid, ferulic acid, syringic acid, catechin, from several aromatic plant extracts following a prior solid-liquid extraction using acidified aqueous MeOH solution. These plant extracts presented a TPC ranging between 2.9 to 28.2 mgGAE/g dry sample, and were found to be more effective on inhibiting the growth of several gram (+) microorganisms (Proestos et al., 2006). Similarly, Qiu et al. 2010 used EtOAc to extract the insoluble phenolic acids following an alkali hydrolysis digestion of a diverse range of rice grains. The obtained extracts presented the highest phenolic content, ranging between 31 to 212 mgGAE/kg of rice dw, and DPPH radical scavenging activities, ranging between 24 to 81 μmol of Trolox equivalents (TE) per 100 g of rice. Moreover, these extracts presented the highest content of hydroxycinnamic and hydroxybenzoic acids, with ferulic acid, sinapic acid, and p-coumaric acid being the most predominant acids (Qiu et al., 2010). Ethyl acetate can also be used in combination with diethyl ether (DE) (1:1 v/v) to fractionate phenolic compounds from aqueous solutions, as DE presented a higher extraction rate for phenolic acids and aldehydes, i.e. 4-hydroxybenzoic acid (4-HBA) aldehyde conjugate, *p*-

CA, whereas EtOAc for acids and aldehydes of low and high molecular mass, such as catechin (dimers, trimers of catechins), hydroxycinnamic esters, thus increasing the extraction yield (de Simón et al., 1990). Other solvents, such as n-butanol and water have been used to extract polar molecules such as phenolic glucosides, peptides, and sugars (Liu et al., 2011). Hexane, on the other hand, is in general used to extract highly nonpolar compounds such as waxes, oils, sterols or for delipidation purposes. Nevertheless, Meneses et al. 2012 showed that hexane fractions still contained low amounts of flavonoids from BSG substrates, and furthermore Socaci et al 2018 showed hexane to be a possible selective solvent for other classes of bioactive compounds such as terpenoids and aroma compounds (Meneses, 2013, Socaci et al., 2018).

1.3.2.2 Chromatography

Beside LLE, chromatographic procedures that are more sophisticated entail the separation of constituents based on their respective affinities for two phases of a solution: a stationary phase and a flowing mobile phase. One of these separation techniques is flash chromatography. For example, the separation of peptides as well as phenolic compounds from natural sources has been accomplished via the use of flash chromatography (Hossain et al., 2014, Lawton et al., 1999, Gangopadhyay et al., 2016). Gangopadhyay et al. 2016 used flash chromatography to chromatographically fractionate the total polyphenol extract from barley grain, further, to select the most antioxidant-active fractions, and finally to determine the identity of the major contributors to the sample's reported antioxidant capacity. By using this approach they managed to identify several flavanols (catechin, procyanidin B, prodelphinidin B, procyanidin C) and a novel substituted flavanol (catechin dihexoside) which showed the highest antioxidant capacity determined by three *in vitro* antioxidant assays, DPPH, FRAP, and ORAC (Gangopadhyay et al., 2016).

Although the procedures may not provide a resolution as high as preparative HPLC and can be time-consuming, it does provide the benefits of cheap cost and large-scale sample separation. Moreover, the limitation of flash chromatography is due to the relatively large particle size of the column material

(approx. 50µm), which makes it difficult to separate chemicals that are extremely nearly eluting to one another.

1.3.2.3 Ultrafiltration - Molecular weight cut-off (UF-MWCO)

Beside the above-mentioned techniques, the recovery, purification, and concentration of targeted compounds, such as phenolic compounds, from plant materials can be performed using ultrafiltration membranes with specific MWCO sizes. Different kinds of macromolecules (such as suspended particles, polysaccharides, proteins, and pectins) may be recovered from a crude plant extract using wide UF membranes with MWCO ranging from 50 to 100 kDa. High molecular weight components (such as tannins, proteins, hydrolysates, some phenolic compounds) can be concentrated using UF membranes ranging from 4 to 30 kDa, while low molecular weight compounds (such as anthocyanins, low molecular weight phenolic compounds and sugars, peptides) can be concentrated using tight UF membranes ranging from 1 to 3 kDa (Cassano et al., 2018). For example, using UF in the separation of several compounds (such as organic acids, sugars, tannins, pectins, phenolic substances) from olive mill wastewater a complete separation of fats from salts, sugars and polyphenols was obtained (Turano et al., 2002). Through an ecologically friendly approach based on water extraction, and membrane separation technology, enriched fractions in phenolic compounds (hydroxycinnamic acids, flavonoids, anthocyanins, carotenoids) were obtained from leaves and pitted olive pulp of *Olea europaea L*. and *Cynara scolymus L*. by-products, raw artichoke extracts, citrus by-products (orange) (Cassano et al., 2014, Conidi et al., 2014, Romani et al., 2017).

It is possible to achieve a high degree of antioxidant activity, for example, from low quantities of the original plant extract by using a purification procedure that excludes fractions with low antioxidant activity. Furthermore, it is essential to get pure extracts to confirm the identification and safety of antioxidant compounds, such as polyphenols, that may be later exploited as health promoting agents. Thus, an efficient fractionation or separation methods are critical to properly screen and analyse the obtained fractions.

1.4 Analysis of bioactive components

Accurate identification of phenolics and their concentration measurements are particularly critical since plant tissues contain a wide variety of primary and secondary metabolites. There are multiple constraints that make the determination of polyphenols difficult, such as the wide varieties of phenolic compounds with a broad range of polarities, stability, low levels of the compounds, and the matrix effect due to interferences by impurities and other chemicals. Developing an efficient approach for extracting multianalytes from a complex matrix is challenging owing to the possible fast interactions of targeted analytes with other matrix elements. The identification of phenolic compounds is further constrained by their structural similarity resulting in identical UV absorption spectra. Several researchers have addressed the issue of developing analytical techniques for these compounds (Hapsari et al., 2021). Thus, analytical methods must be very efficient, selective, and sensitive in order to provide adequate data for the identification of natural compounds such as polyphenols in plants (Ganzera and Sturm, 2018). To achieve this, sample preparation is one of the most important steps prior to analysis, since the analytical technique's sensitivity is reliant not only on the chemical nature of the polyphenols, but also the extraction process, purification procedures, and the concentration of these compounds in the generated plant extracts (Khoddami et al., 2013).

In general, traditional spectrophotometric assays are regularly used for characterisation and quantification of the compounds in plant extracts. However, due to the structural diversity and variation in quantities of the extracted compounds, the spectrophotometric methods' detection and characterisation can be challenging providing little information on the structure and composition of individual components. With the advancing technology, modern high-performance chromatographic separation techniques and hyphenated methods have been developed that enable to identify and quantify the bioactive components (Patel et al., 2010). Over the past 5 decades, liquid chromatography (LC) has become the most popular and reliable technique for analysis the bioactive compound such as polyphenols from different types of plant extracts (López-Fernández et al., 2020).

The separation and purification can be made by using different types of columns (stationary phase) and mobile phases (solvents). Additionally, the separated compounds can be easily identified by LC coupled with different types of detectors (e.g., ultraviolet, refractive index, photo diode array detectors) and quantified against standards or by mass spectrometry (MS). LC coupled with MS offers a unique chance to analyse simultaneously all constituents from an extract together with their derivates, making it one of the most frequently used techniques in analytical chemistry, with a rising application in the qualitative and quantitative analysis of phenolic compounds from different types of plants (Lucci et al., 2017, Jin and Russell, 2010). Organic or inorganic molecules are used to generate gas-phase ions in MS, which are then separated by their mass-to-charge ratio *(m/z)* and used to identify and quantify various components via their corresponding *m/z* and its abundance (Gross, 2006).

In recent years, ultra-performance liquid chromatography (UPLC) has been developed, which use columns with smaller particle sizes (~1.7μm) and can sustain high pressure (10 000psi) thus improving the resolution, speed and efficiency (Churchwell et al., 2005). Furthermore, UPLC provides a cost advantage over traditional LC since it uses around 80% less organic solvent (Lucci et al., 2017). One of the most advanced quantification techniques is liquid chromatography tandem mass spectrometry (LC-MS/MS). This technique employs multiple reaction monitoring (MRM) method, which monitors the process, and detects specific product-to-precursor ions. The advantage of this technique is the selectivity, sensitivity (sensitive down to ppb levels), speed of analysis and accuracy. More details regarding the LC-MS/MS principle and equipment used on the qualitative and quantitative analyses of polyphenols in this research thesis are described in Chapter 2, section 2.5.2. For the volatiles, gas chromatography (GC) techniques have been usually used for their separation and quantification, but due to the derivatization procedures and low volatility of some bioactive compounds (like phenolic acids), LC-based analytical techniques are commonly employed (Siddiqui et al., 2017). Sample clean-up is paramount for proper identification and quantification of bioactive compounds from any matrix. Some of the common sample clean-up methods include sequential

extraction, liquid-liquid partitioning or solid phase extraction (Jin and Russell, 2010), which are also descried in more detail in Chapter 2, section 2.4.3.

In BSG, for the quantification of targeted phenolic compounds, UPLC-interfaced tandem quadrupole detector (TQD) systems are widely used to identify and quantify phenolic compounds based on mass of the compounds. Also used are LC-DAD/PDA detectors (Moreira et al., 2012b, Moreira et al., 2013)by comparing the light absorption spectra of a sample against a standard (at wavelengths of 280 nm hydroxycinnamic acids, procyanidins and flavanols can be detected, while at 320 nm for hydroxycinnamic acids and 360 nm for flavanols) (Watson, 2018b). The data generated by the two approaches, as chromatograms, and comparing the retention times, UV and MS/MS data with that of reference standards, indicated clearly the presence of several hydroxycinnamic acids, *p*-coumaric, ferulic and sinapic acid, respectively, with ferulic acid being the most abundant compound. Moreira et al., 2012 and 2013 also stated that due to the lack of reference standards, the remaining peaks in the generated chromatograms were tentatively described using literature data only, but still managed to reveal the presence of several isomeric ferulate dehydrodimers and one dehydrotrimer (Moreira et al., 2012b, Moreira et al., 2013). Similarly, the HPLC-DAD acquired for the BSG extracts showed the presence of protocatechuic acid and catechin at 280 nm, while caffeic acid, *p*-coumaric acid and ferulic acid, at 325 nm, which were confirmed by comparing their retention times of authentic standards (Barbosa-Pereira et al., 2014). Dvořáková et al., 2008, used the HPLC-DAD systems for the quantification of several flavan-3-ols and cinnamic acid derivatives in barley extracts. The LC-UV chromatograms generated revealed (+)- epi/-catechin and gallic acid at the wavelength of 280 nm, caffeic acid, *p*-coumaric acid, ferulic and sinapic acid at 320 nm, and protocatechuic and vanillic acid at 250 nm, respectively (Dvořáková et al., 2008). Beside the above-mentioned wavelengths, Stefanello et al., 2018 managed to identify diverse phenolic compounds in BSG, corn silage and cereal brans using HPLC-DAD at wavelength of 280, 320 and 360 nm, which were representative for hydroxybenzoates (gallic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid etc.) and proanthocyanidins (myricetin, resveratrol, kaempferol etc.), respectively (Stefanello et al., 2018b). In the study of Kumari et al., 2019,

the characterization of phenolic compounds in BSG extracts was performed by using UPLC-ESI-MS/MS. The data revealed unbound phenolics *p*-coumaric acid and caffeic acid as the main phenolic compounds through the application of MRM monitoring transitions negative ion mode (Kumari et al., 2019). da Coast Maia et al. 2020 also used UPLC-MS/MS to profile phenolic compounds based on the analysis of their molecular structure by classes, in both the hydrolysed and non-hydrolysed extracts of three different BSG types. A total of 93 phenolics have been tentatively identified in all the extracts, among which several were common in both extracts and other were specific either to the free or bound extracts. Flavonoids were the most common kind of compounds discovered (57%), followed by phenolic acids (25%), other polyphenols (6.5%), lignans (6.5%), and stilbenes (2%). These compounds were identified based on the *m/z* ratio, retention time, fragmentation score and isotope similarity against reference standards where available. The most abundant among flavonoids were (+)-catechin, (-)-epicatechin, myricetin, quercetin; among phenolic acids were gallic acid, gentistic acid, caffeic acid, syringic acid, *p*-coumaric acid, *trans*-ferulic acid, sinapic acid, and 4-hydroxybenzoic acid (da Costa Maia et al., 2020). Some of these flavonoids and phenolic acids are commonly found in barley malt and BSG, and were reported by other authors as well (Ikram et al., 2017, Carvalho et al., 2016). Verni et al., 2020 used an analytical hybrid system, i.e. HPLC-PAD-MS/MS, and based on the retention times, experimental and calculates *m/z*, molecular formula, fragmentation pattern, score, and error (at ppm levels) identified 43 compounds in the free and bound BSG extracts. The predominant identified compounds were ferulic acid (*m/z* 193), and its derivates (dimers-*m/z* of 385 and 387, trimers-*m/z* 577, and tetramers-*m/z* 771) in the bound phenolic extracts, whereas quercetin (*m/z* 301), catechin (*m/z* 289) and epicatechin (*m/z* 289) were identified in the free phenolic extracts, respectively. Other phenolic compounds were identified, such as vanillic (*m/z* 167), caffeic (*m/z* 179), *o*-coumaric (*m/z* 163), and sinapic acid (*m/z* 229) (Verni et al., 2020). For example, ferulic acid using the hybrid analytical platform was identified with UV maxima at 325 nm, and the UPLC-MS/MS confirmed with its $[M-H]^-$ ions of *m/z* 193 and its typical generated MS/MS fragment ions at *m/z* 134, 149, 178.

Even though phenolic compounds can be detected by the various analytical methods described above, a significant difference has been observed in their sensitivity. For example, flavan-3-ols (+)-catechin and (−)-epicatechin have low absorptivity in the UV region, thus concentrations of approx. 180-fold lower were achieved by using MS analysis compared to DAD detection (Carvalho et al., 2015). The limit of detection using the HPLC-DAD analysis were > 0.3 mg/mL, whereas for the UPLC-MS/MS-TQD were <1µg/mL, respectively, showing the high sensitivity and selectivity of the LC-MS/MS systems in the analysis of targeted phenolic compounds (Carvalho et al., 2015).

1.5 Biological activities

The scientific community's interest in phenolic compounds has grown due to their high bioactive potential, making them one of the most researched bioactive components in plants-based foods. This corresponds to a vast number of research that assess their bioactive potential, with *in vitro* studies being the most common. These include antioxidant, antimicrobial, antiallergenic, cardioprotective, neuroprotective, antiatherogenic, anti-inflammatory, anticancer, antidiabetic activities, tested using *in-vitro* assays (Jin and Russell, 2010, Faraone et al., 2019, McCarthy et al., 2013a, MacDonald-Wicks et al., 2006, Shahwar et al., 2010).

1.5.1 Bioassays

As a definition, a bioassay is the biological testing procedure for estimating the concentration of an active substance in a formulated or bulk material by measuring its biological response in living organisms (Systems, 2018). There is a vast range of bioassays to determine specific biological activities of an extract, for example *in-vitro*, *in-silico*, *ex*-vivo and/or *in*-vivo studies. *In-vitro* assays include chemical or enzyme linked based studies and are the first choice of analysis towards determination of biological activities. An important aspect that needs to be taken in consideration is purification, identification, characterisation and quantification of the isolated compounds, which are influenced by the extraction techniques, and their association with the biological effects.

1.5.1.1 *In-vitro* antioxidant activity assays

Antioxidants are compounds that react with oxygen or nitrogen species (radicals) by inhibiting oxidation reaction in biological systems. Antioxidants have two modes of action, either they scavenge free radicals and thus oxidizing themselves in a stable form or by donating electrons. It is important to determine the antioxidant capacity of the isolated compounds as they can be used as nutraceuticals or additives in food industry (Tiwari et al., 2013).

Broadly, to measure the *in-vitro* antioxidant activity, two types of reaction mechanisms are outlined for the substrate: i) hydrogen atom transfer (HAT) radical scavenging reactions, ii) single electron transfer (SET) reducing power reactions. Besides, other assays include oxidants as superoxide anion, singlet oxygen, peroxyl radical, peroxynitrite, hydrogen peroxide and hydroxyl radical (Table 1-4).

HAT methods are based on scavenging free radicals by donating hydrogen atoms, hence measuring the chain reaction antioxidant capacity. The antioxidant capacity is determined by measuring the fluorescence intensity obtained because of the competition of a possible antioxidant with the substrate (MacDonald-Wicks et al., 2006). The main bioassays based on HAT are: Oxygen radical absorbance capacity (ORAC), Crocin bleaching, and Total peroxyl radical-trapping antioxidant parameter (TRAP)(Chávez, 2017).

SET methods are based on the capacity of a potential antioxidant to donate an electron and reduce certain compound (carbonyls, metals, and radicals) thus measuring reducing power. The SET methods are end reactions and measure the relative percentage decrease in reaction product (Tiwari et al., 2013). The change of colours of the sample is recorded by a spectrophotometer and the absorbance is plotted against the antioxidant concentration (most commonly Trolox).

The main bioassays based on SET are: The Folin-Ciocalteu reagent (FCR)-based total phenolic (TPC), The ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging, Copper reduction, Trolox equivalent antioxidant capacity/ABTS radical cation decolorization (Chávez, 2017).

Table 1-4 Summary of the related features of some of the methods to evaluate the antioxidant capacity of the phenolic compounds.

Method	Required equipment	Biological relevance	Mechanism	End point
TRAP	Sophisticated	High	HAT	Lag phase
FRAP	Medium	Low	SET	Time varies
Copper reduction	Medium	Low	SET	Time
TEAC/ABTS	Simple	Low	SET	Time
DPPH	Simple	Low	SET	IC50
Folin-Ciocalteu	Simple	Medium	SET	IC50

1.5.1.2 *In-vitro* antidiabetic activity assays

Diabetes is a chronic disease associated with peculiarly high levels of glucose in the blood, affecting more than 422 million people globally, and causing around 1.5 million deaths annually (WHO, 2016). Diabetes is a major health threat across the globe, and currently the antidiabetic treatments are based on synthetic drugs that present various side effects. Diet is the main factor in controlling diabetes, and in fact the consumption of food rich in bioactive compounds, including phenolic acids, have shown to increase glucose uptake and synthesis of glycogen, and also reducing the circulating glucose and lipid levels in various chronic diseases (Vinayagam et al., 2016).

The key enzymes in the carbohydrate metabolism are α-amylase and α-glucosidase, with the main role of converting dietary carbohydrates to glucose. α-glucosidase is responsible for the breakdown and absorption of carbohydrate from the small intestine, and by introducing a competitive inhibitor, like acarbose, the absorption of most carbohydrates will be decreased and thus limit the postprandial increase of glucose (Vinayagam et al., 2016). Dipeptidyl peptidase IV inhibitors promote improved glucose homeostasis by impeding degradation of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1, thus extending the action of insulin while repressing the release of glucagon (Caymachem, 2018).

In the recent *in vitro* studies, it has been proven that phenolic acids extracted from peels and pulps of several apples can inhibit the activity of these enzymes, thus potentially reducing the risk of developing diabetes (Vinayagam et al., 2016). The polyphenol concentrates rich in catechin, epigallocatechin, and epicatechin,

of the persimmon leaf tea had significant porcine pancreas α-amylase inhibitory activity in a concentration-dependent manner, of 24%, and 45% activity inhibition at the concentrations 24 and 48 µg/ml of polyphenol concentrate powders; millet seed coat matter extract containing approx. 10% w/w phenolic compounds (caffeic acid, syringic acid, ferulic acid, among others) the potential for managing hyperglycaemia by inhibiting the activities of porcine pancreatic α-amylase (IC_{50} of 16.9µg extract) and rat intestinal α-glucosidase (IC_{50} of 23.5µg extract)(Ali Asgar, 2013); multiple antioxidant activities were found in the phenolic extracts of raw and processed millets, as well as being effective inhibitors of α-amylase and α-glucosidase, with ferulic acid, vanillic acid and kaempferol being the most predominant phenolics (Pradeep and Sreerama, 2015); rich ferulic acid extracts of several variants of organic rye presented a high amylase inhibitory action, whereas traditional rye varieties contain higher levels of catechin showed inhibitory capacity towards α-glucosidase activity (Mishra et al., 2017); individual phenolics, such as quercetin, ferulic, and *p*-coumaric acids by showing mixed non-competitive inhibition, were found to exhibit substantial glucosidase inhibitory action (Adisakwattana et al., 2009).

The α-glucosidase activity assay is a colorimetric assay, in which α-glucosidase hydrolyses the substrate to release p-nitrophenol that can be measured colorimetrically. The assay is easy, quick and high-throughput capable (Abcam, 2018). The α-amylase activity assay is also a colorimetric assay, where the amylolytic enzymes hydrolyses the starch to reducing sugars, and further the reducing sugar reduces the 3,5-dinitrosalicylic acid to generate red-brown substance. The intensity of the colour is proportional to the enzyme activity. The inhibitory effect of the substrate is tested against the positive control, acarbose (Cohesion, 2018).

1.5.1.3 *In-vitro* Anti-Cholinesterase activity assays

With ageing, people become vulnerable to all kinds of chronic diseases. One of these diseases is Alzheimer's (AD), which is a progressive neurodegenerative disorder of the central nervous system causing dementia, and unfortunately AD is irreversible. The main causes of AD, amongst others, include the loss of cholinergic neurons, increase of butyrylcholinesterase (BChE) and

acetylcholinesterase (AChE) activities, and marked by increased β-amyloid protein deposit in the brain tissue (Szwajgier and Borowiec, 2012). Today the AD treatment is mainly based on cholinesterase inhibitors, thus increasing the brain acetylcholine levels. Examples of anti-ChE include galantamine, Huperzine A and B, Tacrine, Rivastigmine etc. (Giacobini, 2004) Acetylcholine is a chemical messenger with an import role in learning and memory and it needs to be at high levels to support communication between nerve cells, thus cholinesterase inhibitors prevent the breakdown of acetylcholine reducing the development of AD (Alzheimer's-Ass., 2018).

It has been shown that phenolic acids, especially ferulic acid, possess a neuroprotective role towards striatal neuronal cells, act as antidepressant, and increase the *in-vitro and in-vivo* proliferation of neural stem cells of rats (Yabe et al., 2010, Szwajgier and Borowiec, 2012). Moreover, recent research shows that the ß-amyloid peptide-induced oxidative damage in the development of Alzheimer's disease may be mediated by hydrogen peroxide. The neuroprotective properties of polyphenols, the most common dietary antioxidants, are often superior to those of antioxidant vitamins against hydrogen peroxide (Dai et al., 2006). The inhibition of AChE and BChE activity can be determined based on Ellman’s colorimetric method, applicable for microplate reader. This method is rapid, simple, and cheap and also can be modified for high-throughput analysis. The inhibitory effect of the tested extract is dependent on the dilution factor and compared to a positive control, galantamine (Jońca et al., 2015).

1.6 Research objective

Brewer’s spent grain (BSG) is the insoluble residue of the barley malt and the most abundant by-product generated during the beer brewing process. It is known to be a rich source of phenolic compounds, that have demonstrated to possess a diverse range of health benefits. In addition to the health benefits of phenolics, their recovery from the beer brewing process by-products is critical to the sustainable growth of the agro-food industry via the comprehensive usage of its natural raw materials. This significance becomes even more apparent when a sector, such as the brewing industry, has a large impact on a country's economy.

As, phenolic extracts generated using organic solvents and alkaline hydrolysis have previously demonstrated *in-vitro* inhibitory effects on biological enzymes, the objective of this thesis was to investigate the inhibitory potential of phenolic extracts generated from different types of BSG residues, using several classic and novel extraction procedures in combination with organic solvents and acid-base solutions and to assess their potential as inhibitors of enzymatic activities *in vitro.*

Firstly, a classic approach to extract phenolic compounds from two different types of BSG using acetone, methanol, and ethanol to extract free phenolics, and chemical hydrolysis to extract bound phenolics was assessed. Liquid-liquid partitioning was used further to obtained rich phenolic extracts using diethyl-ether: ethyl acetate and as subjected to ultrafiltration using several molecular weight cut-off membrane-based filtration devices. Further, the phenolic composition of the extracts was determined by using conventional Folin-Ciocalteu spectrophotometric method and the more advanced chromatographic UPLC-MS/MS analysis (Chapter 3).

Next, the best extraction and separation parameters from Chapter 3 were used further to compare the classic extraction efficiency to release free and bound phenolics from three different types of BSG aided by novel extraction technologies, such as MAE and UAE, and subsequent liquid-liquid partitioning. Furthermore, our investigation was focused on the quantitative and qualitative analysis of phytochemicals (mainly polyphenols) in the BSG extracts using UPLC-MS/MS and Folin-Ciocalteu methods (Chapter 4).

The inhibitory potential of free and bound phenolic extracts from BSG light and subsequent fractions produced using liquid-liquid partitioning with a diverse polarity range of organic solvents against cholinesterases activities were investigated using *in-vitro* assays. Moreover, based on the phenolic profile obtained using UPLC-MS/MS, individual and blends of individual phenolics at specific ratios were prepared, assessed against the enzymatic activities, and compared with the original BSG extracts. The data obtained were thoroughly scrutinised with statistical analysis to reveal any correlations between the phenolic content and enzymatic activity inhibition (Chapter 5).

Finally, free and bound phenolic extracts from BSG dark were subjected to further fractionation using flash chromatography. The potent flash chromatography fractions for *in-vitro* anti-carbohydrase and -cholinesterases were identified and chemically characterized with spectrophotometric and chromatographic techniques (Chapter 6).

Chapter 2

2. General Materials and Methods

This chapter contains information on the common materials that were used in the experimental work, as well as the principles behind the methodologies applied to be able to fulfil the research objectives of this thesis.

2.1 Chemicals and reagents

A list of commonly used materials in most experiments along with their suppliers are included in Table 2-1. All chemicals and reagents used were of analytical grade and purchased from MERCK IE (formerly SIGMA ALDRICH), unless otherwise stated.

Table 2-1 Commonly used materials with their supplier information

Material	Type	Supplier
Organic solvents	Acetone, diethyl ether, ethyl acetate, *n*-butanol, ethanol, methanol, acetonitrile, petroleum ether, hexane	MERCK (formerly Sigma Aldrich), Arklow, Wicklow
Enzymes and substrates	Acetylcholinesterase, Butyrylcholinesterase, α-Amylaze, α-Glucosidase; acetylcholine iodide; S-butyrylthiocholine chloride; bovine serum albumin, potato starch, 4-nitrophenyl α-D-glucopyranoside	MERCK (formerly Sigma Aldrich), Arklow, Wicklow
Reagents	Folin-Ciocalteu, DTNB - 5,5'-dithiobis-2-nitrobenzoic acid, 3,5-Dinitrosalicylic acid (DNS) reagent	MERCK (formerly Sigma Aldrich), Arklow, Wicklow, IE
Kits	β-glucan mixed linkage assay kit, K-BGLU	Megazyme, Bray, Co. Wicklow, Ireland
Standards	Ferulic acid, *p*-coumaric acid, protocatechuic acid, catechin, caffeic acid, 4-hydoxybenzoic acid, sinapic acid, syringic acid, Acarbose, Galantamine,	Extrasynthese (Genay Cedex, France) and MERCK (formerly Sigma Aldrich), Arklow, Wicklow, IE
Acids and base chemicals	Sodium hydroxide, hydrochloric acid, formic acid; Tris HCl, sodium phosphate, sodium chloride, sodium carbonate	MERCK (formerly Sigma Aldrich), Arklow, Wicklow, IE
Filters	Polytetrafluoroethylene (PTFE) Captiva 0.45 µm;	Agilent (Santa Clara, CA, United States
	Buchi FlashPure ID C18 reverse phase cartridge (40µm)	Thermo Fischer Scientific Ltd, Carrigaline, Co.Cork, Ireland
	Amicon Ultra centrifugal filters (100kDa, 10kDa, 3kDa)	Millipore UFC, Merck DE
	Ceramic membranes (15kDa, 5kDa, 1kDa)	Atech Innovations GmbH, Gladbeck DE
	Atlantis T3 C18 column (100 × 2.1 mm; 3 µm); HSS T3 column (2.1 × 100 mm, 1.8 µm).	Waters Corporation, Milford, MA, USA
Equipment	Flash chromatography system	Analogix IntelliFlash, Modell 310, Varian, CA, USA
	Sigma 2-16KL centrifuge	Sigma, Osterode am Harz, Germany
	Spectrophotometer PharmaSpec UV-1700	Shimadzu Technology, Kyoto, Japan

Alliance 2695 HPLC system coupled to a Q-ToF mass spectrometer; UPLC-TQS system	Waters Corporation, Milford, MA, USA
SPECTROstar Omega microplate reader	BMG Labtech, Offenburg, Germany
Freeze drier Cuddon FD80	Cuddon Ltd., Blenheim, NZ
Blender Robot Coupe R3-3000	Robot-Coupe Ltd., Isleworth, UK
Vacuum packer VamaZS11	Vama Maschinenbau GmbH, Wildpoldsried, DE
Microwave MARS^{TM}-6	CEM, Matthews, NC, USA
Sonication bath Transonic TI-H-10 35 kHz	ELMA Sch., Singen, Germany
Infrared drying balance (Sartorius MA160)	Sartorius Lab Instruments, Germany
Shaker (MaxQ 6000 Shaker)	Thermo Fisher Scientific, MA, USA

2.2 Determination of proximate chemical composition of BSG

Several techniques were used to obtain detailed information regarding the BSG chemical composition, including water, protein, fat, dietary fibre, and ash content. The overall principles are described below whereas the experimental work described in Chapter 3, section 3.3.2.

The protein, fat, dietary fibre and ash content were performed at Food Industry Development Department and Food Quality and Sensory Sciences labs in Teagasc Ashtown.

2.2.1 Moisture content

The moisture content of BSG substrates was determined using an infrared automated drying balance (Sartorius MA160, Sartorius Lab Instruments, Germany). The results were displayed as Moisture in % M (% moisture).

2.2.2 Protein content

Protein content in BSG samples was calculated by measuring the total nitrogen (N) content using a LECO FP628 elemental analyser (Leco Corp., St. Joseph, MI, USA). LECO FP628 uses the Dumas based technique (combustion) to determine the nitrogen content in samples. The Dumas principle is an analytical chemistry method for determining the carbon and nitrogen content in organic matrixes. In principle, a sample of known mass is added to the LECO nitrogen analyser, heated in a furnace, and combusted (950-1,050°C) in a pure

oxygen atmosphere. As a result, chemicals such as carbon dioxide (CO_2), water (H_2O), nitrogen dioxide (NO_2), and nitrogen (N_2) are released. The interfering gases (CO_2, H_2O) are removed from the atmosphere by passing them through specific adsorbent columns, and over a hot copper metal to remove O_2 and convert nitrogen oxides to N_2. The remaining gases are then pass through a column with a thermal conductivity detector (TCD) at the end to measure the nitrogen content. As a result, the thermal conductivity detector's signal may be converted into a nitrogen content (Ebeling, 2020). The device is calibrated using a pure material that has a known nitrogen content, such a EDTA (N_2=9.59%). The crude protein is calculated then by the formula:

$$(\textbf{Equation 1})\ \%Protein = factor\ x\ \%N_2$$

where the factor is 6.25 (number assuming the nitrogen content of proteins to be 16% in cereals) (Jones, 1931), and $\%N_2$ as measured by the LECO equipment.

2.2.3 Fat content

Fat content in BSG samples was determined by using a paired SMART 6 Moisture&Solids Analyser with the ORACLE rapid fat analyser (CEM, Corp, Matthews, NC, USA).

SMART 6™ system is used for a rapid moisture analysis of samples prior to fat analysis. This system uses a dual frequency drying energy source which prevents sample burning and an incomplete drying prior to fat analysis. The SMART 6™ utilizes low frequency microwaves that penetrate the entire sample thus removing the volatile compounds via dipole rotation, and a high frequency infrared energy to heat the sample surface to remove non-polar components via molecular vibration (CEM, 2019b). Following the drying process, the sample is transferred in to the ORACLE and analysed for fat content. ORACLE is a fast fat analyser (30second/sample) that does not require any kind of method development. The system is based on NMR technology that uses a proprietary pulse sequence to completely isolate the detection of the proton signal in fat molecules from all other compositional proton sources, (i.e. proteins, carbohydrate, ash), thus removing any interferences from the measurement and normalizing the fat signal from the samples (CEM, 2019a).

2.2.4 Dietary fibre content

Total dietary fibre content (TDF) in BSG samples was determined by using the ANKOM FBT Dietary Fibre Analyser (ANKOM Technology, NY, USA), automated version of AOAC 991.43 and AACC 32.07.01 methods. The equipment and materials (enzymes, chemicals, bags etc.) used for the TDF analysis were specific for ANKOM FBT analyser. The principle of the method consists in subjecting samples of dried foods (BSG milled, dried, in duplicate) to a consecutive enzymatic digestion by heat stable α-amylase, protease and amyloglucosidase to remove starch and proteins. TDF include both soluble and insoluble dietary fibre.

2.2.5 Ash content

Ash content was determined by charring BSG samples in muffle furnace following the AOAC 923.03 standardised method. The mass of the residual material (ash) was compared to the mass of the original material and expressed as percentage ash by mass.

2.3 BSG sample preparation techniques

Brewer's spent grain (BSG) is the leftover residue of barley malt following the mashing stage of beer production. BSG of various types (Light-L, Dark-D, mixed -L&D) were collected from local Irish brewing companies (Diageo PLC – Guinness Dublin, Ireland, and Rye River Brewing Company, Celbridge, Co. Kildare, Ireland) directly from the hopper and transported to the lab. No information regarding the barley variety or the malting process was provided. The colour of the BSG residue, either light, dark or mixed comes from the roasting of malt at various temperatures during the brewing process (<230ºC). The darker the malt colour the higher the temperature used in roasting of barley. The mixed BSG type was received from the brewery directly as a mix of light and dark malt at 9:1 ratio.

Depending on the availability of the drying equipment, the BSG samples were either lyophilized or oven dried. Lyophilization was performed using a FD80 Cuddon Freeze Dryer, South Island, New Zealand, at a temperature of −54 °C and a pressure of 0.064 mbar for 72 h, and the oven drying was performed using

a Binder E28 oven (Binder GmbH, Germany) for 72 h at 60ºC. Following the drying process, the samples were milled using a Retsch MM400 mixer ball mill (Retsch GmbH, Germany) and further sieved to a particles size of <1mm, vacuum packed and stored in a -28°C freezer until required for further use. The milled BSG material was used for solid liquid extraction and for chemical and functional characterisation The BSG solid/liquid ratios were 1/20 (w/v) (Meneses et al., 2013).

2.4 Extraction and separation methodologies

The term “extraction” refers to a variety of actions but conveys the idea of something being pulled out of something else, and in this thesis, we refer to it as when a liquid solvent is used to solubilize and separate a solute from plant materials (Berk, 2018). Extraction is the initial stage in separating targeted natural compounds from the raw plant matrices. The primary goal of an extraction is to utilize a liquid (solvent) to dissolve (solvate) targeted chemical compounds (solute), separate them from the solid plant matter, and concentrate the solute by solvent removal to obtain an extract. Moreover, the extraction procedure is to optimize target-compound yield with no or minimum effect on target-compound characteristics while reducing extraction of unwanted compounds and leave behind the insoluble material part (Handa et al., 2008).The following steps are involved in the extraction of phytochemicals from plant-based products: (a) the solvent penetrates the plant matrix; (b) the plant compounds dissolve in the solvent; (c) the compounds are diffused out of the plant matrix; (d) the extracted compounds are recovered(Berk, 2018). Any factor that increases the rate of diffusion and solubilization in the preceding phases will enhance the extraction process. This process, depending on the solvent type, may change the chemical structure of the solid material, i.e. hydrolysis of insoluble biopolymers to form soluble molecules (Berk, 2018). The extraction rate is determined by the following factors: the rate at which a solvent is transported into the solid material to be leached; the rate at which soluble components dissolve in a solvent; the rate at which the solute is transported out of the solid material and from its surface to the solution (Handa et al., 2008).

Using solid-liquid extraction (SLE), a minimum amount of solvent is preferred to be used to extract as much of the solute as possible, with the purpose of obtaining a concentrated extract and to lessen the time for drying. Furthermore, in a single extraction process using a minimum amount of solvent, a condition of equilibrium is reached. This condition happens when the tendencies of the solute to pass from the plant matrix to solvent and from solvent to matrix are equal, and as well when both parts present the same concentration of solute. To overcome this issue, a multi-stage extraction process is necessary as one single-stage extraction would not be sufficient to recovery the product of interest. Moreover, during the extraction process several conditions need to be taken into consideration. Conditions, such as temperature, pressure, particle size, agitation, solid-to-solvent mass ratio, extraction length etc., that affect the extraction performance in terms of yield and rate of extraction, as well as the quality of the extracted product. Also, another important factor to consider is the polarity of the targeted compounds when selecting a solvent (Pronyk and Mazza, 2009, Pereira et al., 2016, Berk, 2018).

A smaller particle size during extraction has been found to improve bioactive component recovery by allowing for more solvent penetration and solute diffusion. Nevertheless, reducing too much the particle size of the plant material may cause excessive solute absorbance into the plant material inducing unwanted complications during the filtration step. Temperatures is another important factor that allows for a better solubility and diffusion rate of compounds of interest, although too high temperatures may produce unwanted contaminants or toxic compounds. In terms of extraction length, increasing the extraction time over a certain period may improve efficiency. Longer extraction periods, on the other hand, may have little effect on extraction once an equilibrium is established between the internal and external parts of the plant material (Roohinejad et al., 2017, Berk, 2018, Zhang et al., 2018).

Currently the most extensively utilised extraction methodologies for polyphenols are classic and novel extraction methodologies. The classic method consists in mixing the solid substrate with either organic solvents (i.e., alcohols), acid (i.e., sulfuric acid) or alkali (i.e., sodium hydroxide) solutions, whereas the novel extraction technologies (i.e., microwave and ultrasound assisted

extractions) use the same principal but assisted by microwave and ultrasonic radiations, respectively.

2.4.1 Classic extraction technologies

The classic extraction technologies (conventional solid-liquid extraction) include maceration, percolation, infusion, and hot continuous extraction (Soxhlet), among others, methodologies used for several decades to extract bioactive compounds from different plant or food materials. The basic principles and mechanisms of these classic extraction methodologies are same as the leaching process (see above). Percolation is a continuous process where the saturated solvent containing soluble compounds is constantly substituted with fresh solvent compared, while Soxhlet uses the principles of reflux and siphoning for the extraction process. Infusion on the other hand, uses maceration (see below) of plant material in cold or boiling water for a brief time to obtain fresh infusions that contain the soluble components of interest (Handa et al., 2008, Zhang et al., 2018).

In this research project, maceration, also known as soaking, was used as a classic methodology to extract potential bioactive compounds, such as polyphenols, from BSG substrates and compared to novel extraction methodologies (section 2.4.2).

In general, the maceration process on a small scale consists of placing the whole or milled dry plant material in a stoppered container with a solvent and left at room temperature for at least several days with regular agitation or mixing. Following, the mixture is strained off, the wet solid material is pressed to recover as much as possible of the liquid. Still, a significant amount of the liquid may be left in the marc after the first maceration, thus a repetitive maceration process may be more efficient in recovery of the liquid containing soluble components. Finally, the strained liquids are mixed with the expressed liquid and paper or cloth filtered to obtain a clear solution (Handa et al., 2008).

The dried material is macerated in a suitable solvent to facilitate swelling and hydration, where a combination of osmotic and diffusion processes transfers the soluble components from the plant material into the solvent. The mass transfer rates decrease with increasing the concentration of soluble components

in solvents, until equilibrium is reached, i.e., same concentration of soluble components in the solid plant material and solvent. Afterwards, the mass transfer of the soluble components from plant material to solvent will no longer take place. Mass transfer may be improved by heating the mixture, and as well by replacing the solvent that is in equilibrium with the plant material with a fresh solvent, thus changing the concentration gradient of the soluble components (Handa et al., 2008, Zhang et al., 2018).

Treatment of the BSG substrate to facilitate the extraction of polyphenols can be achieved using maceration with aqueous acid or base solutions of the substrate, followed by partitioning with green solvents. The sequential procedures involved in the extraction of compounds from plant materials, normally, are as follows: drying (oven, lyophilization), size reduction (milling), extraction (acid/base solutions, organic solvents), filtration (syringe or paper filters), concentration and drying (under nitrogen, using a rotavapor) (Handa et al., 2008).

2.4.1.1 Chemical hydrolysis

Acid and alkaline hydrolysis are chemical pretreatments used to enhance solubilization of lignocellulosic biomass into its main components, lignin, hemicellulose, and cellulose, respectively, as well as to decrease cellulose crystallinity and disrupt the biomass structure (Sun and Cheng, 2002). The main objective of the acid pretreatment is to degrade hemicellulose, whereas of the alkali pretreatment to degrade lignin from the lignocellulosic biomasses as to further use a subsequent extraction with organic solvents to recover potential solubilised compounds, i.e. polyphenols, that are interlinked with these polymers in the matrix (Fry, 1979).

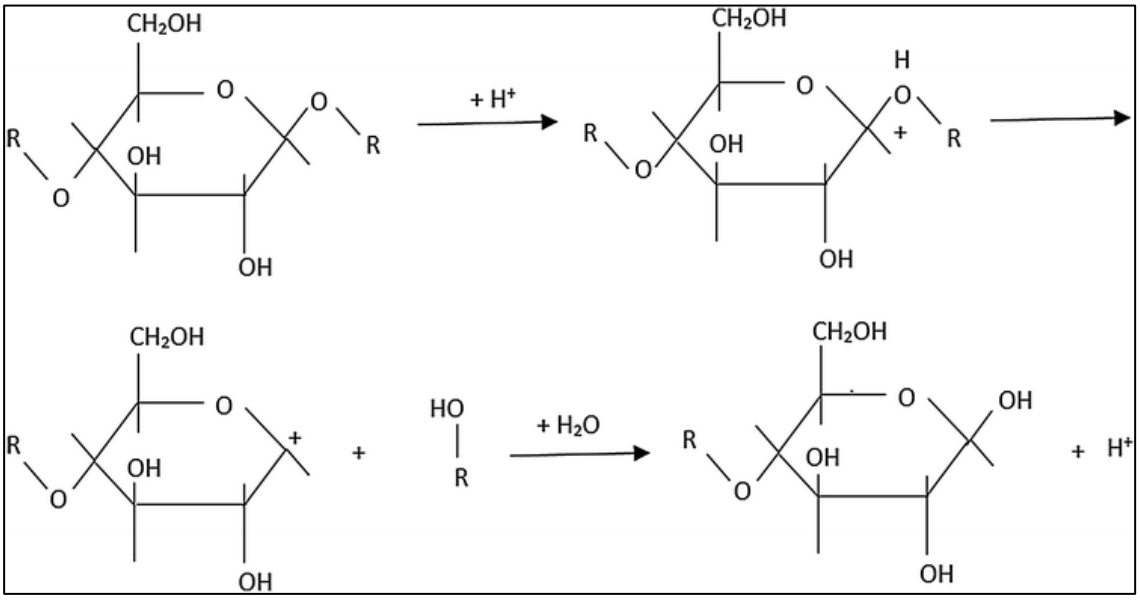
2.4.1.1.1 Acid hydrolysis

Acid pretreatment implicates the use of acids (concentrated or diluted) to degrade the recalcitrant structure of lignocellulosic biomass. Acids, such as hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) are used to depolymerize the complex polysaccharides (i.e. hemicellulose) of lignocellulosic biomass by breaking down the polysaccharides-based glycosidic linkages to their individual monosaccharide constituents, or to form gluco- and xylo-oligomers. HCl, when

added to water will have all the hydrogen (H^+) and chloride ions dissociated (Cl^-), and when mixed with lignocellulosic biomass, particularly the protons (H^+) will migrate to the reactive sites of the lignocellulosic biomass causing molecular disruption. Therefore, when using an acid medium to hydrolyse lignocellulosic biomass, acid acts as a catalyst resulting in protonation of the interglycosidic oxygen atoms. As a result, the charged group exits the polysaccharide chain, enabling hydroxyl group of the water to replace the bond and finally releasing the proton. As a consequence of the glycosidic linkages breakage, the polysaccharides degrade into simple sugar units, such as glucose, xylose, and arabinose, and part of the lignin as well (Harmsen et al., 2010, Loow et al., 2016). The mechanism of action of acid on hemicellulose is shown in Figure 2-1.

Acid hydrolysis was performed on the BSG residues following an organic solvent extraction with 80% methanol (MeOH). The method was adapted from Verardo et al, 2011 (Verardo et al., 2011) with slight modifications and used in Chapter 3, section 3.3.4.2 of this thesis.

Figure 2-1 Mechanism for acid-catalysed hydrolysis of hemicellulose glycosidic bonds as obtained from (Loow et al., 2016)

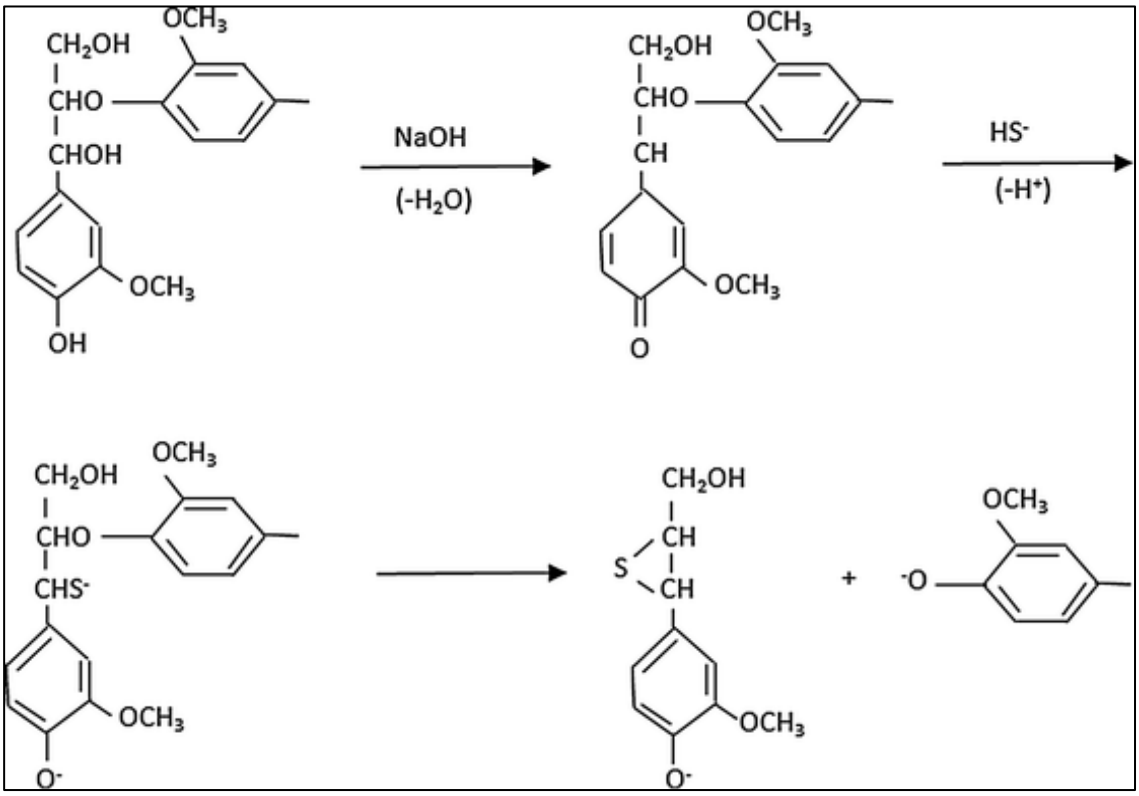


2.4.1.1.2 Alkali hydrolysis

The mechanism of alkaline hydrolysis on lignocellulosic biomass is thought to occur via the saponification of the lignin-carbohydrate complex linkages, specifically the ester bonds that crosslink xylan hemicelluloses and lignin, and

ether bonds in lignin (Ricardo Soccol et al., 2011). Alkaline hydrolysis of lignocellulosic biomass is normally carried out by using aqueous-alkaline solutions of sodium hydroxide (NaOH) or potassium hydroxide (KOH), at diverse concentrations (% w/v or moles/litre). Treatment of lignocellulosic biomass with NaOH solutions cause the breakdown of intermolecular ester bonds, leading to an increased internal surface area and reduce the degree of polymerization of the biomass, and ether bonds, separating the aromatic rings and disrupting the lignin structure, which later lead to the cleavage of lignin from the polysaccharide matrix (Fan et al., 2012, Loow et al., 2016). The mechanism of action of alkali hydrolysis on lignin can be seen in Figure 2-2.

Figure 2-2 Mechanism for NaOH on breaking down the ether bonds of lignin as obtained from (Loow, Wu et al. 2016)



Alkali hydrolysis was performed in two ways, one following the methodology of Verardo et al, 2011 (Chapter 3), and the other following Wand et al., 2008 and Moreira et al., 2012 (Chapters 4, 5 and 6), both with slight modifications (Moreira et al., 2012a, Verardo et al., 2011, Wang et al., 2008).

The first method was used in Chapter 3, where alkali hydrolysis was performed on the BSG residues following a previous organic solvent extraction with 80% methanol (MeOH). The second method was used in Chapter 4 and 5,

and partly in 6, where alkali hydrolysis was performed on milled BSG without a prior extraction process following the methodology of (Moreira et al., 2012a, Wang et al., 2008). In Chapter 6, a sequential bound phenolic extraction was performed. In the first part of the method, 0.75% NaOH was used as described in Chapter 4 and 5, and for the second part of the extraction the residue was further hydrolysed using 7.5% NaOH solution, under nitrogen.

2.4.1.2 Organosolv extraction

Organosolv is an extraction technique that combines the power of organic solvents, or their aqueous solutions, and heat to break down the lignocellulosic structure (lignin and hemicellulose) of plants based materials (Ravindran et al., 2018). The ability of an organic solvent to dissolve a solute depends on its polarity, polarity of the targeted compounds, and solubility of the solute in the solvent. Based on the polarity (a molecule possesses distinct positively and negatively charged end), molecules can range from polar or slightly polar to non-polar. Most of the solvents employed in the organosolv extraction are organic in nature and are categorized from polar to non-polar based on the polarity index parameter. This parameter is defined as the capacity of a solvent to interact with a variety of polar test solutes and its value increases with solvent polarity (polarity index values from 0.0 to 1.0). In other words, polar solutes can be dissolved by using polar solvents and non-polar solutes by non-polar solvents (Gupta et al., 1997, Handa et al., 2008). This phenomenon when compounds with similar chemical characteristics will dissolve in each other is also known as “like dissolves like”.

Extraction from different matrices is performed using chlorinated and non-chlorinated solvents, such as chloroform, acetone, methanol, ethanol, acetonitrile etc. Most organic solvents used are alcohols i.e., ethanol, methanol, due to low cost and fully miscible with water, and as well as ketones such as acetone. Moreover, the use of organic solvents in an extraction process offers the advantage of easy and fast recovery by distillation due to their low boiling point and concomitant low energy requirement for their recovery, and further recycled for extraction (Zhao et al., 2009). In Table 2-2 several parameters are described

when choosing an organic solvent for the extraction of targeted compounds from lignocellulosic materials.

Table 2-2 Factors that need to be taken in consideration when choosing a solvent for extraction of targeted compounds from plant materials (Handa et al., 2008)

Solvent factors	Requirements
Solvent power	High solvent selectivity for the extraction of targeted or desired compounds
Boiling temperature	Low boiling temperature for fast solvent removal
Reactivity	No chemical reactions of the solvent with the extract, nor should it rapidly decompose
Viscosity	Low solvent viscosity leads to a low pressure drop, high mass-transfer and heat
Safety	Non-flammable, non-corrosive, non-toxic, non-hazardous, low environmental risk solvent
Cost	Solvent availability at low cost
Vapor pressure	A low vapor pressure at working temperature is needed to avoid solvent loss through evaporation.
Recovery	Easy removal of the solvent to obtain solvent-free extract

Unfortunately, solvent extraction has several disadvantages that may restrict its use, including the need for large quantities of solvent and lengthy extraction periods.

In this research project, extraction of free (unbound) polyphenols (FP), referred to as crude extracts, from BSG samples was carried out using aqueous-ethanol, -methanol, and -acetone solutions, following the methodologies of several authors, as classic extraction methodology and as well in combination with novel extraction methodologies, such as ultrasound.

In Chapter 3, the extraction of free phenolic was performed following the methodology of Verardo et al, 2011 with slight modifications (Verardo et al., 2011). In chapters 4, 5 and 6, the extraction of free phenolic was performed following the previously optimised method of (Meneses et al., 2013).

2.4.2 Novel extraction technologies

In this research project, the extraction of polyphenols from BSG was performed not only by using classic extraction technologies (section 2.4.1) but as well novel extractions technologies. These novel technologies follow the principles of green chemistry techniques and methodologies, to “reduce or

eliminate the use or generation of feedstocks, products, by products, solvent reagents etc that are hazardous to human health and environment" (Anastas, 1999). Moreover, novel extraction technologies present several advantages over the classic ones, in terms of efficiency, reduced extraction time and solvent volumes, higher extraction yields and lower operating costs. Ultrasound-assisted extraction (UAE), Microwave-assisted extraction (MAE), supercritical fluid extraction (SPE), pressurised solvent extraction (PSE) are some of the currently available extraction technologies that are considered clean, green and efficient alternative to classic extraction technologies (Joana Gil-Chávez et al., 2013).

2.4.2.1 Microwave-assisted extraction (MAE)

MAE is a process that uses microwave radiation to heat polar and non-polar solvents that are in contact with plant substrates to facilitate the separation of organic compounds from plant materials into the solvent (Eskilsson and Björklund, 2000). The microwave produces electromagnetic waves with wavelengths ranging from 1 m to 1 mm, which correspond to frequencies ranging from 300 MHz to 300 GHz (Hitchcock, 2004). These waves interact with polarizable plant materials and dipoles of polar solvent at molecular level through different ways: polar molecule alignments, molecular rotation and vibration and transfer of kinetic energy to release electrons and ions (Hitchcock, 2004). These interactions happen when the electromagnetic waves, by vertical oscillation, create an ionic conduction and a dipole rotation of polar molecules in the matrix and solvent, thus producing friction and collision with nearby molecules. This interaction further results in an increase in thermal energy and pressure, causing structural changes to the plant material and further breaking the cell walls (Hitchcock, 2004). Furthermore, the extraction solvent penetrates the plant material via diffusion, leading in the solubilization of solutes into the solvent until saturation is reached (Li et al., 2012).

MAE differs from classic extraction techniques in that it uses a distinct mechanism, involving a synergistic combination of mass and heat transfers that operate in the same direction, from the inside of the material to outside, whereas in classic extraction techniques, the mass (inside to outside) and heat transfer

(outside to inside) occurs in opposite directions (see Figure 2-3 and Figure 2-4) (Deo et al., 2015).

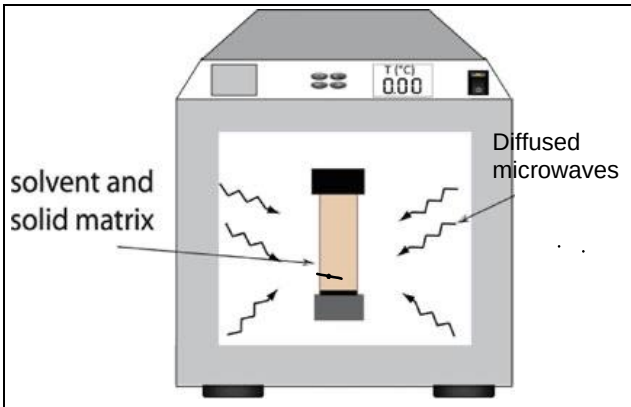


Figure 2-3 Closed-vessel system of Microwave Assisted Extraction as obtained from (Li et al., 2012)

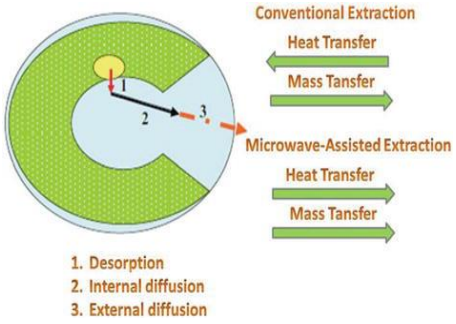


Figure 2-4 The mechanism of heat and mass transfer in classic and microwave assisted extraction as obtained from (Deo et al., 2015)

MAE equipment can be used either in closed extract vessels (Figure 2-3), where temperature and pressure can be controlled, or open vessels which are under atmospheric pressure. Using closed-vessels systems the solvent can be heated beyond its boiling point at atmospheric pressure, maximizing the mass transfer of desired compounds from the plant material. Using open-vessel systems the highest temperature that can be reached is determined by the solvent boiling point at that atmospheric pressure (Li et al., 2012). With the recent advances in the development of microwave reactors, the temperature and pressure can be control during the extraction period. Controlling these parameters can led to a rapid rise in temperature, selective heating, superheat the solvents without boiling in the microwave, thus increasing the reaction rates and further enhancing the extraction efficiency (Levin et al., 2019). In addition, higher extraction rates can be obtained by mixing aqueous alkali or acid solutions, or organics solvents with the samples to the MAE extraction. Stirring, by the addition of magnetic bars to the extraction vessels is also a beneficial factor as it improves the transfer rate between the extraction solution and sample, especially for compounds bound to the plant cell wall (Camel, 2001).

In this research project, Chapter 4, MAE of BSG phenolics was performed according to the method previously optimized and reported by Moreira et al., 2012 (Moreira et al., 2012b). The extraction was carried out in a closed-vessel

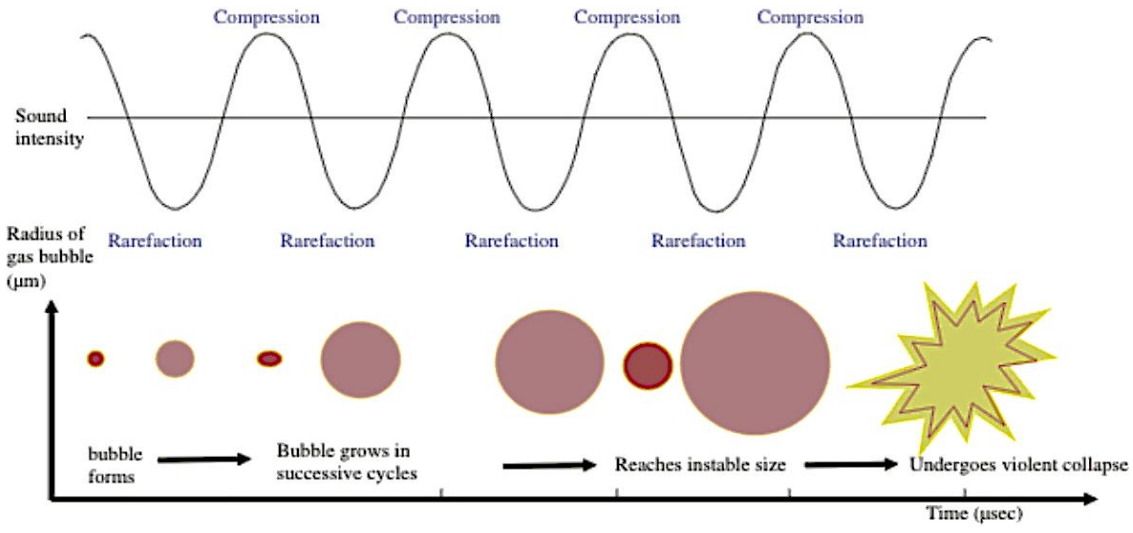
microwave MARSTM-6 (CEM, Matthews, NC, USA) equipped with a 40-position carousel.

2.4.2.2 Ultrasound-assisted extraction (UAE)

UAE is a process that uses ultrasonic waves in the range of 20 to 2000 kHz to create cavitation of micro bubbles while passing through a solution. At the impact with the surface of the plant material, the cavitation bubbles collapse, and the resulting shockwave induces disruption, macroturbulence, high velocity interparticle collisions and perturbation to the plant cell wall, which further facilitates the mass transfer of organic compounds into solution (Roohinejad et al., 2017).

Acoustic cavitation (phenomenon of creation, growth, and implosive collapse of bubbles in liquids under the influence of an ultrasonic field) is the principal driving force for the extraction effects of UAE (Figure 2-5) (Leighton, 1994, Le et al., 2015). Numerous physical and chemical phenomena are responsible for the ultrasonic effect, with the most common being a series of compressions and rarefactions in the molecules of the medium because of exposing the sample to ultrasound. This mechanism is responsible for the formation of bubbles that are subsequently compressed resulting in a local increase of temperature and pressure. The local pressure gradients in the liquid medium causes the formation, growth and lastly the collapse of bubbles (Gallo et al., 2018).

Figure 2-5 UAE cavitation mechanism as obtained from Le, Julcour-Lebigue et al., 2015.

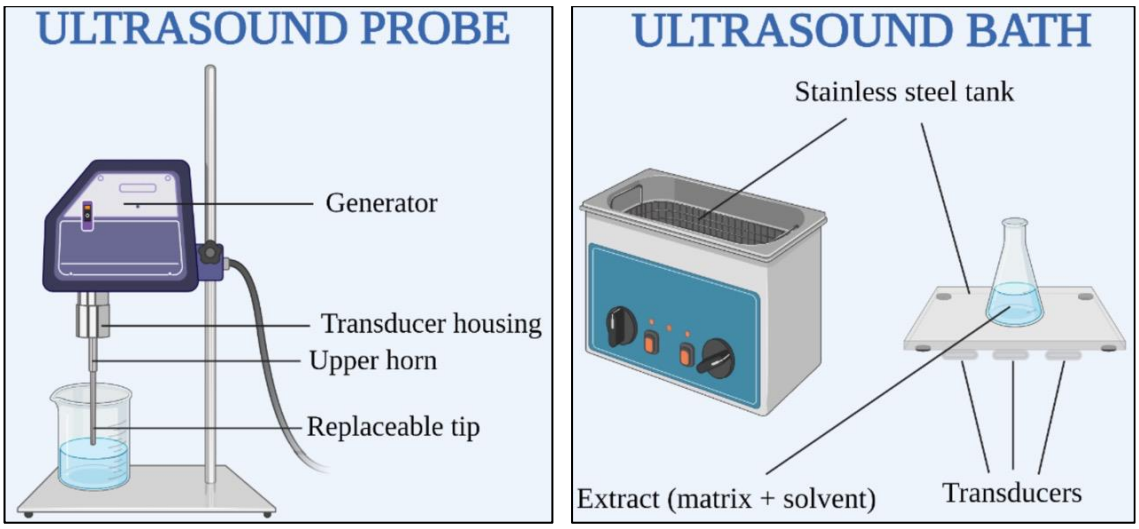


There are two types of cavitation bubbles, transient and stable cavitation. Transient (inertial) cavitation bubbles form under regular acoustic pressures, persist for a relatively brief time (less than a cycle of compression and rarefaction) and followed by an abrupt collapse. On the other hand, stable (non-inertial) cavitation bubbles form under irregular acoustic pressures, persist for a longer period (several cycles of compression and rarefaction) and finally merging during the compression cycle (Tiwari, 2015, Piyasena et al., 2003, Laborde et al., 1998). The ultrasound effect is reduced when stable cavitation bubbles do not reach their critical size to abruptly collapse (Tiwari, 2015, Albu et al., 2004). During the sonication process there are thousands of bubbles formed, of which some are reasonably stable whereas others reach an unstable size and collapse violently generating high temperatures (<5000 K) and pressures (50 MPa) at a microscopic level (Piyasena et al., 2003, Tiwari, 2015). The high temperatures and pressures generated facilitate localised shear disruption, weakening of cell walls and membranes and cell rupture of the plant material. Ultrasonic waves also help with solvent diffusion into the matrix by creating pores in the membranes of the plant material providing access to the underlying tissues. The matrix's hydration and swelling expand pores even further, facilitating the entry of the solvent (Khadhraoui et al., 2021). All these factors contribute to a higher penetration of the solvent into the plant tissue, ultimately leading to an enhanced diffusion of the plant solid components into the solvent. The combination of this action, increased mass transfer, and significant cell rupture results in the liberation of cell components (Tiwari, 2015, Albu et al., 2004, Rostagno et al., 2003).

UAE may be carried out directly (ultrasonic probe) or indirectly (ultrasonic water-bath) (Figure 2-6). Among the two, a probe sonication offers a more consistent and focused ultrasonic power input, making it more effective and powerful than an ultrasonic water-bath (20 000W/L vs 20 W/L), by at least a 1 000x higher in energy input/volume. Also, the input energy is applied directly and uniformly to the plant material with a minimum loss, meaning that the probe outperforms an ultrasonic water-bath (Asadi et al., 2019). In contrast, using an ultrasonic water-bath the ultrasonic energy is applied indirectly to the plant material, thought the walls of the water bath (Medina et al., 2017). When using

UAE, various factors need to be taken into consideration when extracting organic compounds from plant materials, such as type of ultrasonic reactor (water bath or probe), working frequency, ultrasonic power or intensity used, extraction time and temperature, solvent (type, properties and ratio to solid material) and medium properties (viscosity, surface tension) (Tiwari, 2015).

Figure 2-6 UAE systems



Picture adapted from (Carreira-Casais et al., 2021)

In this research project, UAE was carried out on the Transonic TI-H-10 35 kHz (Elma Sch. GmbH, Singer, Germany) ultrasonic unit, with the xtraction parameters previously optimised in similar substrates from (Irakli et al., 2018, Wang et al., 2008), and used in Chapter 4 for the extraction of free and bound phenolics. Another ultrasonic unit, a Branson 3510 43kHz (Branson Ultrasonics Corp., Danbury, Connecticut USA) was used in Chapter 3 for the extraction of free phenolics following the methodology of (Verardo et al., 2011).

2.4.3 Separation and fractionation techniques

Following the extraction process, a vital step in recovery of targeted analytes from plant extracts is separation and fractionation. The components of the crude extract obtained using the techniques described above are complex and include a range of analytes that need additional separation and fractionation to obtain the active fraction with the compounds of interest. Although there are many methods available, liquid-liquid extraction, flash chromatography and molecular-weight-cut-off are some of the most frequently utilised for fractionation. These methods are dependent on the solvent combination used and by the

physical or chemical properties of each compound to separate distinct groups of molecules from plant extracts (Zhang et al., 2018).

2.4.3.1 Liquid-liquid extraction (LLE)

Liquid-liquid extraction (LLE), also called solvent-partitioning (LLP), is a separation process in which a solute from one solvent is transferred to another solvent, with the two solvents being immiscible or partially miscible with each other. Often, water or an aqueous mixture is one of the solvents and the other being a nonpolar organic liquid. A two-step process is followed in liquid-liquid extraction, first is mixing (contact between the two solvents) followed by phase separation. These two steps are important when choosing the partitioning solvents and the mode of action. A dynamic mixing is favourable to facilitate the transfer of extractable compounds from one solvent to another, but this may result in emulsion formation which may impair the subsequent phase separation step. An equilibrium is reached when the chemical potential of the extractable solute is identical in both phases, leading to the so called “distribution coefficient”. The distribution coefficient expresses the relative inclination of a solute for a solvent, and in ideal solution, at a specific temperature, is constant, and independent of the concentration. Adjusting the distribution coefficient, in some cases, can improve the efficiency of the LLE process (e.g., at low pH, the preference of a non-dissociated organic acid for nonpolar solvent; at high pH, in dissociated form a preference for aqueous solvent)(Berk, 2018).

LLE is an advantageous technique due to its ease of operation and the general availability of appropriate organic solvents. In LLE, depending on the type of bioactive compounds to be separated from a crude liquid plant extract, several solvents, such as ethyl acetate, alcohols, and acetone, may be used to extract polar compounds, whereas hexane, chloroform, benzene are used for non-polar type of compounds. For example, if an enriched phenolic extract is required, the removal or separation of lipids (non-polar compounds) would be the necessary. This can be achieved by using a biphasic liquid-liquid system, where hexane (nonpolar organic solvent) is added to the crude extracts which contains an aqueous solvent (polar solvent) (Berthod and Carda-Broch, 2004). Following, by using gentle shaking or agitation to facilitate partitioning, most of the lipids will

migrate then to the nonpolar organic phase, whilst the polar phase will contain the remaining phenolic compounds.

In this research thesis, LLE was used to recover the phenolic compounds following the extraction with either aqueous organic solvent or hydrolysis (saponification) with alkali or acid solutions, and the procedure described in each chapter and just briefly in this section. In Chapter 3, LLP was used to pool the bound phenolic compounds released in the aqueous solutions following an acid and alkali treatment of the BSG light and dark pellet free of unbound phenolic compounds. In Chapter 4, the liquor supernatants were acidified to pH 6.5 and subsequently subjected to liquid-liquid partitioning in EtOAc:Water (1:1 v/v, 3 times) to obtain polyphenol-enriched fractions. For Chapter 5, sequential fractionation of the Free Phenolic (FP) and Bound Phenolic extracts (BP, or Total Phenolic, TP) was performed by using solvents with increasing polarities, method adapted from Tu et al., 2013 with some modifications (Tu et al., 2013). Finally, in Chapter 6, following the filtration step of the extraction of FP and BP extracts, the mixtures were subjected to liquid-liquid extraction using a 1:1 ratio of Diethyl ether and ethyl acetate (DE:EA).

2.4.3.2 Flash Chromatography

Flash chromatography is an improved preparative column chromatography technique that permits the separation and enrichment of specific organic compounds from an extract into their individual constituents. The flash chromatography system is equipped with a column, or a pre-packed plastic cartridge fitted with silica gel particles, a gradient pump, sample injector ports, a UV detector, and a fraction collector to recover the eluent. The automated system is controlled by a software that allows the user to control and adjust the running conditions, while also being able to control the collection of all or only the fractions of interest that contain the organic compounds. Lastly, the software generates a visual chromatogram characteristic for the retention of the compounds in the collected fraction (Ayare et al., 2014, Bickler, 2020).

In general, the principle of flash chromatography is that the eluent is quickly forced through a plastic column under gas pressure (usually nitrogen or compressed air). The eluent is either isocratic or a binary liquid with a mix of

organic solvents, where one solvent has a higher polarity (i.e. water) than the other (i.e. acetonitrile). This mixture allows to adjust the polarity of the eluent thus managing to separate the compounds from an extract based on their polarity. The column of the stationary phase presents a wide inner diameter and is packed with an adsorbent which in general is silica gel of various sizes (15 - 60µm). Particles less than 25 µm should only be utilized with low viscosity mobile phases to avoid poor flow rates. Gel beds are typically 15 cm high, with operating pressures of 1 – 3 bar and solvent flow rates of < 150mL/min. The organic compounds from an extract interact with the silica bed gels based on their charge, relative solubility, and absorption(Ayare et al., 2014, Bickler, 2020, Mossaba et al., 2000). Flash chromatography can be performed in normal-phase (non-polar solvents with polar stationary phase) or reverse phase (polar solvents with non-polar stationary phase). In reverse phase, polar compounds elute sooner and the non-polar later, and opposite for normal phase.

In the current thesis, fractionation of polyphenols from different BSG extracts of free and total polyphenols was used in Chapter 6 and achieved following the methodology of Gangopadhyay et al., 2016 with slight modifications (Gangopadhyay et al., 2016). A Varian 310 flash chromatography system (Analogix IntelliFlash, Modell 310, Varian, CA, USA, Figure 2-7) was used, equipped with a Buchi FlashPure ID C18 reverse-phase flash cartridge (particle size of 40 µm irregular, 12 g), a binary solvent system containing water (mobile phase A), and acetonitrile (mobile phase B) and a UV detector, which was set at wavelengths of 245, 280, 320 and 360 nm to monitor the eluting fractions (see wavelength colour description in Figure 2-6). Following the flash chromatography run, the results are presented in a chromatogram (i.e., Figure 2-7 chromatogram of BSG D crude extract), with running time at the bottom (time-min), eluent gradient concentration on the right (% B – acetonitrile), fraction number on the top (fraction 1 to 30) and absorbance units (AU between 0.00 to 0.39) on the left side of the chromatogram.

Figure 2-7 Flash chromatogram obtained following fractionation of BSG D crude extract; the blue line represents the eluent (B) gradient starting from 0% to 100% acetonitrile over a period of 30minutes; the peak colours represents the wavelengths (nm) of the UV-detector used to scan the fractions at 245nm - yellow, 280nm - red, 320nm - blue and 360nm - green.

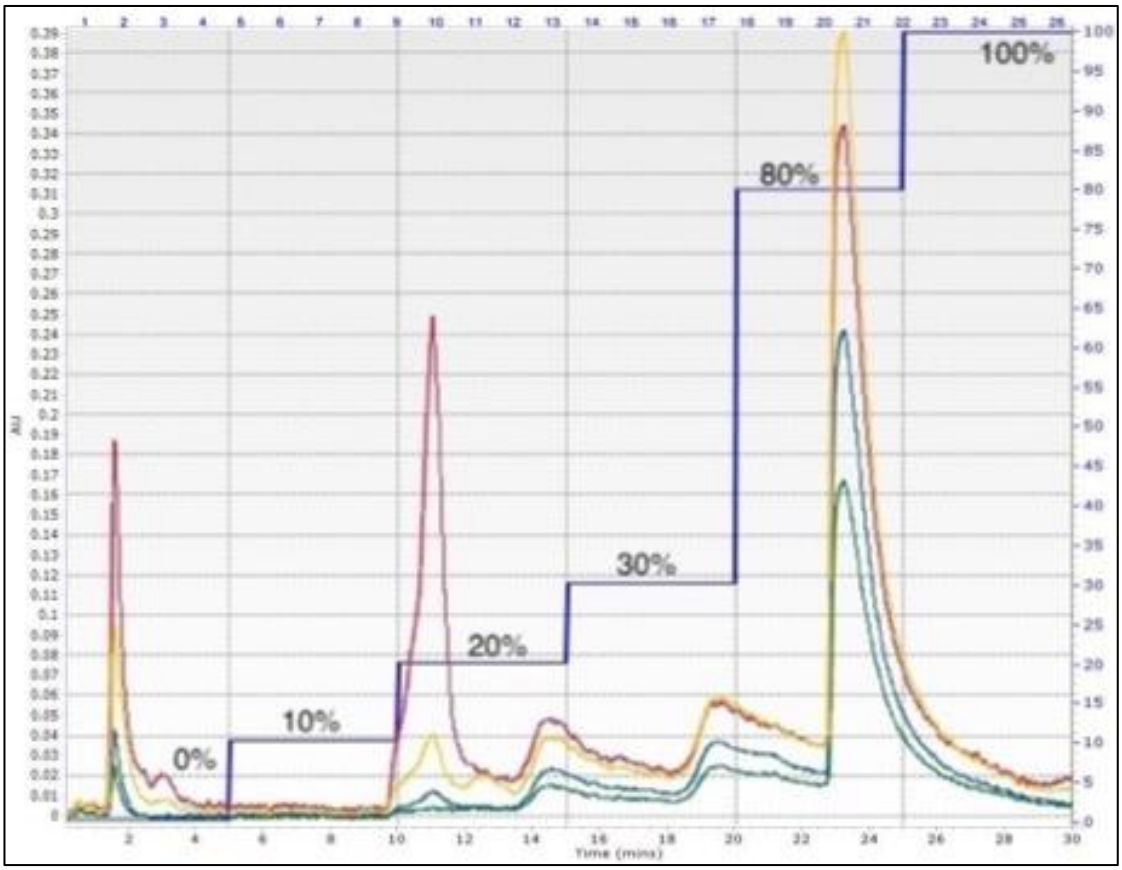


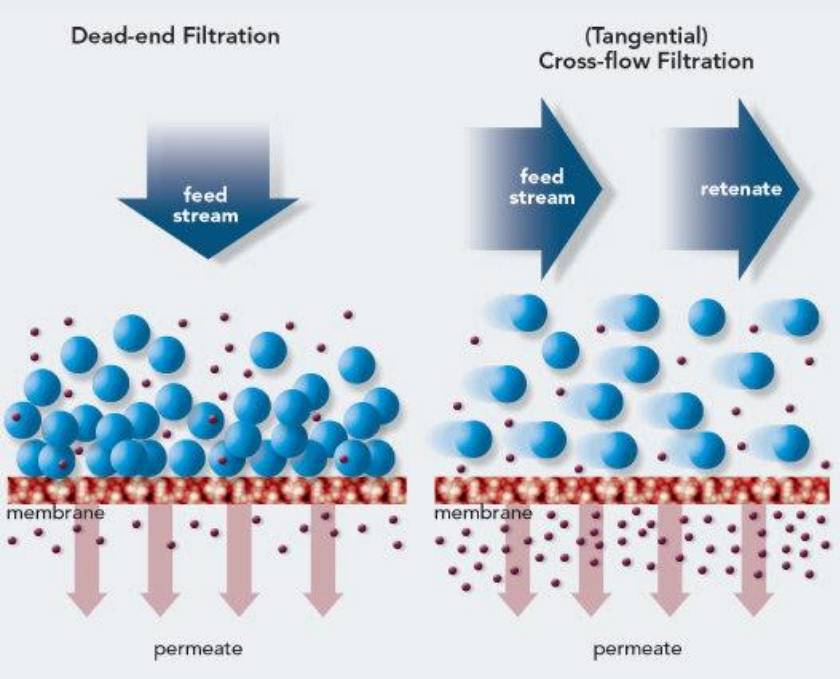
Figure 2-8 Analogix IntelliFlash 310 System Flash Chromatography Workstation

2.4.3.3 Molecular-weight cut-off (MWCO)

The molecular weight cut-off (MWCO) is a method of characterization used in filtration to characterize the pore size distribution and retention capacities of different type of membranes. In the context of solute retention, it is defined as the lowest molecular weight (in Dalton (Da)) at which more than 90% of a solute with a specific molecular weight may be retained by the membrane. There are several factors (sample concentration, composition, molecular shape) and conditions (temperature, pressure, cross-flow velocity) that need to be taken in consideration when performing MWCO membrane filtration (Drioli et al., 2016, Synderfiltration, 2021b).

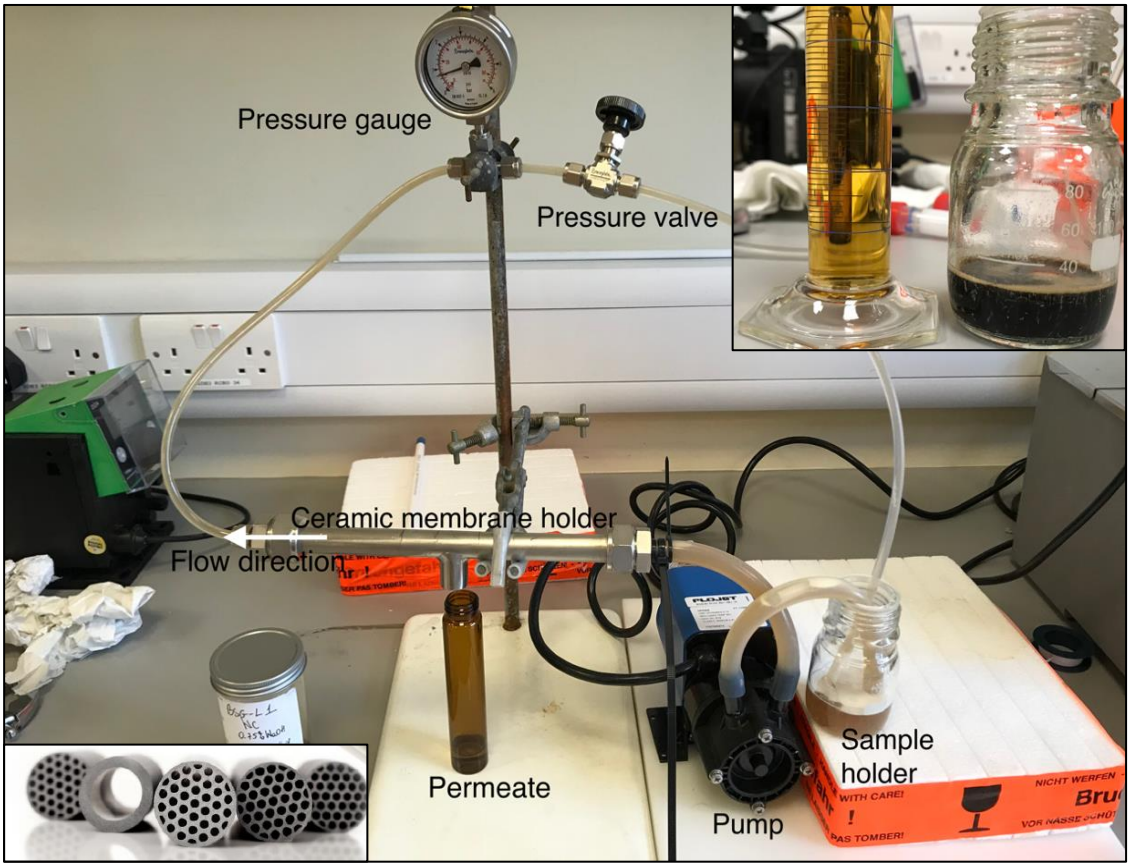
There are different ways to separate compounds from extracts by their MWCO, either using dead-end filtration (centrifugal filtration membranes) or cross-flow filtration (single- /multi-channel ceramic membranes) (Figure 2-9). The dead-end filtration has a normal feed flow that passes through the membrane surface under applied pressure resulting in a build-up layer on the surface of the membrane. In the cross-flow filtration the feed flow is pumped creating a pressure gradient while passing tangentially across to the membrane surface (Paipuri et al., 2015, Synderfiltration, 2021b). Using cross-flow filtration, the build-up of solute on the surface membranes is reduced and as well the overall amount of fouling and cleaning process (Synderfiltration, 2021b). Filtration membranes are of different types according to their MWCO and pore size, as follows: microfiltration (MWCO > 100kDa, pore size 0.1-10µm), ultrafiltration (MWCO 1kDa-500kDa, pore size 0.001-0.1µm), nanofiltration (MWCO 0.2 – 1kDa, pore size 1-10nm) and reserve osmosis (MWCO <200 Da, pore size <1 nm) (Synderfiltration, 2021a). Components having smaller sizes than the smallest pores can travel through the membrane without being hindered. On the other hand, components with a diameter smaller than the biggest pores but bigger than the smallest pores are rejected in accordance to the pore size distribution of the membranes (Kumar et al., 2018).

Figure 2-9 The feed stream in dead-end filtration vs cross-flow filtration (Paipuri et al., 2015)



In this research project (Chapter 3), MWCO centrifugal filters were used to separate BSG compounds according to their MWCO from BSG free phenolic and bound phenolic extracts. BSG free phenolic extracts were obtained following a methanol extraction, and bound phenolic extracts obtained following an acid and alkali hydrolysis extraction and their subsequent partitioning using liquid-liquid extraction with diethyl ether:ethyl acetate. Moreover, single channel ceramic membranes were used in a locally build lab-scale open-flow filtration system composed of a pump (Flojet HPR 6/8, Xylem, Hoddesdon, Hertfordshire, UK), stainless steel channel support containing single channel ceramic membrane (Atech Innovations GmbH, Gladbeck DE) with MWCO of 15 kDa (pore size of 0.2µm), 5 kDa (pore size of 0.1µm) and 1 kDa (pore size of 0.05µm), hoses, pressure gauge and pressure-reducing valve, samples holder for recirculation and permeate collector (see Figure 2-10).

Figure 2-10 Cross-flow lab scale filtration system containing single ceramic membrane, (top right corner) permeate collected after filtration vs remaining sample; (lower left) ceramic membrane models, with single channel being used in the current filtration system



2.5 Identification and Quantification of the extracted compounds

In general, traditional spectrophotometric (colourimetric) assays are regularly used for the characterisation and quantification of the compounds in plant extracts. However, due to the structural diversity and variation in quantities of the extracted compounds, the spectrophotometric methods' detection and characterisation can be challenging providing little information on the structure and composition of individual components. With the advancing technology, high-performance liquid chromatographic separation techniques and hyphenated methods have been developed that enable to identify and quantify the individual bioactive components.

2.5.1 Colourimetric methods to determine the TPC by Folin-Ciocalteu reagent

Colourimetric assays are simple methods in which specific reactions result in products that present optical absorbance at certain wavelengths of light. This allows reactants to be measured using a spectrophotometer or a microplate reader for numerous samples at the same time.

Colourimetric reactions are commonly measured by UV/Vis spectrophotometers. This type of method is easy to perform, fast, low-cost, and routinely applicable in lab use. To measure the total polyphenol content in plant extracts, polyphenols react with redox reagents, such as Folin-Ciocalteu reagent, to produce a blue complex that can be measured in visible-light range. However, colourimetric assays need the use of a reference standard, and only then the total concentration of the hydroxyl groups in plant extracts can be measured (Blainski et al., 2013).

The Folin-Ciocalteu (FC) assay was established in 1927 and is an antioxidant assay based on electron transfer. The method measures the reductive capacity of a sample by measuring the change in colour from yellow to a dark blue chromophore in the presence of phenolic compounds (Folin and Ciocalteu, 1927).

FC reagent consists of a blend of sodium molybdate, sodium tungstate, phosphoric acid, hydrochloric acid, lithium sulphate, bromine, and water. During the assay run, the FC reagent will react with phenolic compounds or non-phenolic reducing compounds to form hexavalent phosphomolybdic/phosphotungstic acid complexes. After the addition of sodium carbonate (Na_2CO_3), at basic pH (~10) the transfer of electrons will take place to form chromogens (colour forming compounds) that absorb light and can be detected spectrophotometrically between 750nm and maximum of 765nm (Singleton et al., 1999a, Folin and Ciocalteu, 1927, Magalhães et al., 2008).

The FC assay only provides an estimate of the total phenolic content (TPC) because in addition to phenols, the FC reagent may react with other reducing agents, thiols, and redox-active metal ions, inflating the final values, while the

existing OH groups and their position in the molecule determine the generated blue colour (Milella et al., 2014, Iswaldi et al., 2011, Birsan et al., 2019).

Several polyphenol standards can be used as reference to quantify the TPC in extracts, with gallic acid being the most used, and the results expressed in milligrams of gallic acid equivalent (mg GAE) per mL or mg of sample.

Total phenolic content (TPC) of BSG extracts was determined by colourimetric assays using FC reagent (Sigma-Aldrich, Arklow, IE), and a Shimadzu PharmaSpec UV-1700 spectrophotometer (Shimadzu Technology, Kyoto, Japan), following the method described by Singleton et all with small adaptation (Singleton et al., 1999a). The results are expressed in milligrams of gallic acid equivalent per gram BSG dry weight or milligram BSG extract (mg GAE/g BSG dw – mg BSGe).

2.5.2 Liquid Chromatographic Mass spectrometry

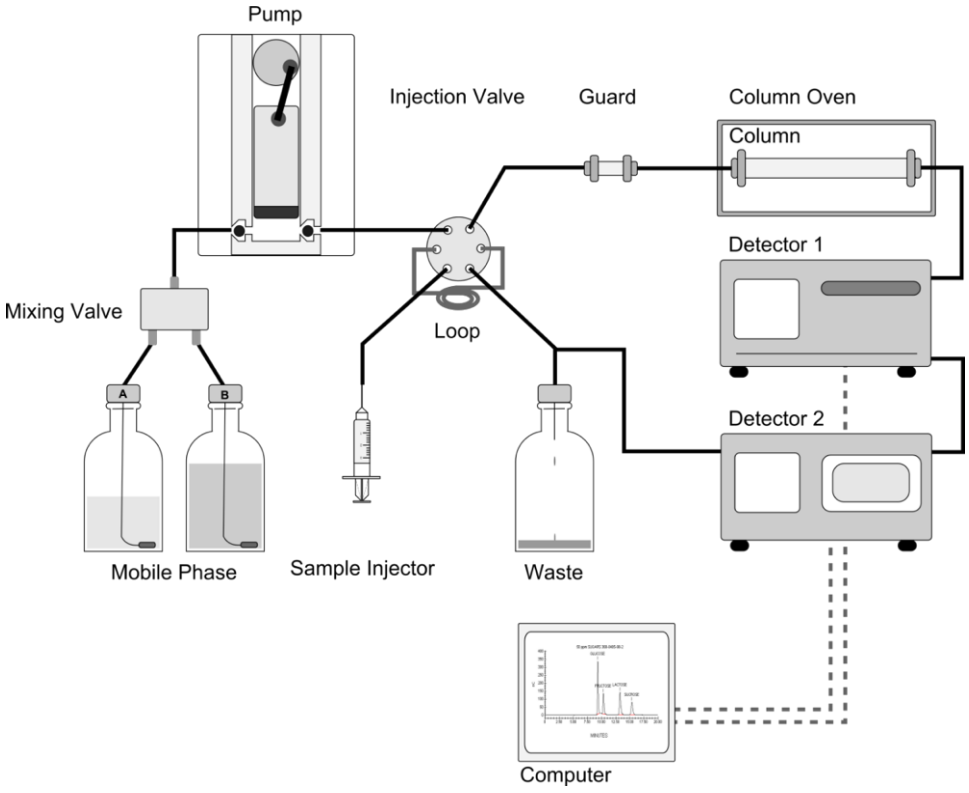
2.5.2.1 Ultra-/High-performance liquid-chromatography (UPLC, HPLC)

Liquid-chromatography (LC) is a well-established method for substance separation from a given sample. High-Performance LC (HPLC) is an effective technology for analysing a wide range of samples, based on a separation principle, with the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (column packed with pellicular or porous particles). The molecules are delayed when passing through the stationary phase, depending on the chemical structure of the analyte, leading to the separation of each component in a sample while they flow out of the column, eluting at different times. The HPLC separation of molecules from a sample is based on the primary characteristics of the molecules, such as polarity, electrical charge, and molecular size. An overall schematic layout of HPLC can be seen in Figure 2-11.

Normal phase and reversed phase are the two commonly used HPLC methodologies, with reversed phase being the most widely used for small molecular weight compounds. The difference between the two is that in normal phase, the silica packed column is polar while the solvent is not, but in reversed phase, the column has non-polar C8 or C18 covalently linked, and the solvent is

polar to mid-polar. Typical mobile phase in reversed phase is an aqueous or organic liquid, such as water, methanol, or acetonitrile. Also, the elution modes used in HPLC separation can be isocratic or gradient. The mobile phase, which may consist of either a pure solvent or a combination, stays constant throughout the run in isocratic elution, whereas in gradient elution the mobile phase composition varies during the separation (Waters(1), 2021). The mobile phase is pushed through the system by a precisely regulated pump that maintains a consistent pressure and flow rate. The stationary phase is chosen depending on the compounds to be separated. Separation is determined by the polarity of the compounds and its affinity to the selected column, which can be packed with silica containing moieties (in order of decreasing polarity) of cyanopropylsilyl- [CN], n-octylsilyl- [C8], and n-octadecylsilyl- [C18, ODS]. Moreover, separations can be made based on the electrical charge of the molecules by using columns containing anion or cation exchanger stationary-phase particles, and molecular size by using columns containing controlled-porosity silica packings (Waters). For a better separation, i.e. phenolic compounds, the addition of an acid (generally formic acid 0.1-0.5% v/v) in the mobile phase improves their separation by reducing ionization of both hydroxyl and carboxyl groups.

Figure 2-11 High-performance liquid chromatography schematic layout



Besides HPLC, Ultra-High Performance Liquid Chromatography (UHPLC or UPLC) is an advanced LC that operates at higher pressures (<15,000psi) compared to HPLC (<4,000psi). Based on the generated pressures between the two systems is the column particle size of the sorbent, where for HPLC the most common are between 5 and 2.5 µm, and for UPLC of <2 µm. The higher pressures and the lower particle size of the sorbent in UPLC offers significant improvement in the resolution, speed and sensitivity compared to HPLC.

Following the separation of the analytes in the column, the compounds can be detected using a variety of detectors, including mass spectrometry (MS), UV, Photo Diode Array (PDA), Refractive index (RI), etc.

2.5.2.2 Mass spectrometry

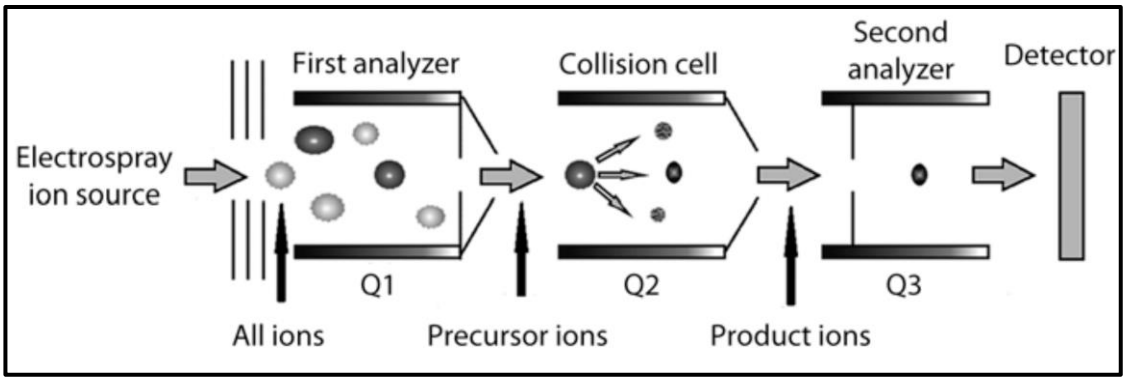
Mass spectrometry (MS) enables the separation of analytes according to the mass-to-charge ratio (*m/*z) of ions (*m/z*). Ions in gas phase are sampled into a mass analyser via a series of apertures for further separation and detection. The signals are displayed as a mass spectrum, which is a plot of intensity of ions (total ion current –(TIC)) against *m/z*.

Quadrupole Mass Spectrometers employ quadrupole mass analysers consisting of four conducting rods arranged in a symmetrical and parallel construction, with a space in the middle along which ions are conducted. The conducting rods are connected to power supplies that produce radiofrequency (RF) and direct current (DC). When a RF voltage is applied between one pair of opposing rods and a DC offset voltage to the other pair of opposing rods inside the quadrupole, an oscillating electric field is produced. While passing through the oscillating electric field in the quadrupole, ions are separated depending on the stability of their flight path. At a given set of RF and DC potentials, only ions of a specific *m/z* will exhibit a stable trajectory and reach the detector while the other ions will strike the rods and be neutralized. The RF and DC voltages may be adjusted such that the quadrupole functions as an ion mass filter or analyte-targeted detector for ions at specific *m/z.* Selective ion monitoring, in which the RF and DC voltages for certain ions of interest are applied, may be utilized to enhance sensitivity. Because fewer ions *m/z* are scanned during a single scan

cycle, a higher percentage of ions of the desired *m/z* reach the detector, resulting in improved sensitivity (Smith, 2013).

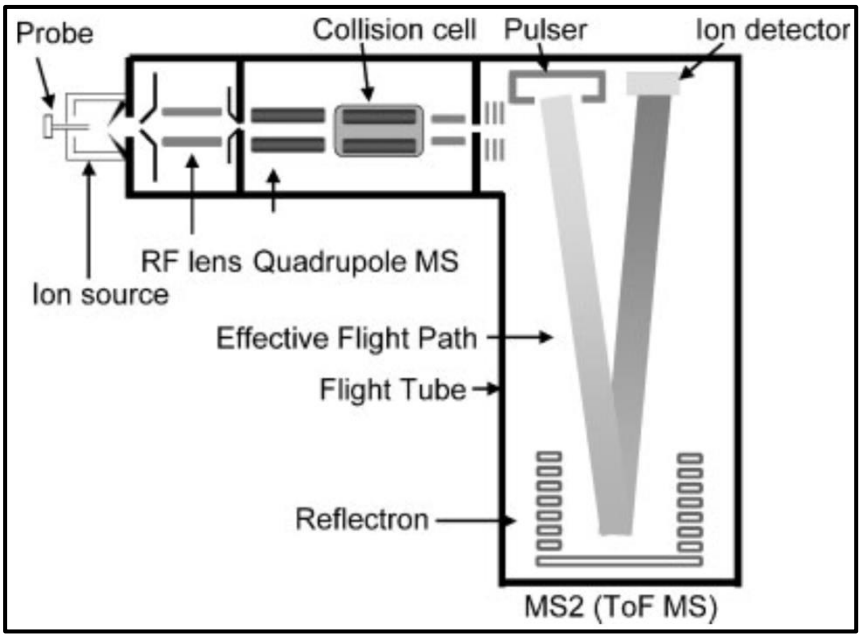
Tandem-Quadrupole Mass Spectrometer (TQD-MS) is a tandem mass spectrometer made up of two quadrupole mass spectrometers that are separated by a collision cell (Figure 2-12). The first quadrupole mass spectrometer selects precursor ions, which are subsequently fragmented in the collision cell through collision-induced dissociation by an inert gas (argon or nitrogen), and the final quadrupole mass spectrometer analyses and selects the product ions before passing them to the detector. This type of data collected is known as selected-reaction monitoring (SRM). However, when several transitions are observed during the chromatographic run, this is referred to as multiple-reactions monitoring (MRM). The benefits of TQD-MS include lower vacuum requirements, relatively fast and easy to operate and high-throughput analysis. Also, improved sensitivity and specificity, resulting in reduced detection and quantification limits.

Figure 2-12 Overall schematic layout of Tandem Quadrupole Detector (TQD) as described by Faktor, Dvorakova et al. (2012)



Time-of-Flight Mass Spectrometer uses an electric field of known strength to accelerate ions to the same kinetic energy, and then subsequently measures the time needed to reach the detector. Similar charged particles will present similar kinetic energies, while their velocities will vary according to their *m/z*. This means that ions with lower *m/z* will reach the detector sooner compared to higher *m/z* ions. Commonly, ToF analysers are preceded with a quadrupole (Q) mass analyser (Figure 2-13), which will allow mass-profiling across a wide molecular weight range, greater mass accuracy and mass resolution, higher sensitivity and dynamic range, fast acquisition, making this hybrid instrument a popular choice for detecting unknown analytes.

Figure 2-13 Overall schematic layout of Q-ToF-MS detector as described by (Lacorte and Fernandez-Alba, 2006)



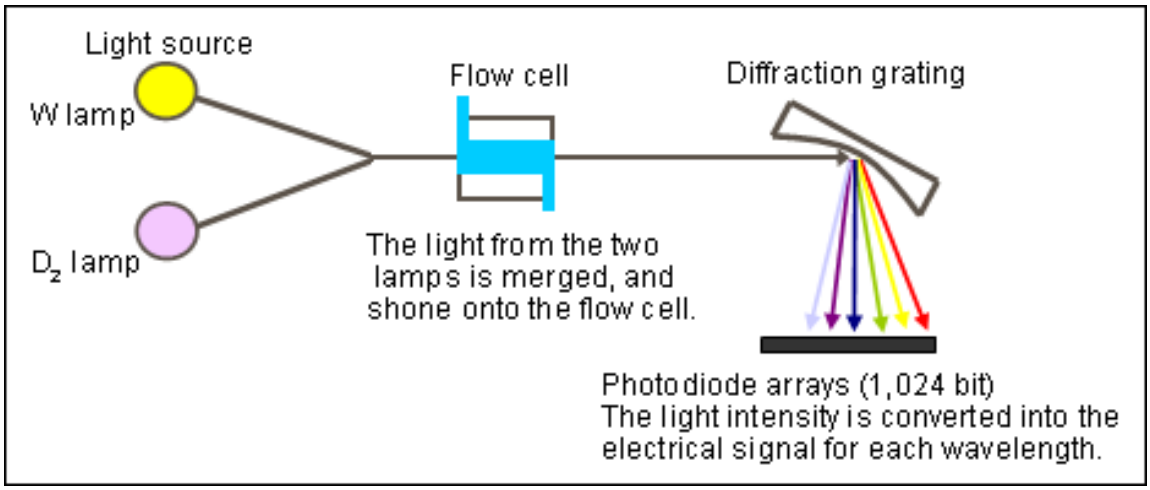
LC–MS analyses are becoming more common in all areas of analytical research, but this technique is often viewed as complex and requiring high operator skill. As a result, several considerations must be made when using an LC-MS system. One of the first issues is contamination, as not only affects the method (i.e. poor results caused by signal suppression) but as well the instrument (i.e. contamination of the ion sources by involatile components). pH is also a critical parameter that needs to be taken in consideration, and this can be done by controlling the pH of the mobile phases by using a volatile acid (i.e. 0.1% formic aid) or base (0.1% ammonium formate), depending on the column rated pH. Other things to be taken in consideration may be the benchmarking method and generated data (system quality control), infusion to obtain optimized parameters for the LC and MS for optimal signal of the analytes, among other (Watson, 2018a).

2.5.2.3 UV/Vis and Refractive index detectors

Photo-Diode Array detector (PDA) is an ultraviolet/visible light (UV/Vis) spectrophotometer, with an operating wavelength range of 190 to 800nm (Figure 2-14). After transiting the LC eluent through an optical flow cell, incident light passing through the sample is subsequently scattered while passing through a diffraction grating. The detector can then detect the quantity of scattered light for a variety of wavelengths. This allows for the recording of full absorbance spectra

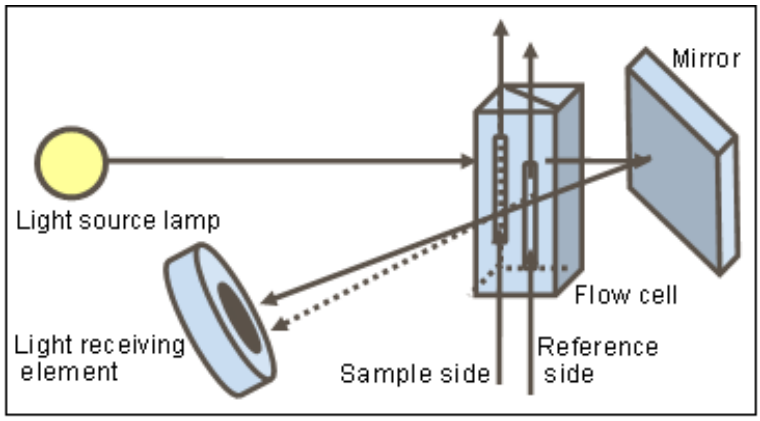
for each time point of the samples. The PDA is therefore able to provide a chromatogram over a wide range of wavelengths (where the sample is assumed to absorb significantly) at one time, and spectra for each time point. This implies that the purity of each peak may be examined to identify issues like co-elution of numerous compounds, and further the spectral profile may aid in the identification of unknown peaks in the chromatograms. This method detects components based on their ability to absorb UV light at any wavelength and can be used to identify a wide range of organic compounds, i.e. phenolic compounds, terpenoids.

Figure 2-14 Optical system layout of Photodiode Array (PDA) detector as obtained from Hitachi (2021)



Refractive index (RI) detector detects analytes based on their refractive index in a solution, and is in general used in the analysis of compounds with poor UV light absorption bands, i.e. carbohydrates, sugars (Figure 2-15). At the start, the RI detector equilibrates a split flow cell with the current utilised mobile phase. When this is finished, the reference side remains static while the sample is sent through the opposing side. The difference in the refractive index between the sample and the reference determines the angle at which the incident light is refracted. A spatial light detector is used to measure the change.

Figure 2-15 Optical system layout of RI detector as obtained from (Hitachi, 2021)



2.5.3 LC-based systems

LC-MS/MS analyses offer the possibility to identify, characterize and quantify chemical compounds present in plant-based extracts. HPLC coupled to a Q-ToF-MS/MS detector provides excellent dynamic range, high mass resolution and mass accuracy and has been used successfully in qualitative analyses, providing not only structural elucidation of compounds but as well characterization of the complex mixtures. On the other hand, UPLC coupled to a TQD-MS/MS detector provides high sensitivity, selectivity, specificity (MRM scan mode), a wide dynamic range of detection and reproducible spectra measurements, thus being successfully used for quantitative analyses of targeted compounds. Combination of LC-Q-ToF-MS/MS and LC-TQD-MS/MS analysers allows for wide screening and complete profiling for targeted and untargeted samples analysis, while the quantification of the chemical compounds can be accurately performed against commercially available standards. ToF-MS monitors the time an ion takes to traverse through a field-free zone, thus the longer the traverse time the better separation of the ions, with times taking up to 1 hour when using the Q-Tof with an HPLC system. On the other hand, triple quadrupole, which uses an electric field to segregate targeted ions with various m/z ratios, runs of even <10minutes can be performed. By using these two analysers, a quick, accurate, and thorough qualitative and quantitative analysis of the different chemical compounds in BSG extracts can be performed.

In this research project the most used LC-MS/MS systems were UPLC-TQD (Waters) for quantification of the BSG phenolic compounds, and HPLC-Q-Tof (Waters, sometimes coupled to a PDA detector) for characterization of the extracted phenolic and other unknown BSG compounds (Figures 2-16 A and B). Also, HPLC-RI was used for screening of monomeric sugars and polysaccharides that might be present in the BSG samples, and HP-SEC for molecular size separation of possible polymers present, respectively. A brief overview of the overall systems is presented below, and a complete description in each research chapter.

2.5.3.1 HPLC-Q-ToF mass spectrometry

Quadrupole time-of-flight (Q-ToF) Premier mass spectrometer coupled to Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) was used to profile various phytochemicals in the BSG fractions following the procedure previously described by (Hossain et al., 2010b).

2.5.3.2 UPLC- TQD mass spectrometry

Ultra-high performance liquid chromatography coupled to tandem quadrupole mass spectrometer (UPLC-TQD, Waters Corp., Milford, MA, USA) was used to quantify the BSG polyphenols by adapting the previous method used in raw barley (Gangopadhyay et al., 2016).

MassLynx^{TM} software was used to control the analytical systems, and Targetlynx^{TM} integration software (Waters Corp., Milford, CT, USA) was used for data acquisition, processing and reporting for the quantitative results of the compounds in the various extracts.

Figure 2-16 LC-MS/MS equipment used: **(A)** HPLC-ESI-Q-ToF, and **(B)** UPLC-ESI-TQD

A

B

2.5.3.3 HP-SEC-PDA/RI

High-performance size exclusion chromatography (HP-SEC) coupled to a RI and PDA detector was used to profile BSG extracts for molecular weight (MW) distribution of proteins and polysaccharides, and monomeric sugars. An Agilent 1100 series chromatography system coupled to a G1311A pump, G1313A automatic samples injector, guard column (SB-G 6; Shodex), a gel filtration column (SB-804 HQ, Shodex) and a RI detector G1362A (Agilent 1200 series) were used to screen for polysaccharides and monomeric sugars in BSG extracts, whereas for proteins a PDA detector (Waters 2996) at a wavelength of 254 nm was used (Figure 2-17). The mobile phase (isocratic) for sugar determination was a 0.1 M Tris HCl buffer (pH 8.0) with a flow rate of 0.5 mL/min and an injection volume of 20 μL for 30 minutes run. The MW was calculated by plotting the retention time vs log (MW) using standard β-glucan polymers with MW 35, 70, 229, 265, 391, and 650 kDa. The mobile phase (isocratic) for protein determination was a 0.1M Tri-HCl at pH7, with a flow rate of 1 mL/min and injection volume of 20 μL for 30 minutes run.

Figure 2-17 Agilent HPLC-SEC system coupled to PDA and RI detector

2.6 *In-vitro* Anti-cholinesterase and Antidiabetic enzymatic assays

To clearly identify possible compounds of optimum health, considerable research data on dietary components from epidemiologic, clinical, and mechanistic (*in-vitro*) perspectives would be necessary. It's possible that indicators will be chosen based on a specific extracted plant component, i.e. phenolics, if the process is shows to be transparent, scientifically robust, and the plant component has public health implications. Many *in vitro* screening assays have been designed to measure biological activities of compounds in specific organs or cell types. *In vitro* biological activity may reveal a mechanism of action or response that can be extrapolated to an in vivo end goal. The most straightforward approach to test a chemical for a specific mechanism of action at molecular level is to see whether it inhibits a specific enzyme or binds to a specific receptor or other biomolecule (National Academies of Sciences, 2015). Thus, *in-vitro* enzymatic assays were used to examine the inhibitive properties of BSG extracts and fractions against several enzymes' activities (cholinesterases and carbohydrases).

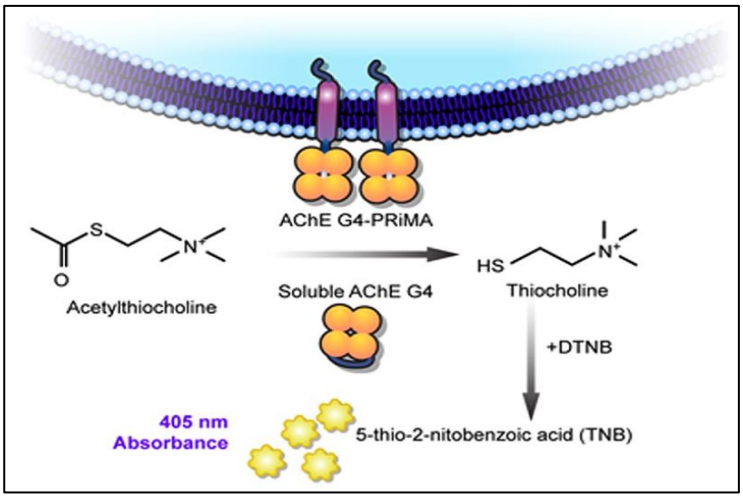
2.6.1 Acetyl- and butyrylcholinesterase activity assays

Cholinergic synapses contain acetylcholinesterase (AChE) which hydrolyses acetylcholine. On the other hand, a non-specific cholinesterase, butyrylcholinesterase (BChE) is primarily synthesized in the liver and found primarily in blood plasma, but as well nervous system cells express BChE. It's the relative preference for substrate that distinguishes AChE from BChE: AChE hydrolyses acetylcholine faster, while BChE hydrolyses butyrylcholine faster.

Ellman's method is widely used as a standard test for assessing acetyl- and butyrylcholinesterase (AChE, BChE) activities. The spectrophotometric method was developed by Ellman et. al in 1961 and is based on the enzymatic hydrolysis of thiocholine derivative (acetyl- or butyryl) by AChE (EC 3.1.1.7) or BChE (EC 3.1.1.8) to thiocholine (Figure 2-18). The resulting product reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), leading to the formation of yellow colouration (TNB - 5-thio-2-nitrobenzoic acid). Measurement of TNB can be done

spectrophotometrically by reading the absorbance between 405 or 412nm (Ellman et al., 1961, Li et al., 2017a). The Ellman's method consists of two stages: the first stage AChE or BChE breaks acetylcholine or butyrylcholine into thiocholine and acetic acid; the second stage DTNB reacts with thiocholine resulting in the production of TNB.

Figure 2-18 AChE activity described by Ellman's assay as obtained from Li, Huang et al. (2017)



Galantamine, a cholinesterase inhibitor and a commonly prescribed drug for treating Alzheimer's disease, is a standard drug that was used to compare the inhibitory effects of AChE and BChE activities with BSG extracts and was used as positive control.

The inhibitory potential of BSG extracts, fractions, blends and individual polyphenol towards anti-AChE and anti-BChE activities was determined in vitro by Ellman's colorimetric method (Ellman et al., 1961) and adapted to cuvettes following the procedure of Faraone et al., 2019 (Faraone et al., 2019).

2.6.2 α-amylase and α-glucosidase activity assays

α-amylase is a glycoside hydrolase enzyme that acts on α-1,4-glycosidic linkages to hydrolyse starch and glycogen, into shorter chain molecules, maltose and dextrins (Figure 2 – 18). The α-amylase (EC 3.2.1.1) hydrolyses the starch chain at different sites resulting in smaller oligosaccharides such as maltose (disaccharide), maltotriose (trisaccharide), and α-limit dextrin (a mixture of polymers of D-glucose units linked by α-(1,4) and α-(1,6) glycosidic bonds)(Jiang et al., 2019). 3,5-Dinitrosalicylic acid (DNS) reagent is commonly used to estimate the reducing sugars in solutions. DNS reagent is an aromatic compound that

reacts with the reducing sugars to form 3-amino-5-nitrosalicylic acid and reading the absorbance at 540 nm. Thus, the α-Amylase activity can be determined using the colorimetric method with DNS reagent.

Similarly, α-glucosidase (EC 3.2.1.20) is a glycoside hydrolase enzyme that acts on products of amylase hydrolysis, on the non-reducing α-1,4-linked D-glucose terminal of oligosaccharides or disaccharides (maltooligosacharides, amylose, amylopectin, soluble starch) with the release of simpler or monosaccharides (e.g., glucose, fructose) (Tomasik and Horton, 2012). The α-Glucosidase activity can be determined by the hydrolysis of 4-nitrophenyl-α-D-glucopyranoside substrate by α-glucosidase, resulting in the formation of a colorimetric (405 nm) product, which is proportional to the α-glucosidase activity present.

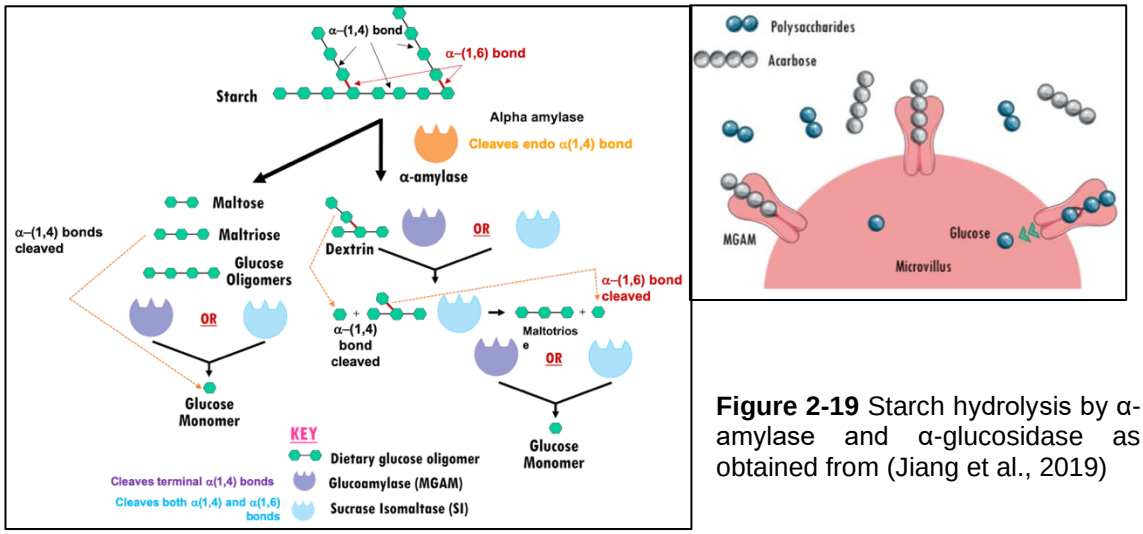


Figure 2-19 Starch hydrolysis by α-amylase and α-glucosidase as obtained from (Jiang et al., 2019)

Acarbose, a glycoside hydrolase enzyme inhibitor and a commonly prescribed drug for treating non-insulin dependent (type II) Diabetes Mellitus, is a standard drug used to compare the inhibitory effects of α-amylase and α-glucosidase activities with BSG extracts and was used as positive control. Both assays were adapted from following the methodology of Faraone et al., 2019 (Faraone et al., 2019).

The final concentration of samples, BSG extracts and positive control, in the final assay mixture was calculated by taking in the account a dilution factor, as follows: acetyl- and butyrylcholinesterase: 10, α-amylase: 30, α-glucosidase: 6.25.

Chapter 3

3. Conventional extraction and quantification of polyphenols from Brewer's spent grain light and dark

3.1 Abstract

The work in this first research chapter is focused on the extraction of phenolic compounds from two by-products of the brewing industry, light and dark BSG, by using a classic extraction approach. The evaluation of these classic extraction methodologies will provide a key for preliminary quantitative predictions of an acceptable extraction process, based on the type of the extraction solvent with the follow-up separation and enrichment techniques. Based on generated extracts, the phenolic content will be determined and ranked using quantitate and qualitative spectrophotometric and chromatographic techniques, to determine not only the total phenolic content but as well separating the extracts to identify and characterize individual BSG phenolic compounds. Three aqueous-organic solvents (80% acetone, methanol, ethanol) were tested for the extraction of free phenolics from BSG, whereas acid (ethanol + 25% HCl *v/v*) and alkali (2 M NaOH) solutions were tested for extraction of bound phenolics, and their enrichment using liquid-liquid extraction with diethyl ether:ethyl acetate and ultrafiltration using MWCO filter membranes. The best extraction parameters were used as benchmarks to further assess the effect of emerging extraction technologies, such as microwave and ultrasound as described in chapter 4, as well as in subsequent chapters.

3.2 Introduction

Food waste is a worldwide issue with negative effects on the environment, society, and economy. Globally, more than 25% of all food produced is wasted, and the society is constantly driving for efficiencies, food sustainability and to minimise waste production and associated pollution. The total amount of food waste generated in the European Union (2018) is estimated to be around 88 million tonnes/year (EC.EU, 2021), with Ireland (2018) generating around 1 million tonnes/ year (EPA, 2021) and UK (2018) around 9.5 million tonnes/year (WRAP, 2018). Food waste contributes significantly to climate change accounting for 8-10% of worldwide greenhouse gas emissions.

There is increasing dismay at the amount of waste generated by the agri-food processing chain. One of the major producers of organic waste from agri-food sector is the brewing industry. Spent grains, spent yeast, spent hops, and wastewater sludges are examples of solid residues and by-products generated after the barley malting and brewing by the beverage industries. It is estimated that for every 1,000 tonnes of beer produced, there is around 10,000 tonnes of liquid waste and ~173 tonnes of total solid waste. In other words, for every 100 L of beer produced results in around 1,000L of wastewater, 20kg of spent grain, 0.3kg of spent yeast, 0.3 kg of spent hops, and 3-5% of germ/roots from the total amount of barley, and other unwanted materials (Amoriello and Ciccoritti, 2021). Spent grains, generally called brewer's spent grains (BSG), is the most abundant beer brewing by-product, accounting for around 85% of all solid by-products generated by the brewing industry. BSG primarily consists of barley husk, parts of the pericarp and endosperm, and other residual components of barley grain (Mussatto, 2006b). In Europe, Germany tops the annual BSG generation with 1.8m tonnes, while UK and Ireland are on 4th (0.8m tonnes) and 11th place (~0.2m tonnes) in 2019 (Mussatto, 2006b, BrewersofEurope, 2019). With such high volumes of BSG generated that fetch an average €35/tonne, the exploitation of its valuable constituents could increase the market value of BSG for the food or pharmaceutical sectors (Lynch et al., 2016). Moreover, BSG is a lignocellulosic material rich in fibres, such as lignin (12-28%), hemicelluloses (20-25%, mainly arabinoxylans) and cellulose (12-25% β-(1,4)-linked glucose*)*, proteins (19-30%),

lipids (<10%), minerals (<5%), and phytochemicals, such as polyphenols (<3%) (Mussatto, 2006b, Lynch et al., 2016).

Phytochemicals in plants usually occur in low concentrations and that the different classes of phytochemicals would respond differently to different extracting solvents. For the extraction of polyphenols, generally aqueous alcoholic solvents are used (Dhanani et al., 2017). Solvent extraction efficiency depends on the solubility of the compounds in the solvent (polarity), particle size, time, temperature, and combined with ultrasound assisted extraction (high intensity and frequency sound waves) or other physical cell-disruption techniques increases the extraction yield of targeted compounds from the biomass (Dorta et al., 2012, Bartolomé et al., 1997). Additional chemical and/or enzymatic hydrolysis steps would release the phytochemicals bound to the cellular-wall components (Bartolomé et al., 1997).

BSG residue is a major source of health promoting compounds, such as β-glucan and phenolic compounds, which are shown to possess anti-obesity, anti-diabetic and antioxidant activity (Szwajgier and Borowiec, 2012, Steiner et al., 2015a, Bravi et al., 2021). The phenolic compounds, especially hydroxycinnamic acids (*p*-coumaric, ferulic, caffeic, sinapic and syringic acid) and hydroxybenzoic acids – (protocatechuic and 4-hydroxybenzoic acid) have been found in high abundance in BSG (Faulds et al., 2002b, Bartolomé et al., 2003). These phenolic acids can occur in free or bound forms, but dominantly in bound forms in BSG, where the phenolics interact with carbohydrates and proteins creating complex biomolecules (Quinde-Axtell and Baik, 2006a, Naczk and Shahidi, 2006b). Moreover, the polyphenol composition changes during the kilning process of the malt, as with increasing temperatures the Maillard reaction promotes the formation of melanoidins (polymers formed by the interaction between proteins and carbohydrates), which sometimes may involve polyphenols (Piggott et al., 2014, Yang et al., 2019). This process may reduce the content of free polyphenols as melanonids can trap polyphenols within their structure during the kilning process (Maillard and Berset, 1995). Depending on the drying or roasting conditions of the malt during the kilning process, a pale or light malt is obtained when mild drying is applied (70-80°C), whereas at higher drying temperatures

(200-230°C) a dark or chocolate malt is obtained, with the resulting BSG residue being called BSG light and BSG dark (Moreira et al., 2013).

Because of biomass recalcitrance and complexity of the lignocellulosic matrix, an efficient extraction method is necessary to generate extracts with high polyphenol yield. To this, a number of extraction processes, such as the conventional solid-liquid extractions and/or liquid-liquid extractions, have been tailored to extract phenolic compounds from BSG including maceration, or physical assisted extraction techniques (microwave or ultrasound-assisted extraction) and generally involved chemical and/or enzymatic hydrolysis to release the bound phenolics (Guido and Moreira, 2017, Naczk and Shahidi, 2006b, Bonifácio-Lopes et al., 2020b). The principles of these extraction methodologies have been described in Chapter 2. Solid-liquid extraction is among the most effective and common approaches used to extract phenolic compounds from BSG by using either organic solvents or acid and base solutions (Meneses, 2013, Mussatto et al., 2007a, Mussatto et al., 2007b). Organic solvents (i.e. ethanol, methanol, acetone) have been successfully used to extract free phenolic acids from BSG (Socaci et al., 2018, Zuorro et al., 2019). Ethanol has long been recognised as an excellent solvent for polyphenol extraction that is also safe to consume, methanol on the other hand has been found to be more effective in extracting low molecular weight polyphenols, whereas aqueous acetone may be use to extract high molecular weight flavanols (Dai and Mumper, 2010). Acid hydrolysis with hydrochloric acid (HCl) and saponification with sodium hydroxide (NaOH) at different concentrations (commonly used range of 0.1 to 2 M) is an efficient method for liberation of ester- and ether-linked phenolics (bound phenolic acids) from xylan, hemicelluloses and lignin components (Soccol et al., 2019, Macheiner et al., 2003). Following the solid-liquid extraction, a subsequent step is necessary to obtain enriched polyphenol extracts by using immiscible mid-polar range solvents, i.e. ethyl acetate, to pool polyphenols from the BSG extraction medium. Similarly, ultrafiltration or dialysis can be used in a parallel approach to fractionate phenolic compounds based on their molecular weight, using membranes with molecular weight cut-offs (MWCO) of different sizes (commonly used MWCO range of 1 to 100 kDa) (Suwal and Marciniak, 2018, Conidi et al., 2018, Tierney et al., 2013).

Furthermore, the determination of the polyphenolic content in BSG has been extensively performed using colorimetric assays, such as Folin-Ciocalteu. Unfortunately, because the assay is difficult to standardise and is non-specific, it has been suggested to be used solely for approximating the phenolic content of an extract. Past studies on BSG have largely shown the effect of the type of solvent on the extraction of antioxidant phenolic compounds using colorimetric methods, which crudely provide the total phenolic content based on the reactions of polyphenolic type compounds and the chemical reagent (Ainsworth et al., 2007, McCarthy, 2013, Meneses, 2013, Spinelli et al., 2016b). Due to this fact, hyphenated chromatographic methods, such as liquid chromatography tandem mass spectrometry (LC-MS/MS), HPLC-UV, have been the method of choice to separate and quantify accurately individual and complex polyphenolic compounds in the extracts(Meneses, 2013, Fărcaş et al., 2013b, Piggott et al., 2014, Zuorro et al., 2019, Singleton et al., 1999a). Generally, spectrophotometric approaches overestimate the phenolic content due to the interaction of other non-polyphenolic molecules, such as reducing sugars, with the FC reagent used in the assay (Escarpa and González, 2001, Sánchez-Rangel et al., 2013). A tandem quadrupole mass spectrometry instrument coupled to an LC system with the multiple reaction monitoring (MRM) scan mode offers higher selectivity, specificity and sensitivity whilst decreasing analysis times compared to spectrophotometric or HPLC-UV methods (López-Fernández et al., 2020, Zhao et al., 2018). This is why researchers have been discouraged in recent years from assessing polyphenols solely by spectrophotometric techniques (Granato et al., 2018, Harnly, 2017).

In this first experimental chapter, a classical approach to extract and enrich polyphenols from two types of BSG (light and dark) that have rarely been described for both free and bound phenolics. Besides the development of extraction procedures that decrease or eliminate the use of energy and petroleum solvents, maintaining a safe and high-quality extract, while following the EU and national regulations is necessary for potential upscaling (EUR-Lex, 2015, Bart, 2011). To this, the use of water with admixtures of acid or base, but also highly volatile solvents such as ethanol, acetone, that are considered safe to use in food has been encouraged. The objectives of this study were (1) to determine the

solvent(s) that yield high phenolic content, with or without chemical hydrolysis, (2) to investigate the suitability of different MWCO membrane filtrations for enrichment of polyphenols.

Hence, the effect of organic solvents (ethanol, methanol, and acetone) and chemical hydrolysis (acid or alkali) on the release of free and bound phenolics from BSG has been explored. Furthermore, to reduce other unwanted co-extracted compounds and further to obtain enriched phenolic extracts, the BSG extracts were subjected to liquid-liquid extraction followed by the application of various MWCOs membrane filtration. Finally, the phenolic contents in the various BSG extracts were estimated using spectrophotometric methods, whilst the quantification of individual polyphenols in these extracts was determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

3.3 Materials and Methods

3.3.1 Samples and Chemicals

BSG Light (L) and, Dark (D) were obtained from River Rye Brewing Company, Celbridge, County Kildare, Ireland. The BSG was taken directly from the hopper and transported to the research centre within 2h and stored at -20^{o}C. The frozen BSG was freeze-dried (Cuddon FD80, Cuddon Ltd., Blenheim, NZ), blended (Robot Coupe R3-3000 blender, Robot-Coupe Ltd., Isleworth, UK) and vacuum packed (VamaZS11, Vama Maschinenbau GmbH, Wildpoldsried, DE) until required.

The organic solvents (ethanol, methanol, acetone, hexane, diethyl ether, ethyl acetate, petroleum ether), and chemical reagents, NaOH, HCl and Folin-Ciocalteu were purchased from Merck (Arklow, Co. Wicklow, Ireland). Polyphenol standards of *p*-coumaric, *trans*-ferulic, sinapic, caffeic, protocatechuic, syringic and 4-hydroxybenzoic acid were purchased from Extrasynthese (Genay Cedex, France).

3.3.2 Proximate composition of BSG L and D

The proximate composition of BSG including moisture, lipid, protein, ash and carbohydrate (including total dietary fibre and β-glucan).

The moisture content of BSG substrates was determined by using an infrared drying balance (Sartorius MA160, Sartorius Lab Instruments, Germany), where raw BSG (triplicate samples) was added to a Sartorius aluminium disposable pan and spread evenly (<1g). The pan was then placed on the pan support of the drying balance and recorded the weight automatically. The drying balance was set at a temperature of 105°C, with automatic end of analysis, and the final weight was recorded when the weight loss per 24 s was below the automatically detected threshold (in mg, constant mass). The results were displayed as Moisture in % M (% moisture).

Protein content was determined in BSG samples by weighing out (0.2g dried, triplicate) into tin foil cup and placed in the carousel on the LECO, and detection of nitrogen content within four to five minutes. A nitrogen-to-protein conversion factor of 6.25 was used to convert the nitrogen content of the BSG samples to their protein content and expressed as percentage protein by mass.

For lipid content, BSG samples of known weight were added on ORACLE quartz fibre pads and dried using the SMART 6™ moisture and solid analyser. After drying, the samples were packed tight, added to ORACLE NMR tubes, and transferred to the ORACLE fat analyser. The BSG samples were tested in triplicate, and the results expressed as percentage fat by mass.

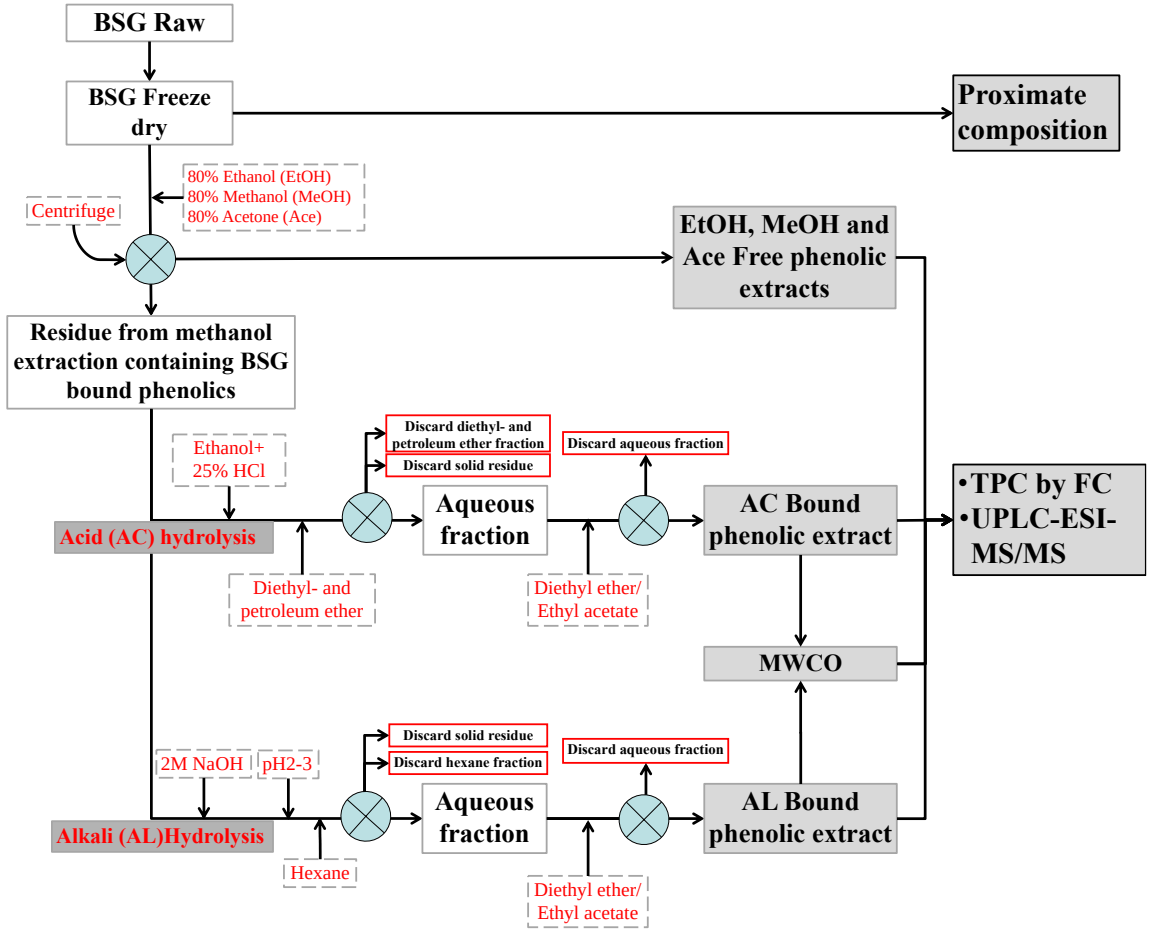
Total dietary fibre content (TDF) in BSG samples was determined by using the ANKOM FBT Dietary Fibre Analyser (ANKOM Technology, NY, USA), automated version of AOAC 991.43 and AACC 32.07.01 methods. To determine the TDF content, the mixture is treated with alcohol (ethanol) to precipitate the soluble dietary fibre prior filtering, followed by washing of the TDF residue with ethanol and acetone, dried and weighed. To determine the insoluble dietary fibre (IDF) content, the enzyme digestate is filtered and the residue, representing the insoluble fibre part, is washed, dried, and weighed. To determine the soluble dietary part (SDF), the filtrate and washes are precipitated with ethanol, filtered, dried, and weighed. The mass of the residual material (TDF) was compared to the mass of the original material and expressed as percentage TDF by mass.

The β-glucan content was determined using a β-glucan mixed linkage assay kit (K-BGLU, Megazyme, Bray, Co. Wicklow, Ireland) following AOAC 995.16 protocol.

Ash content was determined by charring BSG samples (2-3g dried, triplicate) added to pre-dried and pre-weight crucibles in muffle furnace at 600°C for 4 hours (AOAC 923.03). The mass of the residual material (ash) was compared to the mass of the original material and expressed as percentage ash by mass.

A diagram showing the overall experimental approach used in this chapter is shown in Figure 3-1 along with the extraction methodology adapted from the previous work of Verardo et al, 2011, with slight modifications.

Figure 3-1 Methodology of extraction, enrichment, and analysis of polyphenol in free and bound phenolic extracts from Brewer's spent grain light and dark.



BSG – brewer's spent grain, MWCO – molecular weight cut-off, NaOH - sodium hydroxide, HCl – hydrochloric acid, AC – bound phenolic extracts obtained by acid hydrolysis, AL – bound phenolic extract obtained by alkali hydrolysis, TPC by FC – total phenolic content by Folin-Ciocalteu reagent, UPLC-ESI-MS/MS – ultrahigh performance liquid chromatography – electrospray ionisation – mass spectrometry.

3.3.3 Extraction of free phenolics

The extraction of free phenolics from BSG L and D was performed by using 80% organic solvent solutions. The method consisted of mixing 4 grams of freeze dried BSG (freeze drying protocol see Chapter 2, section 2.3) with 40mL of 80% aqueous organic solvent (1:10 w/v) in a 50 mL tube and sonicated (Branson 3510, 42 kHz) for 10 minutes at room temperature to extract the free phenolics. Three different organic solvents were used: ethanol, methanol, and acetone. Following the extraction period, the tubes were centrifuged for 10 minutes at 9 484 g (Sigma 2–16KL, Osterode am Harz, Germany). The supernatants were pooled, and concentrated in a rotatory evaporator at 37°C. The concentrated extracts were reconstituted in 50% methanol, syringe filtered through 0.45 µm PTFE filters, transferred in 30 mL tubes and dried under nitrogen gas. The pellets from the methanolic extraction (called MeOH1 and MeOH2), following the centrifugation step, were dried in the fume hood overnight and used further for chemical hydrolysis to extract bound phenolics (see below). The extraction of free phenolics (crude extract) was carried out in duplicate for both BSG L and D (12 extracts) and stored at 4°C prior to use.

3.3.4 Extraction of bound phenolics

The extraction of bound phenolics from BSG L and D was performed by using acid and alkali solutions, followed by liquid-liquid extraction with organic solvents as illustrated above in Figure 3-1.

3.3.4.1 Alkaline hydrolysis

The dried pellet from MeOH1 extraction (Figure 3-1) was mixed with 200 mL of 2M NaOH aqueous solution, and kept shaking at 170 rpm, 22°C for 20 h (MaxQ 6000 Shaker, Thermo Fisher Scientific, MA, USA). The mixture was then filtered, and the aqueous part was neutralised to pH 2-3 by adding 10 M HCl (50-60 mL) and subsequently subjected to liquid-liquid partitioning. The aqueous fraction was mixed with hexane (2 x 250mL) to remove lipids. The hexane layer was discarded, and the aqueous fraction was further extracted five times with 100 mL diethyl ether:ethyl acetate (1:1 *v/v*). The organic fraction containing free phenolics was syringe filtered and concentrated in a vacuum-rotavapor at 37°C.

The concentrated extract was reconstituted in methanol and dried under nitrogen gas.

3.3.4.2 Acid hydrolysis

The dried pellet from MeOH2 extraction (Figure 3-1) was mixed with 24 mL of 96% ethanol (EtOH) and 120 mL of 25% HCl at 65°C for 30 min. under shaking. Following this, the mixture was cooled to 40-60°C, and 40 mL of 96% EtOH and 50 mL diethyl ether:petroleum ether (1:1 *v/v*) were added. The mixture was let to settle, the organic layer was discarded, and the pellet was washed twice with diethyl ether: petroleum ether (1:1 *v/v*), at 40-60°C to remove lipids. The lipid-free mixture was then filtered, and the aqueous fraction was mixed five times with 100 mL of diethyl ether: ethyl acetate (1:1 *v/v*). The organic fraction containing the bound phenolics was filtered and concentrated in a vacuum rotavapor at 37°C. The concentrated extract was reconstituted in methanol and dried under nitrogen gas and stored in the freezer until further use.

3.3.5 Enrichment of the hydrolysed extracts

The diethyl ether: ethyl acetate extracts from the alkali and acid hydrolysed BSG L and D were subjected to separation using a 100 kDa, 10 kDa and 3 kDa molecular weights cut-off (MWCO) centrifuge filters at room temperature. The dried extracts were reconstituted in 50% methanol and diluted to a final concentration of 10ppm. A 15mL aliquot of the extract was subjected to MWCO separation using 100 kDa, 10 kDa and 3 kDa Amicon Ultra15 centrifugal filters (Amicon®Ultra, Millipore UFC, Merck DE) in a Sigma 2–16KL centrifuge (Sigma, Osterode am Harz, Germany) at 5 000*g* for 15 to 30 min. at room temperature. Following the centrifugation step, the permeate was collected to obtained MWCO fractions of <100 kDa, <10 kDa and < 3kDa, respectively. The MWCO fractions were then stored in a freezer at -28ºC until further use.

Similarly, ceramic membranes with a MWCO of 15 kDa and 1 kDa have been used. The liquid sample (>100 mL) was placed in a glass bottle, the pump was primed with part volume of the sample and turned on. The liquid sample for separation was pumped through the channel containing the active membrane layer. The membrane layer separates all the material being bigger than the membrane pores and the filtrate penetrates through membranes layer and

support. The boundary layer thickness is controlled by cross flow, with the pressure adjusted between 1 and 2 bar, and a running time of <2 hours. The permeate with MWCO of <15 kDa, <5 kDa, <1 kDa was collected in a glass bottle through the orifice in the channel support and stored in in a freezer at -28ºC until further use.

3.3.6 Determination of total phenolic content by Folin-Ciocalteu

The crude and hydrolysed BSG L and D extracts were examined for their total phenolic content (TPC) by colorimetric assays using Folin-Ciocalteu (FC) reagent.

In 1.5 mL Eppendorf tube, 100 µL of extract was mixed with 100 µL each of methanol and FC reagent, and 700 µL of 20% sodium carbonate solution. The tubes were vortexed and incubated for 20 min. in darkness at room temperature. After the incubation, the tubes were centrifuged at 13,000 rpm for 3 min. to remove turbidity. Following this, 200 µL of the reaction mixture was transferred into 96-well micro plate and measured for absorbance at 735 nm using a Shimadzu spectrophotometer. Different concentrations of gallic acid as standards were used (10–300 µg/mL in 50% methanol) to prepare a calibration curve. The results are expressed in milligrams of gallic acid equivalent per gram BSG dry weight.

3.3.7 LC-MS/MS Identification and Quantification of BSG Phenolic Compounds

The identification and quantification of individual free and bound phenolic compounds in the crude, hydrolysed and MWCO extracts were achieved by using an Acquity ultra-high-performance liquid chromatography (Waters, USA) coupled to electrospray ionisation tandem- mass spectrometry (UHPLC-ESI-MS/MS) as mentioned in Chapter 2, section 2.5.3.2.

Separation of the phenolics was carried out on an Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm). The mobile phase consisted of milliQ water (solvent A) and acetonitrile (solvent B) both containing 0.1% formic acid, with the following gradient: 0–2.5min 2%B, 2.5–3min 10-15%B, 3–6min 15% B, 7.5–8.5min 35%B, 8.5–9.5min 98%B and 9.5–10.0min 2%B at a flow rate of 0.5 mL/

min. for 10 min. The injection volume for all the samples was 5 µL. The column temperature was set at 50 ˚C, while the samples were kept at 4 ˚C. The ESI source was set in negative mode and the quantification of each compound was performed using multiple reaction monitoring (MRM) method, where the first quadrupole was set to scan certain precursor ion mass-to-charge (*m/z*) ratio and the third quadrupole to scan structurally distinct product ions (*m/z*). For screening the phenolic compounds in the BSG samples, a pre-existing database of MRM transitions, which included the most often detected phenolics in barley, was employed. The ESI source conditions used: capillary voltage 3kV, cone voltage 35V, extractor voltage 3V, source temperature 120°C, desolvation temperature 250°C, desolvation gas - nitrogen, desolvation gas flow 800 L/h, cone gas flow 50 L/h, and collision gas flow 0.1mL/min.

For the quantification of the most abundant phenolic compounds, 7 phenolic standards were prepared (see Table 3-1 below). A stock solution (1 mg/mL) for each standard was prepared and appropriate dilutions covering the range of 0.05 ppm to 100 ppm were made to obtain the standard curve. Targetlynx^{TM} software (Waters Corp., Milford, USA) was used to process the data and quantify the compounds in the various extracts. The results were expressed as milligram per gram BSG dry weight (mg/g BSG dw). The ferulic acid dimers and trimers were quantified using the standard curve from ferulic acid.

Table 3-1 Phenolic acids prepared for quantification by using an ultra-performance liquid chromatography triple quadrupole mass spectrometry (UPLC-TQD MS) system, with their corresponding molecular weight formula, retention time, multiple reaction monitoring and detector energy separation parameters.

Standards	Molecular Formula	Retention Time (min)	MRM (*m/z*)	Cone Voltage(V)	Collision energy(eV)
Ferulic acid	$C_{10}H_{10}O_4$	5.52	192.9 →133.9	31	16
			→177.9		12
p-coumaric acid	$C_9H_8O_3$	4.70	163.0 →118.9	25	14
Syringic acid	$C_9H_{10}O_5$	4.07	197.1 →152.9	31	12
			→181.9		14
4-hydroxybenzoic acid	$C_7H_6O_3$	3.39	137.0 → 64.9	29	26
			→ 92.9		14
Sinapic acid	$C_{11}H_{12}O_5$	5.76	223.1 →120.9	32	26
			→163.9		14
Caffeic acid	$C_9H_8O_4$	3.80	179.0 → 78.9	35	24
			→134.9		16
Protocatechuic acid	$C_7H_6O_4$	2.63	153.0 → 80.9	29	8
			→108.9		14

3.3.8 Statistical analysis

Differences between means were analysed using one-way analysis of variance with post-hoc Tukey test (IBM SPSS Statistics 24). Where the values *a, b* is missing from the data tables or graphs, no statistical analysis has been performed due only single extraction being performed and thus no mean could be obtained to be able to statistically compare the extraction yields, TPC values and quantification data on the bound phenolic extracts.

3.4 Results and Discussion

3.4.1 Proximate chemical composition

BSG L and D were subjected to proximate chemical composition analysis to determine the moisture, protein, carbohydrate, fat, ash, β-glucan, and total dietary fibre content (see Table 3-2). The results were expressed as % on BSG dry weight basis (% dw), except for the moisture content. Moisture content in the raw BSG L and D were at similar levels of approximately 75% with no statistically significant differences ($p<0.05$). BSG L presented a higher ($p<0.05$) content of proteins (27%) and total dietary fibre (TDF, 56%) compared to BSG D (24% and

45%, respectively). However, the carbohydrate, fat, and ash content were not significantly higher ($p<0.05$) in the BSG D compared to BSG L. The β-glucan content, on the other hand, was significantly higher in BSG D (1.6%) compared to BSG L (0.9%). Differences in chemical composition of BSG may be due to the variations in the brewing process, such as the kilning, wort maceration and filtration processes, and as well differences in the various barley cultivates (Santos et al., 2003).

Table 3-2 The proximate composition of Brewer's spent grain light and dark

Parameters	BSG Light (%dw)	BSG Dark (%dw)	Literature** (%dw)
Protein	27.0±0.2[a]	23.6±0.03[b]	14.2-26.7
Carbohydrate*	66.5±1.0[a]	69.3±0.7[a]	n.a.
Total Dietary Fibre (TDF)	56.2±2.8[a]	44.8±2.2[b]	59.1-84.1
β-glucan	0.9±0.1[b]	1.6±0.1[a]	1.0-2.0
Fat	5.1±0.1[b]	5.6±0.1[a]	3.0-13.0
Ash	1.4±0.7[a]	1.5±0.6[a]	1.1-4.6
Moisture	75.6±0.5 (% w/w)[a]	74.4±0.8(% w/w)[a]	70.0-80.0 (% w/w)

*Means with different letters (a, b) in the same row are significantly different at $p<0.05$ (n=2); carbohydrate content was calculated by difference [%carbohydrate=100-%(protein+fat+ash)]; TDF was calculated by sum of cellulose, hemicellulose and lignin; n.a. = not available; **(Mussatto, 2006b, Lynch et al., 2016)*

The above results show that the main constituent of BSG is carbohydrate, ranging between 66% to 70% in BSG L and D followed by protein (~ 27 to 23%) and fat (~5 to 6%), respectively. TDF is the major constituent of the BSG carbohydrates, representing ~83% in BSG L and ~65% in BSG D, whereas β-glucan was found at the close levels between these two types of BSG.

The micronutrient profiles of BSG samples were found to be in the same range as previously published studies, as shown in Table 3-2. More than half of the BSG dry mass comprises of carbohydrates, followed by a variation in protein (14-27%) and fat (3-13%) content. β-glucan and ash were present at low levels, varying between 1 to 2% and 1 to 5%, respectively.

The high moisture content is expected as BSG soaks up water during the brewing process and acts as a filter to clarify the wort during the lautering step. On the other hand, the reason behind the high variation in the chemical composition of BSG could be related to barley variety and brewing conditions. As the barley variety, growing conditions and harvesting time, and further the specific brewing steps, which include the type and proportion of barley used (mix of

different varieties), the type of adjuncts used, each step being specific for the individual brewing houses (Mussatto, 2006b).

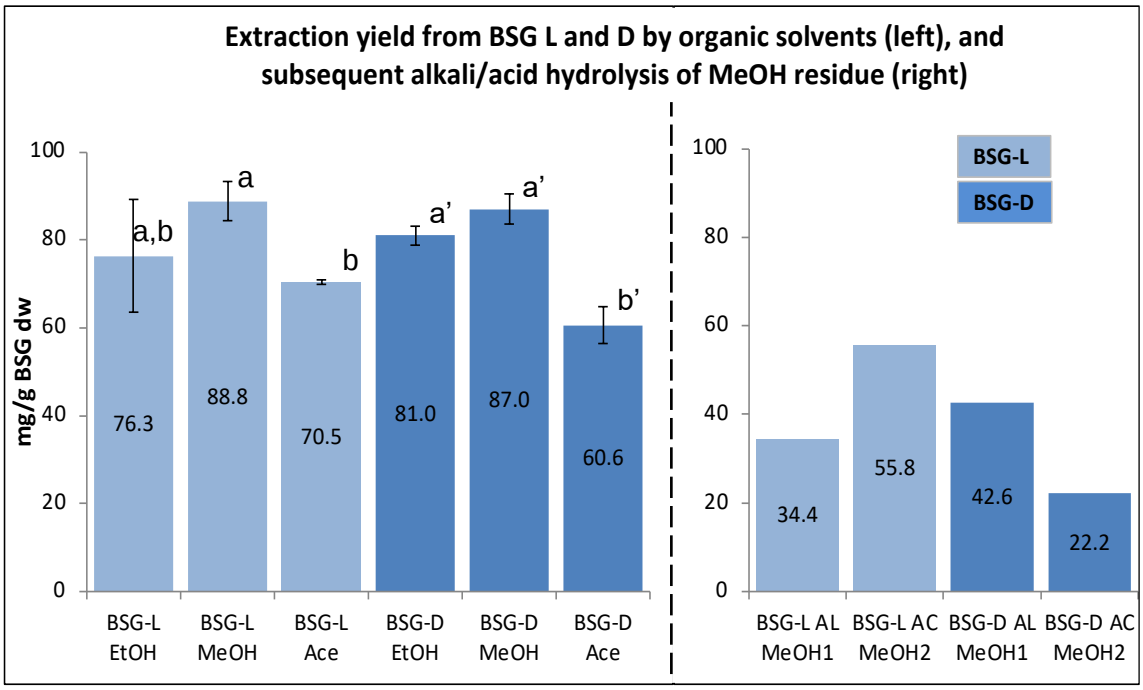
3.4.2 Extraction yield

The major goal of the extraction was to obtain phenolic compounds and increased extraction yields while trying to reduce the concentration of unwanted components from BSG, such as sugars or proteins. As one process does not suffice, pre-treatment, extraction, isolation, and fractionation are all necessary procedures in the recovery of phenolic compounds from BSG. The first two processes are critical since they determine the final product's yield. BSG is better broken down and extracted more effectively when pre-treatment is applied as it facilitates solvent access to the vacuoles and several other storage structures. For example, maceration, grinding, and homogenization of the material until a desired particle size is achieved boosts the solvent accessibility to cell wall structures. To enhance the recovery yields, chemical pre-treatments such as acid or alkaline hydrolysis was coupled with organic solvents or membrane filtration in the extraction of phenolic compounds from BSG.

In this research chapter with the applied extraction methodology, a maximum extraction yield (the dry weight of the solid part obtained after evaporating the solvent used as extractant) of around 10% from BSG (dw) was obtained using organic solvents (Figure 3-2). Figure 3-2 consists of two sets of column graphs showing the obtained extraction yield using organic solvents (left side) and chemical hydrolysis (right side). The extraction yield prior to chemical hydrolysis varied from 60.6±4.2 to 88.8±4.4 mg/g BSG dw basis (Figure 3-2). The highest yield was obtained using methanol as extractant solvent, with 88.8±4.4 mg/g from BSG L and 87.0±3.5 mg/g dw from BSG D, whilst ethanol and acetone showed a lower extraction yield, with 76.3±12.9 and 70.5±0.5 mg/g dw in BSG L, and 81.0±2.1 and 60.6±4.2 mg/g dw in BSG D, respectively. All three organic solvents presented a similar trend on the extraction yield in both BSG L and D, with acetone presenting the lowest extraction yield, whereas methanol and ethanol were significantly higher ($p<0.05$, $n=2$), but at similar level to each other. Since methanolic extracts resulted higher yield from both BSG L and D, chemical hydrolysis on the pellets after the extraction was pursued. Acid hydrolysis

generated better yield from BSG L, but the alkali hydrolysis provided a higher yield in the BSG D. The extracts following chemical hydrolysis were generated in single experiments, thus no statistical analysis could be made.

Figure 3-2 The efficiency of organic solvents on free phenolic extraction and chemical hydrolysis on bound phenolic extraction yield from Brewer's spent grain light and dark.



MeOH1 and MeOH2 are the residual pellets obtained following organic solvent extraction and used further for chemical hydrolysis extraction, with MeOH1 residue used for alkali hydrolysis (BSG-L AL MeOH1 and BSG-D AL MeOH1 samples), and MeOH2 residue used for acid hydrolysis (BSG-L AC MeOH2 and BSG-D AC MeOH2 samples). AC – acid hydrolysis, AL – alkali hydrolysis; Values in the graph (a, b; a', b') on the same column of BSG L (light blue) and BSG D (dark blue) with each individual solvent type which do to share the same letter are significantly different ($p<0.05$)

Organic solvents presented a higher extraction efficiency on a BSG dry weight basis compared with chemical hydrolysis extraction in both BSG L and D. This may be because chemical compounds (i.e., starch, amino acids, peptides, lipids, including polyphenols), that are polar or mid polar, dissolve in water and organic solvents might have been recuperated in the first extraction step. As this was a sequential extraction, the remaining part of BSG consisted mainly of polymers (i.e., cellulose, lignin) that do not solubilize easily due to the plant cell wall recalcitrance. Chemical hydrolysis on the other hand, increases the extraction rate and promote depolymerization that further would release the bound phenolic compounds from the cellular-wall components, and thus obtaining a higher extraction yield. One aspect that needs to be taken in consideration is that the organic solvent extraction was repeated three times for each substrate compared to chemical hydrolysis extraction which was performed

in singles. In this case, if the chemical hydrolysis step was not performed accordingly (best extractions parameters – extraction repeats, temperatures, time etc.), the subsequent liquid-liquid partitioning (water: organic solvent phases) will not improve the polyphenol extraction yield. It is expected that the polyphenols would be present and recovered in the organic solvent phase, whereas the water phase, might have contained the most of the depolymerisation constituents of cellulose, hemicelluloses etc. were discarded. This may also be a factor in the lower extraction yield obtained using the chemical hydrolysis. Other factors that might have affected the extraction efficiency is the acid/alkali concentration, temperature, and the extraction time. Mussatto et al. (2007) in one of their studies showed that a higher concentration (1 to 2% w/v) of alkali and temperatures (80°-120°C) are a better option to obtain a higher extraction yield (Mussatto et al., 2007a). Temperature at which alkali hydrolysis was performed in this extraction methodology was at room temperature and this might have impacted the lower extraction yields as well. Lastly, the surface contact between samples and extraction solutions is enhanced when particle size is reduced. Prior to this extraction process, the only available blender was used to grind the freeze-dried BSG, which would not produce fine powder, but rather into coarse smaller particles. Milling, on the other hand, would transform BSG into powdered sample with smaller and more homogeneous particles, allowing for greater surface contact with extraction solutions and further facilitating to obtain higher polyphenols extraction yields.

Even though higher extraction yields were obtained using organic solvents without hydrolysis, the concentration of polyphenols in these extracts is much lower compared to chemical hydrolysis, as the most phenolic compounds in BSG are present in bound form as supported by data in the subsequent sections (Quinde-Axtell and Baik, 2006a, Naczk and Shahidi, 2006b).

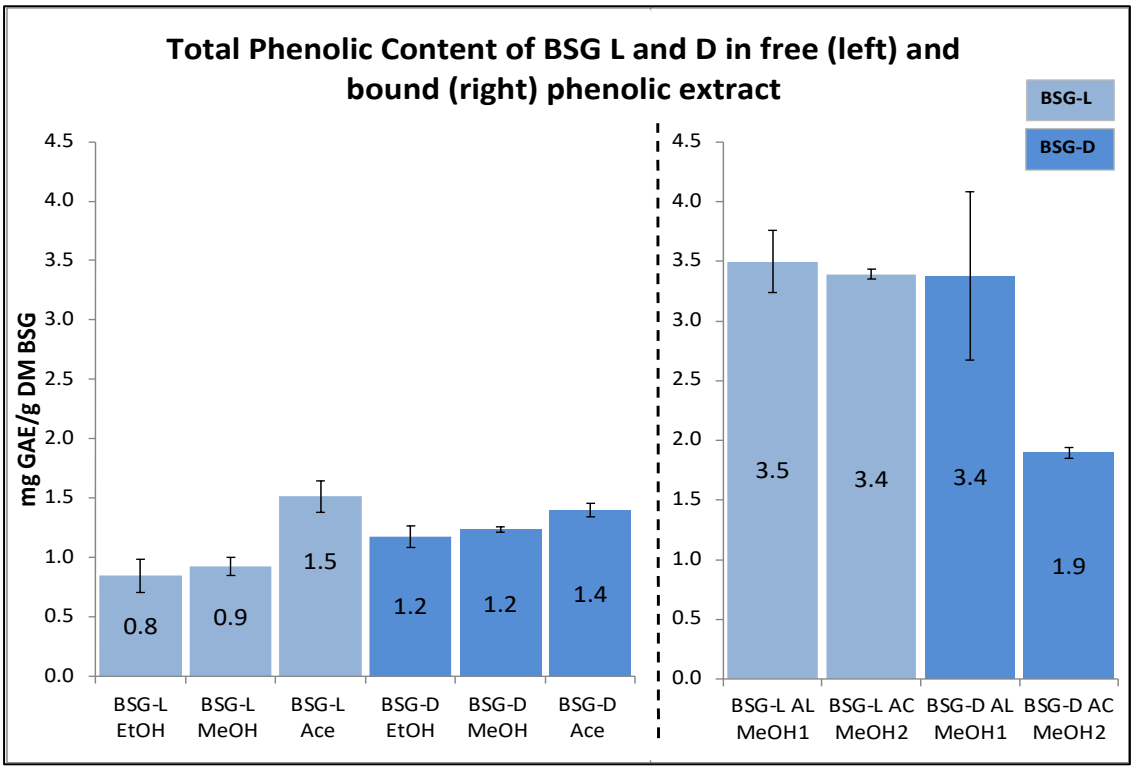
3.4.3 Total antioxidant activity of BSG extracts

As postulated above, a greater extraction yield would not always reflect a larger concentration of phenolic compounds, since certain circumstances may favour the extraction of other matrix components that are solubilized according to the extraction approach. These would include non-phenolic molecules such as

lipids, terpenes, waxes, amino acids, and others, which are undesirable. Thus, a cheap, fast, and relatively simple technique would be to test the BSG extracts for phenolic content using a common colorimetric assay to determine the total phenolic content (TPC) by using the Folin-Ciocalteu (FC) reagent. TPC is an important parameter that also correlates to antioxidant capacity of the extracts (Hossain et al., 2010a, Tzima et al., 2020). TPC forms the frontline assay to determine the content of plant phenolics. Nevertheless, FC reagent does not only react with phenolic compounds present in the extracts but also with other interfering compounds, i.e., sugars, amino acids, which may result in false positive results overestimating the actual phenolic content. For this reason, extra separation steps, MWCO membrane filtration (1kDa, 3kDa, 10kDa, 15kDa and 100kDa), were introduced with the aim of reducing the possible interfering compounds.

The TPC estimates in BSG L and D extracts obtained by using organic solvent (left side) and chemical hydrolysis extraction (right side) are presented in Figure 3-3. TPC levels, expressed in mg GAE/g BSG dw, in BSG L and D varied from 0.8 to 1.5 mg GAE/g, when using organic solvents, and 1.5 to 3.5 mg GAE/g, when using chemical hydrolysis. Among the organic solvents used, acetone performed significantly ($p<0.05$) better (~1.5 mg GAE/g) compared to methanol (0.9 mg GAE/g) and ethanol (0.8 mg GAE/g) in BSG L, whereas no significant differences were observed in BSG D between the three organic solvents used (1.2-1.4 mg GAE/g). TPC levels in both BSG L and D presented a similar trend, with increasing concentration in the order: ethanol < methanol < acetone. On the other hand, alkaline hydrolysis improved the TPC levels in BSG L and D, with almost 4 times than the non-hydrolysed extracts, while TPC in the acid hydrolysed BSG D improved slightly. The TPC levels in BSG L and D were at similar levels of ~3.5 mg GAE when using acid and alkali hydrolysis, whereas acid hydrolysis resulted in almost 2 times lower TPC levels of ~1.9 mgGAE/g, respectively, in BSG D.

Figure 3-3 Total phenolic content in the free and bound phenolic extracts of Brewer's spent grain light and dark.



EtOH -Ethanol, MeOH – Methanol, Ace – Acetone, AL – alkali hydrolysis, AC – acid hydrolysis, mgGAE/g DM BSG – milligram of gallic acid equivalent per dry mass BSG; Values in the left graph (a, b,; a') of the solvent extraction on the same column of BSG L (light blue) and BSG D (dark blue) with each individual solvent type which do to share the same letter are significantly differently ($p<0.05$); no statistical analysis performed on the right graph due to single extractions by chemical hydrolysis, whereas the standard deviation bars are from the technical replicate

The results obtained by using the FC colorimetric technique agrees with the other studies presented in the literature (Bonifácio-Lopes et al., 2020a, Vellingiri et al., 2014, López-Linares et al., 2021, Guido and Moreira, 2017) however, some authors have presented a much higher TPC using similar extraction processes. Amongst all extractions using 80% organic solvents, Meneses et al. reported in one of their studies that methanol resulted in the highest levels of TPC of 6.46 mg GAE/g dw, followed by ethanol and acetone at around similar levels of 5.5 mg GAE/g dw. It is worth mentioning that in their study the generated extracts with 60% (v/v) acetone had the highest level of total phenolics of 9.90±0.41 mg GAE/g dw (Meneses, 2013). Vellingiri et al. (2014) utilised a two-stage solvent extraction with 80% ethanol and reported that the second extraction phase resulted in a less concentrated extract in terms of TPC than the first extraction step, with 0.652 mg GAE/g dw and 1.451 mg GAE/g dw, respectively (Vellingiri et al., 2014). McCarthy et al. (2013) reported TPC values of 1.26 and 4.53 mg GAE/g DW in pale and black BSG, whilst using acidified methanol (McCarthy et

al., 2013b). The aqueous-solvent extraction with 50% methanol and acetone from BSG in Stefanello et al. (2018) study showed three times higher levels of TPC when using acetone (~3.4 mg GAE/g) vs methanolic (~1 mg GAE/g) extraction (Stefanello et al., 2018a). The highest TPC levels reported in the literature were by Moreira et al. (2013) where alkali hydrolysis with NaOH (0.75%) was used in combination with green extraction technologies (microwave assisted extraction, MAE), obtaining as high as 20±1 mg GAE/g dry BSG from BSG L and 16±0.6 mg GAE/g dry BSG D, respectively (Moreira et al., 2013). In their extraction process no organic solvent or other enrichment step was used. Similarly, Stefanello et al. (2018) obtained closer TPC levels to Moreira et al. (2013) using extraction by maceration with alkali-hydrolysis, 17.5±0.4 mg GAE/g of BSG sample. The major causes for the confirmed discrepancies may be attributed to the sample type and origin, as well as the extraction process utilised. Since BSG is a lignocellulosic material with a considerable number of phenolic acids esterified to the cell wall, recovery of these acids is more difficult (Meneses, 2013, Moreira et al., 2012a).

3.4.4 Identification and quantification of individual polyphenols from BSG

The specific molecular weight determination of biologically active chemicals is identified by using molecular ions and their distinctive fragment ions in the LC-MS/MS. Using the precursor to product ion scans or MRM transition ions of the commercial polyphenol standards in the LC-MS/MS, the phenolic compounds in the extracts can be identified by matching their retention time with that of the standard polyphenols. Moreover, these analytical procedures, in particular the use of various column chromatography chemistries, enable the separation of phenolics across a broad range of polarity that is required due to the complex composition of polyphenols in plant matrix such as BSG. The elution of the analytes in the reversed phase chromatography is in decreasing order of polarity, with the less polar compounds eluting towards the end of chromatogram.

The LC-MS/MS has been employed to detect the presence of individual phenolic compounds in BSG extracts by using an in-house MRM list (26 reference standard polyphenols). The in-house MRM list of phenolic compounds include: chlorogenic acid, catechin, epicatechin, quinic acid, caffeic acid, gallic

acid, vanillic acid, protocatechuic acid, 4-hydroxybenzoicic acid, pyrocatechol, procyanidin C1, procyanidin B1, quercetin-3,4'-di-O-glucoside, sinapic acid, syringic acid, ferulic acid, *o*-coumaric acid, *p*-coumaric acid, quercetin, quercetin-3-O-glucoside, luteolin, luteolin-7-O-glycoside, kaempferol-3-O-glucoside, naringenin-7-O-glycoside, quercetin 3-O-arabinoside and oleanolic acid.

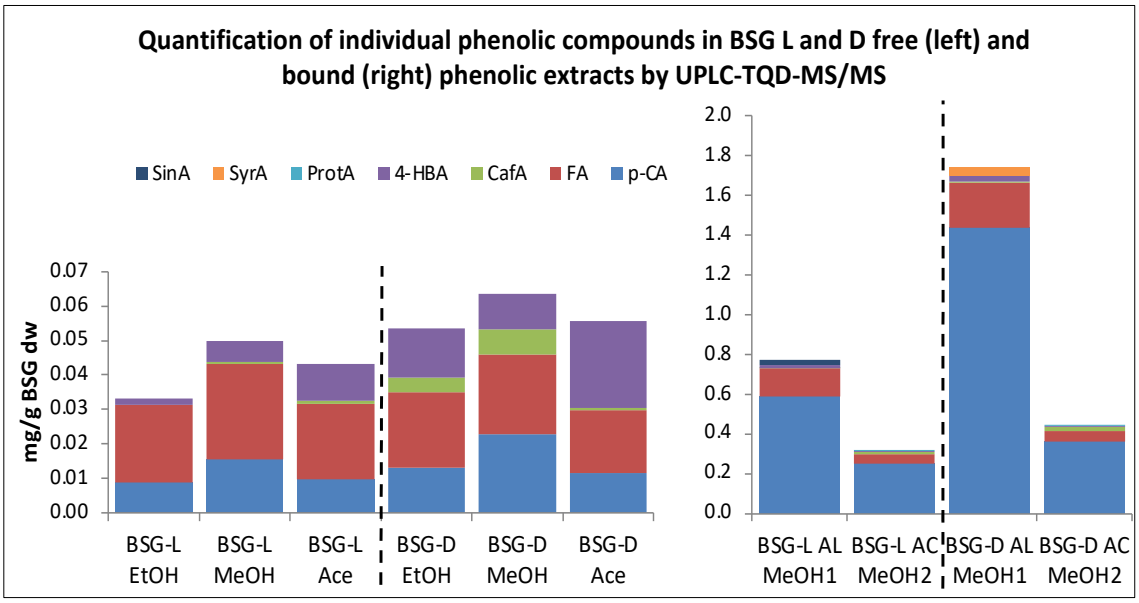
Besides the structural diversity of phenolic compounds, the level and type of phenolic compound also differ depending on the type of barley cultivar and the process it was subjected to; for instance *p*-coumaric and ferulic acids are generally dominant in BSG (Ikram et al., 2017), whereas catechin, gallic acid, vanillic acid, and syringic acid, among others, are found in low amounts in BSG (Mussatto et al., 2007a, Bonifácio-Lopes et al., 2020a). Thus, amongst the 26 compounds screened, several of the phenolic compounds were expected and quantified in the BSG extracts, namely: *p*-coumaric acid (*p*-CA), ferulic acid (FA), protocatechuic acid (ProtA), 4-hydroxybenzoic acid (4-HBA), sinapic acid (SinA), syringic acid (SyrA), and caffeic acid (CafA) (Figure 3-4). The seven phenolic compounds were quantified against standard concentrations ranging between 5 ppb to 50 ppm (n=10). The obtained results are shown in Figure 3-4.

The graph on the left side of the Figure 3-4 shows the UPLC-MS/MS quantification data of individual phenolic compounds in the BSG L and D free phenolic extracts (extraction using organic solvents), whereas the right-side graph of the bound phenolic extracts (extraction using acid and alkali hydrolysis) and expressed in mg/g BSG dry weight. The Figure shows that the concentration of phenolic compounds varies depending on the extraction method applied. The highest sum of total phenolics was obtained using alkali hydrolysis extraction from both BSG L and D compared with either of the organic solvents or acid hydrolysis extraction, with the total sum of polyphenols presenting an increase of approx. twentyfold in BSG L and thirtyfold in BSG D, respectively, compared to extraction using organic solvents. The phenolic compounds in the free BSG L and D extracts were at similar levels, irrespective of the organic solvent used, with the sum of total phenolic varying between 0.03 and 0.06 mg/g BSG dw.

The predominant phenolic compounds in the free phenolic extracts of BSG L and D, were *p*-CA, FA, CafA and 4-HBA, irrespective of the organic solvent used for extraction and with slight variations in their concentration. Among the

three organic solvents in both BSG L and D, MeOH performed slightly better in extracting phenolic compounds with moderately higher concentrations compared to EtOH and Ace; FA (0.03 mg/g BGS dw) and p-CA (0.02 mg/g BSG dw) being present at similar levels and being the most abundant phenolic compounds. Furthermore, CafA and 4-HBA were present at higher levels in BSG D compared to BSG L. These phenolics compounds are found in free form and are directly released and easily recovered by using organic solvents (Galanakis et al., 2013).

Figure 3-4 Quantification of the most abundant polyphenols in brewer's spent grain light and dark free and bound phenolic extracts



SinA – sinapic acid, SyrA – syringic acid, ProtA- protocatechuic acid, 4-HBA – 4 hydroxycinnamic acid, CafA – caffeic acid, FA - ferulic acid, p-CA – p-coumaric acid; AL – alkali hydrolysis, AC – acid hydrolysis. EtOH -Ethanol, MeOH – Methanol, Ace – Acetone, AL – alkali hydrolysis, AC – acid hydrolysis, mg/g BSG dw – milligram per gram BSG dry mass.

In terms of chemical hydrolysis, alkali hydrolysis of BSG L and D resulted 2.6x and 4.2x higher levels of sum of total phenolics, respectively than for acid hydrolysis, reaching a total of approx. 0.8 mg/g BSG dw in BSG L and 1.7 mg/g BSG dw in BSG D. Acid hydrolysis has not been assessed before on BSG to our knowledge, although it has been used on barleys (Gangopadhyay et al., 2016). Similarly, to free phenolic extracts, the predominant phenolic compounds in the bound phenolic extracts of BSG L and D, were p-CA, FA, CafA and 4-HBA. The most abundant phenolics in bound phenolic extracts of BSG L and D were p-CA (0.6 and 1.4mg/g BSG dw, respectively) and FA (0.1 and 0.2 mg/g BSG dw, respectively) using alkali hydrolysis extraction. These are all hydroxycinnamic acids that have been reported by several other authors as being the most

abundant in BSGs (Bartolomé et al., 2003, Faulds et al., 2002b, Mussatto et al., 2007a, McCarthy et al., 2013b). SyrA was quantified only in the bound phenolic extract of BSG D, whereas SinA only in BSG L, both being extracted using alkali hydrolysis. On the other hand, protocatechuic aid was quantified in bound phenolic extracts of BSG L and D using acid hydrolysis. These three phenolic compounds were present at very low levels in these extracts compared to *p*-CA.

The phenolic compounds targeted in this research chapter were hydroxycinnamic acids and hydroxybenzoic acids, as they were mostly described in literature and found either in free form or bound form in BSG (Mussatto et al., 2007a, Bonifácio-Lopes et al., 2020a, Moreira et al., 2013). Among these phenolic acids, it was expected that the most abundant phenolic acid, FA and p-CA, to be found in the BSG bound phenolic extracts as they are mostly found esterified or etherified to the matrix, and interestingly they were the most abundant in the free phenolic extracts as well. There results were similar or in close range with the findings of several authors. For example, Ikram et al. described that ferulic and *p*-coumaric acid were the most abundant hydroxycinnamic acids found as bound phenolic acids, but also as free phenolics (Ikram et al., 2017). Furthermore, using organic solvents, such as 50% acetone and 50% methanol, Stefanello et al. obtained very low levels of ferulic and *p*-coumaric acid from BSG: 0.84 and 0.56 mg/100g BSG respectively. In contrast, by using alkali extraction the levels of ferulic and *p*-coumaric acid increased by approx. 122 and 370 times, respectively (Stefanello et al., 2018a). The highest yield of 1.31±0.04% of BSG dw has been reported for FA, and a 10-fold lower values were reported by the same authors in their later published article following saponification with NaOH (Moreira et al., 2012b, Moreira et al., 2013) and with other authors. *p*-CA has been reported in levels of 2 to 3 fold lower than FA (Moreira et al., 2013), in close range to FA (Mussatto et al., 2007a, McCarthy et al., 2013b) and sometimes only traces were observed (Bartolomé et al., 1997). Moreover, Bonifacio-Lopes et al. showed that ferulic acid and p-coumaric acid were present only in extracts obtained by using 80% ethanol, at 3.9 and 43µg/g BSG, and 60% ethanol, at 19.4 and 26 µg/g BSG, respectively, compared to water or pure ethanol extraction (Bonifácio-Lopes et al., 2020a). Furthermore, 4-hydroxybenzoic acid was present in all the extracts in the Bonifacio et al. 2020

work, with the highest concentration of 105 µg/g BSG by using 60% ethanol. On the other hand, syringic acid and protocatechuic were present in low amounts or not detected in these extracts.

The BSG residues analysed were received directly from the mash tun and dried in an oven without being washed (see the drying protocol in Chapter 2, section 2.3). As BSG is used as a filtration bed for filtering the mash and separate the wort in the brewing process, traces of phenolic acids as described above, might have been present. Szwajgier 2009 determined the concentration of phenolic acids in two experimental worts and showed that FA, *o/p*-CA, 4-HBA, ProtA, SyrA, SinA and CafA were present at concentrations raging from 0.2 to 51 µmol/L of wort, with FA being the most abundant phenolic acid (Szwajgier, 2009). Thus, it is our belief that the overall concentration of phenolic acids in the extracts obtained by using organic solvents are not extracted only from BSG residue but may be also from the wort.

The spectrophotometric data obtained by using Folin-Ciocalteu reagent to estimate the total phenolic content in the free and bound phenolic extracts in BSG L and BSG D, were compared to those produced by the chromatographic method using UPLC-MS/MS. The data showed that the spectrophotometric method overestimated the total phenolic content approx. 18x in the free phenolic extracts, 4x and 2x in the alkali BSG L and BSG D bound phenolic extracts, and 11x and 5x in the acid bound phenolic extracts, respectively. Further to this point, Meneses et al., 2013 extracted antioxidant phenolic acid from BSG using different organic solvents and/or in combination with water and showed that all the extracts presented TPC along with lower amounts of proteins and reducing sugars (Meneses et al., 2013). Meneses et al., 2013 also showed that the antioxidant activity of the extracts correlated with the total phenols and flavonoids and acknowledged that some antioxidant activity contribution came from compounds that were not identified. Similarly, Kähkönen et al., 1999 reported that TPC can be influenced by specific compounds present in mixtures, and therefore can result in a false prediction of the antioxidant activity based only on TPC values (Kähkönen et al., 1999).

The chromatographic method is in general free of interferences thus offering a higher accuracy in the estimation of phenolics, whereas other co-extracted

compounds, such as proteins or peptides etc. may show a higher influence on the total phenols at the concentration ranges detected in the extracts by the spectrophotometric method (Escarpa and González, 2001). On the other hand, an underestimation of the phenolic content in BSG extracts may be shown by the UPLC-MS/MS chromatographic method as we have targeted seven phenolic compounds (see Table 3-1) where other phenolic compounds might have been missed, such as dehydrodimers or -trimers of ferulic acid, catechin, procyanidin, etc. (Moreira et al., 2013, Birsan et al., 2019, Patrignani et al., 2021, Verni et al., 2020), which would bring the overall phenolic content close the results obtained by the spectrophotometric method. Thus, it is our belief that these methods should be compared like-per-like when referred to the phenolic content or when data sets are compared with other data published in similar research papers.

Regardless of the extraction process used to extract polyphenols from plant materials, the obtained extracts contain not only phenolic compounds but also other components such as polysaccharides, peptides, waxes etc. (Ignat et al., 2013). An extra step to remove any unwanted compounds and to obtain phenolic-rich fraction is necessary, which will also reduce the interference in the LC-MS/MS analysis.

3.4.5 Separation and enrichment of polyphenols from BSG

A solution to the above problem would be to use membrane-based technologies that enable various compounds to be separated from a solution by applying a hydrostatic pressure difference between the two sides of a permselective barrier. As a consequence, the solution is separated into a permeate fraction, which contains all compounds that have passed through the membrane, and a retentate fraction, which contains all compounds that have been rejected by the membrane while still retaining part of the solvent (Cassano et al., 2018).

The molecular weight cut-off (MWCO) defines the membrane's pore size range, which in turn determines the separation of compounds. MWCO has shown to be one of the most effective technologies for ultrafiltration (UF), with pore sizes between 2 and 100 nm that are able to retain compounds with molecular weights from 1 kDa to 350 kDa (Singh, 2006, Conidi et al., 2018, Cassano et al., 2018).

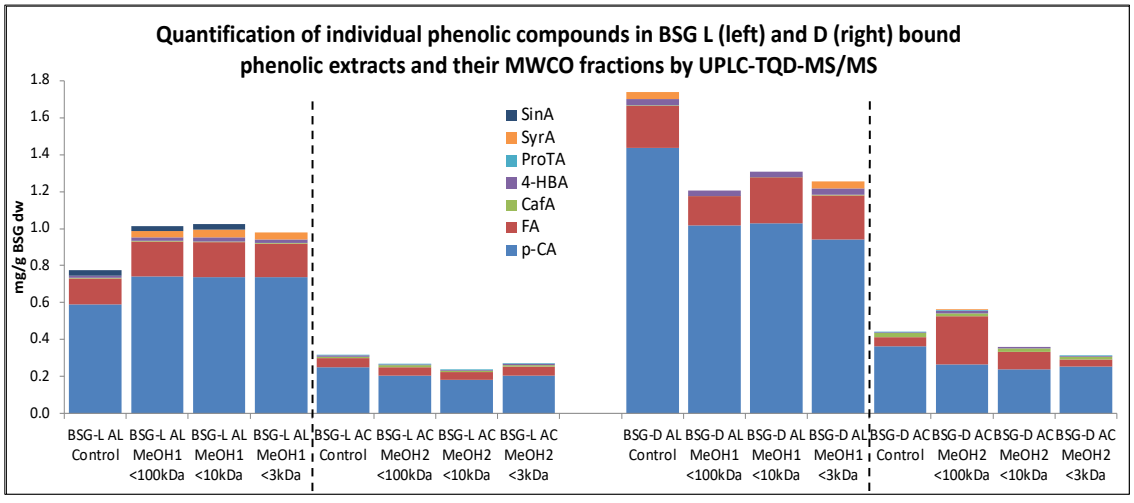
Centrifugal membrane devices have demonstrated good capabilities to separate high from low or moderate size compounds in diverse plant extracts. Even though they are suitable for use of small volumes of samples (2-20mL), they provide a rapid sample handling (1-2h, 6-24 samples/ centrifugation) in mild operating circumstances, while making it feasible to collect the permeate and retentate fraction from the centrifugal membranes (Prodanov et al., 2008). There is a broad applicability of the membrane filters that have been successfully applied to a recovery of a wide range of food and plants primary or secondary metabolites. Different kinds of macromolecules (such as carbohydrates, proteins, and pectins) may be recovered using membranes with MWCO ranging from 50 to 100 kDa; high molecular weight components, such as tannins, hydrolysates, proteins and even phenolic fractions can be concentrated using membranes ranging between 4 to 40 kDa, while other low molecular weight compounds such as phenolic compounds, anthocyanins, peptides etc can be concentrated using membranes ranging from 1 to 3 kDa (Cassano et al., 2018, Tang et al., 2009, Castro-Muñoz et al., 2019). Depending on the MWCO and the type of membranes used, low molecular phenolic compounds, in most cases, may be recovered at amounts ranging from 65 to 100% from several agri-food wastewaters, such as winery effluents, olive mill wastewaters, orange press liquor, grape seeds etc (Castro-Muñoz et al., 2016).

The individual phenolic content levels in the original BSG L and D extracts (control) obtained using alkali and acid hydrolysis extraction, with an initial fractionation step of the extracts based on diethyl ether: ethyl acetate 1:1 organic solvent mix, was used to determine the effectiveness of the fractionation across the 3 kDa, 10 kDa and 100 kDa MWCO membranes. Figure 3-5 shows the quantification of phenolic compounds in BSG L and D of the bound phenolic extracts obtained by using alkali and acid hydrolysis, and their MWCO fractions with the molecular weight of the separating membranes of 100 kDa, 10 kDa and 3 kDa. The graph in Figure 3-5 is divided in two parts: the left side shows the quantification data of phenolic compounds in BSG L by using alkali (BSG-L AL Control) and acid (BSG-L AC Control) solutions, each with their <100 kDa, <10 kDa, and <3 kDa fractions, respectively, whereas the right side shows the same fractions but of the BSG-D residue, (BSG-D AL Control, BSG-D AC Control). The

targeted phenolic compounds in the MWCO fractions were the same 7 polyphenols as described in figure above, Figure 3-4. The MWCO of <100 kDa, <10 kDa and <3 kDa fractions of the BSG-L AL Control showed an overall improvement of approx. 30% in the sum of individual phenolics (from 0.8 to 1mg/g BSG dw), and with the most abundant phenolics, *p*-CA and FA, being increased by 25% (0.59 to 0.76 mg/g BSG dw) and 32% (0.14 to 0.19 mg/g BSG dw) higher respectively compared with the levels in the BSG-L AC Control. A higher increase in abundance was observed in 4-HBA (56%) and CafA (143%) compared to Control, but their levels were very low (<0.02 mg/g BSG dw) in all the analysed samples compared to *p*-CA and FA. On the other hand, SyrA was not detected by the UPLC-MS/MS in the control sample, whereas in all MWCO fractions could be detected and quantified, reaching levels of approx. 0.04 mg/g BSG dw. On the other hand, SinA was not detected in the MWCO of <3 kDa fraction compared with the Control, <100 and < 10 kDa fractions, where it was found at similar levels of approx. 0.13 mg/g BSG dw. Contrary to the MWCO fractions of the BSG-L AL Control, the overall sum of total phenolic levels in BSG-D AL Control and its fractions reduced by approx. 28%, from 1.75 to 1.26 mg/g BSG dw. As per the most abundant individual phenolics, the levels of *p*-CA decreased by approx. 31% compared with the Control (from 1.4 to 1 mg/g BSG dw) in all the MWCO fractions, whereas FA was present at similar levels in the Control, <10 and <3 kDa MWCO fractions, approx. 0.24 mg/g BSG dw, and a drop of 31% was observed in the <100 kDa MWCO fraction to 0.16 mg/g BSG dw. The other phenolic compounds, ProtA, SyrA, SinA, CafA, 4-HBA, were also present at very low levels in the BSG-D AL Control and its MWCO fractions (<0.03 mg/g BSG dw). Contrary with the above observation when an improvement or decline was observed in the levels of phenolic compounds in the two sets of samples of the BSG-L AL and BSG-D AL, when using centrifugal membranes, in the BSG-L AC Control and its MWCO fractions, the phenolic compounds were at similar levels. An additional observation that is worth mentioning is that in <100 kDa fraction of BSG-D AC Control, a 5x increase in the FA levels was observed (from 0.06 to 0.26 mg/g BSG dw), whereas the p-CA levels dropped by 30% (from 0.37 to 0.25 mg/g BSG dw) in all MWCO fractions. Overall, the use of centrifugal membranes with MWCO sizes of 100 kDa, 10 kDa and 3 kDa showed little variation, irrespective of their MWCO sizes, in the separation and enrichment of

polyphenols from the BSG L and BSG D extracts. The polyphenol content was determined in the permeate part rather than retente part of the BSG MWCO fractions, and an increase in the detector response of the UPLC MS/MS method was expected as any interfering compounds with MWCO sizes >100 kDa were removed. This can be observed in the MWCO fractions of BSG-L AL Control, where an increase in recovery of phenolics was obtained. On the other hand, UPLC-MS/MS methods are susceptible to ion suppression of the ESI unit, which is vulnerable to the matrix effect leading to a decrease in the metabolite signal. This can be observed in the BSG-D AL and AC MWCO fractions as the phenolic content decreased to the same level irrespective of the MWCO sizes of the filtration membranes. This may be because by using chemical hydrolysis extraction, the acid and alkali solutions might have hydrolysed melanoidins, tannins or proteins that interfere and inhibit polyphenol ionization in the UPLC-MS/MS analysis. Moreover, acid hydrolysis extraction in both BSG-L and BSG-D extracts showed a low extraction phenolic yield with no significant improvement in the separation of phenolics by the MWCO membranes, and therefore not recommended. Similarly, for the BSG-L AL and AC, where the MWCO separation did not significantly increased the phenolic content in the fractions and reduced slightly their content in the AC fractions.

Figure 3-5 Individual phenolic compounds quantified in Brewer's spent grain light and dark bound phenolic extracts, and their molecular weight cut-off fractions



MWCO – molecular weight cut-off fractions obtained by ultrafiltration (centrifugal filter membranes) with MWCO sizes of less than 100, 10, and 3 kilodaltons (kDa); SinA – sinapic acid, SyrA – syringic acid, ProtA- protocatechuic acid, 4-HBA – 4 hydroxycinnamic acid, CafA – caffeic acid, FA - ferulic acid, p-CA – p-coumaric acid; AL – alkali hydrolysis extraction, AC – acid hydrolysis extraction.

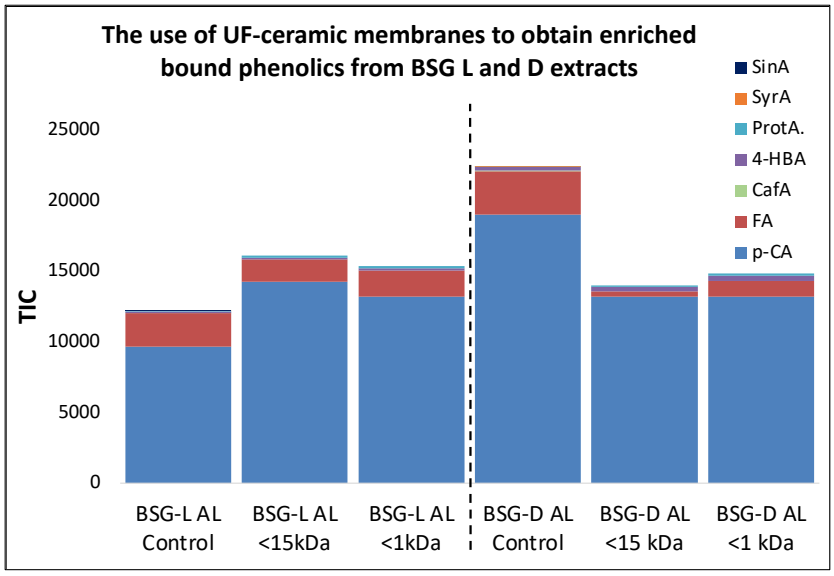
It was expected that by using the MWCO separation approach to obtain a better uniformity in the overall separation and recovery of phenolic compounds compared to Controls. For example, in MWCO fractions of BSG-L AL Control an increase in the recovery of phenolic was obtained with approx. 30% in all the fractions, whereas the opposite was observed in MWCO fractions of the BSG-D AL Control, and a smaller decrease in the fractions of BSG-L AC Control and BSG-D AC Control. Although the MWCO centrifugal membranes for the separation of phenolics in BSG extracts has shown to be a simple and quick pre-treatment process, the inclusion of this step has shown variable results in BSG polyphenol recovery. Overall, MWCO filtrations significantly enhanced the phenolic content of BSG-L AL extracts, whereas in others, BSG-L AC and BSG-D AL extracts, an opposite effect was observed. Due to this variability in the recovery and enrichment of the phenolic compounds from BSG extracts, the ceramic membrane filtration as an alternative to the membrane centrifugal filters was also explored.

Ceramic membranes with 1 kDa and 15 kDa MWCO were used to determine the effectiveness of the fractionation process across the BSG L and D extracts obtained by alkali extraction, while targeting the same 7 polyphenols as described in the Figure 3-4 and 3-5, respectively. Figure 3-6 shows the total ion chromatogram (TIC) peak areas of each phenolic compound in BSG-L and BSG-D controls and their MWCO fractions. TIC was used to compare the peak area of each of the targeted phenolic compounds in the BSG samples, rather than preparing a complete set of dilutions series of standards with a known concentration and calculate their concentration in the samples based on the graph equation. The graph in Figure 3-6 is divided in two parts: the left side shows the TIC data of phenolic compounds in BSG L obtained using alkali (BSG-L AL Control) with its <1 kDa and <15 kDa fractions, respectively, whereas the right side shows the same fractions but of the BSG-D AL Control sample.

Overall, the use of ceramic membranes with the specific MWCO showed a similar trend in the fractionation of the phenolic compounds from BSG extracts obtained by using alkali extraction as with the membrane centrifugal filters. The MWCO of <1 kDa and <15 kDa fractions of the BSG-L AL Control showed an overall improvement of approx. 32% in the sum of TIC of individual phenolics,

whereas in same MWCO fractions of the BSG-D AL Control showed an overall decrease of 37%, respectively. This increase and decrease in the sum of phenolic compounds in the both ceramic membrane fractions of BSG-L and BSG-D was observed in the fractions obtained by membrane centrifugal filters, as seen in Figure 3-5. Moreover, the same trend was observed in the most abundant phenolic compounds, for example the p-CA levels increased by approx. 47% in both MWCO fractions of the BSG-L AL Control, whereas in the same fractions of BSG-D AL Control, p-CA levels decreased by approx. 30%, respectively. FA on the other hand, showed similar levels in both controls, whereas a slight decrease of approx. 20% was observed in the MWCO fractions of the BSG-L AL Control, and an approx. 75% decrease in the BSG-D AL fractions. The other phenolic compounds, SyrA, SinA, CafA, 4-HBA and ProtA, were as well present in very low levels in all the BSG samples compared to p-CA and FA, and as observed above, Figure 3-5, in the MWCO fractions generated by the membrane centrifugal.

Figure 3-6 Screening of phenolic compounds in the BSG L and D bounds phenolic extracts using ceramic membrane filtration



Ceramic membranes with molecular wight cut-off (MWCO) size of <15 kDa and <1 kDa; TIC – total ion chromatogram; SinA – sinapic acid, SyrA – syringic acid, ProtA- protocatechuic acid, 4-HBA – 4 hydroxycinnamic acid, CafA – caffeic acid, FA - ferulic acid, p-CA – p-coumaric acid; AL – alkali hydrolysis extraction, TIC – total ion chromatogram.

The ceramic membranes fractionation system offers several advantages, such as chemical, mechanical, and thermal stability, wide pH-range, long operational life, high membrane flux, large mechanical strength etc. (Scott, 1995). However, the in-house MWCO ceramic membrane system used to fractionate the

individual phenolic compounds of BSG-L and BSG-D extracts presented several disadvantages compared to membrane centrifugal filters, as observed while running the experimental setup. These disadvantages include a high volume of at least 100 mL is necessary; only one sample can be processed at a time due to the ceramic membrane cylinder that can hold only one ceramic membrane at a time; the time to run a sample takes approx. 2hours; the ceramic membranes can be quite expensive; they need washing and rinsing after each use; inability to use it in the dark, and not being able to maintain a stable temperature of the solution while pumping through.

The separation of compounds is not always straightforward. In theory, separation methods utilising filtration membranes may separate certain substances using a sieving mechanism or a cross-flow filtration based on the MWCO; unfortunately, the MWCO of the membranes is not the only factor to consider. For example, asymmetric membrane pore manufacturing may not necessarily constitute a limited MWCO range; moreover, other phenomena, such as the concentration of the extracts, polarisation, membrane fouling, may occur (Cassano et al., 2018).

3.5 Conclusions

Of the BSG L and D substrates, the 80% methanol and 80% acetone extracts of BSG L had the highest free phenolic content, whereas alkali hydrolysis resulted highest yield of bound phenolic content as determined by spectrophotometric analysis using FC reagent and further complemented by the chromatographic analysis using UPLC-MS/MS. Interestingly, the hydroxycinnamic acids, namely ferulic acid and *p*-coumaric acid, were the most abundant phenolic compounds in both free and bound phenolic extracts. Moreover, liquid-liquid extraction using diethyl ether:ethyl acetate was sufficient to obtain enriched phenolic BSG extracts and that the use of ultrafiltration and ceramic membranes to further purify these extracts showed no significant improvement in the phenolic levels. Overall, BSG light presented a higher content of phenolic compounds compared to dark BSG.

The goal of this chapter was to see how the organic solvents and acid/base solutions used with specific parameters (solid:liquid ratio, temperature, time),

influences the release of phenolic compounds from light and dark BSG substrates. As two types of phenolic compounds were released, free and bound, the best organic solvent (acetone) and NaOH will be both used in the following chapters to generate BSG extracts. Furthermore, as UAE and MAE are frequently employed as alternatives to traditional extraction procedures to improve plant bioactive recovery, the next stage in this study will be to investigate these novel extraction techniques with the above-mentioned solutions that may significantly decrease extraction time, energy, and solvent usage while still extracting larger levels of phenolics from BSG. This should be paired with technologies and methodologies that are suitable for commercial use, as well as low-cost and easy-to-scale-up processes.

Chapter 4

4. Application of novel extraction technologies in recovery of polyphenols from BSG

This research investigation resulted in the publication of the following research article:

***Birsan, R.I.**; Wilde, P.; Waldron, K.W.; Rai, D.K. Recovery of Polyphenols from Brewer's Spent Grains. Antioxidants 2019, 8, 380. https://doi.org/10.3390/antiox8090380*

4.1 Abstract

The recovery of antioxidant polyphenols from light, dark and mix brewer's spent grain (BSG) using conventional maceration and novel microwave and ultrasound assisted extraction was investigated. Total polyphenols were measured in the crude (60% acetone), liquor extracts (saponified with 0.75% NaOH) and in their ethyl acetate (EtOAc) partitioned fractions both by spectrophotometry involving Folin–Ciocalteu reagent and liquid-chromatography-tandem mass spectrometry (LC-MS/MS) methods. Irrespective of the extraction methods used, saponification of BSG yielded higher polyphenols than in the crude extracts. The EtOAc fractionations yielded the highest total phenolic content (TPC) ranging from 3.01±0.19 to 4.71±0.28 mg gallic acid equivalent per g of BSG dry weight. The corresponding total polyphenols quantified by LC-MS/MS ranged from 549.9±41.5 to 2 741.1±5.2 µg/g of BSG dry weight. MAE and UAE methods with the parameters and equipment used did not improve the total polyphenol yield when compared to the conventional maceration method. Furthermore, the spectrophotometric quantification of the liquors overestimated the TPC, while the LC-MS/MS quantification gave a closer representation of the total polyphenols in all the extracts. The total polyphenols were in the following order in the EtOAc fractions: BSG light > BSG Mix > BSG dark, and thus suggested BSG light as a sustainable, low-cost source of natural antioxidants that may be tapped for applications in food and phytopharmaceutical industries.

4.2 Introduction

As previously stated in Chapters 1 and 3, Brewer's spent grain (BSG) is generated in millions of tonnes every year as the major by-product of the brewing industry, with an annual global production estimated to be 39 million tonnes, of which the EU generates ~8 million tonnes (Conway, 2019, TBOE, 2019). BSG is used as a low-value animal feed with a market value of ~35 Euro/tonne and thus making it an ideal substrate from which to recover high value compounds (Lynch et al., 2016). In addition to cellulose, hemicellulose, lignin, protein and lipids as the main components, BSG also contains low molecular weight phenolic compounds that have been associated with a wide array of health-benefiting properties (Jay et al., 2008, Shahidi and Yeo, 2018).

Several extraction methods, optimized and applied towards the recovery of polyphenols from BSG, have been comprehensively reviewed by several authors (Lynch et al., 2016, Brglez Mojzer et al., 2016). Depending on the types of BSG produced because of different cooking temperatures (70–250 ºC), the polyphenol contents also differ between the lightly roasted malt producing light or pale BSG and the deeply roasted malts producing dark or black BSG. A common practice in breweries is to mix the light and dark malts in the ratio ~9:1 *w*/*w* to obtain the desired caramel colour and aroma of the beverage. Since BSG predominantly contains bound phenolics, chemical or enzymatic hydrolysis protocols are routinely used to release the phytochemicals bound to the cell-wall components (Krygier et al., 1982, Sancho et al., 2001, Mussatto et al., 2007a). Solvent extraction or chemical hydrolysis combined with ultrasound (UAE) or microwave assisted extraction (MAE) or other physical cell-disruption techniques have been shown to increase the extraction yield of targeted compounds from BSG and similar biomass (Guido and Moreira, 2017, Bartolomé et al., 1997, Kumari et al., 2017, Naczk and Shahidi, 2006a). For example, in the recovery of BSG polyphenols, an optimised MAE method has been reported to result in a five-fold higher ferulic acid yield than the conventional solid–liquid extraction techniques (Moreira et al., 2012b). In contrast, the same MAE parameters were also applied by Stefanello et al. (Stefanello et al., 2018a) on BSG and corn silage, but the MAE yielded significantly lower total phenolic content than the conventional maceration method. In a separate study, mathematical models were used to

optimize three extraction parameters (i.e., substrate to solvent ratio, extraction temperature and solvent composition) for MAE and UAE to recover maximum yield of unbound polyphenols from the unsaponified BSG. The subsequent experiments performed using the optimum parameters also resulted in higher polyphenolic contents by UAE (4.1 mg GAE/g BSG dw) and by MAE (3.9 mg GAE/g BSG dw) compared with the maceration method (3.6 mg GAE/g BSG dw) (Carciochi et al., 2018). Both MAE, based on rapid heating of the solvent through microwave energy (that causes molecular motion via ionic conduction and dipole rotation), and UAE based on acoustic cavitation, increase the solvent penetration into the substrate leading to improved mass transfer rates. There is, however, a limited number of studies that focus on the UAE, MAE and conventional extraction methods to recover polyphenols from saponified BSG despite the presence of optimisation studies on individual methods in BSG (Moreira et al., 2012b, Carciochi et al., 2018, Meneses et al., 2013) or similar substrates (Irakli et al., 2018, Wang et al., 2008).

In addition, several of the aforementioned and other BSG polyphenol extraction studies were quantified spectrophotometrically using the Folin–Ciocalteu (FC) chemical method (Carciochi et al., 2018, Meneses et al., 2013, Piggott et al., 2014, Zuorro et al., 2019, Spinelli et al., 2016a, Fărcaş et al., 2013a) either alone or with hyphenated chromatographic methods (Carciochi et al., 2018, Meneses et al., 2013, Piggott et al., 2014, Zuorro et al., 2019, Fărcaş et al., 2015, McCarthy et al., 2012, Moreira et al., 2013, Stefanello et al., 2018b). Both above polyphenol quantification methodologies have been used in Chapter 3, and due to the differences observed in the polyphenols levels I will continue to combine the two approaches for further analyses of BSG extracts. Moreover, this approach will offer a better knowledge of the extract's phenolic profile, a more accurate quantification of the polyphenolic compounds extracted and the efficacy of the different extraction methodologies.

As it was shown in Chapter 3, water-acetone mixture was a better choice, among the organic solvents used, to extract free phenolics from BSG L and BSG D based on the obtained TPC results. On the other hand, saponification with NaOH was the best choice to extract bound phenolics, based on both TPC (spectrophotometric analysis) and quantification results of individual phenolics

(chromatographic analysis), compared to HCl hydrolysis. Moreover, liquid-liquid partitioning proved to be a sufficient follow up separation step to obtain enriched phenolic BSG L and BSG D extracts as neither the MWCO centrifugal filters nor the ceramic membranes showed a consistent separation and enrichment of phenolics from both types of BSG extracts. Besides, these MWCO based separations added cost and time in the extraction process. Several other authors have also reported the superiority of aqueous acetone mixtures to extract free phenolics and sodium hydroxide to extract bound phenolics from BSG residues (Meneses et al., 2013, Zuorro et al., 2019, Mussatto et al., 2007a, Moreira et al., 2012b, Stefanello et al., 2018b).

Also in the previous chapter, we mentioned that the spectrophotometric methods generally overestimate the true phenolic contents, whereas a liquid chromatography system coupled to a tandem quadrupole mass spectrometry detector (TQD) provides a much more accurate quantification of the phenolic compounds in the analysed BSG extracts. Based on the results obtained in Chapter 3, this observation has been further confirmed. Thus, we believe that a complete screening of BSG extracts using a high-resolution mass spectrometer, such as quadrupole-time-of-flight (Q-ToF) coupled to a HPLC system, would elucidate other analytes, e.g. dimers of ferulic acid, are present in the BSG extracts in addition to the targeted phenolics. Even though the spectrophotometric methods overestimate the total phenolic content in BSG residues, the TPC by Folin-Ciocalteu assay provides good quantitative comparability between matrices based on similar works presented in the literature (most of the phenolic content estimation in food extracts are based on the Folin-Ciocalteu assay), which could be used further to compare the phenolic content in similar substrates.

In this second experimental chapter, we have investigated and compared the recovery of polyphenols from saponified light (BSG L), dark (BSG D) and Mixed (BSG Mix) BSG using maceration, MAE and UAE techniques, but as well using aqueous organic solvents mixtures. The parameters for the various extraction methods have been adapted from the literature for maceration and UAE, whereas previously optimised parameters were applied for MAE (Moreira et al., 2012b). Apart from developing and introducing sustainable alternatives to

classic extraction, the novel extraction techniques might present the ability to boost the extraction yield while reducing extraction time and solvent use, thus improving the economical and environmental aspects of the process. The objectives of this study were (1) to examine the application of novel extraction technologies on BSG phenolic yield, (2) to identify and quantify various phenolic compounds in BSG extracts following novel extraction methods.

4.3 Materials and Methods

4.3.1 Samples and Chemicals

BSG L and D were provided by Diageo Ireland, Dublin. BSG Mix (light:dark, ~9:1 *w*/*w*) was obtained from the River Rye Brewing Company, Cellbridge, County Kildare, Ireland. The BSG samples were directly transported to the research centre within 30 min., oven-dried (see the drying protocol in Chapter 2, section 2.3), milled (<1 mm) and vacuum packed until required.

The organic solvents (methanol, acetone, ethyl acetate (EtOAc), formic acid, acetonitrile), and sodium hydroxide (NaOH) were purchased from Merck (formerly Sigma Aldrich, Arklow, Co. Wicklow, Ireland). Polyphenol standards of gallic acid, *p*-coumaric acid, ferulic acid, sinapic acid, caffeic acid, protocatechuic acid, 4-hydroxybenzoic acid and +(-)catechin; and the chemicals FC reagent, hydrochloric acid and sodium carbonate were purchased from Merck (Arklow, Co. Wicklow, Ireland). Leucine-enkephaline was purchased from VWR International Ltd. (Blanchardstown, Dublin, Ireland).

4.3.2 Solid-liquid extraction

A schematic flow of the extraction procedures used is illustrated below in Figure 4-1.

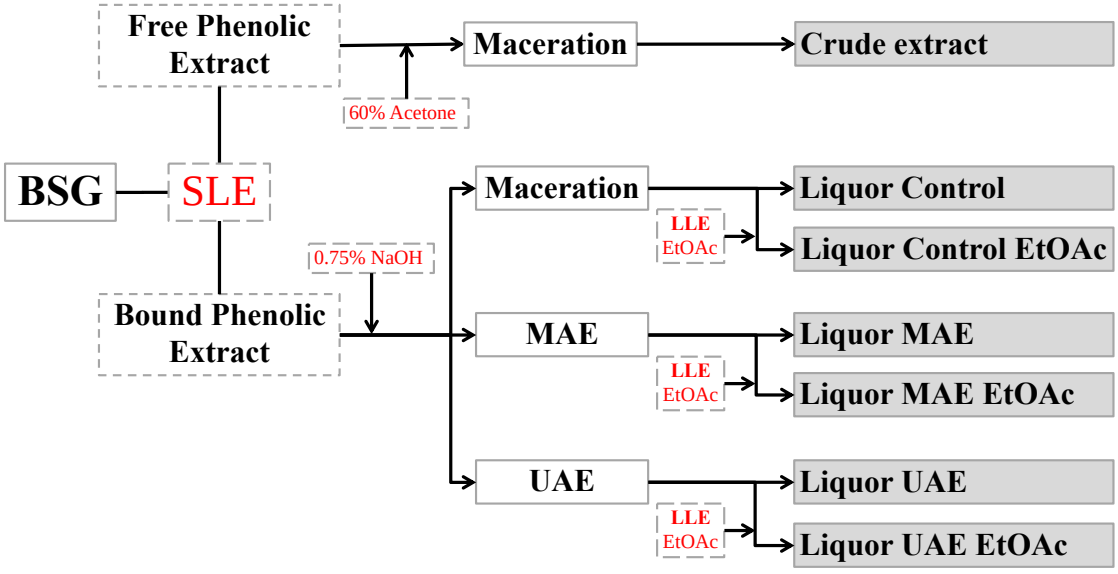
4.3.2.1 Maceration

Extraction of free (unbound) polyphenols, referred to as crude extracts, from BSG samples was carried out as in the previously optimised method of (Meneses et al., 2013). Milled BSG was mixed with 60% aqueous-acetone solution (1:20 *w/v*) in a sealed amber glass bottle, at 60ºC in a water bath for 30 min. with constant stirring. After the extraction times were complete, all the extracts were

left to cool at room temperature followed by centrifugation at 9484*g* for 10 min. (Sigma 2–16KL, Osterode am Harz, Germany). The supernatants were pooled, and syringe filtered through 0.45 µm PTFE filters. The extracts were then stored in a freezer at -28ºC until further use.

For the extraction of bound phenolics, 0.75% NaOH aqueous solution at 80°C for 30 min. with constant stirring was used (Moreira et al., 2012b, Wang et al., 2008). After the extraction, all the extracts were left to cool at room temperature followed by centrifugation at 8 400 rpm for 10 min. (MegaStar 600, VWR, Leuven, Belgium). The supernatants were pooled and filtered through PVDF filters under vacuum. Aliquots of the liquor supernatants were acidified by adding 37% hydrochloric acid drop-wise until the pH reached 6.5 and subsequently subjected to liquid-liquid partitioning in EtOAc:water (1:1 *v*/*v*, 3 times) to obtain polyphenol-enriched fractions. The EtOAc fractions were evaporated to dryness under nitrogen and reconstituted in 20 mL 50% methanol. All the extractions were carried out in triplicate and stored at −25 ◦C until further use.

Figure 4-1 Flow chart showing the extraction procedure for brewer's spent grain (BSG) samples light, dark and mixed for free and bound phenolics.



SLE – Solid-liquid extraction using acetone and sodium hydroxide (NaOH); LLE – Liquid-liquid extraction of alkali-hydrolysed fractions (liquors) partitioned with ethyl acetate (EtOAc); MAE – microwave assisted extraction; UAE – ultrasound assisted extraction.

4.3.2.2 Microwave Assisted Extraction

MAE of BSG phenolics were performed according to the method previously optimized and reported by Moreira et al. (Moreira et al., 2012b). Briefly, 2 g BSG samples were transferred to 55mL MarsExpress TFM extraction vessels (tetrafluoroethylene modified, vessels) with 40 mL of 0.75% NaOH solution (1:20 w/v in triplicate), capped and tightly sealed using a simple hand tightening tool. Extraction was carried out for a duration of 15 min. at constant temperature (100ºC). In all the vessels magnetic stirrers were added and used at maximum stirring speeds, while the pressure-leak and temperature were monitored for each vessel using an electronic sensors-pressure (ESP-1500 Plus system) to measure pressure inside the vessels, and infrared sensors (MTS-300 system) for recording inside and outside temperature of the vessels, respectively.

After the extraction times were complete, all the extracts were left to cool at room temperature, transferred in 50 mL centrifugal tubes after cooling and centrifuged at 8400 rpm for 10 min. (MegaStar 600, VWR, Leuven, Belgium). The supernatants were pooled and filtered through PTFE filters. The supernatants were acidified to pH 6.5 by adding hydrochloric acid solution (HCl 37%), and aliquots (20 mL) of the supernatants were subsequently subjected to liquid-liquid partitioning in EtOAc:water (1:1 *v*/*v*, 3 times) to obtain polyphenol-enriched fractions (section 2.4.3.1). The EtOAc fractions were evaporated to dryness under nitrogen and reconstituted in 20 mL 50% aqueous-methanol solution. All the extractions were carried out in triplicate and the extracts stored at −25ºC until further use.

4.3.2.3 Ultrasound Assisted Extraction

Ultrasound assisted extraction was carried out by using a Transonic TI-H-10 35 kHz sonication bath (ELMA Sch., Singen, Germany) at ~80 ºC for 30 min., adapting the parameters previously optimised in similar substrates (Irakli et al., 2018, Wang et al., 2008). The substrate to solvent ratio (1:20 w/v) and the alkali concentration were maintained as used in the MAE and maceration methods, where 2.5 g each of milled BSG D, L and Mix samples were mixed with 50 mL 0.75% NaOH solution in 100 mL amber bottles. The bottles were sealed to avoid

any loss of solvents. After the extraction times were complete, the samples were processed and subjected to LLE.

4.3.2.4 Preparation of Samples Following Maceration, MAE and UAE Treatments

After the extraction procedures were complete, all the extracts were left to cool at room temperature followed by centrifugation at 8400 rpm for 10 min. (MegaStar 600, VWR, Leuven, Belgium). The supernatants were pooled, and syringe filtered through 0.45 µm PTFE filters for free phenolics and bound phenolic extracts. Aliquots (20 mL) of the liquor supernatants were acidified by adding hydrochloric acid solution (37%) until the pH reached 6.5 and subsequently subjected to liquid-liquid partitioning in EtOAc:Water (1:1 v/v, 3 times) to obtain polyphenol-enriched fractions. The EtOAc fractions were evaporated to dryness under nitrogen and reconstituted in 20 mL 50% methanol. All the extractions were carried out in triplicate and stored at −25 ºC until further use.

4.3.3 Determination of total phenolic content by Folin-Ciocalteu

Total phenolic content of BSG extracts was determined by colorimetric assay using FC reagent following (Singleton et al., 1999b), and overall methodology described in Chapter 2, section 2.5.1. Briefly, a mixture of BSG extract, methanol and 20% sodium carbonate were prepared and incubated in darkness for 20 minutes at room temperature. The mixture was then vortexed, centrifuged and an aliquot of the supernatant was transferred to 96-well plate and the absorbance measured at 735nm. The recorded absorbance of the samples was calculated using a calibration curve of known concentrations of gallic acid. The results are expressed in milligrams of gallic acid equivalent per gram BSG dry weight (mg GAE /g BSG dw).

4.3.4 LC-MS/MS Identification and Quantification of BSG Phenolic Compounds

Quadrupole time-of-flight (Q-ToF) Premier mass spectrometer coupled to Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) was used to profile various phytochemicals in the BSG L EtOAc fraction following the

procedure previously described (Hossain et al., 2010b). Accurate mass measurements of the molecular ions were achieved using an internal reference compound (Leucine–Enkephalin). The separation of the compounds was achieved on an Atlantis T3 C18 column (100 × 2.1 mm; 3 µm) using milliQ water (solvent A) and acetonitrile (solvent B) both containing 0.1% formic acid at a flow rate of 0.3 mL/min. at 40 ºC. Electrospray ionisation (ESI) mass spectra were recorded on a negative ion mode for a mass range *m/z* 70–1000. Capillary and cone voltages were set at 3 kV and 30 V, respectively. Collision-induced dissociation (CID) of the analytes was performed using argon as the collision target.

Ultra-high performance liquid chromatography coupled to tandem quadrupole mass spectrometer (UPLC-TQD, Waters Corp., Milford, MA, USA) was used to quantify the BSG polyphenols by adapting the previous method used in raw barley (Gangopadhyay et al., 2016). Separation of the phenolics was carried out on an Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm). The mobile phase consisted of milliQ water (solvent A) and acetonitrile (solvent B) both containing 0.1% formic acid. The UPLC separation was performed by an increasing organic solvent gradient from 2% to 98% B at a flow rate of 0.5 mL/min. for 10 min. The column temperature was set at 50 ºC, while the samples were kept at 4 ºC. The ESI source was set in negative mode and the quantification of each compound was performed by multiple-reactions monitoring (MRM) (Table 4-1).

Table 4-1 Multiple reaction monitoring (MRM) transitions, cone voltage and collision energies used for the UPLC-TQD quantification of Brewer's spent grain polyphenols

Standards	MRM (*m/z*)	Cone Voltage (V)	Collision energy (eV)
Ferulic acid	192.9 → 133.9	31	16
	→ 177.9		12
p-coumaric acid	163.0 → 118.9	25	14
Catechin	289.1 → 245.0	40	16
Syringic acid	197.1 → 152.9	31	12
	→ 181.9		14
4-hydroxybenzoic acid	137.0 → 64.9	29	26
	→ 92.9		14
Sinapic acid	223.1 → 120.9	32	26
	→ 163.9		14

Caffeic acid	179.0 → 78.9	35	24
	→ 134.9		16
Protocatechuic acid	153.0 → 80.9	29	8
	→ 108.9		14

For the quantification of polyphenols, a stock solution (1000 ppm) for each standard was prepared and appropriate dilutions covering the range of 0.098 to 100 ppm were made to obtain standard curves. Targetlynx^{TM} integration software (Waters Corp., Milford, CT, USA) was used to quantify the compounds in the various extracts.

4.3.5 Statistical Analysis

Results are expressed as means of the triplicates±standard deviation (SD). Differences between means were analysed using one-way analysis of variance with post-hoc Tukey test (SPSS Statistics 24). The statistical analysis on the different groupings was carried out using Minitab 18.0 (Minitab, Inc., State College, Pennsylvania, USA). The values were considered significantly different when $p < 0.05$.

4.4 Results and Discussion

4.4.1 Extraction yield

In this research chapter, a simplified extraction methodology was used to obtain free and bound phenolics from BSG residues by using the best conditions previously identified in Chapter 3, such as organic solvent (acetone), and alkali solutions. Also, the follow up liquid-liquid extraction was simplified by using a one-step extraction with ethyl acetate as the organic solvent. Moreover, the potential of ‘green’ and ‘smart’ extraction methodologies, such as MAE and UAE, have been tested in combination with the above-mentioned chemical methods. Ultrasound is commonly employed to enhance traditional solvent extraction, although microwaves are recognised for their capacity to remove components without the need of solvents (J Mason et al., 2011). Additionally, several parameters have been increased, such as temperature and time, that have shown to improve the extraction yield (Bonifácio-Lopes et al., 2020b).

In this study the extraction yield was recorded for the crude extracts, which were obtained by using 60% acetone-aqueous solution, and the ethyl-acetate (EtOAc) fractions of the NaOH saponified BSG L, D and Mix extracts (Table 4-2). The extraction yield of the raw liquors (the liquid phase following alkali hydrolysis – liquors) were not recorded as the overall process required several extra steps, such as dialysis and freeze drying, time and the equipment that was not available at that moment. The extraction yield (the dry weight of the solid part obtained after evaporating the solvent used as extractant) among the recorded extract and fractions varied between 73.9±3.3 and 98±4.9 mg/g BSG dw, respectively (Table 4-2). The maximum extraction yield of approx. 10% from BSG Mix (98.04.9 mg/g BSG) was obtained by using 60% acetone. Similar levels ($p>0.05$) were obtained as well from BSG L and BSG D, of 95.3±2.9 and 90.6±5.4mg/g BSG dw, respectively. The extraction yields of liquors were slightly lower ($p>0.05$) compared to the crude extracts of the BSG L, D and Mix, except for the BSG D- and Mix Liquor MAE EtOAc fractions that were significantly lower ($p<0.05$), of 73.9±3.3 and 74.0±5.7 mg/g BSG dw, respectively. The extraction yield of BSG L fraction was consistent among the extraction technologies used, with a coefficient of variation <0.5%. On the other hand, the extraction yield of BSG D- and BSG Mix EtOAc fractions presented a much higher coefficient of variation of 12% and 6%, respectively meaning a less consistent extraction repeatability among the BSG D and BSG Mix type of samples.

The extraction yield of BSG L and BSG D obtained by using 60% acetone-aqueous solution were slightly higher compared with the ones presented in Chapter 3, section 3.4.2. This may be because of the difference in concentration of acetone (60% vs 80%), temperature (60°C vs room temperature) and time (30min vs 10 min) used, resulting in a higher extraction yield. Furthermore, the extraction yield from BSG L and D obtained by using maceration with alkali hydrolysis was also much higher, approx. 2x, compared to the yields obtained in Chapter 3, section 3.4.2. In Chapter 3, BSG residue were first mixed with organic solvents to recover free phenolics, and the residual pellet was further treated with alkali solutions to release bound phenolics (sequential extraction). In this chapter, the free phenolic extraction using 60% acetone and bound phenolic extraction using alkali hydrolysis were performed individually on the BSG residues, thus the

extraction yield recorded for the bound phenolic extracts will also contain part of the free phenolic extracts. It is worth mentioning that the BSG were obtained from different breweries, without knowing the type of barley used in the malting process, as well the overall brewing process parameters, factors that may also affect the extraction yield.

Table 4-2 Extraction yield from Brewer's spent grain light, dark and mix by acetone and ethyl acetate fractions of liquors obtained by alkali hydrolysis combined with novel extraction technologies.

Samples	Extraction yield *(mg/g BSG)*		
	BSG L	BSG D	BSG Mix
Crude	95.3±2.9^{a}	90.6±5.4^{ab}	98.0±4.9^{a}
Liquor Ctrl EtOAc	89.9±1.4^{ab}	80.6±10.9^{ab}	80.0±7.3^{ab}
Liquor MAE EtOAc	89.2±6.1^{ab}	73.9±3.3^{b}	74.0±5.7^{b}
Liquor UAE EtOAc	89.0±4.8^{ab}	94.2±1.7^{a}	82.8±9.1^{ab}

Ctrl EtOAc – control ethyl acetate; values bearing different letters (a, b) are significantly different ($p < 0.05$) from each other.

4.4.2 Total antioxidant activity of BSG extracts

The total phenolic content (TPC) from the crude extracts for the L, D and Mix BSG were 2.84±0.11, 2.81±0.14 and 3.85±0.04 mg GAE/g BSG dw, respectively (Table 4-3). Past studies, by other authors, on the crude extracts of light and dark BSG have also shown TPC in a similar range (McCarthy et al., 2012, Kumari et al., 2019). These relatively low TPC levels in the crude extracts are because of the fact that the BSG contains a high amount of lignin ranging from 19.4–49.2 g/100 g that is connected to its cell wall polysaccharides by phenolic acids (Mussatto et al., 2007a, Meneses et al., 2013, Aura et al., 2013). Therefore, it is essential to hydrolyse the rigid lignocellulose structural components to release the phenolic acids. Alkali hydrolysis is commonly used with BSG and other similar substrates. The TPC of the hydrolysed fraction (liquor) prior to acidification and partitioning is often reported, which is four- to five- times higher than the TPC values of the crude extracts (McCarthy et al., 2012, Stefanello et al., 2018b). For example, McCarthy et al. (McCarthy et al., 2012) recorded 16.0 mg GAE/g BSG dw and 18.3 mg GAE/g BSG dw for the light and dark BSG liquors, respectively. This trend is also evident from our study, where

TPC values for the liquors ranged from 15.42 to 19.20 mg GAE/g BSG dw as opposed to the crude extracts (2.81 to 3.85 mg GAE/g BSG dw). Generally, the dark BSG have previously shown higher levels of TPC values than the light BSG owing to the presence of high molecular weight melanoidins (Piggott et al., 2014), which are accumulated as by-products of the Maillard reaction produced during the malting and brewing process (see Chapter 1 section 1.1.3). The melanoidins mostly consist of sugar degradation products and amino acids (Friedman, 1996) that can also react with FC reagent and thus contributing to the higher levels of TPC.

Table 4-3 Total phenolic contents in the NaOH saponified Brewer's spent grain extracts (liquors) and their subsequent ethyl acetate fractions following acidification.

Samples	TPC *(mgGAE/g BSG dw)*		
	BSG L	BSG D	BSG Mix
Crude	2.84±0.11^{c}	2.81±0.26^{c}	3.85±0.04^{c}
Liquor Ctrl	16.67±0.87^{b}	17.27±0.41^{ab}	19.20±0.40^{a}
Liquor Ctrl EtOAc	4.67±0.27^{c}	3.08±0.15^{c}	4.71±0.28^{c}
Liquor MAE	15.42±1.16^{b}	15.55±0.56^{b}	16.94±1.84^{b}
Liquor MAE EtOAc	3.85±0.19^{c}	3.01±0.19^{c}	4.24±0.22^{c}
Liquor UAE	15.76±0.72^{b}	16.72±0.96^{b}	16.99±0.32^{b}
Liquor UAE EtOAc	4.17±0.21^{c}	3.43±0.46^{c}	4.62±0.27^{c}

Total phenolic contents (TPC) in mg GAE/g BSG dw; ethyl acetate fractions following neutralisation (EtOAc); Ctrl represents maceration method, microwave assisted extraction (MAE), ultrasound assisted extraction (UAE) of light (L), dark (D) and Mix BSG. For each substrate, total phenolic content (TPC) values bearing different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

However, the acidification of the liquors and subsequent partitioning with EtOAc, the TPC values of the EtOAc ranged between the crude and the liquor fractions (Table 4-3). Interestingly, the TPC of EtOAc fractions in the BSG D averaging 3.17 mg GAE/g dw is significantly lower than those of the L and Mix BSG averaging 4.23 and 4.52 mg GAE/g dw, respectively. Similar findings where the phenolics were lower in the hydrolysed dark BSG compared to light BSG have been reported by Moreira et al. (Moreira et al., 2013). Although the application of MAE and UAE techniques resulted, in general, slightly lower TPC in the BSG EtOAc fractions than the conventional maceration method. The possible reason for this decrease is due to the structural characteristic of the BSG as it predominantly contains a high lignin content (Jay et al., 2008, Mussatto et al., 2007a). It has been suggested before that the MAE is not able to promote

sufficient molecular movement and rotation to overcome the lignin-barrier in contrast to constant stirring in the maceration method (Moreira et al., 2012b, Carciochi et al., 2018). Furthermore, the high temperature in MAE may induce the degradation of thermolabile polyphenols. A study on the effect of temperature on the extraction of polyphenols from *Gordonia axillaris*, an edible wild fruit, has shown a decrease in antioxidants' recovery with higher temperatures in MAE (Li et al., 2017b). In general, high temperature has a positive effect on the extraction yield due to enhanced solubility and diffusivity of materials, however in UAE the high temperature has a negative effect on the extraction yield (Bimakr et al., 2017). The high temperature increases the solvent vapour pressure and results in a decrease in surface tension that affect the cavitation bubble formation, which may explain the low TPC in the UAE treated samples.

The TPC results of the Crude BSG L and D extracts obtained in this research chapter were 2x higher compared with the TPC levels obtained in Chapter 3 section 3.4.3 from a similar BSG type and organic solvent used to extract free phenolics. On the other hand, the bound phenolic extracts obtained from BSG L and D presented a 1.4x higher TPC levels compared to the results obtained in Chapter 3 for similar extracts. An overall explanation is further discussed at the end of section 4.4.3.2.

4.4.3 Identification and Quantification of BSG Polyphenols

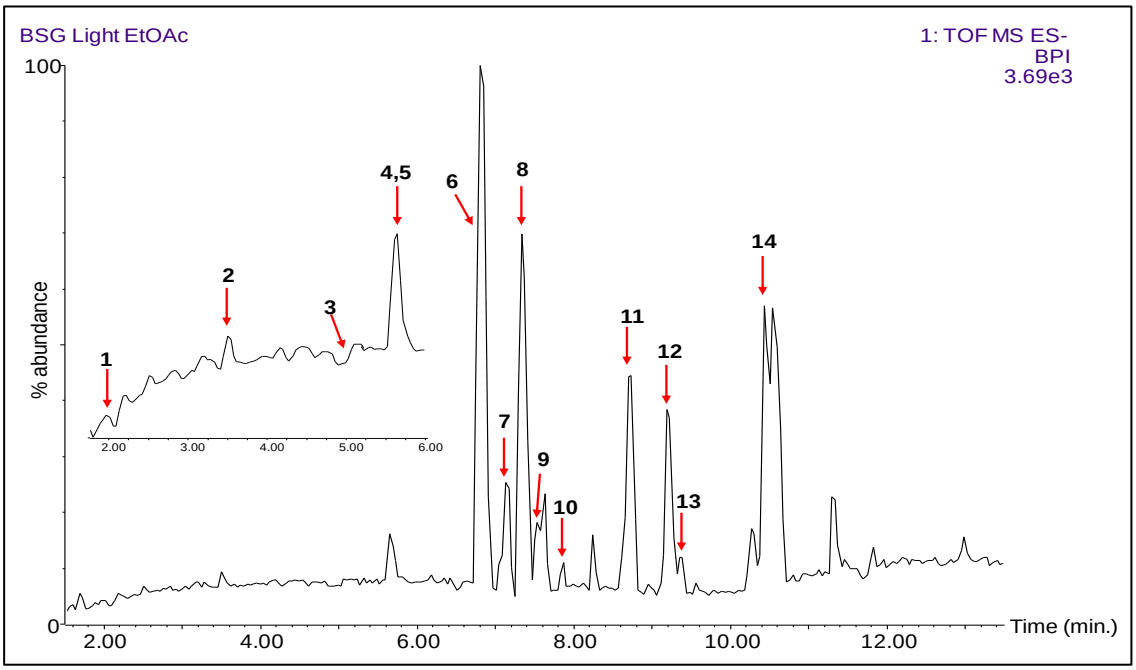
Individual BSG polyphenols were quantified using LC-MS/MS systems. The aim was to explore the presence of various phenolic compounds and then to undertake a qualitative and quantitative analysis of phenolics in BSG extracts and fractions. Using the HPLC-Q-ToF the predicted chemical formula and compound identification based on the accurate molecular weights of the components using full scan mass spectral as well as tandem MS (MS/MS) data were obtained. For those polyphenols that the authentic standards were commercially available were used for identification and quantification of polyphenols in the BSG extracts.

4.4.3.1 Identification of BSG polyphenols

As many as 14 different polyphenols were tentatively identified in the BSG L EtOAc extract using the accurate mass measurements, fragment ions and in conjunction with the literature (Figure 4-2, Table 4-4). Some of these polyphenols

(protocatechuic acid and caffeic acid) were present in low amounts or co-eluted (syringic acid) with other phenolic acids as illustrated in the magnified inset in Figure 4-2 and the extracted ion chromatograms for these compounds in Figure 4-3. Seven phenolic acids (ferulic acid, protocatechuic acid, 4-hydroxybenzoic acid, caffeic acid, syringic acid, *p*-coumaric acid and sinapic acid) and a flavonoid (catechin) were identified using commercially available standards and subsequently quantified using UPLC-TQD (Table 4-4). Several of the ferulic acid dimers and trimers listed in Table 4-4 have been identified previously in BSG using HPLC-DAD-MS/MS methods (Jay et al., 2008, Moreira et al., 2012b, Hernanz et al., 2001).

Figure 4-2 The high-performance liquid chromatography coupled to a quadrupole/time-of-flight chromatogram of ethyl acetate fraction of Brewer's spent grain light showing the polyphenols (peaks 1–14) as assigned in Table 4-4. Shown in the inset is close-up figure for the minor peaks 1–5. The elution time for peaks 1, 3 and 4 are demonstrated in their extracted ion chromatograms in Figure 4-2



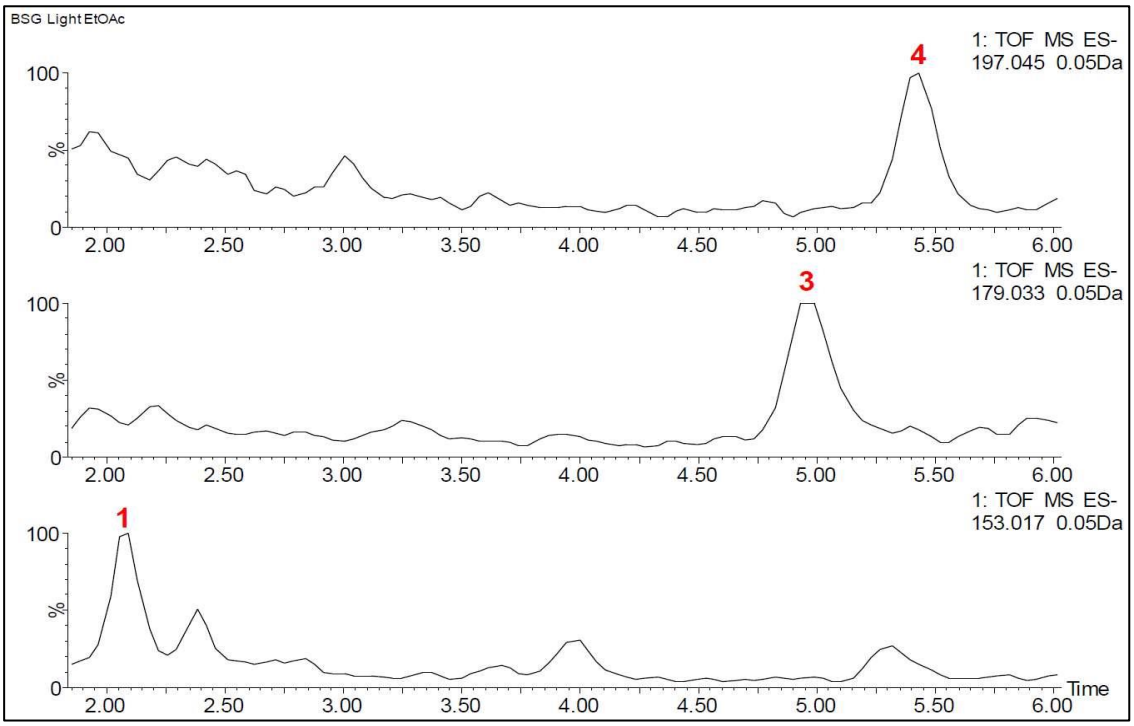
In this study, an additional peak eluting at 7.13 min (peak 7) contained a cluster of two molecules of ferulic acids corresponding to *m/z* 387.1073 [predicted molecular formula ($C_{20}H_{20}O_8$)]. On subjecting this molecular ion to MS/MS, the fragment ions *m/z* 343.1 [*m/z* 387.1 – *m/z* 44.1 (CO_2)], ferulic acid at *m/z* 193.1, *m/z* 149.1 [(*m/z* 193.1 – *m/z* 44.1 (CO_2)] and *m/z* 134.0 [(*m/z* 193.1 – *m/z* 59.1 (CO_2CH_3)] further supported the detection of dimeric ferulic acid (Figure 4-4).

Such non-covalent dimers generally form when the monomeric units are abundant in the sample, i.e., ferulic acid in this case.

Table 4-4 HPLC-Q-ToF identification of polyphenols in the ethyl acetate fraction of hydrolysed light BSG.

Peak No.	RT (min.)	Observed $[M-H]^-$ (*m*/*z*)	Calculated $[M-H]^-$ (*m*/*z*)	Chemical Formula	MS/MS Fragment Ions (*m*/*z*)	Tentative Identification
1	2.05	153.0169	153.0188	$C_7H_6O_4$	109.03	protocatechuic acid
2	3.50	137.0227	137.0239	$C_7H_6O_3$	93.04	hydroxybenzoic acid
3	4.93	179.0331	179.0344	$C_9H_8O_4$	135.04	caffeic acid
4	5.43	197.0452	197.0450	$C_9H_{10}O_5$	153.03	syringic acid
5	5.65	121.0282	121.0290	$C_7H_5O_2$	92.03	benzoic acid
6	6.80	163.0380	163.0395	$C_9H_8O_3$	119.05	coumaric acid
7	7.13	387.1073	387.1080	$C_{20}H_{20}O_8$	343.13, 193.05, 178.03, 149.07, 134.05	ferulic-ferulic acid dimer
8	7.34	223.0614	223.0606	$C_{27}H_{30}O_{16}$	179.02	sinapic acid
9	7.54	341.1019	341.1025	$C_{19}H_{18}O_6$	267.08, 193.05, 134.04	decarboxylated diferulic acid
10	7.87	385.0915	385.0923	$C_{20}H_{18}O_8$	282.09, 267.07 (100%), 239.08, 148.06	diferulic acid
11	8.73	385.0909	385.0923	$C_{20}H_{18}O_8$	325.09/326.09, 282.11/281.11 (100%), 267.08 (75%).	diferulic acid isomer
12	9.19	193.0516	193.0501	$C_{10}H_{10}O_4$	178.03, 134.04	ferulic acid
13	9.39	577.1342	577.1346	$C_{30}H_{26}O_{12}$	533.17, 355.09,	triferulic acid
14	10.44	341.1035	341.1025	$C_{19}H_{18}O_6$	326.09, 311.07, 282.09, 267.08 (100%), 239.08	decarboxylated diferulic acid isomer

Figure 4-3 Extraction ion chromatograms for peak 1 (*m/z* 153.017 [M-H]-), peak 3 (*m/z* 179.013321 [M-H]-) and peak 4 (*m/z* 197.045 [M-H]-)



The LC-Q-ToF MS analysis of the BSG L extracts showed the presence of [M – H]- ions at *m/z* 577.1 eluting at ~ 9.4 min (peak 13 in Table 4-4, and Figure 4-5 (a)). This molecular ion (*m/z* 577.1) can be dimers of procyanidin B as previously reported to be present in BSG (Verardo et al., 2015, Quinde-Axtell and Baik, 2006b), but could as well represent the trimeric form of ferulic acids (Underlin et al., 2020). The chemical formula ($C_{30}H_{26}O_{12}$) predicted by accurate mass measurement (*m/z* 577.1342) also could not differentiate between the procyanidin B and ferulic acid trimer as both have identical chemical composition. Further LC-QToF MS analysis of authentic procyanidin B1 and B2 (Figure 4-5 (b, c)) showed different retention times (2.3 min and 4.0 min, respectively) than the peak eluting from the sample suggesting that this compound cannot be procyanidin B type. Further MS/MS analysis of the *m/z* 577.1 ions from the procyanidin B1 and the sample (Figure 4-5) were performed.

Figure 4-4 Electrospray ionisation (ESI)-MS/MS of *m/z* 387.1 showing the fingerprint fragment ions of the ferulic acid dimer (FA = ferulic acid).

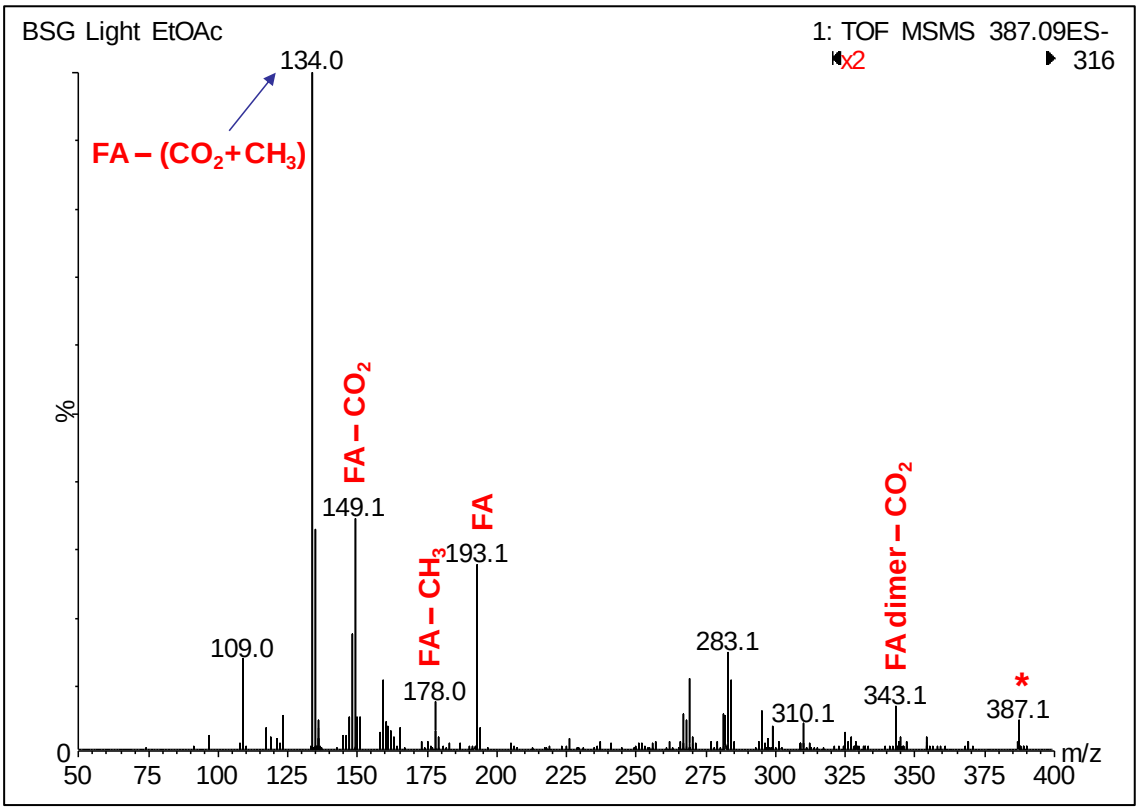
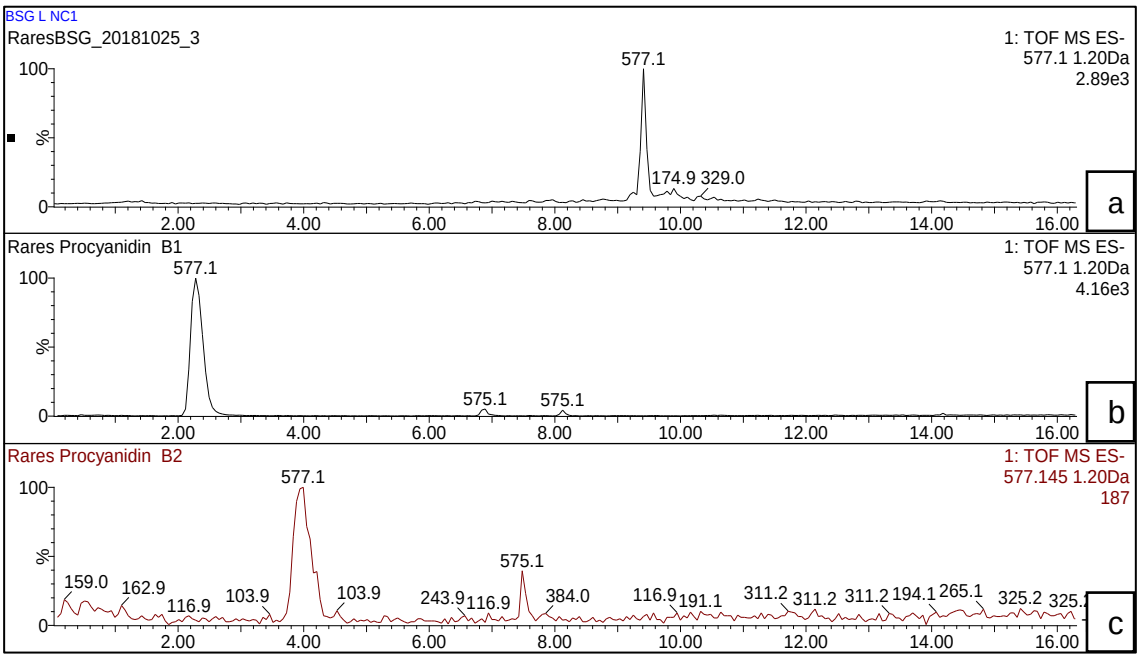


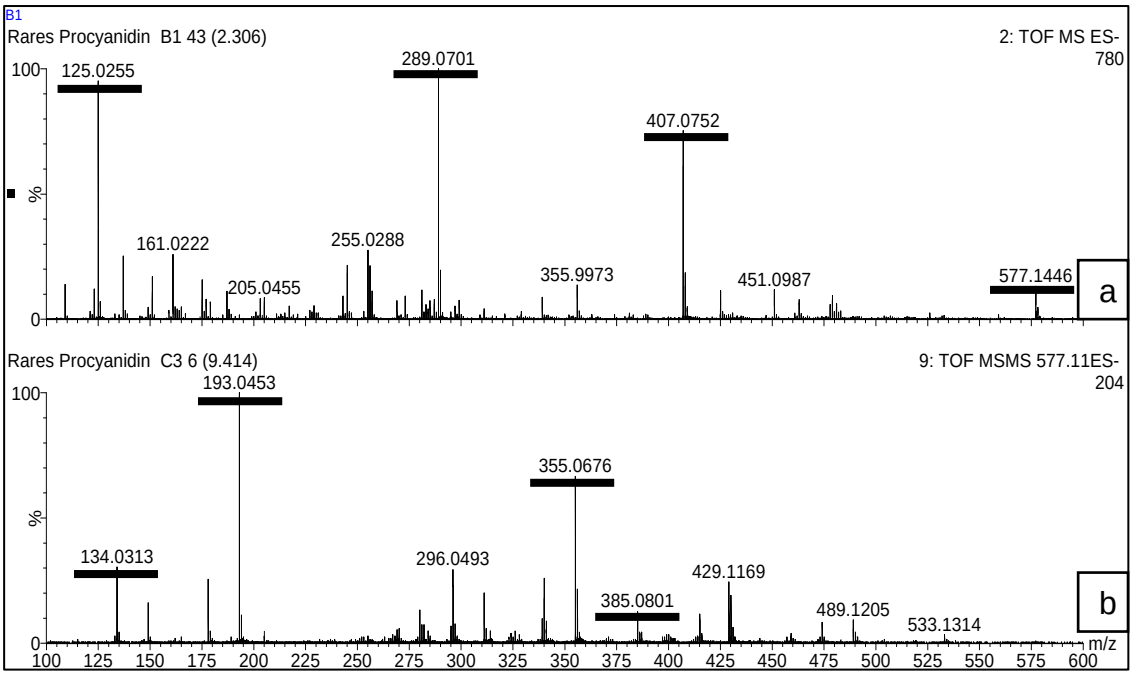
Figure 4-5 Extraction ion chromatograms for the peaks at *m/z* 577.1 $[M-H]^-$ of BSG L extract (a), Procyanidin B_1 (b) and -B_2 (c) (Note: peak at *m/z* 577.1 from (a) also corresponds to peak 13 from Figure 4-2).



The fragmentation pattern of Procyanidin B_1 showed its characteristics product ions at *m/z* 407, 289 and 125 in the MS/MS spectrum, where the fragment

ions at *m/z* 289 represents to the monomeric (epi)catechin. However, the MS/MS of *m/z* 577.1 from the samples (Figure 4-5b) showed different product ions at *m/z* 429, 385, 355, 193 and 134 in the MS/MS spectrum. These *m/z* ions suggest the characteristic fragmentation pattern of ferulic acid (*m/z* 193), its dimer (*m/z* 385) revealing that the *m/z* 577.1 in the samples is a ferulic acid trimer (Jay et al., 2008, Moreira et al., 2013). Moreover, neither catechin nor procyanidins have been report in the saponified bound phenolic extracts of BSG residues (Ikram et al., 2017).

Figure 4-6 Electrospray ionisation (ESI)-MS/MS of *m/z* 577.1 showing the fingerprint fragment ions of the Procyanidin B1 (a) and trimer of ferulic acid (b).



4.4.3.2 Quantification of BSG Polyphenols

Total polyphenols, the aggregate sum of individual polyphenols measured by UPLC-MS/MS, in each of the BSG EtOAc fractions, were found in decreasing levels of abundance in the following order: BSG L > BSG Mix > BSG D (Table 4-5). Statistically significant differences were found (in the same direction of abundance as TPC by FC) between the total polyphenols of BSG L, D, and the Mix. The BSG L (2 741 µg/g BSG dw) contained more than four times the total polyphenols found in BSG D (693 µg/g BSG dw), which is in contrast to the TPC values where the dark BSG contained similar levels to light BSG as in this study (Table 4-6) or exceeded those in the light BSG (Piggott et al., 2014, Moreira et

al., 2013). The BSG Mix showed intermediate total polyphenol levels, i.e., between the BSG L and the BSG D as expected. Since BSG Mix constituted both the L and D (~9:1 w/w) BSG, we also measured the polyphenols in its crude and various 'liquor' fractions (prior to neutralisation and EtOAc partitioning) by UPLC-MS/MS. The crude extract of the BSG Mix contained low levels of polyphenols (~26 µg/g BSG dw), of which catechin constituted more than 50% of the total free polyphenols. This was 45- to 54- fold less than the total polyphenols present in the various EtOAc fractions (1 170–1 387 µg/g dw) of the same sample. McCarthy et al. 2012 also reported low levels of total polyphenols (30.6 µg/g in light and 27.2 µg/g in dark BSG dw) using HPLC coupled with diode array detector (DAD)-mass spectrometry analysis of the crude extracts (McCarthy et al., 2012). Stefanello et al. 2018, on the other hand, recorded 82.4 µg/g total polyphenols in the crude BSG extract, of which catechin constituted 83% of the total polyphenols. The TPC for these two studies ranged from 0.98–4.53 mg GAE/g BSG dw, which corroborate our findings (Stefanello et al., 2018b). An even more interesting finding is that the total polyphenols in the liquors of BSG Mix were significantly lower than in the corresponding EtOAc fractions despite the fact that the TPC values for all 'liquor' fractions were very high (Tables 4-3 and 4-5). A similar observation was made by Stefanello et al. 2018, where the TPC for the liquor was 17.4 mg GAE/g BSG dw, whilst the HPLC-DAD quantification of total polyphenols for the same liquor was 3 195 µg/g dw (Stefanello et al., 2018b). The HPLC-DAD value was closer to the TPC value of their crude BSG extract (3.43 mg GAE/g BSG dw). The high TPC values in the liquor fractions must have been attributed by other non-polyphenolic compounds such as reducing sugars, amino acids and peptides (Jay et al., 2008) that are fractionated in the water phase during the EtOAc:water partitioning.

In all the saponified BSG extracts, ferulic acid was the most predominant phenolic acid comprising in excess of 50% of the total polyphenols, followed by *p*-coumaric acid. When the most abundant phenolic acid, i.e., ferulic acid is considered, there is no significant difference between the efficiency of the different extraction methodologies for the same type of BSG substrate. Several other studies have also established that the dominant polyphenols in BSG are ferulic acid and *p*-coumaric acid (Mussatto et al., 2007a, Stefanello et al., 2018b)

and thus had become the target compounds of recovery in several studies (Sancho et al., 2001, Mussatto et al., 2007a, Bartolomé et al., 1997, Zuorro et al., 2019, Hernanz et al., 2001, Dobberstein and Bunzel, 2010, Faulds et al., 2002a, Szwajgier et al., 2010).

Other abundant polyphenols in the BSG were sinapic acid and syringic acid, which have also been reported by other authors (Moreira et al., 2013, Szwajgier et al., 2010). The UPLC-MS/MS determination of total polyphenols from MAE and UAE of the BSG EtOAc fractions showed a similar trend to their TPC values (Tables 4-3 and 4-5), where MAE and UAE yielded lower total polyphenols than the conventional maceration method. The lowest recovery of total polyphenols was by the MAE method. As explained earlier in Section 4.4.2, the MAE technique was not able to overcome the lignin-rich barrier, and that the extraction parameters used in the MAE and UAE may have induced thermal degradation of polyphenols. The UPLC-MS/MS quantification of polyphenols in the various BSG EtOAc fractions was closer to the spectrophotometric FC-method (Table 4-5 vs. Table 4-3). Athanasios et al. have used gas chromatography-mass spectrometry (GC-MS) and showed total polyphenols ranged between 2 688 to 4 884 µg/g dw in the four different batches of BSG, although the authors did not perform spectrophotometric analysis but these values are very close to TPC values of BSG in general (Athanasios et al., 2007a).

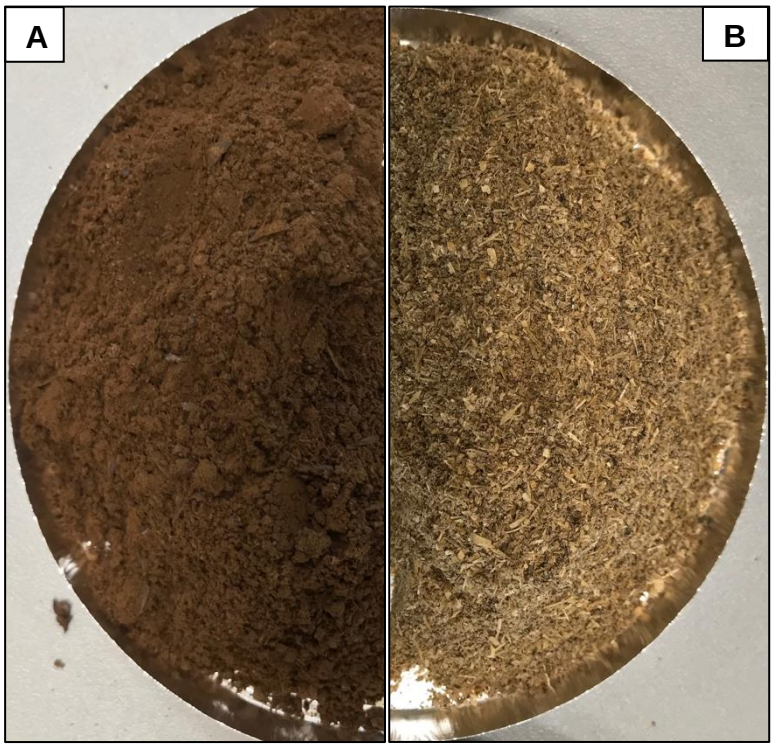
As in Chapter 3 we have used similar substrates as in this chapter (BSG L and BSG D), and partly similar extraction methodologies (maceration with organic solvents and sodium hydroxide) it is worth to mention several key differences between the obtained results in the TPC and the quantification of individual phenolics.

The UPLC-TQD quantification of total polyphenols in the BSG L Ctrl EtOAc extract (approx. 2.7 mg/g BSG dw) obtained by maceration under alkali hydrolysis in this chapter (Table 4-6) was 3.5x higher compared with the levels obtained in Chapter 3 section 3.4.3 of the similar BSG L extract (0.8 mg/g BSG dw), and 2.4x lower in the BSG D Ctrl EtOAc extracts vs BSG D AL Control extract (0.7 vs 1.7mg/g BSG dw), respectively. Similarly, to the results obtained in Chapter 3, the most abundant polyphenols were FA and *p*-CA. In this chapter, FA was approx. 2.5x higher compared to *p*-CA in both BSG L- and D Ctrl EtOAc extracts.

The opposite was observed in Chapter 3, where *p*-CA was the predominant phenolic compound, at a 6x and 7x higher levels compared to FA in the BSG L and D extracts, respectively. Moreover, in this chapter, the crude extracts were abundant in catechin, whereas in chapter 3, FA and *p*-Ca were the predominant phenols.

There are several possible explanations for these variations in the phenolic content observed in the two research chapters. In Chapter 3, for example, the extraction of free and bound phenolics was performed sequentially, whereas in this chapter each free and bound phenolic extraction was performed individually, meaning that the bound phenolic extracts also contain free phenolics. This may be one reason for the increase in the TPC, and the individual phenolic levels in the bound phenolic extracts in this chapter. The extraction temperature was also a factor that needs to be mentioned, as in this chapter, the extraction temperature applied was close to the boiling point of the organic solvent used to extract free and bound phenolics, whereas in Chapter 3 the extraction was performed at room temperature. Higher temperatures favour solvent diffusion in the sample matrix, thus potentially allowing for a better extraction and an increased yield of phenolic compounds (Alara et al., 2021). There was a difference in the same substrate types, for example the BSG dark used in Chapter 3 (River Rye Ireland) was less dark than the one used in this chapter (Diageo Ireland), based on appearance (see Figure 4-7). The kilning temperatures of malt used by Diageo in producing Guinness stout beer are around 232°C obtaining a completely black coloured malt (Guinness, 2018), whereas we believe that River Rye used a slightly lower roasting temperatures, to obtain a malt of a caramel colour.

Figure 4-7 BSG D milled provided by Diageo Ireland (A) and by River Rye Ireland (B)



This observation is of importance as it has been shown in the literature that the levels of phenolic compounds decrease with the increase in the roasting temperatures (Moreira et al., 2013, Samaras et al., 2005). The increase in extraction temperatures and times and a lower volume of acetone seemed to promote the extraction of free phenolics, including the flavan-3-ol, catechin, as observed in the higher TPC and the individual phenolic levels in the free phenolic extracts (crude extracts). This observation was also made by Meneses et al. 2013, where the use of 60% acetone almost doubled the TPC values compared to 80% acetone extraction (Meneses et al., 2013). Finally, breweries use different sources and batches of barley malt to produce beer and is well now that the phenolic content varies with the barley variety, malting and brewing process and if other natural adjuncts (e.g. wheat or oats) are being added to the brewing process, resulting in untraceable sources for BSG residues (Wannenmacher et al., 2018, Kunze, 1996). The problem with this last part is that the brewing companies do not want to share information regarding barley malt variety used, malting process or even if adjuncts are added to the brewing process, thus making the use of BSG residues and the extraction process inconsistent, meaning the use of a standardised extraction process would not yield similar

phenolic levels. Based on the above explanations, these are some of the hypotheses I have developed to explain why this chapter's phenolic levels of the BSG extracts are greater than Chapter 3.

Table 4-5 UPLC-TQD quantification of BSG polyphenols

Samples	Ferulic Acid	*p*-Coumaric Acid	Catechin	4-Hydroxybenzoic Acid	Sinapic Acid	Syringic Acid	Protocatechuic Acid	Caffeic Acid	Total
BSG L Ctrl EtOAc	1809.5±272.8^{a}	686.6±59.0^{a}	2.11±0.23^{b}	16.66±4.45^{a}	14.63±2.48 ^{a}	33.9±10.44 ^{b}	3.46±1.04 ^{ab}	0.147±0.065 ^{d}	2741.1±5.2 ^{a}
BSG L MAE EtOAc	1545.6±157.3^{a}	499.1±31.2^{bc}	1.43±0.48^{b}	9.41±1.15^{bcd}	11.02±3.99 ^{ab}	18.9±7.26 ^{bc}	1.38±0.72 ^{cd}	0.370±0.031 ^{b}	2087.2±196.8 ^{a}
BSG L UAE EtOAc	1669.7±21.8^{a}	579.2±22.7^{b}	1.05±0.07^{b}	10.76±0.99^{bcd}	10.36±1.52 ^{ab}	17.8±3.68 ^{bc}	2.29±0.83 ^{bc}	0.176±0.013 ^{d}	2291.2±42.7 ^{ab}
BSG D Ctrl EtOAc	404.7±51.0^{cd}	185.3±8.3^{f}	1.66±1.01^{b}	13.12±0.38 ^{ab}	7.63±1.92 ^{bc}	76.4±28.84 ^{a}	3.83±0.63 ^{a}	0.407±0.065 ^{b}	693.0±85.7 ^{de}
BSG D MAE EtOAc	351.0±33.9^{d}	155.3±7.5^{f}	1.23±0.33^{b}	11.36±2.28 ^{bc}	4.68±0.67 ^{c}	21.7±4.84 ^{bc}	4.09±0.55 ^{a}	0.547±0.079 ^{a}	549.9±41.5 ^{e}
BSG D UAE EtOAc	413.6±135.8^{cd}	173.4±56.6^{f}	2.18±0.74^{b}	10.69±1.39 ^{bcd}	8.28±0.46 ^{bc}	17.3±5.91 ^{bc}	4.85±0.47 ^{a}	0.389±0.052 ^{b}	629.9±190.9 ^{de}
BSG Mix Ctrl EtOAc	894.6±82.8^{b}	476.4±35.1^{bcd}	nd	6.02±0.93 ^{de}	9.59±0.23 ^{abc}	nd	0.062±0.012 ^{d}	0.226±0.049 ^{cd}	1387.0±119.0 ^{c}
BSG Mix MAE EtOAc	796.8±68.1^{b}	355.4±33.0^{e}	0.47±0.82^{b}	6.88±0.30 ^{cde}	10.23±0.68 ^{ab}	nd	0.015±0.026 ^{d}	nd	1169.8±66.4 ^{c}
BSG Mix UAE EtOAc	848.5±15.2^{b}	386.9±6.7^{de}	nd	6.59±0.55 ^{de}	11.33±1.54 ^{ab}	nd	0.174±0.085 ^{d}	0.328±0.005 ^{bc}	1253.8±11.3 ^{c}
BSG Mix Crude	2.8±2.41^{e}	nd	14.05±1.19^{a}	0.11±0.12 ^{f}	8.28±0.14 ^{bc}	nd	0.49±0.17 ^{d}	nd	25.7±1.97 ^{f}
BSG Mix Liquor Ctrl	714.1±76.7^{bc}	423.3±17.6^{cde}	1.09±0.98^{b}	4.24±0.50 ^{ef}	12.29±1.09 ^{ab}	nd	nd	nd	1155.0±93.2 ^{c}
BSG Mix Liquor MAE	647.4±40.7^{bcd}	330.6±49.5^{e}	1.86±0.36^{b}	4.26±0.33 ^{ef}	9.52±0.29 ^{bc}	nd	nd	nd	993.6±74.8 ^{cd}
BSG Mix Liquor UAE	739.1±22.3^{b}	371.9±30.9^{de}	nd	4.12±0.37 ^{ef}	11.11±0.^{39} ^{ab}	nd	nd	nd	1126.3±53.2 ^{c}

Values are expressed as µg/g BSG dw (mean±SD); nd—not detected; For each substrate, the values reported, for individual and total polyphenols in liquors and their ethyl acetate (EtOAc) fractions bearing different letters (a, b, c, d, e, f) are significantly different ($p < 0.05$) from each other

4.5 Conclusions

UAE and MAE treatments did not improve the BSG polyphenol yield indicating possible thermal degradation of polyphenols with the extraction parameters used in these systems. The findings also suggest that ultrasonic bath operating at 35 kHz is less efficient in aqueous solution for the extraction of polyphenols from BSG. However, these techniques may improve the polyphenol yield and efficacy with further optimisation and when used with other systems, such as ultrasonic probes, and in combination with appropriate organic solvents.

The UPLC-MS/MS data have shown that the saponification followed by acidification and subsequent liquid-liquid partitioning (EtOAc) is the best procedure for polyphenol recovery and enrichment from BSG, irrespective of extraction method. Without neutralisation and partitioning, the colourimetic chemical TPC method falsely overestimates the total phenolic content and levels quantified by related assays in the liquors. Hyphenated chromatographic quantification methods such as LC-MS/MS is therefore necessary to accurately portray levels of total BSG polyphenols present. Moreover, LC-MS/MS is a strong analytical tool to elucidate the presence of unknown compounds, such as diFA, triFA, and differentiate between triFA and Procyanidin B as exemplified in this chapter.

Due to these findings, in the following chapters the extraction of polyphenols for functional testing will be made using the classic extraction approach, with acetone 60% and 0.75% NaOH at elevated temperatures, and the follow-up enrichment using liquid-liquid extraction with diethyl ether: ethyl acetate.

Chapter 5

5. Anticholinesterase Activities of Different Solvent Extracts of Brewer's Spent Grain

This research investigation resulted in the publication of the following research article:

> ***Birsan, R.I**.; Wilde, P.; Waldron, K.W.; Rai, D.K. Anticholinesterase Activities of Different Solvent Extracts of Brewer's Spent Grain. Foods 2021, 10, 930. https://doi.org/10.3390/ foods10050930*

5.1 Abstract

Cholinesterases, involved in acetylcholine catabolism in the central and peripheral nervous system, have been strongly linked with neurodegenerative diseases. Current therapeutic approaches using synthetic drugs present several side effects. Hence, there is an increasing research interest in naturally-occurring dietary polyphenols, which are also considered efficacious. Food processing by-products such as brewer's spent grain (BSG) would be a potential bio-source of polyphenols. In this study, polyphenol-rich BSG extracts using 60% acetone and 0.75% NaOH solutions were generated, which were further subjected to liquid–liquid partitioning using various organic solvents. The water-partitioned fractions of the saponified extracts had the highest total polyphenol content (6.2±2.8 mgGAE/g dw) as determined by Folin–Ciocalteu reagent, while the LC-MS/MS showed ethyl acetate fraction with the highest phenolics (2.9±0.3 mg/g BSG dw). The best inhibitions of acetyl- (37.9±2.9%) and butyryl- (53.6±7.7%) cholinesterases were shown by the diethyl ether fraction of the saponified extract. This fraction contained the highest sum of quantified phenolics (99±21.2 µg/mg of extract), and with significant ($p < 0.01$) inhibitory contribution of decarboxylated-diferulic acid (30.6±0.8 µg/mg of extract). Amongst the standards, caffeic acid presented the highest inhibition for both cholinesterases, 25.5±0.2% for acetyl- and 52.3±0.8% for butyryl-cholinesterase, respectively, whilst the polyphenol blends that mimicked the BSG free and bound phenolic extracts insignificantly inhibited both cholinesterases. The results showed that polyphenol rich BSG fractions have potentials as natural anti-cholinesterase agents.

5.2 Introduction

Evidence in the current literature suggests a strong link to the protective effects of dietary polyphenols towards the prevention of so called “diseases of civilization”, i.e., chronic non-communicable diseases, and protective effects justified via the “biochemical scavenger theory” (Cory et al., 2018, Koch, 2019). Not only being the most abundant antioxidants present in human diet, researchers, food companies as well as consumers, consider dietary polyphenols to be one of the core groups of dietary preventive agents (Teixeira et al., 2013).

Alzheimer’s disease (AD), the most common type of dementia, is a progressive neurodegenerative disease that is commonly characterized by the presence of amyloid-β deposits, τ-protein aggregation, low levels of acetylcholine and oxidative stress (Atta-ur-Rahman, 2015). More than 115 million people worldwide are estimated to be affected by this disease by 2050 with most of individuals aged over 65 years (Atta-ur-Rahman, 2015). Even though the AD pathogenesis has not been fully understood, the main mechanistic theory proposed is the “cholinergic hypothesis” (Cavdar et al., 2019). Choline is an important quaternary amine responsible for the structural integrity and signalling functions of cell membranes, which directly affects the cholinergic neurotransmission (Zeisel, 2003). Acetylcholine and butyrylcholine are important metabolites of choline; acetylcholine is the main neurotransmitter at autonomic preganglionic nerve terminals and mostly prevalent in cholinergic synapses of the central and peripheral nervous system (Geula, 2004, Westfall, 2009). A decrease of acetylcholine levels in the cholinergic synapses in the brain regions seems to be a critical element in the development of AD. Cholinesterases, i.e., acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), are enzymes that hydrolyse acetylcholine and butyrylcholine, respectively, and their inhibition is a current therapeutic target (Atta-ur-Rahman, 2015). Synthetic drugs prescribed to inhibit cholinesterase’s activity have known to have side effects including nausea, vomiting, headache, etc. However, some plant-derived alkaloids such as galantamine, tacrine and physostigmine are also used and have shown symptomatic improvement in AD (Cavdar et al., 2019).

Research studies in recent years have led to the belief that the polyphenols, as natural antioxidants, play a role in prevention and management of numerous degenerative diseases by reducing the oxidative stresses generated by the presence free radicals and oxidants (Hussain et al., 2016, Idehen et al., 2017). Several studies have shown a solid association between foods rich in polyphenols and the reduction of oxidative stress and amyloid accumulation in AD patients (Kim et al., 2007, Hartman et al., 2006, Hu et al., 2013). Specific phenolic acids such as ferulic acid, caffeic acid, *p*-coumaric acid, and 4-hydroxybenzoic acid among others have received considerable attention as anti-inflammatory agents in the pathogenesis of chronic diseases including cancer and cardiovascular diseases (Bouzaiene et al., 2015, Kumar and Pruthi, 2014, Yabe et al., 2010, Winter et al., 2017). Coincidently, the aforementioned phenolic acids are present in high abundance in brewer's spent grains (BSG) (Bartolomé et al., 1997, Faulds et al., 2002b). The main hurdle in BSG is that these phenolic acids are generally bound to other cell wall components requiring hydrolysis by chemical or enzymatic methods for their extraction (Guido and Moreira, 2017, Naczk and Shahidi, 2006a, Nardini et al., 2002). Saponification with sodium hydroxide (NaOH) at different concentrations is an efficient method for liberation of ester- and ether-linked phenolics from xylan, hemicelluloses and lignin components as described in the literature (Soccol et al., 2019) as well as our own studies in chapters 3 and 4. In order to obtain extracts rich in polyphenols, solid–liquid extraction and subsequent liquid–liquid extraction are the most frequently used procedures for this purpose due to ease of use, efficiency and broad applicability (Stalikas, 2007).

In chapters 3 and 4, several solid-liquid extraction methodologies, in combination with both organic solvents, acid / alkali solutions and even with novel extraction technologies, such as microwave and ultrasound were tested. The best outcome to obtain a high polyphenol extraction yield was shown by using the classic approach, maceration with 60% acetone to recover free phenolics and 0.75% sodium hydroxide with the follow-up ethyl acetate liquid-liquid extraction for bound phenolics, methodologies used with specific extraction parameters, such as temperature, time, solid-liquid ratio. Due to the variability in the type of phenolics extracted, both organic solvent (60% acetone) and saponification

(0.75% sodium hydroxide) were used to generate free and bound phenolic extracts and tested further for their potential to be used as bioactive compounds. Moreover, among the tested BSG substrates in this research project, Brewer's spent grain light extracts presented the highest content of phenolic compounds thus it was selected to be tested as a potential source of bioactive compounds using *in vitro* bioactivity assays.

In this research chapter, in addition the classic extraction approach, for the liquid-liquid extraction several extra organic solvents of different polarities have been used. As a result, the liquid-liquid extraction was employed to broaden the scope of the biphasic liquid-liquid process for the extraction and separation of bioactive compounds of varied polarities. The separation and enrichment of these hydrolysed constituents depend greatly on the suitability of the extraction process, phase separation of the initial solvents besides other extraction parameters (temperature, time, pH, etc.) However, a great influence on the recovery of the constituent compounds is the choice of solvent used. Laws of similarity and miscibility suggest that it is more likely for a solute to dissolve in a solvent close to its polarity. Phenolic acids are categorized as hydrophilic or polar compounds and have been successfully fractionated and purified from complex mixtures by using mid-polar range solvents, i.e., ethyl acetate.

With a hypothesis that phenolic rich extracts from BSG could be efficient inhibitors against AChE and BChE activities, this study aimed: (1) to assess the efficiency of four different organic solvents (hexane, diethyl ether, ethyl acetate, butanol) in recovery of phenolic compounds from free and bound phenolic extracts; (2) to determine the phenolic content of the extracts and generated fractions and asses their anti-cholinesterase activities along with the individual and mixtures of quantified phenolic compounds detected; (3) to determine any associations between the phenolic composition and the inhibition of the AChE and BChE activities.

5.3 Materials and Methods

5.3.1 Samples and Chemicals

Brewer's spent grain Light (BSG) was provided by Diageo Dublin, Ireland, which was directly transported to the research centre within 30 min, oven-dried (see the drying protocol in Chapter 2, section 2.3), vacuum packed and stored at -28ºC until required. The BSG used in this research chapter was from a different batch to the one used in Chapter 4.

All the chemicals, organic solvents, polyphenol standards, reagents used in this research chapter were purchased from Merck (formerly Sigma Aldrich, Arklow, Co. Wicklow, Ireland). Materials used for the *in vitro* cholinesterase inhibitory activities: substrates (acetylthiocholine iodide, s-butyryl thiocholine iodide), enzymes (acetylcholinesterase from electric eel, butyrylcholinesterase from equine serum), inhibitor standard (galantamine hydrobromide from *Lycoris sp*.), proteins (bovine serum albumin), Ellman's or 5,50 –Dithiobis (2-nitrobenzoic acid) (DTNB) reagent, chemicals tris hydrochloride, tris base). The organic solvents used for solid-liquid and liquid -liquid extraction: methanol, ethanol (EtOH), acetone (Ace), n-hexane (Hex), diethyl ether (DE), ethyl acetate (EtOAc), n-butanol (BuOH), acetonitrile, formic acid, hydrochloric acid (HCl), and sodium hydroxide (NaOH); Polyphenol standards: *p*-coumaric acid (p-CA), ferulic acid (FA), caffeic acid (CafA), protocatechuic acid (ProA), 4-hydroxybenzoic acid (4-HBA) and +(-)catechin (Cat); Total phenolic content assay: sodium carbonate, gallic acid, Folin Ciocalteu reagent. All chemicals used were of analytical grade and all solutions were prepared with milli-Q water.

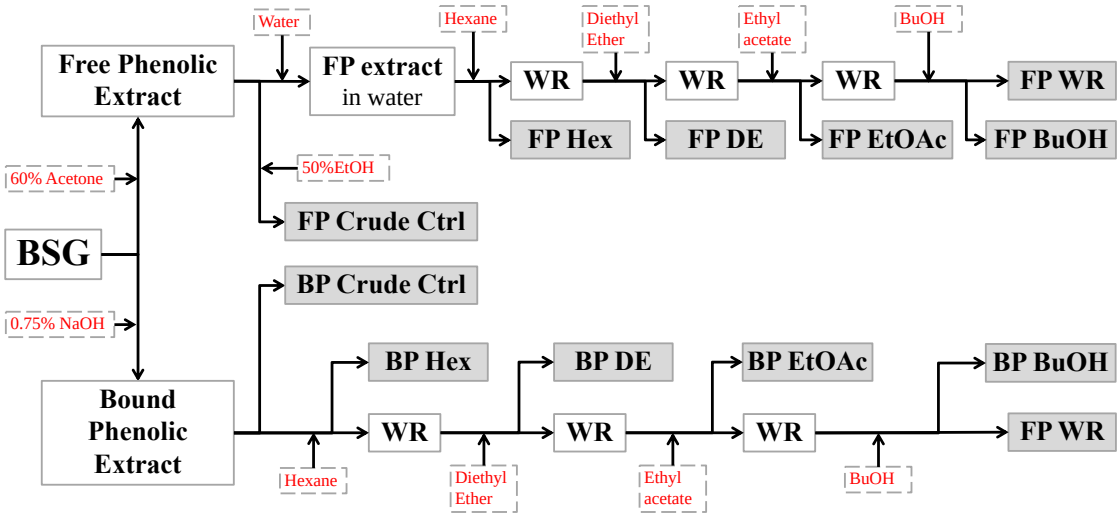
5.3.2 Solid-liquid extraction of free and bound phenolic

A schematic flow of the extraction procedure is illustrated in Figure 5-1.

Extraction of free- (FP) and bound-phenolic (BP) compounds from BSG was done by maceration in combination with 60% acetone and 0.75% NaOH solution, respectively, and as previously described in Chapter 4. Briefly, milled BSG was mixed with 60% acetone (1:20 w/v) in a sealed amber glass bottle and kept in a water-bath at 60°C to extract free phenolics and mixed with 0.75% NaOH solution at 80°C to extract bound phenolics, for 30 min with constant stirring. After the

treatment time, all the extracts were left to cool at room temperature and centrifuged. The supernatants were pooled, followed by syringe filtration, whereas the BP extracts were neutralized and paper-filtered under vacuum. The FP and BP extracts were stored at −28 ◦C until required. The extraction of FP and BP from BSG was carried out in quadruplicates, from which three were used further for liquid–liquid partitioning (fractions FP-, BP- Hex, DE, EtOAc, BuOH, WR) and one as control (FP-/BP- Crude Ctrl) as illustrated in (Figure 5-1).

Figure 5-1 Extraction process of free and bound phenolic compounds from BSG followed by their partitioning using different organic solvents and analysis of the obtained extracts.



FP – free phenolics, BP- bound phenolic; Ctrl – control, WR – water residue, EtOH – ethanol, Hex – hexane, DE – diethyl ether, EtOAc – ethyl acetate, BuOH– butanol, NaOH – sodium hydroxide.

5.3.3 Liquid–Liquid Partitioning of Free and Bound Phenolic Extracts

The fractionation of the FP and BP extracts with solvents of different polarity was adapted from (Tu et al., 2013) with some modifications.

Both the BSG FP and BP extracts were fractionated by using solvents with increasing polarity (empirical parameters of normalized solvent polarity shown in brackets after each solvent) as shown in Figure 5-1 as follows: n-hexane (0.009), diethyl-ether (0.117), ethyl acetate (0.228), n-butanol (0.586) saturated by water (1.0), and the residual water as the remaining fraction. One of the four free phenolic extracts was concentrated under vacuum (Rotavapor R-100, Buchi, Switzerland) to evaporate the acetone and the concentrate was reconstituted in 50% ethanol (FP Crude Ctrl), whereas the rest of three extracts were concentrated to evaporate only the acetone and the remaining water part was used for liquid–liquid partitioning (Figure 5-1). The recovered volumes of aqueous

FP and BP extracts were sequentially pooled three times with each organic solvent in equal volumes of water. The organic layer was recovered and concentrated under vacuum (38ºC), whereas the remaining residual water fraction (WR) was freeze dried. The recovered dried material was reconstituted in a minimal volume of ethanol (98%, v/v) and further diluted with double distilled water to a final concentration of 20 mg/mL, which served as stock solution. The final fractions were syringe filtered as above and stored in a freezer at −28ºC until further use.

5.3.4 Determination of Polyphenolic Content

Total phenolic content was estimated by Folin–Ciocalteu and quantification of BSG polyphenols in the FP and BP extracts and fractions was performed by LC-MS/MS, and as previously described in Chapter 3 and Chapter 4.

5.3.4.1 Total Phenolic Content (TPC) by Folin–Ciocalteu (FC)

Total phenolic content of BSG extracts was determined by colorimetric assay using FC reagent. Briefly, a solution of BSG extract, methanol and 20% sodium carbonate were prepared and incubated in darkness for 20 minutes at room temperature. The mixture was then vortexed, centrifuged and an aliquot of the supernatant was transferred to 96-well plate and the absorbance measured at 735nm. The recorded absorbance of the samples was calculated using a calibration curve of known concentrations of gallic acid. The results are expressed in milligrams of gallic acid equivalent per gram BSG dry weight (mg GAE /g BSG dw).

5.3.4.2 Individual Polyphenol Quantification by UPLC-MS/MS

Ultra-high performance liquid chromatography coupled to a tandem quadrupole mass spectrometer (UPLC-MS/MS) was used to quantify the most abundant polyphenols. For the quantification of polyphenols, appropriate dilutions (0.098 to 50 ppm) of each standard (FA, *p*-CA, Cat, CafA, 4-HBA, ProA) were prepared to obtain a standard calibration curve. TargetlynxTM (Waters Corp., Milford, MA, USA) software was used to quantify the compounds in the various extracts. The ferulic acid dimers and trimers were quantified using the standard curve from FA ($y = 1064.59x + 12.24$, $r^2 = 0.99$).

5.3.5 Preparation of Polyphenol Blends

In order to associate the anti-cholinesterase activity of the BSG fractions to their polyphenol content and composition, blends that mimic the polyphenol profile in the BSG fractions were prepared and tested separately (Table 5-2). Thus, six polyphenols were used in combination to prepare three blends that mimic their abundance in BSG fractions. The blends were prepared at a specific polyphenol concentration as calculated by their UPLC-MS/MS quantification to a final concentration of 1000 µg/mL. The values (µg/mg BSG extract) obtained for each polyphenol from the quantification data were summed and the % content of each polyphenol was calculated from the sum (Table 5-1). The % result was used to calculate the amount of each polyphenol (µg) to be added to a blend mix totalling 1000 µg/mL. For this purpose, the fractions that presented the highest content of quantified polyphenols were selected, namely Blend FP1 EtOAc, Blend BP1 DE, Blend BP3 EtOAc; the number 1 or 3 following Blend FP or BP represents the replicate fraction number that was used to prepare the blend. The specific polyphenols combinations are presented in the Table 5-2 below.

Table 5-1 The individual polyphenol concentrations (µg/mg) of BSG extracts in the FP and BP BSG EtOAc fractions from which the blends had been prepared

Sample	Total (µg/mg)	FA	p-CA	Cat	CafA	4-HBA	ProA
FP EtOAc 1	2.1	0.076	0.007	1.42	0.049	0.074	0.451
BP DE 1	93.5	59.2	33.16	0.0	0.67	0.42	0.04
BP EtOAc 3	65.7	46.2	17.57	0.0	1.31	0.33	0.28
Calculated % of Total (µg/mg)							
FP EtOAc 1	100%	3.7	0.3	68.4	2.4	3.6	21.7
BP DE 1	100%	63.3	35.5	0.0	0.7	0.4	0.05
BP EtOAc 3	100%	70.3	26.7	0.0	2.0	0.5	0.4

Table 5-2 Blends of individual polyphenols at 1 000µg/mL mimicking their abundance in BSG fractions (Table 5-1).

Sample	Total (µg/mL)	FA	p-CA	Cat	CafA	4-HBA	ProA
Blend FP EtOAc 1	1000	36.6	3.7	683.7	23.5	35.5	217
Blend BP DE 1	1000	633.4	354.5	-	7.1	4.5	0.5
Blend BP EtOAc 3	1000	703.2	267.6	-	19.9	5	4.3

FP—free phenolic extract, BP—bound phenolic extract followed by the fraction replicate number; µg/mL—microgram per millilitre; FA—ferulic acid, p-CA—coumaric acids, Cat—catechin, CafA—caffeic acid, 4-HBA—hydroxybenzoic acid, ProA—protocatechuic acid.

5.3.6 Anti-Cholinesterase Assays

The inhibitory potential of BSG extracts, fractions, blends and individual polyphenol towards anti-AChE and anti-BChE activities was determined in vitro by Ellman's colorimetric method and adapted to cuvettes following the procedure of Faraone et al., 2019 (Faraone et al., 2019). BSG extracts being reconstituted in minimal volume of ethanol, various % ethanol solutions (blanks) were tested separately and any interference with the enzyme activity, were subtracted from the final calculations % inhibition generated by the extracts.

For the AChE assay, 75 µL of sample (1 mg/mL extract in final assay mixture), 150 µL of 50 mM Tris HCl buffer (pH 8 with 0.1% bovine serum albumin), 375 µL of 3 mM DTNB reagent and 75 µL of 15 mM acetylcholine iodide substrate were added in a cuvette and pipette mixed. The reaction was initiated by adding 75 µL of 0.18 U/mL AChE enzyme solution and pipette mixed. A blank solution containing 75 µL of 50 mM Tris HCl buffer instead of enzyme solution for each individual sample was used to zero the spectrophotometer prior to reaction initiation. Similar steps were followed for BChE assay, where the substrate (75 µL of 15 mM S-Butyrylthiocholine chloride) and the enzyme (0.1 U/mL of BChE) were used instead. The change in absorbance at 405 nm was recorded for every minute up to 5 min using Shimadzu PharmaSpec UV-1700 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Galantamine, a cholinesterase inhibitor and a commonly prescribed drug for treating AD, was used at different concentrations (1.56 to 50 µg/mL for AChE and BChE in 50% ethanol or otherwise specified) as positive control, and the required concentration to inhibit the activity of AChE and BChE by 50 percent (IC50) was calculated by nonlinear regression analysis. The rate of reaction over time (slope) was calculated for each recorded sample in duplicate against negative control (NC, 50 mM Tris HCl buffer instead of sample/inhibitor), and the results were expressed as percentage of inhibition following the equation:

(**Equation 2 - 1**):

$$\%Inhibition = (1 - \frac{Abs_{Slope}Sample}{Abs_{Slope}NegativeControl}) * 100$$

5.3.7 Statistical Analysis

Results are expressed as means of the triplicates ± standard deviation (SD). The datasets were evaluated for normality and homogeneity of variance by Shapiro–Wilk and Levene's test. Normally distributed data sets were evaluated using one-way ANOVA and Tukey's post hoc tests, whereas non-normal distribution by nonparametric Kruskal–Wallis and Dunn's post hoc test ($p < 0.05$). Welch analysis followed by Games-Howell post hoc test were performed when Levene's test (homogeneity of variance) was significant ($p < 0.05$). The correlation coefficients between the measured variables were calculated using Pearson correlation ($p < 0.05$), and the relation was assessed by regression model (dependent variables: AChE and BChE, independent variables: quantified phenolic compounds and their quantification methods, i.e., TPC by FC and SQP by UPLC-MS/MS). The statistical analytical steps were followed as proposed by Granato et al., 2014 (Granato et al., 2014). Principal component analysis (PCA) was carried out with the standardized data sets to disclose any association between the quantified phenolic compounds in the extracts and the enzymatic assays. Statistical analysis, Pearson's correlation and linear regression were carried out using SPSS v.25 (IBM corp.), while PCA using Minitab v.17 (Minitab, Inc., Coventry, UK).

5.4 Results and Discussion

5.4.1 Extraction Yield

The extraction yields were measured first for the crude extracts with and without saponification, and then for the different solvent fractions employed in liquid–liquid partitioning (Table 5-3). Extraction yield defined as "Total" yield in (Table 5-3) was determined by summing the extraction yield of each of the various liquid–liquid fractions. As exemplified by the 60% acetone extract, the yield of total fractions (80.2±3.4 mg/g BSG dw) was lower than that of the crude extract (94.9±9.2 mg/g BSG dw) indicating the occurrence of losses during the liquid–liquid partitioning, such as emulsion formation, filtration, as well the variation of the sample material, particle size, solubility of the immiscible solvent, when extractions were done in replicates (Watson, 2017). The extraction yield of

the total fraction following saponification (0.75% NaOH) showed a 5-fold higher yield (424.2 mg/g BSG dw) than the non-saponified (60% acetone, 80.2 mg/g BSG dw). More than 80% of the saponified material was recovered in the WR fraction followed by 9% in EtOAc and 6% in BuOH fractions. On the other hand, for the unsaponified (60% acetone) extract, recovery in the Hex, DE and WR fractions were in similar range amounting to 32%, 28%, and 25%, respectively of the total recovered material.

Table 5-3 Extraction yield, TPC and SQP levels (means±SD) of the crude extract, free (FP) and bound phenolic fractions (BP) obtained from BSG using 60% acetone and 0.75% NaOH

Samples	Extraction Yield *(mg/g BSG)*		TPC *(mg GAE/g BSG)*		SQP *(mg/g BSG)*	
	FP	BP	FP	BP	FP	BP
Hex F.	19.4±16.8^{a}	12.2±7.1^{b}	0.09±0.09^{a}	0.02±0.0^{b}	n.d.^{b}	<0.01^{c}
DE F.	25.8±14.2^{a}	8.3±1.2^{b}	0.23±0.09^{a}	0.67±0.02^{b}	<0.01^{a}	0.8±0.0^{ab}
EtOAc F.	6.6±4.0^{a}	34.6±6.9^{a}	0.12±0.07^{a}	3.5±0.5^{a}	<0.01^{a}	2.9±0.3^{a}
BuOH F.	6.2±4.4^{a}	23.8±8.3^{ab}	0.09±0.004^{a}	1.0±0.4^{ab}	<0.01^{ab}	0.07±0.03^{bc}
WR F.	22.2±7.8^{a}	345±162.5^{a}	0.11±0.02^{a}	6.2±2.8^{a}	n.d.^{b}	0.03±0.04^{c}
Total	80.2±3.4	424.2±179.9	0.64±0.07	11.3±3.6	0.013±0.02	3.80±0.2
Crude (Control)	94.9±9.2	n.t.	1.7±0.2	n.t.	<0.02	n.t.

Total represents the sum of each solvent fraction (F.) in the column. Fractions generated by Hex—hexane, DE—diethyl ether, EtOAc—ethyl acetate, BuOH—butanol and WR (water residue); "n.t." means not tested, "n.d." means not detected. The corresponding polyphenols content in FP and BP samples represented by Total Phenolic Content (TPC) by Folin–Ciocalteu in mg of gallic acid equivalent per gram of BSG (mg GAE/g BSG) and sum of quantified polyphenols (SQP) by UPLC-MS/MS in mg/g BSG. Values in the same column for each type of extracted phenolics (FP and BP) with each solvent fraction (Hex, DE, EtOAc, BuOH, WR) bearing different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

The results presented in Table 5-3 were generated by solid–liquid and liquid–liquid extractions, followed by paper filtration and concentrated under vacuum or freeze-dried. As the extractions were carried out in triplicate, the steps of washing the solid extraction residue (crude extracts) and separation of the immiscible solvents (Hex, DE, EtOAc, BuOH, and water) had influenced the extraction yield levels. Other parameters that may influence the variations in the extraction yield include extraction time, temperature, solvent-to sample ratio, the number of extractions of the samples and the solvent type (Khoddami et al., 2013). BSG is comprised of about 80% lignocellulosic material mainly consisting of polymers, such as cellulose, hemicellulose and lignin, originating from the cell wall material, whereas the remaining 20% comprises mainly proteins (Jay et al., 2008, Mussatto et al., 2007a). Saponification with NaOH facilitates the

delignification of BSG and degradation of other constituents including hemicellulose and proteins (Modenbach and Nokes, 2014, Connolly et al., 2013), and thereby solubilizing up to 60% of the current total BSG constituents (Table 5-3), BP total extraction yield).

Solvent extraction is a suitable method for pooling free base forms of non-saccharide components such as phenolics and other components (Guido and Moreira, 2017), where a recovery of up to 9% of total BSG constituents was observed in this study (Table 5-3, FP total extraction yield). Several authors have also showed alkaline treatment is more effective than organo-solvent method in populating high extraction yields (Guido and Moreira, 2017, Macheiner et al., 2003, Forssell et al., 2008). Beside delignification, dilute alkali solutions are predominantly used to hydrolyze hemicelluloses to mono-sugars/oligomers or proteins into its constituent amino acids and peptides, which can be recovered in the water phase (Macheiner et al., 2003). The presence of such non-polyphenolic molecules could explain for the high variation in the standard deviation and the data is being skewed by the water fraction as it contains all the precipitates of polysaccharides, proteins, etc.

Comparing the extraction yield obtained in this chapter for the crude extract (Table 5-3) with the one obtained in Chapter 4, section 4.5.1, Table 4-2 of BSG-L, the results show a consistent extraction yield of approx. 95mg/g BSG, with a slightly higher variation observed in this chapter. The extraction yield of the EtOAc fraction, on the other hand, is much lower (approx. 35 mg/g BSG) compared with the extraction yield (approx. 90mg/g BSG) obtained in Chapter 4. An explanation to the high difference in the extraction yield may be due to the overall steps used in the liquid-liquid extraction. For example, in Chapter 4 the neutralized liquors were mixed directly with EtOAc solvent, whereas in this chapter, EtOAc was used post hexane and diethyl ether extractions and part of the extraction yield might have eluted in the hexane and/or diethyl ether fractions thus the lower extraction yield of EtOAC fraction. It has been also shown in the literature that with increasing the number of steps in the partitioning process the higher the losses in the extraction yield but as well in the concentration of chemical compounds, and further losses during transfer and evaporation (Juhascik and Jenkins, 2009).

Also, the challenges with emulsion formation, which prevents the extract from being fully recovered (Watson, 2017).

Values on the extraction yield in this study are similar to those reported by other authors (Xiros et al., 2008, Dehnavi et al., 2011). It is essential to obtain a consistent extraction yield so that the extraction process is economically feasible (Wahlström et al., 2017).

5.4.2 Total phenolic content

Like in the other research chapters, two different methods have been used to determine the total polyphenols, colorimetric method for total phenolic content (TPC) using FC reagent, and sum of quantified polyphenols (SQP) by UPLC-MS/MS method (Table 5-3). The results revealed a considerable variability in the TPC and SQP values, where TPCs were always higher than SQP, among the BSG extracts and various solvent fractions. Interestingly the total bound phenolics (BP) presented almost 20 times higher TPC than free phenolics (FP), which was further supported by the SQP values. The TPC and SQP data from Table 5-3 of the Crude extract and FP and BP EtOAc fractions in this chapter are comparable with the ones obtained din Chapter 4, section 4.4.2, Table 4-3 for the TPC and section 4.4.3.2, Table 4-5 for the BP extracts from BSG-L. The TPC levels (1.7mg GAE/g BSG) of the Crude (Control) in this chapter were 40% lower compared with the TPC levels of the similar substrate (2.84 mg/g BSG) in Chapter 4. Similarly, a 25% decrease was observed in the EtOAc F. of the BP extract (~3.5 mg GAE/g BSG) compared to the Liquor Ctrl EtOAc fraction in chapter 4 (~4.57 mg/g BSG). Furthermore, the SQP of the BP EtOAc F. (~2.9mg/g BSG) in this chapter, and Total of the BSG L Ctrl EtOAc fraction (~2.7mg/g BSG) in Chapter 4, showed only an increase of ~7% in the sum of individual phenolics. Among the results obtained in the two chapters, some variations were expected in the TPC and SQP levels of individual phenolics, as the same BSG L substrate used in this chapter was of a different batch compared with the one used in Chapter 4.

Overall, the highest TPCs were observed in the WR and EtOAc fractions of the alkali-hydrolysed extracts with 6.2±2.8 and 3.5±0.5 mg GAE/g BSG dw, respectively. Amongst the FP fractions, the highest TPC was in the DE fraction

(0.23±0.09 mg GAE/g BSG dw) and the TPC values below 0.12±0.07 mg GAE/g BSG dw were observed for the other solvent fractions. On contrary, the highest SQP was found in the EtOAc and lesser in DE fractions with 2.9±0.3 and 0.8±0.05 mg/g BSG dw, respectively. The FP fractions presented a very low SQP (<0.04 mg/g BSG dw) or at not-detectable levels in the Hex and WR fractions. The different sample-type would factor in TPC variation alongside its background such as barley variety, harvesting time, brewing process, extraction process, etc. (Guido and Moreira, 2017). A significant ($p < 0.01$) correlation has been observed between the extraction yield and TPC ($r = 0.896$) using both FP and BP methods of extraction with their independent fractions. There was a high variation between the TPC values reported in the literature as well by numerous authors either in BSG extracts or fractions generated using alkali hydrolysis or organic solvents; the TPC values varying between 0.6 to 10 mg GAE/g dw when using organic solvents and up to 20 mg GAE/g dw when using alkali hydrolysis (Guido and Moreira, 2017). Results from this study fall within this range (Table 5-3). On the other hand, LC-MS/MS quantification of individual phenolics, expressed as Sum of Quantified Phenolics (SQP), in the bounds phenolic (BP) extracts showed that DE and EtOAc extracts accounted for approximately 21% and 76% of the Total SQP, respectively, which corresponded to 6% and 30% of the total TPC values, respectively. In addition, DE and EtOAc fractions of BP extracts showed similar TPC and SQP trends suggesting both organic solvents were able to efficiently extract phenolic compounds from aqueous solutions. For the BP (Hex, BuOH, and WR) fractions, the SQP values were extremely low, which were also noted low in the corresponding FP fractions for both the SQP and TPC values (Table 5-3).

The overestimation of the spectrophotometric over chromatographic method on total polyphenol content is a well-known phenomenon as the former crudely estimates endproducts by both phenolic and non-phenolic compounds, subject discussed in previous chapters. One must use organic solvents such as DE or EtOAc or in combination to pool phenolic compounds from aqueous extracts, which further can be more accurately determined by spectrophotometry (TPC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

5.4.3 UPLC-MS/MS Quantification of BSG Free and Bound Polyphenols

As previously described in Chapter 4, 14 different polyphenols were tentatively identified in the EtOAc fraction of the saponified BSG extract, of which 8 were confirmed using commercially available standards in the UPLC-MS/MS method. In the current work, a total of 9 different polyphenols were quantified, five phenolic acids (ferulic acid, *p*-coumaric acid, caffeic acid, 4-hydroxybenzoic acid, and protocatechuic acid) and a flavonoid (catechin), along with two ferulic acid oligomers, (decarboxylated diferulic acid (DeCa-DiFA), diferulic acid (DiFA), and a trimer, triferulic acid (TriFA)), as ferulic acid equivalents (Table 5-4). The most predominant phenolic acid, i.e., ferulic acid, was measured in the BP fractions, specifically in the DE and EtOAc fractions constituting in excess of 42% and 48%, respectively of the total polyphenols. The next abundant phenolic acid was *p*-coumaric acid with 26% and 19% in the DE and EtOAc fractions, respectively. DeCa-DiFA was the most abundant ferulic acid dimer in the BP DE fraction (31% of the total polyphenols), whereas it was present in traces in the rest of fractions. DiFA and TriFA were found in similar quantities in the BP EtOAc fraction constituting approximately 15% of the total polyphenols, but very low or not detected in the other BP solvent fractions. Catechin was the most abundant polyphenol in FP fractions, representing more than 72% and 61% of the total polyphenols in the EtOAc and DE fractions, respectively. DE and EtOAc showed to be the best solvents to recover phenolic (FP and BP) compounds from BSG. Both DE and EtOAc, due to their ability to form biphasic system with water, where the extraction of mid-polar to non-polar BSG polyphenols is facilitated. Almost 98% of the total phenolic compounds in BSG, as quantified by the UPLC-MS/MS, were present in bound form, whereas the rest 2% were in the free form. These results are in similar range with previous published papers (Forssell et al., 2008).

Stalikas 2007 comprehensive review on general polyphenols and flavonoids noted several authors had successfully used DE and EtOAc to extract phenolic compounds from aqueous solutions (Stalikas, 2007). de Simon et al., 1990 showed there was not a very large difference in the extraction rate of EtOAc compared to DE (de Simón et al., 1990). EtOAc presented a greater extraction rate for acids and aldehydes of low and high molecular mass, such as catechin

(dimers, trimers of catechins), hydroxycinnamic esters, whereas DE showed a superior reproducibility for the extraction of aldehydes and phenolic acids, i.e., 4-HBA aldehyde, p-CA (de Simón et al., 1990). It is for this reason some authors used a ratio of 1:1 (EtOAc:DE) to fractionate phenolic compounds from aqueous solutions (Sosulski et al., 1982). Meneses et al., 2013 showed that hexane was able to extract flavonoids from BSG in low amounts (Meneses et al., 2013), although hexane is mainly used to extract highly nonpolar compounds such as waxes, oils, sterols or for delipidation purposes (Guido and Moreira, 2017). Socaci et al., 2018 had shown hexane to be a possible selective solvent for other classes of bioactive called terpenoids and aroma compounds (Socaci et al., 2018). *n*-Butanol and water are usually used to extract polar compounds such as phenolic glucosides, peptides and sugars (Liu et al., 2011).

Table 5-4 Individual phenolic compounds quantified by UPLC-MS/MS in the BSG extracts and fractions using several extraction solvents

Standards \ Samples		Hex F.	DE F.	EtOAc F.	BuOH F.	WR F.	Crude Ctrl
FA	FP	n.d.	0.053±0.03^{a}	0.054±0.03^{a}	n.d.	n.d.	n.d.
	BP	0.04±0.07^{b}	41.5±15.3^{a}	40.6±7.7^{a}	1.2±1.0^{ab}	n.d.	5.33*
p-CA	FP	n.d.	0.03±0.02^{a}	n.d.	n.d.	n.d.	n.d.
	BP	0.05±0.06^{c}	25.9±6.3^{a}	16.1±1.9^{ab}	0.44±0.4^{b}	n.d.	2.28*
Cat	FP	n.d.	0.33±0.10^{a}	0.88±0.67^{a}	0.06±0.06^{a}	n.d.	0.15*
	BP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CafA	FP	n.d.	0.02±0.01^{a}	0.03±0.02^{a}	n.d.	n.d.	n.d.
	BP	n.d.	0.50±0.2^{ab}	1.04±0.3^{a}	0.03±0.02^{bc}	n.d.	0.08*
4-HBA	FP	n.d.	0.05±0.05^{a}	0.04±0.03^{a}	n.d.	n.d.	0.01*
	BP	n.d.	0.30±0.1^{ab}	0.33±0.05^{a}	0.02±0.01^{b}	n.d.	0.03*
ProA	FP	n.d.	0.06±0.03^{ab}	0.21±0.04^{a}	0.03±0.03^{ab}	n.d.	0.02*
	BP	n.d.	0.04±0.00^{ab}	0.24±0.06^{a}	0.03±0.01^{b}	n.d.	0.02*
DeCa-DiFA	FP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP	0.08±0.08^{b}	30.6±0.8^{a}	0.95±0.6^{ab}	0.08±0.04^{b}	n.d.	0.58*
DiFA	FP	n.d.	n.d.	n.d.	n.d.	n.d.	-
	BP	n.d.	0.04±0.01^{b}	12.9±2.4^{a}	0.92±0.6^{ab}	0.09^{*b}	1.09*
TriFA	FP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	BP	n.d.	n.d.	12.8±2.3^{a}	0.70±0.7^{ab}	0.11^{*b}	0.78*
Total	FP	n.d.	0.5±0.3^{a}	1.0±0.9^{a}	0.07±0.1^{a}	n.d.	0.19*
	BP	0.15±0.2^{c}	99.0±21.2^{a}	84.9±14.6^{ab}	3.3±2.8^{bc}	0.1±0.1^{c}	10.2*

Individual phenolic compounds in microgram per mg of BSG extract (µg/mg BSGe) represented by ferulic acid (FA), p-coumaric acid (p-CA), catechin (Cat), caffeic acid (CafA), 4-hydroxybenzoic acid (4-HBA), protocatechuic acid (ProA), decarboxylated diferulic acid (DeCa-DiFA), diferulic acid (DiFA) and triferulic acid (TriFA), in BSG Free Phenolic (FP) and Bound Phenolic (BP) extracts and their organic solvent fractions (F.), hexane (Hex), diethyl ether (DE), ethyl acetate (EtOAc), butanol (BuOH), water residue (WR), and Crude control (Ctrl). "n.d."—not detected, ""—identified in one of the extracts. The values reported for each individual polyphenols and Total in FP and BP extracts with their solvent fractions bearing different letters (a, b, c) are significantly different (p<0.05) from each other.*

As we have mentioned in the previous chapters on the quantification of phenolic compounds, BSG is a good source of phenolic acids, with the most abundant being FA, *p*-CA, while the remaining phenolic acids (caffeic acid, 4-hydroxybenzoic acid and protocatechuic acid) were mostly reported in literature at very low levels compared to FA or *p*-CA (McCarthy et al., 2013b, Ikram et al., 2017) and smaller amounts of flavan-3-ols such as catechin have been reported (Meneses et al., 2013, Moreira et al., 2012b, Mussatto et al., 2007a, McCarthy et al., 2013b). It can be clearly seen (Table 5-4) that DE and EtOAc were the best solvents to recover the above-mentioned variety of polyphenols, either using extraction with 60% acetone or saponification with NaOH, whereas only traces or

low amounts could be found in Hex, BuOH, and WR, respectively. Several authors observed the loss of phenolic acids during harsh alkali hydrolysis (2–4M NaOH solution), but not beyond 10% of the initial values of ferulic and *p*-coumaric acids. However, a stronger alkali condition led to 67% and 36.5% losses of caffeic and sinapic acids, respectively (Krygier et al., 1982, Maillard and Berset, 1995). Beside the above quantified polyphenols, procyanidin B, and chlorogenic acid have been detected in FP EtOAc and BuOH fractions, and sinapic acid in BP and EtOAc fractions. Martín-Garcia et al., 2019 extracted high yield of proanthocyanidin compounds (catechins, procyanidins) from BSG using aqueous acetone, where up to 0.1% BSG dw proanthocyanidins was extracted (Martín-García et al., 2019).

The quantification data of phenolic compounds presented in Table 5-4 are presented as µg/mg BSG extract, whereas to be able to compare with the results obtained for the similar extracts and fractions in Chapter 4, as mg/g BSG, the extraction yield needs to be taken in consideration. Thus, the most abundant phenolic compounds, FA and *p*-CA were found in the BSGL BP EtOAc F. at 1 370±122.4 and 548.7±55.7µg/g BSG, respectively, whereas in Chapter 4 Table 4-5 - BSGL Ctrl EtOAc F, the concentrations were slightly higher, of 1 809±272.8 and 686.6±59.0µg/g BSG, levels increased by 32% and 25%, respectively. Analysing the Table 5-4 of this chapter we observe that the BP DE fractions also contain good amounts of FA and *p*-CA, thus summing up the phenolic levels found in these two fractions, the results are much closer with the ones obtained Chapter 4, with FA levels of 1 701±71.7 and *p*-CA of 758.3±38.97µg/g BSG, respectively. The other phenolic compounds were either below the detection limit or not present (sinapic acid and syringic acid) or at much lower levels (CafA, ProA, 4-HBA compared to Fa and *p*-CA) of <1% of the total of individual phenolics, as observed as well in Chapter 4.

Therefore, depending on the bioactive compounds of interest, different optimized extractions and a variety of organic solvents are required to obtain high extraction yields of the targeted compounds.

5.4.4 Anti-AChE and -BChE Activities

The inhibitory activities of BSG free and bound phenolic extracts along with their various solvent fractions on AChE and BChE were evaluated *in-vitro*. The inhibition results are summarized in (Table 5-5) along with the TPC and SQP (µg GAE/mg and µg/mg of BSG extract or fraction) contents of the tested samples with their corresponding inhibitory potential (in %) of AChE and BChE activities. Samples were tested at a concentration of 1 mg/mL BSG extract in the final assay mixture, unless otherwise stated. It is worth to mention that the sum of quantified polyphenols in FP1 EtOAc, BP1 DE and BP3 EtOAc fractions (fractions chosen for blend preparation) represented 2.08, 123.4, and 96.5 µg/mg of BSG fraction respectively, whereas by difference to 1 mg of extract comprises of other unidentified compounds. All the tested samples exhibited some degree of inhibition on both AChE and BChE with the overall highest inhibitions coming from the BP fractions. FP WR fraction was the only fraction that did not present BChE inhibition. BP DE fractions showed the highest and similar TPC and SQP values with BP EtOAc fraction, while showing 4 and 2-fold higher inhibitions for AChE and BChE activities, respectively. In contrast, FP BuOH fraction showed significantly lower TPC and SQP compared to BP DE, whilst presenting similar inhibitory activities for both AChE and BChE. BP DE fraction presented similar levels of individually quantified phenolic acids with BP EtOAc fraction, except for ferulic acid dimers. DeCa-DiFA was the most abundant polyphenol in BP DE fractions, whereas DiFA and TriFA were present only in BP EtOAc (Table 5-4). The presence of DeCaDiFA only in BP DE fraction may be responsible for the higher inhibitory potential of this fraction towards AChE and BChE activity. This is supported by a significant correlation observed between DeCa-DiFA and anti-AChE/BChE activities (Table 5-7). Pure FA standard was tested individually for anti-AChE and BChE activity (Table 5-6) but neither the dimers nor trimers of FA could be tested individually as they are not commercially available.

Adelakun et al., 2012 showed that ferulic acid dimers have higher antioxidant capacity than the ferulic acid (Adelakun et al., 2012). The FA dimers have four free hydroxyl groups compared to FA (two groups) which could contribute to antioxidant efficacy (Garcia-Conesa et al., 1997). Even though multiple hydroxyl groups in the phenolic compounds are thought to boost the

inhibitory action of AChE through strong ionic binding capacity, unfortunately not all follow the same mode of action due to conformational variation (Jabir et al., 2018). Based on the molecular interactions between the enzymes and phenolic compounds, it has been identified that noncovalent forces, most commonly van der Waals forces, hydrogen bonds, hydrophobic bonds, and other electrostatic forces regulate their interactions. As a result of these interactions, it was found that enzymatic activities were mainly inhibited by non-competitive types of inhibitions (Martinez-Gonzalez et al., 2017).

Table 5-5 Total phenolic content (TPC), sum of quantified polyphenols (SQP) of free phenolic (FP) and bound phenolic (BP) extracts and their anticholinesterase activities in different solvent fractions tested at 1 mg/mL.

Samples at 1mg/mL		TPC µgGAE/mg Extract	SQP µg/mg Extract	AChE %Inhibition	BChE %Inhibition
FP	Hex F.	4.1±0.6^{e}	n.d.	11.7±1.3^{b}	17.5±1.8^{cd}
	DE F.	9.8±1.9^{e}	0.5±0.3^{b}	10.7±3.6^{b}	16.4±3.1^{cd}
	EtOAc F.	20.3±3.4^{de}	1.0±1^{b}	8.7±0.6^{b}	15.7±2.9^{cd}
	BuOH F.	11.4±2.5^{e}	0.07±0.1^{b}	34.9±6.4^{a}	40.5±11.2^{b}
	WR F.	5.1±1.1^{e}	-	12.8±0.7^{b}	-
	Crude Ctrl	17.3±0.7^{de}	0.19*^{b}	20.8±2.2^{b}	17.2±1.2^{cd}
BP	Hex F.	1.8±0.4^{e}	0.15±0.2^{b}	13.8±3.5^{b}	25.1±1.5^{c}
	DE F.	82.9±13.2^{b}	99.0±21.2^{a}	37.9±10.4^{a}	53.6±7.7^{a}
	EtOAc F.	102.3±14.1^{a}	84.9±14.6^{a}	10.3±2.9^{b}	25.3±3.3^{c}
	BuOH F.	40.7±1.6^{c}	3.3±2.8^{b}	14.3±2.9^{b}	16.9±3.1^{cd}
	WR F.	18.0±0.9^{de}	0.1±0.1^{b}	11.6±1.3^{b}	9.4±3.8^{de}
	Crude Ctrl	31.7±0.8^{cd}	10.2*^{b}	10.2±1.4^{b}	11.4±0.4^{de}

*Hex—hexane, DE—diethyl ether, EtOAc—ethyl acetate, BuOH—butanol, WR—water residue, F- fraction, Crude Ctrl—crude control, n.d.—not detected. TPC by Folin–Ciocalteu; SQP by UPLC-MS/MS; Acetyl -, Butyrylcholinesterase (AChE, BChE) inhibition activity expressed as % inhibition and compared to galantamine at IC50 (50% inhibition by 3.4±0.23µg/mL for AChE and 11.9±1.67µg/mL for BChE). The data with an * in the SQP column is given as a single result. The values reported on the column for each TPC, SQP, AChE and BChE in FP and BP crude extracts with their solvent fractions bearing different letters (a-e) are significantly different (p < 0.05) from each other.*

Table 5-6 The potential of six individual polyphenols at 0.1 and 1 mg/mL towards the inhibition (%) of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities.

Standards	AChE % Inhibition		BChE % Inhibition	
	0.1 mg/mL	1 mg/mL	0.1 mg/mL	1 mg/mL
Ferulic A.	1.0±0.9^{b}	15.4±0.1^{ab}	14.6±1^{ab}	27.2±0.9^{ab}
p-Coumaric A.	5.2±0.4^{a}	14.4±0.5^{bc}	6.4±0.6^{b}	22.1±1.3^{bc}
Catechin	3.8±1.1^{ab}	14.9±0.2^{ab}	12.2±0.6^{ab}	31.6±0.4^{ab}
4-Hydroxybenzoic A.	1.0±0.2^{b}	5.2±0.9^{c}	n.d.	11.9±0.6^{bc}
Caffeic A.	3.3±0.4^{ab}	25.5±0.2^{a}	15.4±1.3^{a}	52.3±0.8^{a}
Protocatechuic A.	n.d.	13.8±0.7^{bc}	n.d.	7.6±2.4^{c}
Blends	TPC µgGAE/mg	SQP µg/mL	AChE %Inhibition	BChE %Inhibition
FP EtOAc1	260.6±11.9^{ab}	1000	n.d.	16.7±1.5^{a}
BP DE1	243.8±1.4^{b}	1000	11.1±0.6^{a}	9.9±0.2^{b}
BP EtOAc3	267.4±8.4^{a}	1000	8.3±0.1^{a}	11.2±1.1^{ab}

Three blends (FP EtOAc1, BP DE1, BP EtOAc3), that mimic the polyphenol content in the BSG fractions with the highest Total phenolic content (TPC) and sum of quantified polyphenols (SQP) were tested as well. The values reported for each AChE and BChE at specific concentrations with their individual polyphenols bearing different letters (a, b, c) are significantly different ($p < 0.05$) from each other. n.d. = not detected.

Even though various structural isomers of ferulic acid dimers and trimers obtained from several sources had been described in the literature, there is a lack of information on their antioxidant capacity or as potential enzyme inhibitors, especially of DeCa-DiFA (Pedersen et al., 2015).

Jia et al., 2018 synthesized and evaluated several diferulic acids for antioxidant activity and showed DeCa-DiFA as the best antioxidant among other ferulate dimers examined. Unfortunately, no conclusive explanation was found for the higher inhibitory capacity of DeCa-DiFA, and rather a mix of associated structural characteristics and physiochemical properties of the compounds (Jia et al., 2018). Furthermore, decarboxylation of ferulic acid changes the antioxidant capacity of ferulic acid, and the product formed (4-vinylguaiacol) is a potent antioxidant comparable to α-tocopherol (Nenadis et al., 2003). It has been demonstrated that in homogenous polar mediums, ferulic acid presents a greater antioxidant capacity compared to its vinyl derivate 4-vinylguaiacol, whereas in emulsion systems the antioxidant capacity of 4-vinylguaiacol is much greater (Terpinc et al., 2011). Further investigations are needed as to understand how DeCa-DiFA present a higher inhibitory capacity against both AChE and BChE activities compared to other related compounds.

Ouattara et al., 2013 showed that inhibitions of AChE activity decreased in the order BuOH > EtOAc fractions of *Nelsonia canescens*, even though the EtOAc fraction presented considerable higher polyphenol content (hydroxycinnamic acids) as well as antioxidant activity (Ouattara et al., 2013). Due to low recovery in one of the FP BuOH replicate fractions, a solution of 0.1 mg/mL fraction was tested that showed an AChE inhibition of 11.1±0.95% and 12.1±1.25% for BChE inhibition. Another fraction, i.e., BP DE was tested at 0.5 mg/mL and showed an inhibition of 9.5±2.05% towards AChE and 38.95±3.94% for BChE inhibitions. This fraction presented the highest SQP content and was tested at a 2-fold dilution to check if the % inhibition is concentration dependent.

Several authors have shown that extracts with considerably higher polyphenols content and antioxidant activity (EtOAc extracts), obtained from different plant sources did not exhibit higher inhibitory potential for AChE and BChE activities (Ouattara et al., 2013, Gonçalves et al., 2017). It may be that the contribution of other unidentified bioactive compounds that constitute up to 99% and 90% of FP BuOH and BP DE fractions, respectively, account for the inhibition of AChE and BChE activities. Therefore, further separation of these fractions is required to assign their individual involvement in inhibition of AChE and BChE activities. In an earlier study on extracts rich in hydroxycinnamic acids from 26 medicinal plants of the *Lamiaceae* family were tested at 0.25, 0.5, and 1 mg/mL against AChE activity have shown above 75% inhibitions at 1mg/mL, but decreased to <25% for most extracts at 0.25mg/mL (Vladimir-Knežević et al., 2014).

The BSG fractions and extracts tested for anti-AChE and BChE activities showed high and low inhibitory potential and corresponded to high or low contents of TPC and SQP (Table 5-5). This suggested that the phenolic compounds are possibly effective natural inhibitors against AChE and BChE activities. Hence, the individual polyphenol and their blends were investigated for the enzyme inhibition studies.

Table 5-6 shows the AChE and BChE inhibitory potential (%) of individual phenolic compounds prepared at a specific concentration along with three blends that replicate their concentrations in BSG fractions to investigate potential synergy between the compounds. The activity of the various standard

polyphenols at 1mg/mL concentration was in the order: Caffeic acid > ferulic acid > *p*-coumaric acid, catechin, protocatechuic acid > 4-HBA for AChE inhibition, whereas for BChE the order of activity were caffeic acid > catechin > ferulic acid > *p*-coumaric acid > 4-HBA > protocatechuic acid. All the tested polyphenols at a 10-fold lower concentration presented an insignificant inhibition activity of <5% for AChE and <15% for BChE with some polyphenols expressing no inhibition at all. In general, the individual polyphenol showed a stronger inhibition against BChE than AChE at 1mg/mL. Caffeic acid showed the most potent inhibitory activity with 52.3±0.75% at 1mg/mL against AChE and 25.5±0.30% against BChE activity. The prepared polyphenol blends presented insignificant inhibition against both AChE and BChE activities at 1mg/mL and lower inhibitions compared to their actual counterparts. The individual phenolic compounds were tested at mg/mL rather than molar concentration against the enzyme activities because our interest was to use BSG extract rich in phenolic compounds as an inhibitor, rather than to purify these phenolic compounds from the BSG extract. For the later, the molar concentration required to inhibit the activity of the enzyme would be necessary.

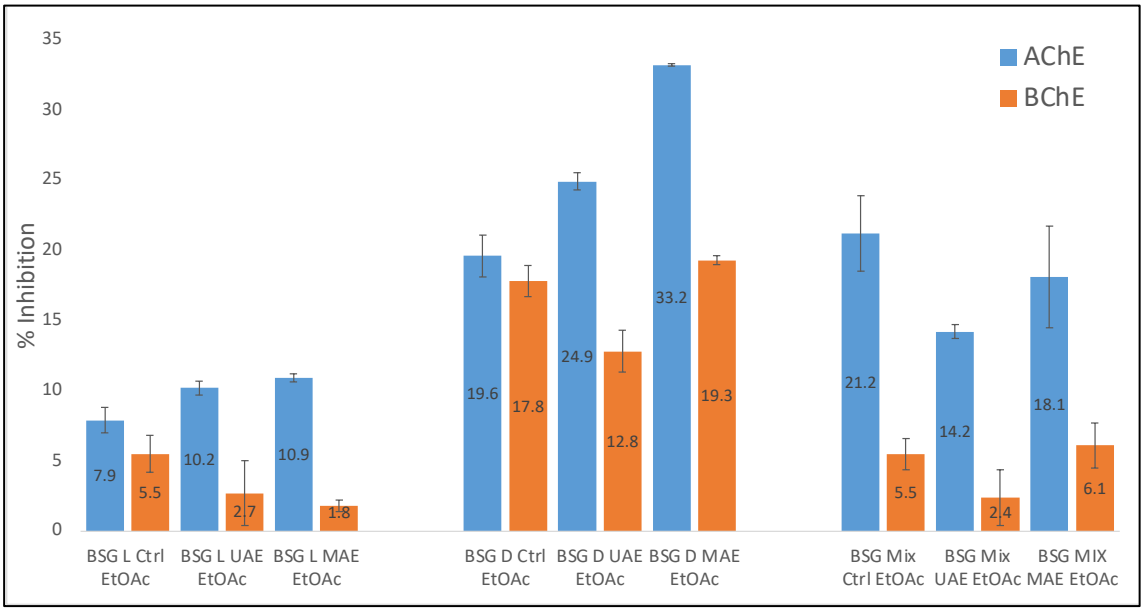
The composition of blends mimicked only the quantified individual polyphenols in the BSG FP EtOAc, BP DE, and BP EtOAc fractions, whereas the original BSG fractions could contain other unidentified compounds, i.e., peptides, amino acids, lipids. The difference in composition together with the quantified ferulic acid dimers and trimers in the fractions may explain the lower inhibitory potential of the BP DE and BP EtOAc blends against both AChE and BChE activities. The FP EtOAc fraction presented very low quantifiable polyphenols with catechin being the most abundant and representing 68% of the total quantified polyphenols. The FP EtOAc blend contained the same % of catechin but at higher content when tested against AChE and BChE activity. Both the fraction and blends showed similar inhibition for BChE activity, whereas low or no inhibition detected against AChE activity. Additionally, an explanation of the higher inhibitory potential of the fractions vs blends would be that the identified and unidentified compounds might present a synergistic effect in the fractions compared to blends, thus increasing their potency towards the inhibition of cholinesterases' activities.

BSG L, among other types of BSG residues (BSG D and BSG Mix) used in this research project, was chosen as substrate to extract phenolic compounds and further test their potential against AChE and BChE activities. The reason behind using BSG L was that, according to the quantification data obtained in Chapter 4, showed the highest levels of phenolics compared to BSG D and BSG Mix, respectively, and those levels were confirmed as well in this chapter.

Several similar fractions from BSG D and BSG Mix were still available from the previous work of Chapter 4 and were tested as well. The results (Figure 5-2) show the inhibition potential towards the AChE and BChE activities of several EtOAc fractions of BSG L, BSG D and BSG Mix at concentrations of 300 µg/mL in final assay mix (3.33x lower compared to the levels used in this chapter). The fractions exerted inhibitions towards both enzymatic activities in the order: BSG D > BSG Mix > BSG L. The BSG D fractions showed the highest inhibitory potential against both AChE and BChE activities, exerting between 19.6% to 33.2% against AChE activity, and 12.8% to 19.3% against BChE activity. On the other hand, BSG Mix fractions showed mid-level inhibitions compared to BSG D and BSG L fractions, between 14.2% to 21.2% against AChE activity, and 2.4% to 5.5% against BChE activity. Lastly, BSG L fractions presented the lowest inhibitory potential among the three types of fractions analysed, against both AChE and BChE activities, between 7.9% to 10.9% against AChE activity, and 1.8% to 5.5% against BChE activity. In chapter 4, Table 4-5 the quantification data of phenolics in the same fractions was in the direction of BSG L > BSG-Mix > BSG D. Even though BSG D fractions presented the lowest concentration of phenolics, it exhibited the highest inhibitory potential among the three tested samples, the opposite was observed for BSG L, while BSG Mix showed mid-levels of phenolics and inhibitions among the three tested BSG types. An explanation to the BSG D higher inhibitory potential may be due to the presence of non-enzymatic browning Maillard reaction products (MRPs, melanoidins) which may act as inhibitors towards AChE and BChE activities. It has been showed in the literature that melanoidins exert antioxidant effect, inhibitors of lipid peroxidation, prevent oxidative damage of DNA, bacteriostatic potential, suppress cancer cell growth *in vitro*, immunomodulatory effects etc. (Langner and Rzeski, 2014, Sharma et al., 2021, Samaras et al., 2005, McCarthy et al., 2012).

The roasting process changes the composition of barley malt, hence the levels of phenolic acids vary depending on how the barley is treated during the kilning process. While certain phenolic compounds with anti-AChE and -BChE capabilities could have been affected during roasting, the creation of additional compounds, such as Maillard reaction products, may help BSGs to preserve or even enhance their anti-AChE and -BChE (Moreira et al., 2013).

Figure 5-2 Anti-acetylcholinesterase and -butyrylcholinesterase activities of ethyl acetate fractions of Brewer's spent grain light, dark and mix



5.4.5 Pearson Correlation, Multiple Regression Model of Variables and PCA

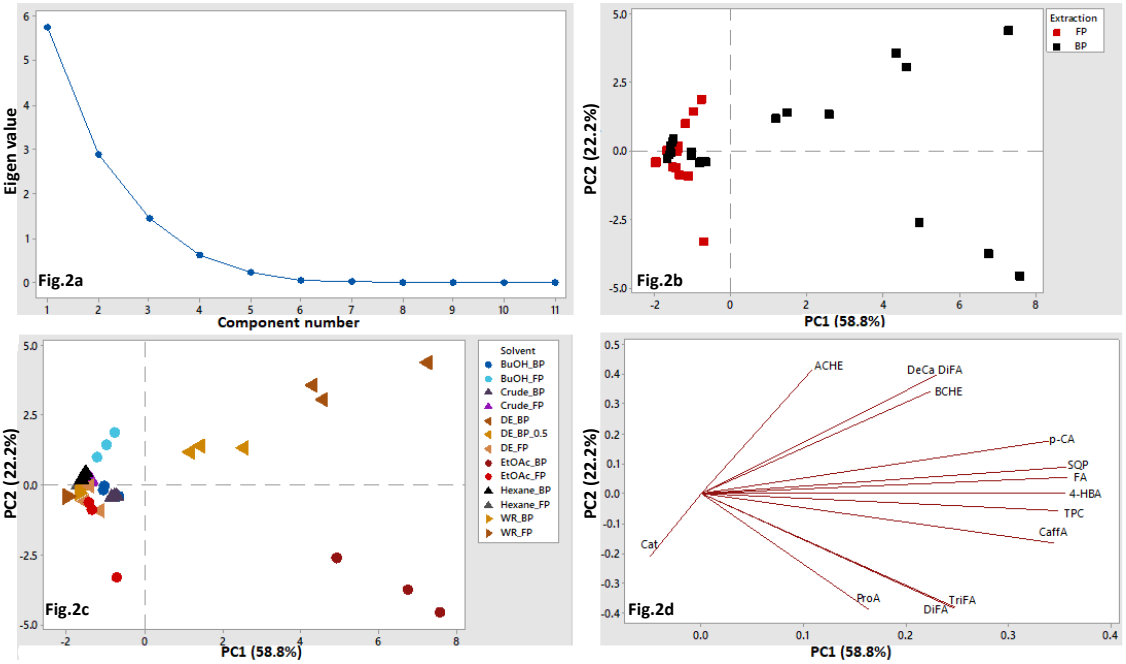
The most used methods for identifying patterns and finding commonalities across different data sets are principal component analysis, Pearson Correlation and Multiple Regression Model of Variables. These approaches are often used to analyse vast amounts of data sets, bioactive substances, functional qualities etc, by evaluating the feasibility of employing several explanatory factors to explain variations in the dependent variable. In order to understand whether there is an association between the above data sets specifically among pairs of variables, i.e., enzymatic assays AChE vs BChE, polyphenols quantification methods TPC vs SQP, or within sets of variables in particular FA versus *p*-CA, or FA versus AChE etc., a number of statistical tests were performed.

Correlation tests were performed to identify any relationships between variables (AChE, BChE, TPC, SQP, and individually quantified polyphenols) either in the BSG FP and BP extracts, or individual polyphenols and their blends. Depending on the independent variables used, the correlation values may increase or decrease. For example, the FP, BP extracts, individual standards and blends were tested for both AChE and BChE, whereas TPC and SQP were analyzed without the individual standard as no data was available. The correlation between the individual phenolic standards was analyzed only in the BP extracts.

AChE and BChE enzymatic assays presented a significant ($p < 0.01$) correlation of 0.687 ($n = 84$) determined by FP and BP extracts, polyphenol standards and blends. The quantification methods of polyphenols content in BSG extracts, TPC and SQP, presented a significant ($p < 0.01$) correlation of 0.974 ($n = 48$) determined by FP, BP extracts and blends. Furthermore, significant ($p < 0.01$) correlation was observed in BP extracts ($n = 21$) between individual polyphenols, i.e., FA and *p*-CA (0.958), 4-HBA (0.994) and CafA (0.887), respectively. Moreover, the FA dimer, DeCa-DiFA presented significant correlation ($p < 0.01$) with the enzymatic assays AChE and BChE of 0.754 and 0.896, respectively ($n = 21$). Other related correlations are shown in (Table 5-7). The multiple correlation coefficient R indicated a very high correlation of 0.842 and 0.984 between the response variables, AChE and BChE, and the explanatory variables (TPC, FA, CafA, 4HBA, ProA, pCA, Cat, DeCa-DiFA, TriFA), with the BP extracts. Further, the coefficient of determination (R^2) indicates that the model fits the data reasonably well, where 70.9% (AChE) and 97.6% (BChE) of the variation could be explained by the fitted model. The adjusted R^2 value of the dependent variable AChE considerably reduced the estimated proportion to 0.471 and slightly to 0.941 for BChE. A regression model has also been presented using FA and *p*-CA (most abundant polyphenols) as variables to explain the anti-AChE and BChE activity (Table 5-8).

Principle component analysis (PCA) was performed on standardized datasets to explore a potential differentiation among BSG FP and BP extracts and their follow-up fractions (Figure 5-3) based on individual polyphenol content (i.e., FA, p-CA, CafA etc.), polyphenol quantification methods (TPC, SQP) and enzymatic assays (AChE and BChE).

Figure 5-3 (a) Score plot of BSG FP and BP extracts and fractions; (b) Score plot for the first two components (PC) separated by the type of extractions FP and BP; (c) Score plot for the first two components separated by the type of organic solvent used for fractionation of the FP and BP extracts; (d) Loading plot of the first two components



PC1 retained about 59% of data variation, while PC2 explained an extra 22% of overall variability leading to a total cumulative variation of 81%. Two score plots for PC1 and PC2 are presented in Figure 5-3, where the variables were separated according to the type of extraction, FP and BP (Figure 5-3/2b), and further partitioning of the extracts by organic solvents, Hex, DE, EtOAc, BuOH, and WR, respectively. In Figure 5-3/2b, it can be observed the formation of a cluster close to the origin of the plot by both FP and BP fractions, and part separation of several BP fractions, in the upper and lower right-hand side of the plot. In 5-3/2c, the part separation is represented by the EtOAc BP fractions in the lower right-hand side, and DE BP fractions in the upper side. These two BP fractions seemed to have a stronger impact on the model as they are the furthest away from the plot's origin.

The loading plot (Figure 5-3/2d) shows the relations between the analysed variables including quantified phenolic acids, quantification methods and enzymatic assays, explained in combination with the eigen values (Table 5-9). Three sets of associations between variables were observed in the loading plot (Figure 5-3/2d). PC1 positively differentiated the BSG FP and BP fractions according to the contents of FA, *p*-CA, CafA, 4-HBA, and the polyphenol

quantifications methods, i.e., TPC and SQP. This positive association was an expected result as a strong and significant Pearson correlation was observed between these variables (Table 5-7). FA and p-CA were the most abundant polyphenols in the BSG extracts and fractions, and with CafA and 4-HBA brought a higher contribution to TPC and SQP quantification methods compared to catechin. PC2 differentiated the BSG FP and BP fractions according to the contents of ProA, DeCa-DiFA, DiFA, TriFA, and the enzymatic assays AChE and BChE. The positive association between DeCa-DiFA and the enzymatic assays, AChE and BChE was an expected result too as among the quantified polyphenols, DeCa-DiFA presented a strong and significant Pearson correlation with both enzymatic assays compared to DiFA, TriFA, and ProA. Moreover, DeCa-DiFA was present only in the DE BP fraction, which presented the highest inhibition among the analysed fractions for both AChE and BChE activities. DiFA and TriFA were present only in EtOAc BP fraction, which presented a 4- and 2-fold lower inhibitions for AChE and BChE activities, respectively.

Table 5-7 Correlation coefficients among analysed variables of BSG BP fractions.

Pearson Correl.	*AChE*	*BChE*	*TPC*	*SQP*	*FA*	*p-CA*	*Cat*	*CafA*	*4-HBA*	*ProA*	*DeCa-DiFA*	*DiFA*	*TriFA*
AChE	1												
BChE	.687**	1											
TPC	.375	.511*	1										
SQP	.543*	.787**	.904**	1									
FA	.502*	.736**	.916**	.990**	1								
p-CA	.645**	.869**	.826**	.980**	.958**	1							
Cat	-.036	-.013	-.293	-.194	-.189	-.187	1						
CafA	.163	.428	.922**	.856**	.887**	.739**	-.163	1					
4HBA	.461*	.715**	.927**	.985**	.994**	.941**	-.190	.907**	1				
ProA	-.113	.094	.801**	.610**	.656**	.439*	-.139	.922**	.700**	1			
DeCa-DiFA	.754**	.896**	.432	.703**	.621**	.817**	-.135	.256	.585**	-.095	1		
DiFA	-.229	-.052	.702**	.481*	.538*	.298	-.107	.854**	.585**	.983**	-.253	1	
TriFA	-.227	-.040	.700**	.488*	.545*	.306	-.102	.858**	.595**	.985**	-.245	.999**	1

*Correlation is significant at the 0.01** level and at the 0.05* level (2-tailed).*

Table 5-8 Summary of multiple regression model of AChE and BChE.

Model	*R*	*R* Square	Adjusted R Square	Std. Error of the Estimate
AChE	.842[a]	.709	.471	7.55749
AChE	.763[b]	.582	.536	7.08868
BChE	.984[a]	.967	.941	3.74240
BChE	.933[b]	.871	.856	5.82498

aPredictors: (Constant), FA, p-CA, Cat, CafA, 4HBA, ProA, DeCA-DiFA, TriFA, TPC; bPredictors: (Constant), FA, p-CA.

Table 5-9 The first four factor loadings for illustrating the interpretation of 5.4-2. Note: Numbers in bold represent the factor loading higher than 0.30

Variable	PC1	PC2	PC3	PC4
FA	**0.357**	0.053	0.020	0.113
p-CA	**0.340**	0.176	-0.041	0.205
Cat	−0.049	−0.209	−0.765	0.004
CafA	**0.343**	−0.167	0.096	−0.028
4−HBA	**0.356**	−0.001	−0.068	0.111
ProA	0.163	−0.388	−0.488	−0.125
DeCa−DiFA	0.229	**0.395**	−0.164	0.362
DiFA	0.247	−0.383	0.226	−0.223
TriFA	0.248	−0.383	0.222	−0.224
ACHE	0.108	**0.410**	−0.087	−0.737
BCHE	0.223	**0.339**	−0.154	−0.344
TPC	**0.347**	−0.057	0.034	0.035
SQP	**0.356**	0.087	−0.004	0.148
Eigenvalue	7.600	2.882	1.4534	0.636
Explained variance (%)	58.5	22.2	11.2	4.9
Cumulative variance (%)	58.5	80.7	91.9	96.8

The statistical analysis showed significant correlations and strong associations between the analyzed variables of BSG fractions. A clear differentiation between BSG bound phenolic polyphenol-rich fractions and free phenolic fractions was observed based on the performed statistical analysis with the most abundant polyphenols (i.e., FA, *p*-CA) being associated with the polyphenol quantification methods, and the decarboxylated FA dimer of BSG BP

DE fraction associated with the anti-AChE and BChE activities. Szwajgier et al., 2012 have associated phenolic compounds from malt as potential cholinesterase inhibitors due to their similar structure to the well-known anti-cholinesterase, in terms of molecular weight, phenol rings and hydrophobic moieties. The highest anti-ChE activities was exhibited by *p*-coumaric acid at 0.38 mM/L, whereas the second best ferulic acid presented a 120-fold lower inhibition at 1 mM/L. In the same study, sinapic and 4-hydroxybenzoic acid (0.03 and 0.01 mM/L) presented similar inhibitions to ferulic acid (Szwajgier and Borowiec, 2012). In a subsequent study by Szwajgier et al., 2013, ferulic acid and *p*-coumaric acid showed similar level of anti-AChE and anti-BChE activities at 0.2mM, whereas caffeic acid showed slightly higher inhibitory potential against AChE and lower for BChE (Szwajgier, 2013). The work of Shahwar et al., 2010 have showed ferulic acid to exhibit AChE inhibitions of 12.38 to 42.65% at varying concentrations (50 to 250 µg/mL) and was found to be strongly dose dependent and with no significant change in inhibition at concentrations above 250 µg/mL (Shahwar et al., 2010). As it can be seen in Table 5-6, FA and *p*-CA presented similar levels of inhibitions towards both enzymes at 0.1 and 1 mg/mL, respectively. Contrary to Szwajgier et al., 2012 study, Ouattara et al., 2013 showed no inhibitory effect on AChE activity by *p*-coumaric acid (Ouattara et al., 2013). Interestingly, caffeic acid at 1 mg/mL showed no inhibition against AChE or BChE in the study by Orhan et al., 2007 (Orhan et al., 2007), whereas in this work caffeic acid showed the highest activities against both cholinesterases. Caffeic acid has been previously shown to present a higher antioxidant activity than other hydroxycinnamic acids, i.e., FA, p-CA (Kikuzaki et al., 2002)], thus may explain the higher inhibitory potential towards the cholinesterases. Vladimir et al., 2014 also examined individual hydroxycinnamic acids, and they presented a stronger AChE inhibition than the hydroxycinnamic acid rich plant extracts. For example, ferulic acid showed a ~50%, ~75%, and ~87% AChE inhibition, and caffeic acid, like in our present study, showed a ~30%, ~85, and ~90%, at 0.25, 0.5 and 1 mg/mL concentrations, respectively (Vladimir-Knežević et al., 2014).

The insignificant anti-AChE and BChE activities of prepared blends would need to be investigated further as the interactions among phenolic compounds could be synergistic or antagonistic, and those studies are sparse and lacking.

As an example, the interaction between *p*-coumaric and ferulic acid in respect to antioxidant capacity is additive, but when caffeic acid is present, the type of interaction changes to antagonistic (Salazar-López et al., 2017). It is suggested that these types of interactions, synergistic or antagonistic, between the phenolic compounds may be related to their chemical structure, the number of the hydroxyl or methoxy group and their position in relation to the carboxyl group, and even their concentration in a mixture (Skroza et al., 2022).

Galantamine, an alkaloid isolated from *Galanthus Woronowii* currently used in AD treatment, is a centrally acting reversible and competitive inhibitor of cholinesterases. Galantamine has shown a 53-fold greater inhibitory activity for AChE than BChE (IC_{50} values ranging from 0.1 to 5.3 μg/mL) (Lilienfeld, 2002). High anti-AChE potency of alkaloids is attributed to the binding of its quaternary nitrogen to an aspartate residue at AChE peripheral anionic site (Pereira et al., 2010), or the ability to build hydrogen bonds with Tyr130 (Lai et al., 2013), and also due to a hydroxyl group at the alkaloid C-2 position (Santos et al., 2018). On the other hand, polyphenols and terpenes bind to the peripheral anionic site of AChE acting as non-competitive inhibitors (Santos et al., 2018). Santos et al. 2018 have reviewed several papers related to anti-ChE activities in which a total of 54 plants species with 36 isolated bioactive compounds were investigated; the authors revealed that alkaloids and coumarins presented a higher potency (IC_{50} <20μg/mL) than galantamine (IC_{50} of 5 μM), whereas flavonoids and phenolic acids presented low potency (IC_{50} 50–1000μM) (Santos et al., 2018). Furthermore, phenolic compounds with close molecular weights (254.24–354.40 Da) have showed that the enzyme-inhibitory activity decreased by the presence 3-hydroxyl group, whereas other hydroxyl groups, their position and number, played a minor role in this context (Szwajgier, 2013).

Roasting temperatures (>150 °C) have shown to increase the levels of catechin and proanthocyanidin hexamers and heptamers in cacao processing, which further improved the inhibitory potential of extracts against enzyme activity (Stanley et al., 2018). High temperature roasting (<232 °C) is also applied in barley malt to produce stout beer resulting in BSG dark residues. Extracts obtained from BSG dark may possess increased levels of homogenous and

heterogenous oligomers of phenolic compounds, which may attribute to improve their potential as enzyme inhibitors.

Another important observation in this study was that the EtOH at concentrations of <10% in the final assay mixture interfered with the enzymes activity and resulted in inhibitions of up to 30% for AChE and less than 4% for BChE activity, respectively. As the tested extracts had been reconstituted in 0 to 50% ethanol (0 to 5% in final assay mixture), the observed EtOH inhibition was subtracted from the final calculations. Several authors have presented numerous polar and non-polar organic solvents that might decrease or enhance the cholinesterase activities (O'Brien, 1956, Pohanka et al., 2013), but this was not the purpose of the current study.

With regard to AD management, the AChE and BChE inhibition are still attractive targets owing not only to the cholinergic hypothesis but as well to several functions in pathogenesis and development of AD (Musial et al., 2007). Approximatively 95% of the cholinesterase activity is due to AChE in normal human brain, whereas its level decreases to 10–15% in the brain of a person with AD, and interestingly BChE activity increases to 120% (Musial et al., 2007).

The molecular mechanism of interaction of alkaloids is similar to the currently used drugs for this purpose, i.e., huperzine, galantamine, thus the high anti-AChE potency (Santos et al., 2018). Phenolic compounds are currently considered as a noticeable agents of reduced risk and management of AD due to their antioxidant and anti-inflammatory properties, low toxicity and abundant sustainable natural sources (Kumar and Pruthi, 2014, Winter et al., 2017, Fardet and Boirie, 2014)].

5.5 Conclusions

BSG represent a clear opportunity to be exploited as a potential source of bioactive compounds if processed in the right way, and further its corresponding polyphenolic extracts be accepted and utilized in health well-being and food processing.

In the current study, BSG extracts and their sub-fractions along with commercially pure phenolic compounds and blends of identified BSG

polyphenols were tested for their potential to inhibit AChE and BChE activities in vitro. Saponification with NaOH (bound phenolic extract) presented the highest polyphenol content per gram of BSG in DE and EtOAc fractions as revealed by TPC (FC reagent) and SQP (UPLC-MS/MS). Ferulic- and *p*-coumaric acids were the most abundant polyphenols, with the highest levels in the DE and EtOAc bound phenolic fractions, whereas catechin was the most abundant in the same solvent fractions but as free phenolics. These results indicate the necessity of using alkali hydrolysis followed by liquid–liquid partitioning with DE and EtOAc to obtain high polyphenol yields.

The in vitro enzymatic assays revealed that not only polyphenol rich fractions (BP DE and BP EtOAc) significantly inhibited AChE and BChE activities, but low polyphenolic-containing fractions (FP BuOH fraction) also had significant impact. Among the individually tested polyphenols, caffeic acid presented the highest inhibitory potential; however, its content in BSG is low. There seems to be a synergistic interaction between polyphenols and other co-extracted compounds in the BSG BP (DE and EtOAc) fractions, whereas little or no synergistic effect between the selected polyphenols in the blend for cholinesterase inhibition. It is claimed that polyphenols presenting synergistic activities are powerful inhibitors against oxidation, peptic ulcers, tumours, anti-inflammatory activities, and other types of conditions, such as Alzheimer's disease (Mitra et al., 2022, Szwajgier, 2013).

The PCA analysis showed a strong inhibitory influence of the presence of a single compound DeCa-DiFA in DE fractions. Significant correlations ($p < 0.01$) have been observed between the enzymatic assays AChE and BChE, as well as between analysis methods TPC and SQP, normally used in concomitance in this type of research investigation and between the individual polyphenols (FA and *p*-CA). The inhibitory effect of BSG extracts and fractions, including their individual polyphenols, on AChE and BChE activity would require further studies such as an additional separation (flash chromatography or preparatory chromatography) of compounds to identify the most potent compound or group of compounds in BSG(s). To this, BSG D would make a substrate of interest to forward investigate this approach due to the possibility of containing other non-phenolic compounds, beside the already characterized phenolic compounds above, that may provide a

significant inhibition against cholinesterases activities responsible for the advances of Alzheimer's disease, and as well other type of enzymes, such as carbohydrases, to manage the blood glucose levels in type 2 diabetes.

Chapter 6

6. Evaluation of dark BSG fractions for anti-cholinesterase and antidiabetic potential: A Flash Chromatography approach

6.1 Abstract

In this final research chapter, a combination of solid- and liquid-liquid extraction, flash chromatography, high-performance liquid chromatography, and *in-vitro* bioassays was used to separate phytochemicals endowed with anticholinesterase and anti-carbohydrase activity in several extracts from dark BSG. Three BSG D free phenolic extracts and two bound phenolic extracts were subjected to flash chromatography to generate >150 fractions. The fractions' inhibitory capacity was tested against α-amylase and α-glucosidase enzymes implicated in diabetes and acetylcholinesterase and butyrylcholinesterase enzymes used to treat Parkinson's or Alzheimer's disorders. The TPC levels in the generated flash fractions resembled the flash chromatograph, with F24 of the FP DE:EA extract showing the highest levels, whereas the quantitative analysis showed F03 and F02 of the BP M and H DE:EA to contain the highest levels of quantified phenolics, with ferulic acid and *p*-coumaric acid as the most abundant. All the tested flash fractions showed inhibitions of up to 80% against AChE and up to 70% for BChE activities, with the highest potential shown by FP DE:EA extract. Against α-amylase and α-glucosidase activities not all the tested fractions presented inhibitions, with the an overall higher potential observed in the fractions generated using 0 to 30% acetonitrile, and BSG D M and H DE:EA extracts in general showing the highest levels of inhibition. Based on the overall results, BSG D seems to present a higher inhibitory potential against the activity of carbohydrase compared to cholinesterase, which may be due to the presence of the identified and quantified phenolic compounds in BSG dark. Considering these findings, BSG D extracts could be a promising source of enzyme inhibitors.

6.2 Introduction

To date the present thesis has mainly focused on the extraction of phenolic compounds from BSG residues by using a combination of classic and novel extraction technologies, with a later application of liquid – liquid extraction to obtain enriched BSG phenolic extracts. These extracts were subjected to quantitative and qualitative analysis for the presence of individual and total phenolic compounds (Chapter 3 and 4), which were then tested for their potential to inhibit the cholinesterase activities (Chapter 5). In Chapter 5 the use of *in vitro* enzymatic assays revealed that not only polyphenol rich fractions (i.e. bound phenolics in diethyl ether and in ethyl acetate fractions) significantly inhibited AChE and BChE activities, but low polyphenolic-containing fractions (free phenolics in butanol fraction) also had significant impact. Moreover, dark BSG (BSG D) has shown moderate inhibitions of both these enzymes at 3-fold lower concentration compared to BSG light fractions. Even though, liquid-liquid extraction showed good separation of BSG polyphenols in the partitioned organic solvent fractions, a further fractionation of these organic fractions allows for the separation of phenolic and non-phenolic compounds and associate the compounds with the biological activity. Flash Chromatography fractionation is a valuable technique used to isolate and enrich individual or classes of phenolic compounds in plant extracts. It includes a rapid separation, isolation, and purification of compounds within a particular fraction (Gangopadhyay et al., 2016).

Due to the common genetic and physiological characteristics of insulin resistance, memory problems, and cognitive decline in aged adults, scientists have dubbed Alzheimer's disease as "Type-3-Diabetes". There is a significant yet complicated connection between type 2 diabetes and Alzheimer's disease. Insulin resistance, insulin growth factor signalling, glycogen synthase kinase 3β signalling pathway, oxidative stress, inflammatory response, amyloid beta development, neurofibrillary tangle formation, and acetylcholine esterase activity control, are all interconnected. Due to the similar pathways between type-1 diabetes, type-2 diabetes, and Alzheimer’s, it is necessary to create medicines capable of performing many functions by blocking these vital pharmacological

targets (Kandimalla et al., 2017). Thus, this being one the reason to evaluate the potential of BSG extracts and fractions as α-amylase, α-glucosidase, acetylcholinesterase, and butyrylcholinesterase inhibitors.

Therefore, the final study of this thesis aimed to use flash chromatography to dissect the BSG D extracts into fractions based on polarity and to further test the potential of the generated flash fractions against the activity of two sets of enzymes *in-vitro*: α-amylase and α-glucosidase (antidiabetic) and acetyl- and butyrylcholinesterase (anti-Alzheimer's) activities. Moreover, to assign the BSG phenolic compounds as potential enzyme inhibitors, the flash fractions had to be screened using non-targeted analysis and quantified using targeted analysis. This was achieved by using Folin-Ciocalteu assay to reveal the total phenolic content of all flash fractions, whereas the targeted individual phenolic compounds quantification was performed using an UPLC-TQD-MS/MS approach, and for screening using LC-Q-TOF-MS/MS analysis. To the best of our knowledge, this is the first study that evaluates the antidiabetic and anticholinesterase activity of BSG dark phenolic-rich fractions.

6.3 Materials and Methods

6.3.1 Samples and chemicals

Brewer's spent grain dark (BSG D) was provided by Diageo Dublin, Ireland, and it was from the same batch as BSG D used in Chapter 5, with the same drying and storage conditions.

All the chemicals, organic solvents, polyphenol standards, reagents, enzymatic substrates used in this research chapter were purchased from Merck (formerly Sigma Aldrich, Arklow, Co. Wicklow, Ireland), unless otherwise stated, and as described previously in Chapter 5 section 5.3.1, including the materials used for the *in vitro* cholinesterase inhibitory activities. For carbohydrases assay, sodium phosphate, sodium chloride, potassium sodium tartrate, 3,5-dinitrosalicylic acid, sodium hydroxide, α-amylase from hog pancreas starch, α-glucosidase from *Saccharomyces cerevisiae*, potassium phosphate monobasic, 4-nitrophenyl α-D-glucopyranoside and acarbose were purchased from Merck (formerly Sigma Aldrich, Arklow, Co. Wicklow, Ireland).

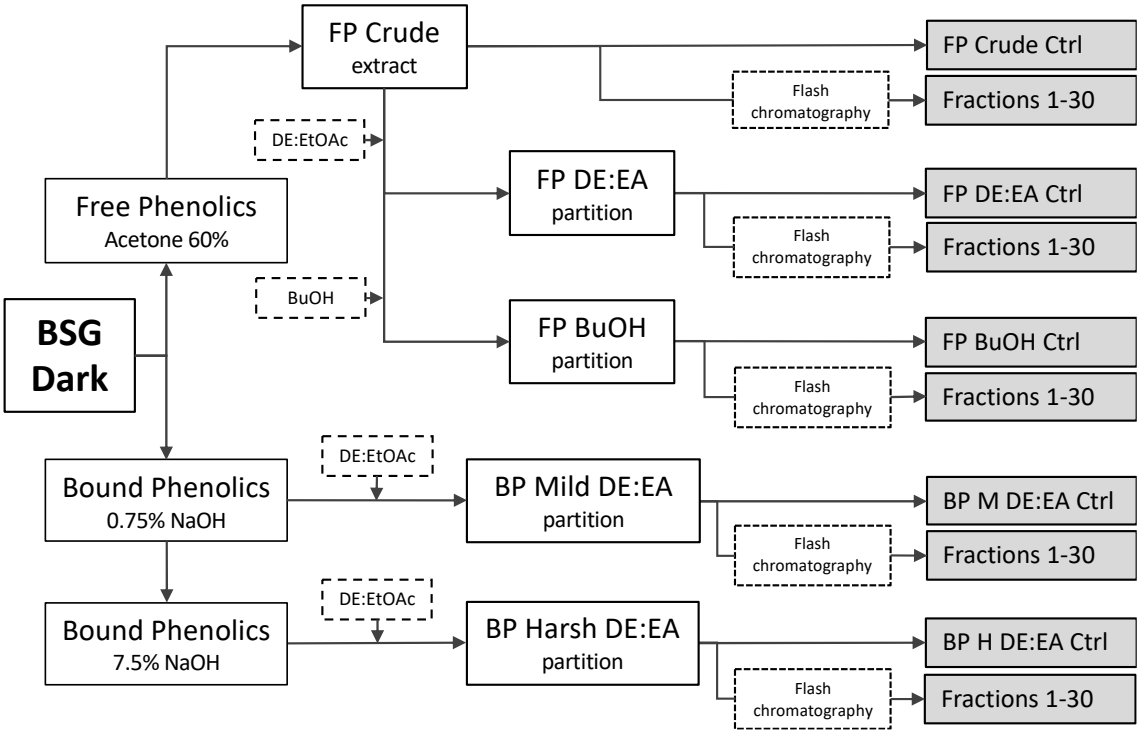
6.3.2 Extraction and fractionation of free and bound phenolics

A schematic flow of the extraction and fractionation procedure used in this chapter is illustrated below in Figure 6-1.

To obtain **BSG D FP extracts**, a single bulk extraction was prepared. Briefly, BSG D free phenolic extracts were obtained by mixing 15 g of milled BSG D with 300 mL of 60% acetone (1:20 w/v) in a sealed amber glass bottle and kept in a water-bath at 60°C for 30 min with constant stirring. After the treatment time, the extract was left to cool at room temperature, centrifuged and the supernatant was syringe filtered. The pellet was then extracted again 2x with 60% acetone (total of 3 extractions), and after filtration the recovered supernatants were mixed (approx. 852mL solution). The recovered free phenolic extraction solution was split in two parts: (1) 284 mL was concentrated under vacuum (859.3 mg dry extract obtained), from which part was kept as **FP Crude Ctrl**, and 200 mg was subjected to flash chromatography partitioning; (2) the remaining free phenolic extraction solution, 568 mL, was concentrated under vacuum to remove the acetone and the remaining water part was subjected to liquid-liquid extraction

with each DE:EA and BuOH. The recovered volumes of DE:EA and BuOH extracts were concentrated under vacuum to obtain **FP DE:EA** (482.2 mg) and **FP BuOH** (365.17 mg) extract. Part of the FP DE:EA extract was kept separately as **FP DE:EA Ctrl** whereas 384mg was subjected to flash chromatography partitioning, and similarly for FP BuOH extract as **FP BuOH Ctrl** and 300 mg subjected to flash chromatography partitioning as well. The FP controls extracts were dissolved in MeOH to a final concentration of 10 mg/mL and stored at −28ºC until required.

Figure 6-1 Extraction process of free and bound phenolic compounds from BSG D followed by their liquid-liquid partitioning and flash chromatography



NaOH – Sodium hydroxide; DE:EA – diethyl ether : ethyl acetate 1:1 v/v; BuOH- n-Butanol saturated with water; FP – free phenolic extract; BP – bound phenolic extract; M – mild hydrolysis 0.75%NaOH; H – harsh hydrolysis 7.5% NaOH; Ctrl – control (part of the extract taken prior being subjected to flash chromatography);

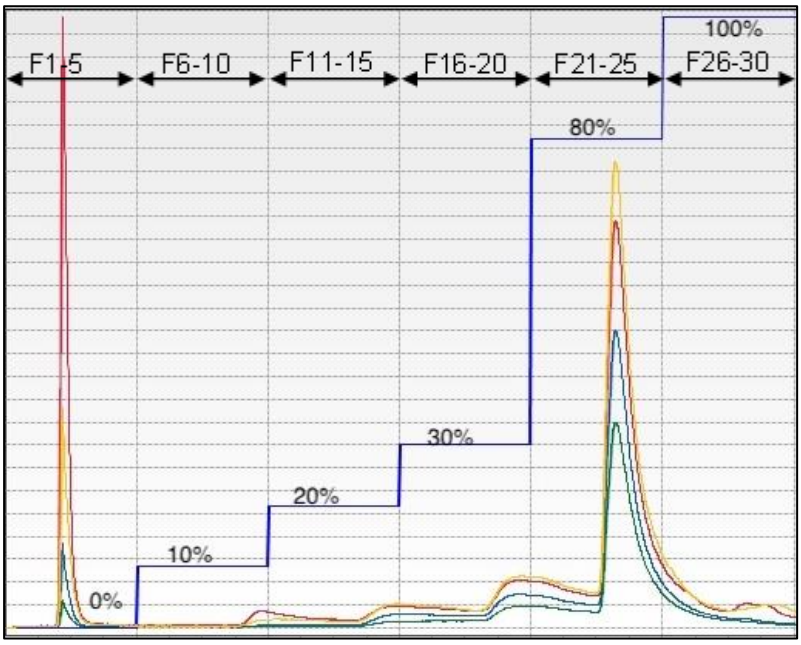
To obtain **BSG D BP extracts**, a sequential alkali treatment was used. For the first extraction, 10 g of milled BSG D was mixed with 200 mL of 0.75% NaOH (1:20 w/v, mild hydrolysis) in an amber glass bottle and kept in a water bath at 80ºC for 30 min with constant stirring. After the treatment time, the mixture was left to cool at room temperature, centrifuged, and the recovered supernatant was neutralised, and paper filtered under vacuum. The BSG D residue following the filtration step was subjected to a second alkali treatment by mixing the pellet with 200mL of 7.5% NaOH (harsh hydrolysis), extracted overnight at room

temperature under nitrogen. After the treatment time, the mixture was centrifuged, the recovered supernatant was neutralised, and paper filtered under vacuum. Following the filtration step, both solutions were subjected to liquid-liquid extraction using DE:EA. The recovered volumes of DE:EA were concentrated under vacuum and the recovered amounts (424.5 mg for the **BP M DE:EA**, 297.1 mg **BP H DE:EA**) were subjected to flash chromatography fractionation to obtain BP M DE:EA and BP H DE:EA fractions. Controls were prepared using the same extraction procedure, **BP M DE:EA Ctrl** and **BP H DE:EA Ctrl,** and prepared stock solution in MeOH to a final concentration of 10mg/mL and stored at −28ºC until required.

For the fractionation of the BSG D FP and BP extracts using flash chromatography, the dried BSG extracts of either free and total phenolics were each dissolved in methanol and mixed with C18 silica sorbent powder (1:4 *w/w*) for a homogenous extract distribution in the sorbent. The mixture was left to dry in a fume hood at room temperature, then added and packed tightly in a loading column, and run-on a Varian 310 flash chromatography system. The column used for flash chromatography was a Buchi FlashPure ID C18 reverse-phase flash cartridge (particle size of 40 µm irregular, 12 g) with a sample loading capacity between 15 mg to 600 mg, in combination with a binary solvent system containing water (mobile phase A), and acetonitrile (mobile phase B). A stepwise gradient (100%A for 5min, 90%A from 5-10 min, 80%A from 10-15 minutes, 70%A from 15-20 min, 20%A from 20-25 min and 0%A from 25-30 min) at a flow rate of 15mL/min (30min) was used to separate polyphenols of pooled BSG extracts, and the fractions were collected every minute over a 30 min run, resulting in 30 fractions of <15mL each, with a total volume of ~450 mL per BSG extract (example of fractionated extract and a flash chromatogram are presented in Figure 6-2 and 6-3). The UV detector was set at 245, 280, 320 and 360 nm to monitor the eluting fractions.

Figure 6-2 Brewer's spent grain dark free phenolic butanol fraction subjected to flash chromatography showing the elution pattern of 30 fractions generated with decreasing solvent polarities

Figure 6-3 Flash chromatogram example of the BSG D FP DE:EA extract (yellow, red, light blue, green lines indicated the chromatograms obtained at 245nm, 280nm, 320nm and 360nm, the stepwise blue line indicating eluent acetonitrile at 0% to 100%, total of 30 fractions and set of 5 fractions per % stepwise acetonitrile).



6.3.3 Determination of Polyphenolic Content

Total phenolic content was estimated by Folin–Ciocalteu and quantification of BSG D polyphenols in the FP and BP extracts and fractions was performed by LC-MS/MS as described in the previous chapters.

6.3.3.1 Total Phenolic Content (TPC) by Folin–Ciocalteu (FC)

Total phenolic content of BSG D extracts was determined by colorimetric assay using FC reagent and described in the previous research chapters. Briefly, the TPC levels were recorded for the BSG D FP and BP controls and their flash fractions and expressed in micrograms of gallic acid equivalent per milligram of

BSG D extract (µgGAE/mg BSG D extract), whereas in the enzymatic assays as µgGAE/mL in the final assay mixture. The 'Sum of TPC levels of fractions' (Figure 6-4) was calculated by first measuring the TPC levels in each mL of fraction (µg GAE/mL), then multiplied with the total volume generated per fraction (14 to 15mL per fraction). The obtained results, µgGAE/fraction, were summed (1 to 30) and the sum was divided by the total weight of the extract used for flash chromatography, obtaining mg GAE/mg of BSG D extract.

6.3.3.2 Quantitative and qualitative analysis of BSG D extracts

Ultra-high performance liquid chromatography coupled to a tandem quadrupole mass spectrometer (UPLC-MS/MS) was used to quantify the most abundant polyphenols, methodology described in Chapter 3. Briefly, for the quantification of polyphenols, appropriate dilutions (0.098 to 50 ppm) of each standard (ferulic acid, *p*-coumaric acid, catechin, caffeic acid, 4-hydroxybenzoic acid, protocatechuic acid, gallic acid, sinapic acid) were prepared to obtain a standard calibration curve. TargetlynxTM (Waters Corp., Milford, MA, USA) software was used to quantify the compounds in the various extracts. The ferulic acid dimers and trimers were quantified using the standard curve from FA ($y = 413.8x + 197.7$, $r^2 = 0.99$).

Quadrupole time-of-flight (Q-ToF) Premier mass spectrometer coupled to Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) was used to profile various phytochemicals in the BSG D extracts and fractions, following the procedure previously described by (Hossain et al., 2010b) and depicted in Chapter 3 and 4. Moreover, in this study phenolics in the flash fractions were screened with direct injection in the LC-Q-TOF-MS/MS system, using a 2 min isocratic solvent (50% ACN). ESI mass spectra were recorded in the negative ion mode with the *m/z* range between 100–2000.

6.3.4 Samples prepared for the enzymatic assays

The fractions, 1 to 30, of each of the BSG extracts (Figure 6-3) were generated using a stepwise gradient with acetonitrile and water. Thus fractions 1-5 eluted at 0% acetonitrile, 6-10 at 10% acetonitrile, 11-15 at 20% acetonitrile, 16-20 at 30% acetonitrile, 21-25 at 80% acetonitrile and finally 26-20 at 100% acetonitrile. In chapter 5 we showed that ethanol at concentrations of <10% in

the final assay mixture interfered with the enzymes AChE and BChE activities and resulted inhibitions of up to 30% for AChE and less than 4% for BChE activity. Thus, prior to testing the BSG D flash fractions against the enzymatic activities, solutions at % acetonitrile like the ones in the fractions were tested as well to check if there might be any interference from this solvent. The results showed that at concentrations of 10%, 8%, 3%, 2% and 1% acetonitrile in the final AChE assay mixture, the activity of the enzyme was inhibited by 73.8±2.9%, 63.0±0.7%, 24.8±1.1%, 13.3±0.1%, and 4.1±1.2%, respectively. On the other hand, methanol was the only solvent that showed the lowest interference with AChE and BChE activity, where at the tested concentrations of 9% and 5% an inhibition of 8.7±1.0% and 2.3±0.2% was observed against AChE activity, and no inhibition was observed against BChE activity.

Due to the above observations, 10mL of each of the 6 to 30 BSG D flash fraction generated with 10%, 20%, 30%, 80% and 100% acetonitrile was dried under nitrogen and reconstituted in 2mL solutions at % MeOH mimicking the same polarity as the original fractions (see Table 6-1). Thus, the fractions 6-10 were reconstituted in 8.6%, 11-15 in 17.2%, 16-20 in 25.8%, 21-25 in 68.5% and finally 26-20 in 85.7% MeOH. Polarity of water was 100, of methanol 51 and of acetonitrile 58, respectively.

Table 6-1 Polarity calculation in the reconstituted samples

Fractions	**1-5**	**6-10**	**11-15**	**16-20**	**21-25**	**26-30**
%Water	100	90	80	70	20	0
%ACN	0	10	20	30	80	100
Calculated Polarity (0-100)	100.0	95.8	91.6	87.4	66.4	58.0
Volumes to reach Calculated Polarity (0-100)						
%Water	100	91.4	82.8	74.2	31.5	14.3
%MeOH	0	8.6	17.2	25.8	68.5	85.7

*Calculated polarity=(%Water*WaterPolarity)+(%ACN*ACNPolarity)*

6.3.5 Anti-Cholinesterase Assays

The overall enzymatic procedure to test the various BSG D extracts and fractions towards anti-AChE and anti-BChE activities was determined *in vitro* by Ellman's colorimetric method and adapted to cuvettes following the procedure of Faraone et al., 2019 (Faraone et al., 2019), and described in Chapter 5, section 5.3.6.

6.3.6 Anti-Diabetic Assays

The anti-diabetic assays comprise of using two types of enzymes, α-amylase, and α-glucosidase, and the BSG D extracts, and fractions were assessed following the methodology of (Faraone et al., 2019).

For the **α-amylase**, the assay mixtures were prepared in Eppendorf tubes, 10 µL of samples at different concentrations (BSG extracts, positive control) and 10 µL of α-amylase solution (0.5 mg/mL) prepared in 20 mM sodium phosphate buffer (pH 6.9 with 6 mM sodium chloride), were added in a tube and incubated at 25°C for 10 minutes. Following the preincubation, 10 µL substrate (1% potato starch in sodium phosphate buffer) was added to each tube and incubated at 25°C for 10 min. Following the second incubation, 20 µL of dinitrosalicylic acid colour reagent (DNS) was added to stop the chemical reaction. The test tubes followed a third incubation by boiling for 10 minutes and cooled to room temperature after. 300 µL of distilled water was added to each to tube to dilute the mixture, aliquots were transferred to a 96 clear bottom plate and the absorbance measured at 540 nm using a SPECTROstar Omega microplate reader (BMG Labtech, Offenburg, Germany). Blank solutions for each sample (enzyme added during the boiling step), and negative control (buffer instead of sample) were prepared, and the absorbance recorded as well. The absorbance of the blank of each of the sample was subtracted to obtain the final samples absorbance, and the results expressed as percentage of inhibition following the equation:

(**Equation 2 - 2**):

$$\%Inhibition = \frac{Abs_{540}NegativeControl \; - \; Abs_{540}Sample}{Abs_{540}NegativeControl} * 100$$

For the **α-glucosidase** assay, the experimental work was performed in 96 clear bottom well plates. In each well was added 60 µL of 2.5 mM 4-nitrophenyl α-D-glucopyranoside, 130 µL of 10 mM phosphate buffer (pH 7) and 40 µL of sample at different concentrations. The plate was incubated for several minutes at 37°C for equilibrium, and 20 µL of α-glucosidase solution (0.28 U/mL in 10 mM phosphate buffer) was added to each well to start the reaction ($T_{0'}$), and further incubated for an extra 10 minutes ($T_{10'}$). The absorbance at 405 nm was measured at ($T_{0'}$) and ($T_{10'}$). Negative control was prepared as well with buffer

instead of sample. The results were expressed as percentage of inhibition following the equation:

(**Equation 2 - 3**):

$$\%Inhibition = \frac{Abs_{405}NegativeControl_{(\mathrm{T10'-T0'})} - Abs_{405}Sample_{(\mathrm{T10'-T0'})}}{Abs_{405}NegativeControl_{(\mathrm{T10'-T0'})}} * 100$$

Acarbose was used at different concentrations (0.156 to 10 mg/mL, and in 50% methanol or otherwise specified) as positive control, and the required concentration to inhibit the activity of α-Amylase and of α-Glucosidase by 50 percent (IC_{50}) was calculated by nonlinear regression analysis. Buffer and organic solvent (%MeOH) instead of sample were recorded as negative control.

For this assay, the BSG D FP and BP controls are not presented as the samples were prepared in MeOH at concentrations of >90%, and when tested separately, MeOH at a 90% concentration showed high inhibitions of approx. 70% against the α-glucosidase activity. Similarly, the fractions 26 to 30 of all the BSG D FP and BP extracts were not tested due to similar issue. For this assay, the remaining fractions (1 to 25) a separate solution was prepared containing only MeOH at % concentrations as shown in Table 6-1 and was used as negative control.

The final concentration of samples, BSG extracts and positive control, in the final assay mixture was calculated by taking in the account a dilution factor, as follows: acetyl- and butyrylcholinesterase: 10, α-amylase: 30, α-glucosidase: 6.25.

6.3.7 Statistical Analysis

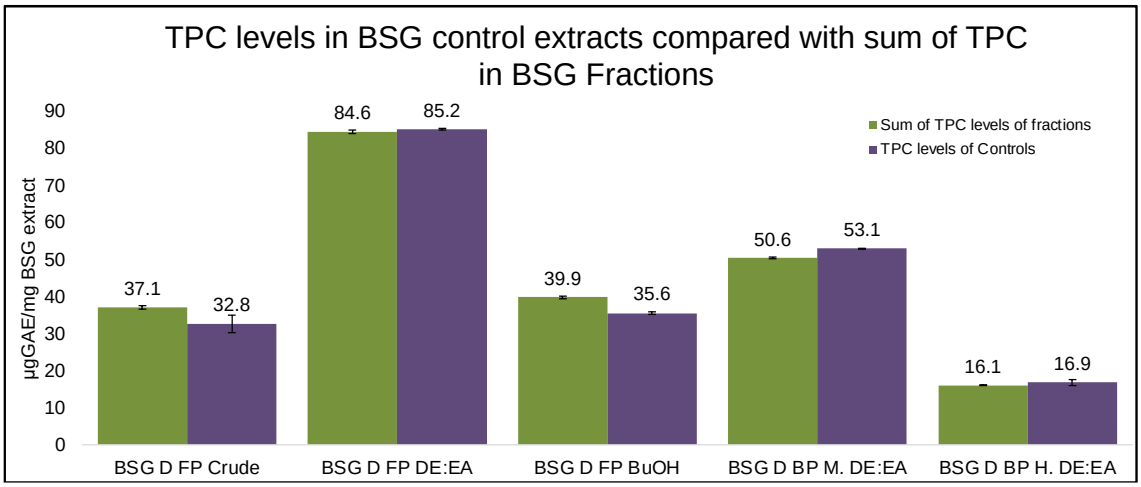
Results are expressed as means of duplicates±standard deviation (SD). The BSG D free and bound phenolic with the follow up flash fractions were generated in singles. TPC, anticholinesterase and anti α-amylase and α-glucosidase activities were measured two times in each flash fraction, whereas the phenolic compounds were quantified once. In chapter 5, a comprehensive statistical analysis has been performed using three independent extracts and the analysis was conducted three times on the same extract. As a result, statistical analysis of the samples was impossible in relation to the "hypothesis of real replicates" (Nunes et al., 2015).

6.4 Results and discussion

6.4.1 TPC levels in BSG D fractions

In Figure 6-4, the TPC levels of the BSG D FP and BP controls were recorded prior to being subjected to flash chromatography, and then compared with the sum of TPC levels recorded for each set of flash fractions (1 to 30) of the representative extract (Figure 6-5). The results showed that between the 'TPC levels of controls' (purple bars) and 'Sum of TPC levels of fractions' (green bars), minor differences were observed in the BSG D FP Crude and BSG D FP Crude samples, whereas for the rest of the samples the results were not that different from each other (Figure 6-4). The TPC determination of the free phenolic samples, showed that the highest TPC levels were recorded for the DE:EA fraction (BDG D FP DE:EA) of the crude extract at 85.2±0.2 μgGAE/mg BSG D extract, whereas in the bound phenolic samples the DE:EA fraction of the mild hydrolysis extraction showed the highest TPC (BSG D BP H DE:EA) at 53.2±0.7 μgGAE/mg BSG D extract (Figure 6-4).The application of a 2nd alkali hydrolysis, with 10x increase in the concentration of NaOH, showed a release of additional 33% in TPC levels of the BSG D BP extract.

Figure 6-4 TPC determination of the BSG D FP and BP controls and sum of TPC levels of representative flash fractions.



Each of the BSG D samples presented in Figure 6-4 were partitioned into 30 fractions during a total run time of 30 min. by using a flash chromatography system equipped with a C18 column to separate the compounds based mostly on polarity. TPC levels for these 30 fractions were determined and the values

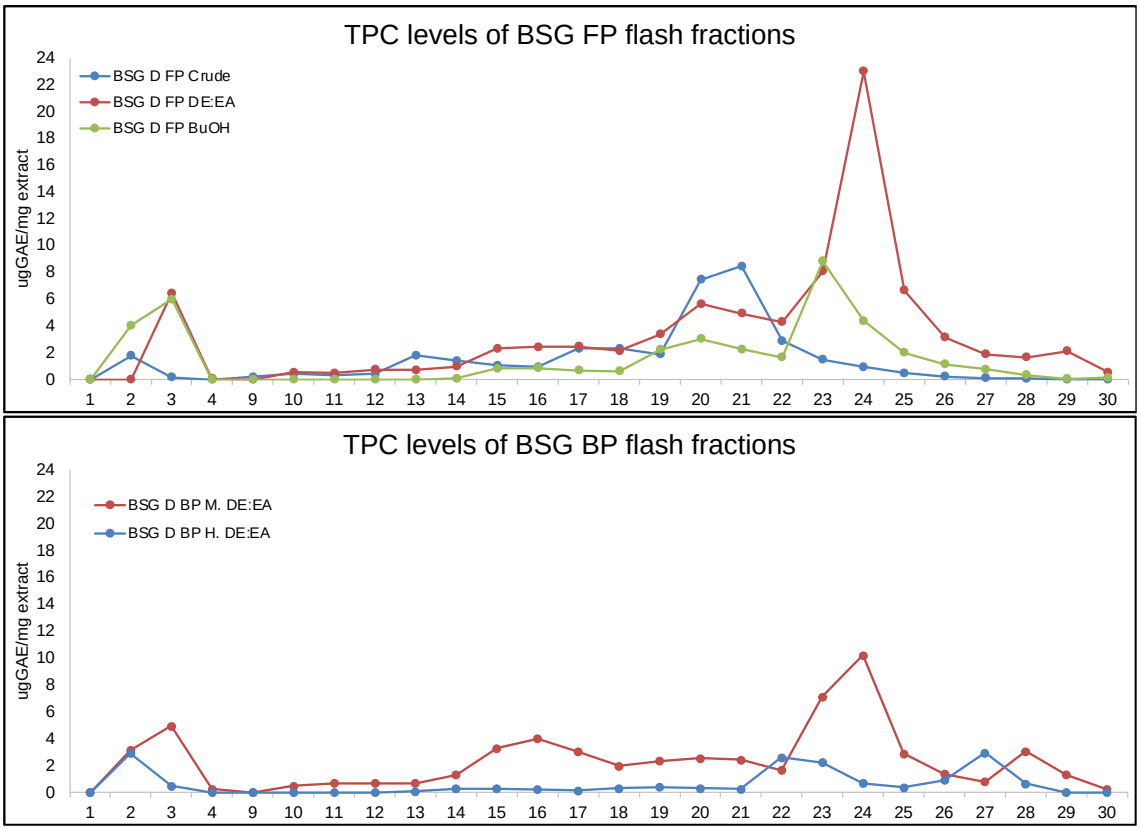
were plotted against the % inhibition of enzymatic inhibitions by each of the flash fractions (Figure 6-7). The TPC graphs of the flash fractions of the partitioned extracts (Figure 6-5) mirrored the recorded flash chromatograms (see Appendices, Figure 6-1) of each set of fractions indicating a strong link among the components of the peaks, mostly influenced by phenolic compounds.

Based on the TPC assay data (μgGAE/mg BSG D extract), flash fractions with most abundant polyphenols in various partitioned BSG D extracts could be ranked in the following order (Figure 6-5):

- BSG D FP Crude fractions: 21 > 20 > 22> 18 > 17 > 19 > 13 > 2
- BSG D FP DE:EA fractions: 24 > 23 > 25 > 3 > 20 > 21 > 22 > 19
- BSG D FP BuOH fractions: 23 > 2 > 24 > 2 > 20 > 21 > 19 > 25
- BSG D BP M DE:EA fractions: 24 > 23 > 3 > 16 > 15 > 2 > 28 > 17
- BSG D BP H DE:EA fractions: 27 > 2 > 22 > 23 > 26 > 24 > 28 > 3

As outlined above and illustrated in Figure 6-5, the highest TPC containing flash fractions were fraction 21 (8.5±0.06 μgGAE/mg) from the BSG D FP Crude, fraction 24 (23±0.04 μgGAE/mg) from the BSG D FP DE:EA, fraction 23 (8.8±0.06 μgGAE/mg) from the BSG D FP BuOH, fraction 24 (10.2±0.03 μgGAE/mg) from the BSG D BP M DE:EA, and fractions 2 and 27 (2.9±0.02 μgGAE/mg) from the BSG D BP H DE:EA extract. Among the BSG D flash fractions, the highest TPC were noted in the fraction containing 80% (fraction 24), 30% (fraction 16) and 0% acetonitrile (fraction 3), with a calculated polarity of 66.4, 87.4 and 100 (scale of 0 - 100 from non-polar to polar; Table 6-1).

Figure 6-5 Total phenolic content determination of the 30 representative flash fractions of each BSG D extracts



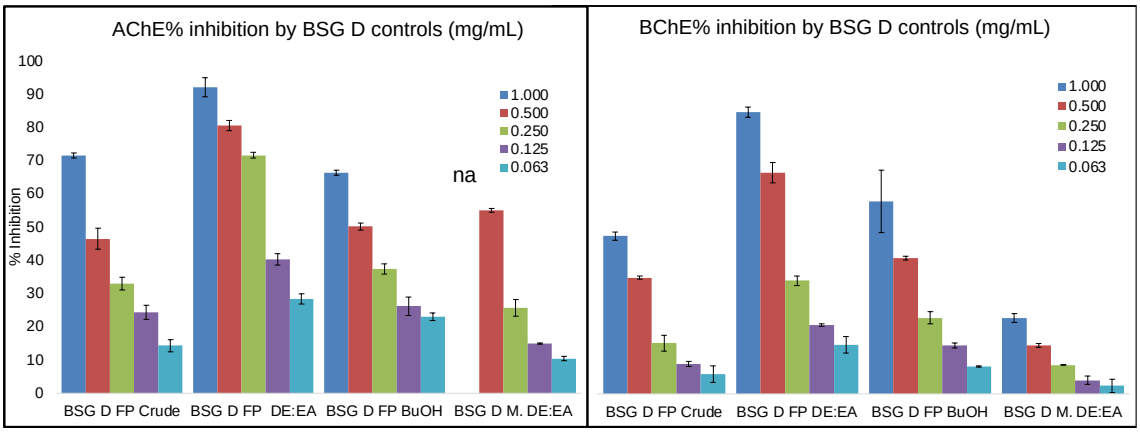
6.4.2 Inhibition of acetyl- (AChE) and butyrylcholinesterase (BChE) activities

The positive control, galantamine, showed inhibitions at an IC50 against the activities of AChE and BChE of 1.9±0.2 μg/mL and 16.5±4.9 μg/mL, respectively. These IC_{50} values were at similar range with the ones obtained in Chapter 5, Table 5-5 (50% inhibition by 3.4±0.23μg/mL for AChE and 11.9±1.67μg/mL for BChE) and with other previous authors (Faraone et al., 2019).

The BSG D control samples had a concentration-dependent activity on both AChE and BChE enzymes (Figure 6-6). In particular, BSG D FP DE:EA showed the highest potential to inhibit the activity of AChE *in-vitro* by approx. 90% at a concentration of 1mg/mL and decreasing moderately to reach a 30% inhibition at 0.063 mg/mL concentration. A similar trend was observed for BChE activity inhibition for this extract showing a slightly lower inhibitory potential at the same concentrations. The BSG D M DE:EA fractions presented the lowest inhibitions in both AChE and BChE, respectively. To reach a 50% enzyme activity inhibition, an approx. 85x higher concentrations of the BSG D FP DE:EA fraction was

necessary compared to galantamine for AChE and approx. 22x for BChE, respectively.

Figure 6-6 Inhibition of Acetylcholinesterase and Butyrylcholinesterase activity by Brewer's spent grain dark controls at various concentrations: 1, 0.5, 0.25, 0.125 and 0.063 mg/mL, respectively



Enzymes: Acetyl- and Butyrylcholinesterase enzymes (AChE and BChE); Brewer's spent grain dark (BSG D) extracts and fractions: free phenolic extract (BSG D FP crude), its diethyl ether fraction (BSG D FP DE:EA) and butanol fraction (BSG D FP BuOH); and the diethyl ether:ethyl acetate fraction of mild (0.75% NaOH) hydrolysed BSG (BSG D M DE:EA); na – not applicable

The AChE and BChE inhibitory activity of BSG D FP and BP flash fractions are presented in Figure 6-7.

A total of seventeen flash fractions generated from the BSG D FP Crude extract were tested. The largest inhibitions (>50% for AChE, >30 for BChE) were observed in the group of flash fractions collected using 80% acetonitrile (fractions 18 to 22), among which fraction 20 (F20) showed the highest inhibition of ˜75% against AChE activity, and an ˜49% against BChE activity by fraction 21 (F21). These two fractions contained the highest levels of TPC among all the tested fractions. Interestingly, F12 which contained compounds collected in 20% acetonitrile showed an inhibition of ˜55% and ˜30 against AChE and BChE activities, respectively, but with 18x lower TPC levels compared to fraction 20. Moreover, a trend was observed (fractions 20 to 26) where a gradual decrease in TPC corresponded with a decreased % inhibition of both enzymes' activities.

A total of seventeen flash fractions generated from the BSG D FP DE:EA extract were tested, where eight fractions showed inhibitions >50% for AChE and six from the same group for BChE activities, respectively. Among these eight fractions, seven fractions were collected in 30% (F19-20) and in 80% acetonitrile (F21-25), which showed TPC levels ranging between 10 and 64 µgGAE/mL,

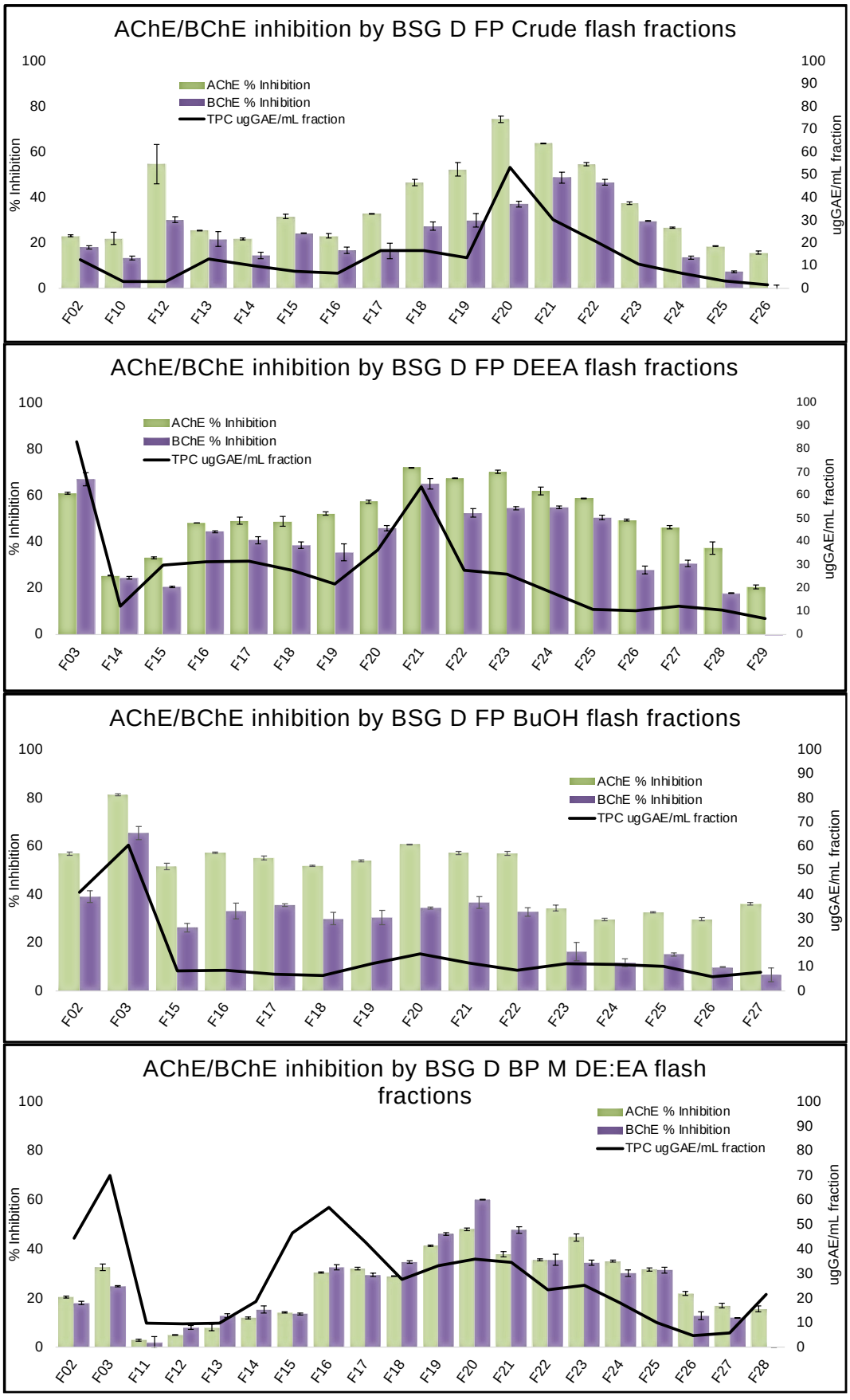
while fraction (F03) was collected at 0% acetonitrile possessing the highest TPC (83 µgGAE/mL).

A total of fifteen flash fractions from the BSG D FP BuOH extract were tested, among which ten showed inhibitions >50% against AChE (F02 - F22), and eight from the same group of fractions showed >30% inhibition against BChE activities. Interestingly, fraction 03 collected presented the highest inhibition of ˜81% against AChE activity, and ˜65% against BChE activity, respectively and also presented the highest TPC levels of ˜60 µgGAE/mL. The other fractions from the group (F15 to F22) were collected at 20%, 30% and 80% acetonitrile showed consistent lower inhibitions (˜32% for AChE and ˜40% for BChE) compared to F03, but at 6x lower TPC levels than in the F03. Although the fractions (F23 to F27) showed similar levels of TPC with that of the fractions F15 to F22, but the former group's inhibiting potential was reduced to almost half compared with the F15 to F22 fractions.

A total of twenty flash fractions from the BSG D BP M DE:EA extract were tested, where fraction 20 presented the highest inhibition of 48% against AChE activity, and 60% against BChE activity, and at TPC levels of 35 µgGAE/mL. Most fractions with a higher inhibitory potential (<50% for both enzymes) were generated using 30% and 80% acetonitrile (F16 to F25) and TPC levels between 10 and 56 µgGAE/mL.

Several observations were made based on the findings presented in Figure 6-7: (1) the BSG FP fractions showed in general a higher potential to inhibit AChE activity, compared to BChE, whereas the majority of BP M DE:EA fractions showed the opposite; (2) it is not always that a fraction with high TPC will indicate a high AChE and BCHE inhibition (e.g. F12 vs F20 of BSG D Crude extract); (3) the majority of the fractions showing the best inhibitions were those collected at 30% and 80% acetonitrile, whereas (4) the lowest being generated with 100% and 10% acetonitrile; (5) BSG FP BuOH fractions showed consistent inhibitions.

Figure 6-7 Inhibition of AChE and BChE activity by BSG D free and bound phenolic flash fractions, and their corresponding TPC levels in the final assay mixture



6.4.3 Inhibition of α-amylase and α-glucosidase activities

Inhibiting the α-amylase and α-glucosidase enzymes activity is an important therapy technique for obese and/or diabetic individuals. Different dilutions of BSG D extracts (controls) and fractions were tested for their ability to inhibit the activity of both enzymes. The concentration of the samples varied, and acarbose served as a positive control. Using nonlinear regression analysis, the concentration of the positive control necessary to inhibit the enzyme's activity by 50% (IC_{50}) in µg /mL was determined. Thus, acarbose presented an IC_{50} of 22.8±0.6 µg/mL towards the inhibition of α-amylase activity, and 399±20 µg/mL towards α-glucosidase activity, respectively. These inhibitory values of acarbose towards the both α-amylase and α-glucosidase enzymes are in similar range with the data presented by (Faraone et al., 2019). Unfortunately, the testing of BSG D controls and some fractions (F26-30) towards the anti α-glucosidase activity was not possible as the concentration of MeOH in the extracts was higher than 90% and showed interference with the enzyme's activity (see section 6.3.4).

The BSG D controls were tested against α-amylase activity (Table 6-2) at concentrations of 166.7 and 333.3µg/mL in the final assay mixture (20x dilution of the 5 and 10 mg/mL stock BSG D solutions), and showed inhibitions between 3 and 22% and 10 to 35%, with FP DE:EA fraction being the most potent. To reach an approx. 50% enzyme inhibition, a concentration higher than 500 µg/mL of the FP DE:EA extract would be necessary (25x higher concentration vs acarbose).

Table 6-2 α-amylase inhibition by BSG D FP and BP controls at 166.7 and 333.3µg/mL

Samples	Concentration in the final assay mixture	
	166.7µg/mL	*333.3µg/mL*
BSG D FP Crude	13.6±1.8	29.8±1.0
BSG D FP DE:EA	22.5±0.3	35.5±0.8
BSG D FP BuOH	7.2±0.4	10.2±2.5
BSG D BP M DEEA	3.4±0.8	20.6±2.5

The results in Figure 6-8 with several stacked column graphs represent selected flash fractions (five or six), with at least one representative group of samples with the same polarity (see Appendices, Figure A6-3 for all tested flash fractions). The BSG D FP and BP fractions were tested at different dilutions (0,

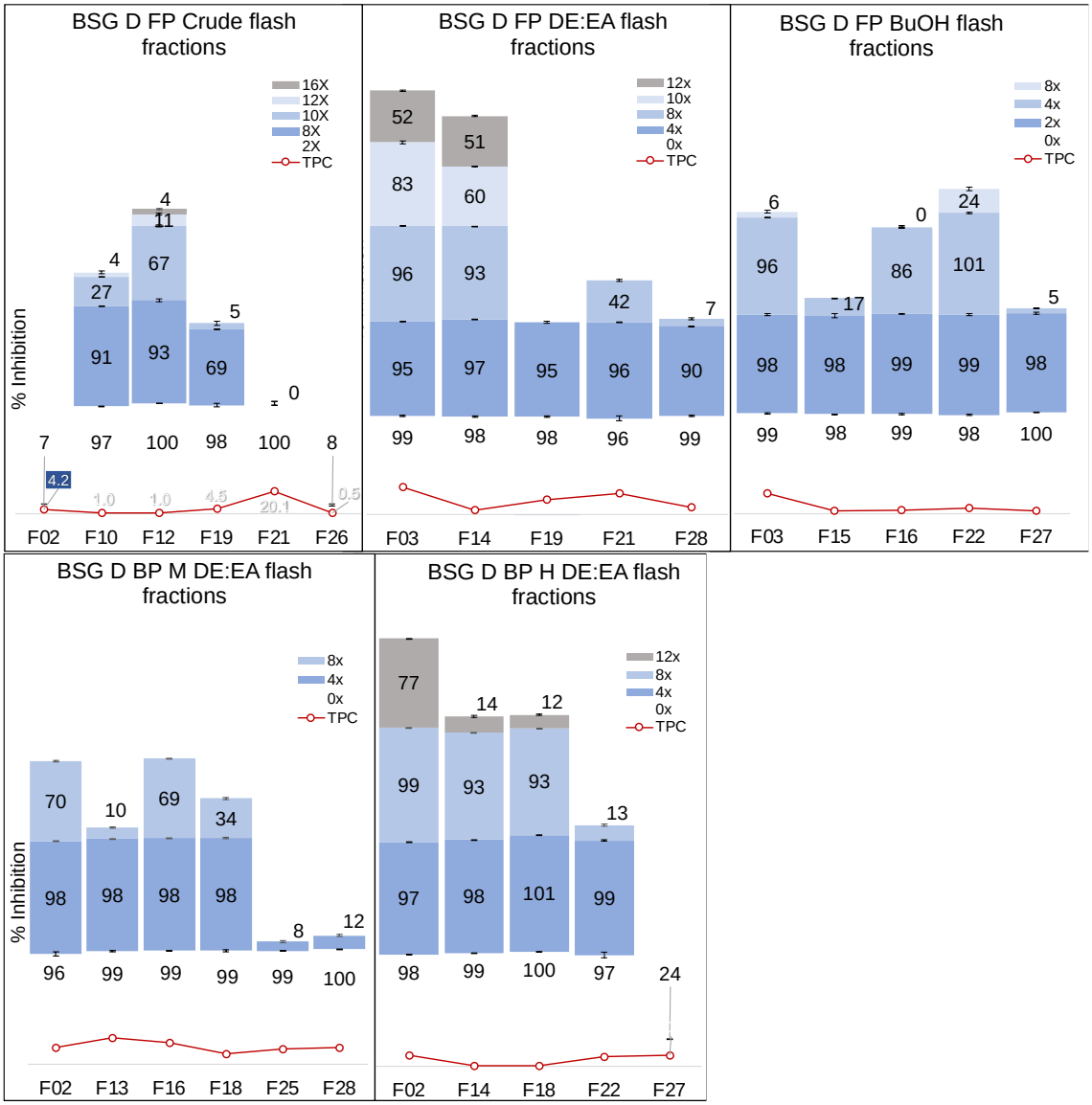
2, 4, 8, 10, 12 and 16x respectively) of the stock solutions, with the black numbers in each coloured column representing % inhibition of α-amylase activity by the selected BSG D fractions tested at specific dilutions. The TPC levels are shown for the stock or 0x dilution of fractions (red line with white numbers) and a further 2x, 4x, 8x 12x or 16x needs to the taken in consideration if the TPC levels need to be calculated. Also, the TPC levels showed for each fraction was calculated taking in account a 30x dilution of the stock sample in the assay mixture and representing the TPC concentration (μgGAE/mL) in the final α-amylase assay mixture, and a 6.25x dilution for α-glucosidase. Some of the samples for α-glucosidase had a 2x, 4x, 8x or 16x dilution prior being used in the enzymatic assay and this dilution was taken in consideration as well to express the final TPC levels.

In the first set of the tested BSG D FP flash fractions, F12 and F10 of BSG D FP Crude showed the highest inhibition against α-amylase activity, with stock F12 fraction showing inhibitions up to five levels of dilution (16x), and with starting TPC levels of ˜1μgGAE/mL. F02 presented a TPC of 4.2 μgGAE/mL and showed insignificant inhibitions, and similarly for fraction 26. F21, after a 2x dilution, the inhibition dropped to 0, even if this fraction presented the highest TPC (20.1μgGAE/mL) among the other tested fractions. The second set of tested fractions of the BSG D FP DE:EA extract, all showed inhibitions >90% at a 4x dilution of the stock samples, with F03 and F14 being able to show inhibitions of >50% at 12x dilutions. F03 and F14 followed a similar trend of inhibition, with the main differences being the TPC levels, where F03 presented a 7x lower TPC levels compared to F14. Furthermore, the third set of tested fractions of BSG D FP BuOH extract, all showed inhibitions >90% at a 2x dilution of the stock samples, with F03, F16 and F22 being able to show inhibitions of >80% at 4x dilutions, and up to 8x for F22. F03 presented similar inhibitions with F16 and F22, but the TPC content of F03 was 7x and 3.5x higher compared with the other two fractions.

The set of six flash fractions tested for the BSG D M DE:EA extracts, and they all showed >90% inhibition at 0x and 2x dilution. While the F02 and F16 at an 8x dilution of the stock solution, and TPCs of ~1.9 and ~2.4 μgGAE/mL, respectively inhibited α-amylase activity by approx. 70%. The last set of five flash

fractions tested were those of the BSG D H DE:EA extract, which showed better inhibitions compared with the previous set of BP flash fractions. Interestingly F02 showed inhibitions of 77% at 12x dilutions at TPC level of 10μgGAE/mL. F14 and F18 showed similar levels of inhibitions at same TPC levels, whereas the last fraction, i.e. F27, presented an insignificant inhibition at similar TPC levels as F02.

Figure 6-8 Inhibition of α-amylase activity by BSG D free and bound phenolic flash fractions, and their corresponding TPC levels at stock or 0x dilution in μgGAE/mL



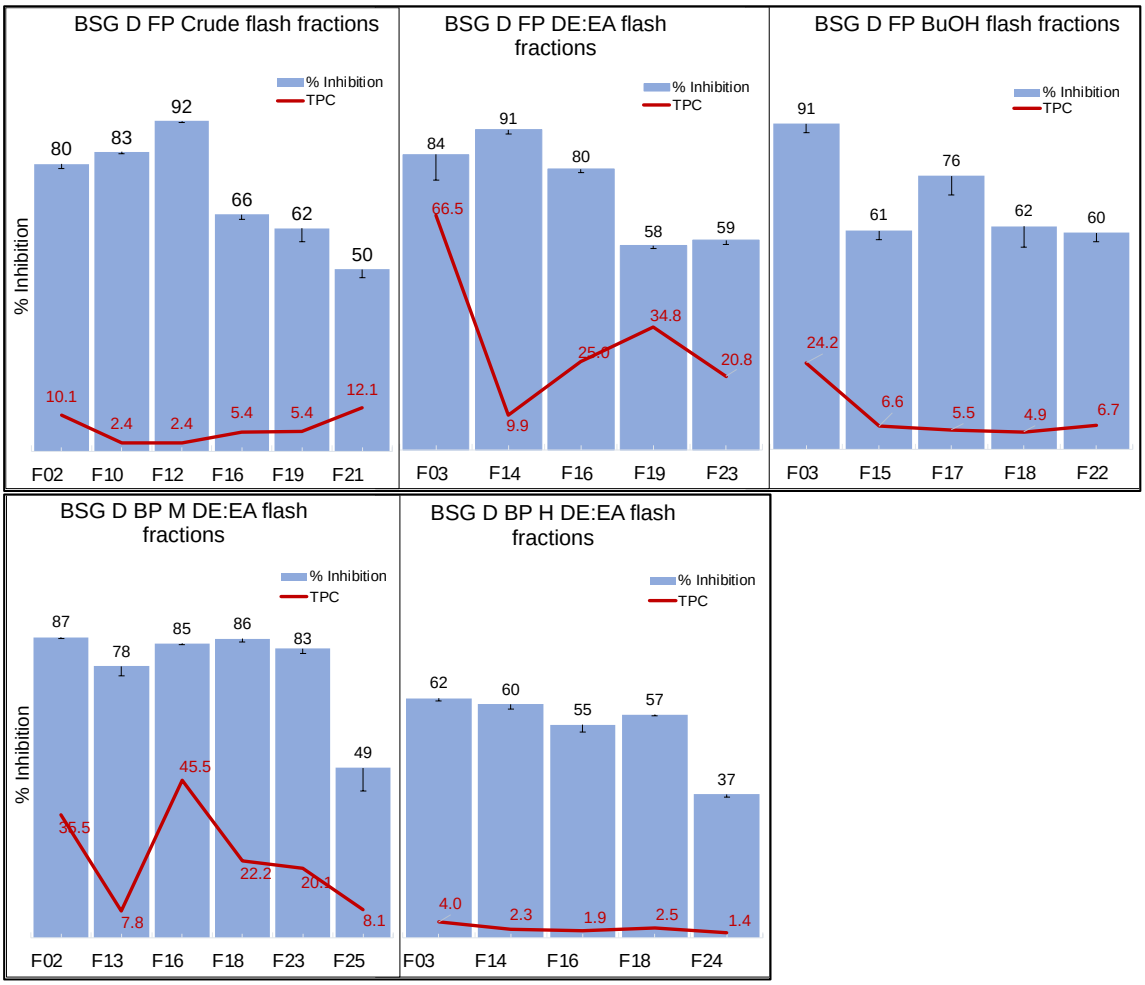
The results showing the inhibitory capacity of the BSG D FP and BP flash fractions against α-glucosidase are shown in Figure 6-9. The TPC levels are plotted for each fraction representing the final concentration of TPC (μgGAE/mL) in the sample used in the assay.

Among the flash fractions of the BSG D FP extracts, all fractions showed inhibition against the activity of α-glucosidase, varying from 50% and up to 92%. The highest inhibitions of >90% were observed in the same group of fractions collected in 20% acetonitrile (F12 of FP Crude, F14 of the FP DE:EA and F14 of the FP BuOH extracts) with TPC levels varying from low (2.4 µgGAE/mL) to medium (9.9 µgGAE/mL), and high (24.2 µgGAE/mL), respectively. In the flash fraction of FP Crude extract the most potent fractions were collected in 0% to 20% acetonitrile (80-92% at TPC of 10.1 to 2.4 µgGAE/mL, respectively), whereas their inhibitory potential was decreasing with slightly increase in TPC levels (66% to 50% inhibition at TPC of 5.4 to 12.1 µgGAE/mL, respectively). A similar trend was observed in the FP DE:EA and FP BuOH where after the F15 fractions the inhibitory potential levels were slight similar whereas the TPC levels varied from high to low in the FP DE:EA and FP BuOH fractions.

Similar to BDG D FP fractions, the BSG D BP M and H DE:EA fractions, all showed carbohydrases inhibitory potential varying between 37% to 87% and TPC levels of 1.4 to 45.4 µgGAE/mL. Compared with the flash fractions of the FP extracts, the BP fractions showed decreasing inhibitory potential after F24 fractions collected in 80-100% acetonitrile. The fractions of the BP M DE:EA extract, namely F02 to F23, showed similar levels of inhibitions (approx. 84%) but at varying TPC levels, with fraction F13 showing the lowest of 7.8 µgGAE/mL and F16 the highest of 45.5 µgGAE/mL. Interestingly, the similar fractions tested from the BP H DE:EA extract showed lower inhibition but consistent (approx. 59%), and the TPC levels were 10 to 20x lower too.

Several observations were made based on the findings presented in Figure 6-9: (1) all the flash fractions presented good inhibitions against α-glucosidase, but not all fractions against α-amylase; (2) some fractions presented high inhibitions at low and high TPC levels (e.g.F12 vs F21 of BSG D Crude extract, F03 and F14 of the BSG D FP DE:EA); (3) in general, the majority of the fractions showing the best inhibitions were collected in < 30% acetonitrile, whereas (4) the lowest inhibition of carbohydrases collected in the fractions containing 80-100% acetonitrile.

Figure 6-9 Inhibition of α-glucosidase activity by BSG D free and bound phenolic flash fractions, and their corresponding TPC levels in the final assay mixture (μgGAE/mL)



Among the tested fractions against α-amylase and α-glucosidase activities some of the fractions performed best against both enzymes: F10 and F12 of the six tested fractions of the FP Crude extract at the lowest TPC levels; F03 and F14 of the five tested fractions of the FP DE:EA extract at the highest and lowest TPC levels; F03 of the FP BuOH extract of the five tested fractions at the highest TPC levels; F02 and F16 of the BP M DE:EA of the six tested fractions at higher TPC levels; and lastly fraction F02 and F03 of the BP H DE:EA extract of the fives tested fractions at the highest TPC levels.

6.4.4 Identification and quantification of the major phenolic compounds in the BSG flash fractions

The BSG D FP and BP flash fractions were subjected to liquid chromatography-tandem mass spectrometry for qualitative (LC-Q-ToF-MS/MS) and quantitative (UPLC-TQD-MS/MS) analysis to profile the compounds

responsible for the different bioactivities evaluated above. First approach was a targeted metabolite approach using UPLC-TQD-MS/MS analysis on the fractions that showed (1) high peak intensity in the flash chromatograms, (2) the highest TPC levels and (3) the ones presenting the highest levels of inhibitions against the enzymes' activities. Subsequently, LC-Q-ToF-MS/MS analyses were performed to screen several fractions from above for other types of phenolic and non-phenolic compounds that were not accounted through the targeted metabolite approach (see Appendices, Figure A6-5, A6-6, and Table A6-7).

As previously described in this thesis chapters, the targeted phenolic compounds available to quantity against reference standards were catechin (Cat), caffeic acid (Caff.A), gallic acid (GalA), 4-hydroxybenzoic acid (4-HBA), sinapic acid (SinA), ferulic acid (FA), *p*-coumaric acid (*p*-CA), whereas decarboxylated ferulic acid, di-ferulic acid and triferulic acid were quantified as ferulic acid equivalent. The results are presented in Figure 6-5 and shows the concentration of each phenolic compound (µg/mL) in the stock flash fractions prior to being used in the different enzymatic assays. Moreover, other three compounds are mentioned with the results being shown as Total Ion Chromatogram (TIC, sum of the intensities of ions), with 5,7-dihydroxychromone (5-chromone) being identified and quantified in BSG by Verni et al. (Verni et al., 2020), whereas the other two unknown compounds, with *m/z* 295 and 329, were mentioned in the previous chapters.

Even though the quantification of phenolic compounds was performed as in the previous chapters, the flash fractions of the three BSG D FP extracts presented very low levels of phenolic compounds (see Figure 6-10). The enriched extract of BSG D FP DE:EA presented the highest levels among the three BSG D FP extracts, with F3 showing the highest levels of catechin, protocatechuic acid and ferulic acid. On the contrary, in the FP BuOH flash fractions only traces of catechin were observed. Interestingly, 5-chromone presented high TIC intensities in the same F15 fraction of both FP Crude and FP DE:EA, with the later showing an approx. 5x higher intensities. Peak 295 was predominant in the F23 and F26 of FP Crude and FP DE:EA. As observed in the previous chapters of this thesis, the BSG D BP extracts presented high levels of ferulic acid and coumaric acid, reaching levels of approx. 203 µg/mL and approx. 57 µg/mL in F3 of the BP

ME:EA extract. Using the harsh alkali hydrolysis extraction, an additional 28% of ferulic acid and 39% of *p*-coumaric acid were released in the same group of fractions (F02). These levels were present in the flash fractions collected in 0% acetonitrile, but their elution continues in decreasing levels up to F24 (collection in 80% acetonitrile). Among the ferulic acid dimers and trimers (DiFA, TriFA), decarboxylated di-ferulic acid was the most predominant in the fractions of BP M DE:EA generated using 80% acetonitrile (F23 and 24), at levels of up to 82 µg/mL, whereas the other polymers of ferulic acid, DiFA was present at low levels and only traces of TriFA were found in the fractions collected at lower % acetonitrile. Similar to F15 of the FP DE:EA, 5-chromone was released early (F03 of BSG D M DE:EA) at almost half the intensity when mild alkali hydrolysis extraction was used (BP M DE:EA) and observed in decreasing levels up to F24 of the same extract. Similarly, unknown compound with *m/z* 295 eluted between 80-100% acetonitrile (F23 to F27) with its concentration increasing as organic solvent increased. The ion at *m/z* 295 in fraction 26 (F26) of the BP M DE:EA and in fraction 25 (F25) of the BP H DE:EA extracts was at 7x and 10x higher compared with the fraction 26 (F26) of the FP DE:EA extract. On the other hand, the same fractions showed compounds with *m/z* 329 eluting at high levels in F23 of the BGS M DE:EA and then decreased with increasing % acetonitrile content.

Figure 6-10 Quantification of phenolic compounds in the BSG D FP and BP flash fractions

Fractions	Concentration of phenolic compunds (µg/mL) in BSG fraction prior enzymatic assay dilution											Total Ion Count - peak area		
BSG D FP Crude	**Cat**	**CaffA**	**GalA**	**ProtA**	**4-HBA**	**SinA**	**FA**	***p*-CA**	**DeCA DIFA**	**DiFA**	**TriFA**	**5-Chromone**	**Peak 295**	**Peak 329**
F2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0
F10	0.0	0.0	0.07	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	2	0
F12	0.16	0.15	0.0	0.23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0
F15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.15	0.0	0.0	0.0	3560	0	0
F17	0.0	0.0	0.0	0.0	0.0	0.0	0.66	0.50	0.0	0.0	0.0	474	0	10
F21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	113
F22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	21	54	39
F23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	1000	48
F24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	157	0
BSG D FP DE:EA														
F3	16.28	2.65	3.86	8.90	3.84	0.0	9.53	0.02	0.0	0.0	0.0	2487	35	22
F15	0.0	5.36	0.0	0.0	0.46	0.0	4.13	1.38	0.0	0.0	0.0	18715	0	25
F18	0.0	0.00	0.0	0.0	0.0	0.0	0.00	0.34	0.0	0.0	0.0	933	0	16
F21	0.0	0.00	0.0	0.0	0.0	0.0	3.85	0.0	0.0	0.0	0.0	124	0	59
F24	0.0	0.22	0.0	0.0	0.0	0.0	4.47	0.0	0.0	0.0	0.0	191	20	301
F25	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	463	421
F26	0.11	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	2577	535
F27	0.08	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	147	23
BSG D FP BuOH														
F3	0.25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	17
F17	0.19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	47
F20	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	4	39
F23	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	29	57
F24	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	0	24
F25	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	63	32
F26	0.16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	34	42
F27	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	7	0
BSG D BP M DE:EA														
F3	0.1	9.5	0.1	8.7	14.0	2.3	203.6	67.5	0.0	0.0	0.0	11243	0	0
F14	0.1	2.3	0.0	0.0	1.7	0.8	70.4	46.9	3.2	14.3	0.0	5786	0	0
F20	0.0	0.0	0.0	0.0	0.0	0.0	5.9	2.5	32.5	3.1	0.9	326	0	87
F23	0.0	0.0	0.0	0.0	0.0	0.5	2.5	2.4	82.2	12.6	2.9	307	178	7798
F24	0.2	0.0	0.0	0.0	0.0	0.0	3.5	2.4	77.1	4.3	0.0	433	317	6380
F25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	3.3	0.0	0.0	62	7751	983
F26	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.1	0.0	0.0	0	17001	547
F27	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0	1102	503
BSG B BP H DE:EA														
F2	0.3	0.3	0.1	2.3	5.8	0.3	55.2	25.7	0.0	0.0	0.0	894	3	754
F14	0.0	0.0	0.0	0.0	0.4	0.0	3.9	6.1	0.0	0.0	0.2	297	0	0
F19	0.0	0.0	0.0	0.0	0.0	0.0	4.4	1.7	5.6	0.0	0.0	0	7	443
F23	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	8.6	0.0	10.9	0	80	4701
F24	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	11.7	0.0	0.0	0	1582	3551
F25	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	23769	1743
F26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	16622	497

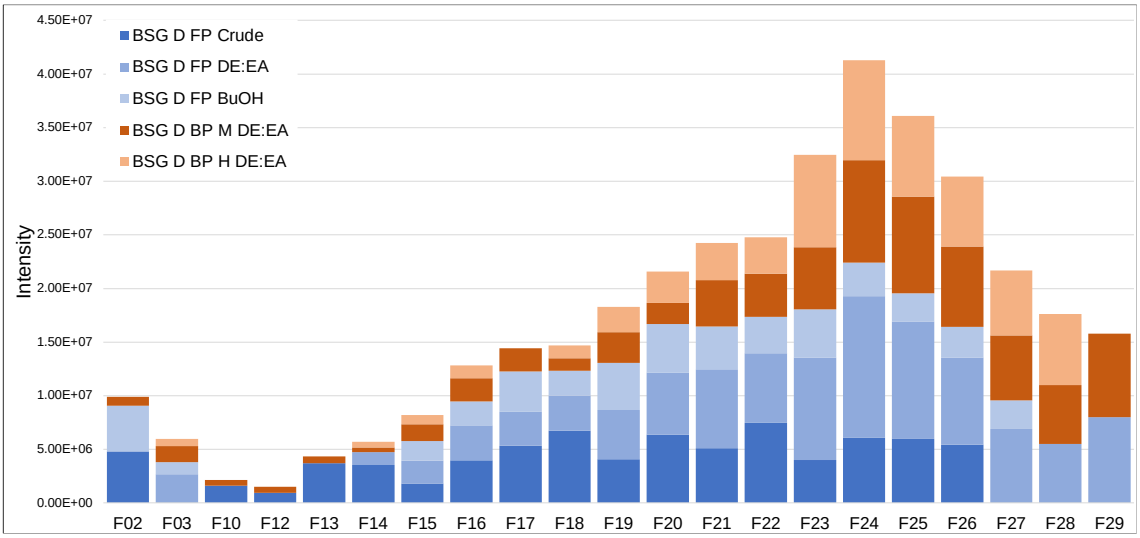
In the non-targeted metabolite profiling, each BSG FP and BP flash fractions were screened in the LC-Q-ToF-MS system, using an isocratic solvent (50% ACN) for 2 min, and the ESI mass spectra were recorded in the negative ion mode for a mass range (*m/z*) 100–2000. The mass spectra of the screened fractions are presented in the Appendices Figure A6-5. Using the MS^e feature, (also called data independent data acquisition) of the LC-Q-ToF, all ions transmitting through the collision cells were fragmented without selecting any particular precursor ion, and thus providing fragmentation patterns for all the transmitted precursor ions. Because of the enriched metabolite in flash chromatography, the total number of generated ions with *m/z* ranging between 100 to 2000 for some of the flash fractions reached up to 16,127; this was graphically presented where the sum of ion intensities to portray the distribution of ion abundance in the fractions. An observation is that the plot of the ion

intensities (Figure 6-11) for each flash fraction resembled the corresponding flash chromatogram (see Appendices, Figure A6-1), where the highest peak on both graphs generated at the flash fractioned collected at 80 to 100% acetonitrile. Due to the high number of unknown compounds generated in the non-targeted approach, it will be a challenging and tedious to elucidate and/ or predict their chemical structures. We therefore focussed on analysing MS data for the most active fractions and the most abundant *m/z* ions recorded in them.

In Figure 6-11 the sum of ion intensities in several BSG D FP and BP flash fractions is presented. The highest ion intensities were observed in fractions generated between 80-100% acetonitrile, with the highest abundancy in the BSG D FP DE:EA and the lowest in the BSG D FP BuOH extracts, whereas the BSG D FP Crude its ion intensities fluctuated across the fractions. For example, the most dominant ions in the BSG D FP DE:EA were at *m/z* of 308.1 (F03, not identified (n.i.)), 179.1 (F14 – caffeic acid), 193.1 (F16, ferulic acid), 341.1 (F19, n.i.), 577.1 (F24, tentatively identified as procyanidin B due to the presence of its unique fragments at *m/z* of 407.1, 451.1, 289.1 (catechin) and 125.1). The later fraction containing the ions *m/z* of 577.1 was previously described in Chapter 4, Figure 4-5. Other dominant ions in the BSG D BP M DE:EA fractions were *m/z* 163.1 (*p*-coumaric acid), 179.1 (caffeic acid) and 193.1 (ferulic acid) in F02 to F18, whereas after this fractions the dimers of ferulic acids at *m/z* of 385.1, 371.1 start to become dominant up to F21, followed by the dominance of an unknown compound with *m/z* 329.1 in the later fractions. This compound was observed in general in all the generated fractions at 80% and 100% acetonitrile. The ions generated at with specific *m/z* were cross checked with Phenol explorer database (http://phenol-explorer.eu/).

An observation is that the All Ion MS/MS intensities shape (Figure 6-11) of the extracts resembles the flash chromatogram spectra (see Appendices, Figure A6-1), with the highest peak spectra being for the fractions generated the region of 80% and start of 100% acetonitrile.

Figure 6-11 Sum of All Ion MS/MS intensity in the BSG D FP and BP flash fractions generated by LC-Q-ToF-MS



In chapter 5 of this thesis, we have observed that BSG D presented a better inhibition against AChE and BChE activity compared with BSG L, even though BSG D presented lower levels of phenolics (see Chapter 4). Moreover, as I mentioned in the introduction of this chapter, and recent investigations imply a cause-and-effect relationship between hyperglycaemia (diabetes) and dementia (Alzheimer's disease), we have decided to introduce in our study an extra set of enzymes to test BSG D extracts against, α-amylase and α-glucosidase. Thus, in this last research chapter we investigated several dissected fractions, obtained from BSG FP and BP extracts, against the two sets of enzymes, AChE/BChE and α-amylase/α-glucosidase, respectively. There were five BSG extracts generated that were subjected to flash chromatography which generated approx. 150 flash fractions to be analysed and tested. Approx. 80% of these fractions have been screened for TPC, and approx. 50% were tested against the activities of AChE/BChE and α-glucosidase/α-amylase, respectively. Beside testing the fractions against enzymatic activities, our aim was to try and associate specific BSG phenolic compounds with bioactivity, however the results are much more complex than expected. Overall observations suggest that there does appear to be some association between total phenolic content (TPC) and some bioactivity in certain fractions; but, in other fractions, there are sometimes instances of high TPC and low activity, and vice versa. Also, there is a wide variety of behaviour that occurs in between, so to rationalise the discussion and cut down on its breadth of coverage, a small number of discussion points that reflect the most

intriguing of these behaviours and attempt to draw some conclusions regarding the relationship between extraction, fractionation, and bioactivity.

Among the tested fractions of the BSG D Crude extract, Fraction 12 showed good inhibitions against both cholinesterases and carbohydrases at the lowest levels of TPC among all the fractions. On the other hand, the highest levels of TPC were observed in F20, which showed the best inhibitions against AChE and the next fraction, F21, against BChE, whereas against α-amylase/α-glucosidase activities, this fraction did not perform as well. The quantitative analysis of F12 showed low levels of catechin, caffeic acid and protocatechuic aid, whereas in F21 the targeted analytes were below detection limit or not present. Moreover, ion abundance plot for the flash fractions showed F12 to contain the lowest ions of all the fractions whereas F20 and 21 the highest. The most abundant ions $[M-H]^-$ were at *m/z* of 194.9 (hydroxycaffeic acid) and 216.9 (not identified) in F12, whereas in F21 ion at *m/z* 577.1 (procyanidin B). The low TPC levels in F12 might suggest non-phenolic compounds to be responsible for the high inhibitions, and the phenolic compounds in F21 due to high levels of TPC. A similar situation was observed for the fractions F03 and F14 of the BSG D FP DE:EA. F03 presented the highest levels of inhibitions against both carbohydrases and cholinesterases activity at the highest TPC levels, whereas F14 presented similar inhibitions with F03 for carbohydrases and AChE but not for BCHE, at 7x lower TPC levels. UPLC-MS/MS quantitative analysis of these fractions showed catechin, protocatechuic acid and ferulic acid at <16µg/mL in F03, and F14 with caffeic acid and ferulic acid levels <6µg/mL. Moreover, these fractions showed low and high TIC of 5-chromone. The ion-abundance plot showed 2x higher levels of ions in F03 compared with F14, and the most abundant ions were *m/z* 163.1 (p-coumaric acid) and 308.1 (n.i.) for F03 and 179.1 (caffeic acid) and 177.1 (5-chromone) in F14. Among the individual standards tested in chapter 5 against AChE and BChE activity, caffeic acid was the most potent and *p*-coumaric was less potent, suggesting that these compounds interact synergistically and antagonistically with other compounds in the extracts. Lastly, among the generated extracts and flash fractions, the BP M DE:EA fraction F03 presented the highest levels of targeted analytes, with ferulic and *p*-coumaric acid being the most abundant, including high TIC of 5-chomone, with decreasing levels up to F24. Based on the

hypothesis that BSG phenolic rich extracts are potentially good inhibitors against enzymatic activities, this fraction should have presented the highest inhibition against both cholinesterases and carbohydrases. Unfortunately, this was not the case, as expected this fraction presented highest TPC levels among all the fractions, some inhibition against carbohydrases but not the highest, whereas for cholinesterase only half of the most potent fraction (F20). Interestingly, the fractions F19 to F25 of the BSG D BP M DE:EA presented the highest inhibitions against AChE and BChE, and the quantitative and qualitative analysis of these fractions showed the highest levels of the DiFA and DeCA-DiFA (dimers of ferulic acid) among the analysed fractions and as well the most abundant ions (*m/z* of 385.1, 343.1, 341.1). Furthermore, we have showed in Chapter 5 that from a statistical point of view, DeCa-DiFA presented significant correlation ($p < 0.01$) with the enzymatic assays AChE and BChE of 0.754 and 0.896, respectively ($n = 21$).

6.5 Conclusions

The objective of this last research paper was to assess the potential of the BSG dark generated flash fractions to inhibit the activities of two sets of enzymes considered as a treatment strategy for Alzheimer's disease and diabetes, namely acetylcholinesterase and butyrylcholinesterase, and *α*-amylase and *α*-glucosidase enzymes. Moreover, the aim was to try and identify phenolic fractions with bioactivity, however the findings were more complex than anticipated.

Based on the findings several conclusions could be drawn. Regarding the extraction of phenolic compounds from dark BSG, a second alkali extraction using NaOH at a higher molarity released approx. an extra 33% of ferulic and *p*-coumaric acid, thus the extraction methodology would need to be optimised to obtain a complete extraction of either free or bound phenolic compounds. Further, the purification of the BSG extracts using flash chromatography can be an initial purification step to produce BSG phenolic-rich extracts, either for evaluating the efficacy of different mixture of phenolic and non-phenolic compounds or for use as antioxidant ingredients in food compositions.

The majority of the BP M DE:EA fractions showed opposite behaviour, in contrast to the majority of the BSG FP fractions, which had a larger potential to

inhibit AChE activity as compared to BChE. Moreover, it is not always the case that a fraction with a high TPC will indicate a high AChE and BCHE inhibition, which may be an indication that not only phenolic compounds might be responsible for the enzymatic inhibition but as well other non-phenolic compounds. The preponderance of the fractions that showed the best inhibitions against AChE and BChE activity were those that were collected at 30% and 80% acetonitrile, whilst the fractions that exhibited the lowest levels of inhibition were those generated with 100% and 10% acetonitrile. For the *α*-amylase and *α*-glucosidase enzymes, was shown that almost all the flash fractions were effective at inhibiting *α*-glucosidase, however not all the fractions were effective against *α*-amylase. Like AChE and BChE, certain fractions demonstrated a high level of inhibition at both low and high TPC concentrations, with most of the fractions that showed the best inhibitions were obtained in acetonitrile concentrations of less than 30%, and the less potent were the ones generated at 80% and 100% acetonitrile.

In summary, considering the findings of this research, BSG dark extracts showed greater efficiency in inhibiting *α*-glucosidase and *α*-amylaze activity, compared to the effects on acetyl- and butyrlcholinesterase activity, which may be due to the phenolic content. However, further studies are necessary which may include additional purification with semi- or preliminary high-performance liquid chromatography (HPLC) for a better isolation and identification of the natural components, followed by structural elucidation that can be accomplished using high-resolution mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

Chapter 7

7. Remarks and Future Perspectives

7.1 Concluding remarks

From both an economic and environmental aspect, more emphasis is being placed on the reuse of agro-industrial by-products. The brewing industry produces a significant number of by-products, the most prevalent of which are spent grains, discarded hops, and yeast. However, because most of these by-products are agricultural, they may be easily recycled and reused. Despite all the potential applications listed, its utilisation is still restricted to animal feed, fertilisers, while the vast majority are discarded in landfills. As a result of microbial degradation, disposal creates environmental difficulties and increases the expense for food processors. Therefore, innovative strategies for utilising these agro-industrial by-products are of tremendous interest. Additionally, delivery of renewable biological resources and converting the resource and its by-product streams into food, feed, and bio-based goods is part of the design for a sustainable European bioeconomy. Moreover, in the last several decades, these by-products have been identified as potential reservoirs of nutritional and functional food ingredients. Due to their propensity to promote a multitude of health benefits, bioactive antioxidants, particularly bioactive phenolic compounds, have received a lot of attention in recent years. Increasing efforts have been stepped up to identify natural resources with antioxidant activity that can effectively replace synthetic antioxidants that have been linked to harmful and carcinogenic effects. The significance of these bioactive compounds resides in their potential health effects, especially in reducing the risk and/or prevention of degenerative diseases such as cardiovascular and neurological disorders, diabetes, cancer, which all include oxidative stress and inflammation as part of their pathophysiology. Hence, the potential of antioxidants and anti-inflammatory compounds, such as polyphenols, to lower the risk of various diseases is critical for public health. These health boosting characteristics could form the basis for the development of functional and nutraceutical ingredients with considerable economic potential.

The goal of this study was to extract bioactive compounds from the brewing by-products, such as brewer’s spent grain, that might be used as a novel source of natural antioxidants against cholinesterases and carbohydrases. The presence

and abundance of phenolic acids in BSG were clearly confirmed in this study. The primary components contributing to the phenolic profile of those samples were hydroxycinnamic acids (caffeic, sinapic, *p*-coumaric, and ferulic) and lower levels of flavanols and catechin. Furthermore, because BSG is the primary by-product of the brewing sector, its use as a source of added-value products may be more cost-effective and warrants further exploration.

The most common method for recovering natural antioxidants from plant materials is to use classic extraction procedures with organic solvents or acid and base chemicals. This type of extraction, on the other hand, is laborious, energy, and solvent consuming, and produces more waste. In this thesis, in Chapter 3, two types of classic extraction methodologies were tested and involved the use of organic solvents to obtain free phenolic extracts, and acid/base solutions to obtain bound phenolic extracts. These methods enabled us to see how the organic solvents and acid/base solutions used with specific parameters (solid:liquid ratio, temperature, time), influences the release of phenolic compounds from light and dark BSG substrates. The outcomes of the chapter showed that acetone and NaOH to be best to extract free and bound phenolics respectively. Furthermore, due to the length, chemical variation, and complex extraction procedure used in Chapter 3, UAE and MAE were employed in Chapter 4 as alternatives to traditional extraction procedure to improve plant bioactive recovery, that may significantly decrease extraction time, energy, and solvent usage while still extracting larger levels of phenolics from BSG. Unfortunately, the use of MAE and UAE as novel extraction technologies with the preoptimized parameters described by (Moreira et al., 2012a) did not improve the extraction yield of phenolic compounds compared with classic extraction method using the same parameters. Thus, classic extraction methodology was used in the next chapters to generate free and bound phenolic extracts. Before using a specific extraction technology and methodology, it should be taken into consideration if it is suitable for commercial use, as well as low-cost and easy-to-scale-up processes.

Industrial BSG by-product contains phenolics that include multiple isomeric dimers (also decarboxylated) and trimers of ferulic acid, according to HPLC-Q-ToF-MS and UPLC-TQS-MS investigations, but as well it has shown to contain a

multitude of other types of non-phenolic compounds. These findings showed that the BSG phenolic extracts may be partitioned and fractionated using either the classic liquid-liquid extraction or by using a more complex system such as flash chromatography, permitting the isolation of bioactive compounds that could be employed as natural and cost-effective antioxidant replacements.

The brew houses use a diverse range of barley varieties for beer production, and as barley type and origin can affect the content of phenolic compounds used in the brewing process, the effect of several malt types (light, dark, and mix), from two different brew houses, on the extracted phenolics was also studied. The findings indicate that the barley malt used, the malting process, specifically the kilning regimes and the roasting temperature (from 70°C to 230°C) may have a significant impact on the phenolic composition and as well on its potential to be used as a bioactive compound source. Phenolic rich extracts from light and mix BSG showed higher levels of total and individual phenolics compared to BSG dark. However, when the extracts were tested *in-vitro* against cholinesterase and carbohydrase activities, BSG dark showed a higher inhibitory potential. This suggests that other types of compounds, such as melanoidins, may be formed during the more severe roasting process of barley malt that results in BSG dark. This knowledge will undoubtedly be useful in the development of new applications for various types of BSG. The findings show that BSG light and dark extracts can be used as a low-cost, high-quality natural source of useful phenolic compounds, and bioactive compounds for human health and the food sector.

The anti-Alzheimer's and anti-diabetic activity of the BSG extracts may be linked to its total and individual phenolic content but as well to other types of compounds present in the extracts, which was determined by the sample's ability to inhibit the activity of acetylcholinesterase, butyrylcholinesterase, α-amylase, and α-glucosidase, respectively, by using *in-vitro* enzymatic assays. The results revealed that BSG dark extracts at approx. 4x lower concentrations, could reduce the activity of AChE and BChE compared with BSG light. Here the phenolic compounds having a low contribution to the inhibitory potential of the extracts, as evidenced by the higher phenolic levels in BSG light. Moreover, caffeic acid showed the highest inhibitory capacity among the tested individual phenolic compounds, of <26% inhibitions against AChE and <53% for BChE at 1mg/mL,

which represents a concentration of 300x and 80x higher concentrations compared to galantamine at IC_{50}. BSG dark extracts were shown to inhibit the activity of α-amylase, and α-glucosidase at very low TPC levels of approx. 1μgGAE/mL fraction. These findings imply that BSG's inhibitory effects are due not only to phenolic compounds but could be also due to non-phenolics, possibly melanoidins, which as are mostly found in dark malts, created by the higher times and temperatures in the roasting of dark malt. Melanoidins have been shown to present reducing capacities, form iron chelates, exert antioxidant effects, inhibit lipid peroxidation, prevent oxidative damage of DNA and immunomodulatory effects. Furthermore, there seems to be some clear correlations between the TPC levels and the measured AChE and BChE inhibitions of BSG flash extracts, whereas there was no obvious correlation for the α-amylase, and α-glucosidase. This may suggest that different molecules are responsible for the observed results, or this is more specific to individual phenolic compounds. Furthermore, decarboxylated diferulic acid was present in high abundance in both light and dark BSG extracts and presented significant correlation ($p < 0.01$) with the enzymatic assays AChE and BChE, and probably with α-amylase, and α-glucosidase, but this would require further investigations. Overall, good results have been obtained in this research project that could open the door to a whole new set of research ideas and projects.

Despite recent breakthroughs in recycling by-products, the recovery of antioxidants from BSG appears to be a particularly challenging yet stimulating subject of research. Indeed, these BSG extracts, which are primarily rich in phenolic acids and have antioxidant capabilities, could be employed as food additives or dietary supplements with potential health benefits.

7.2 Future perspectives

Because of the multidisciplinary character required to progress this field of work, there is still a significant need for additional research, of which a few proposals are provided below:

- How optimise the extraction parameters for each type of BSG substrate prior to extracting bioactive compounds, such as phenolic compounds. Moreover, to use the environmentally friendly extraction technologies, either MAE, UAE, pulsed electric field, alone or in combination with enzymatic assisted hydrolysis by using carbohydrases and/or proteinases, which may enhance the extraction efficacy. The later studies must be assessed based on the cost-benefit analysis of the process. Moreover, when considering how to apply a specific extraction method to obtained BSG phenolic extracts, we suggest that industrial scale to be taken into consideration. For example, using industrial scale microwave extraction reactions capable of handling <100 kg/batch, thus producing large amount of BSG phenolic extracts.
- Due to the nature of phenolic compounds in BSG (free, conjugated and bound to peptides or polysaccharides) the bioactive fractions are unlikely to be bioavailable thus by processing or by using a pretreatment prior incorporating in food products may increase the availability and hence the potential health benefits of these bioactive compounds.
- To confirm the inhibitory potential of bioactive compounds from BSG dark extracts and to evaluate their bioavailability in the body by conducting *in-vivo* investigations and human studies.
- To identify and pinpoint the natural compounds, a further purification using semi- or preparative HPLC would be necessary. Structural elucidation of the purified compounds can then be achieved with high resolution mass spectrometry and NMR spectroscopy.
- Beside the bioactive potential of BSG extracts, the remaining material can be exploited as a rich source of dietary fibre, bioactive proteins, and carbohydrates through a biorefinery approach for functional food ingredients or biofuel.

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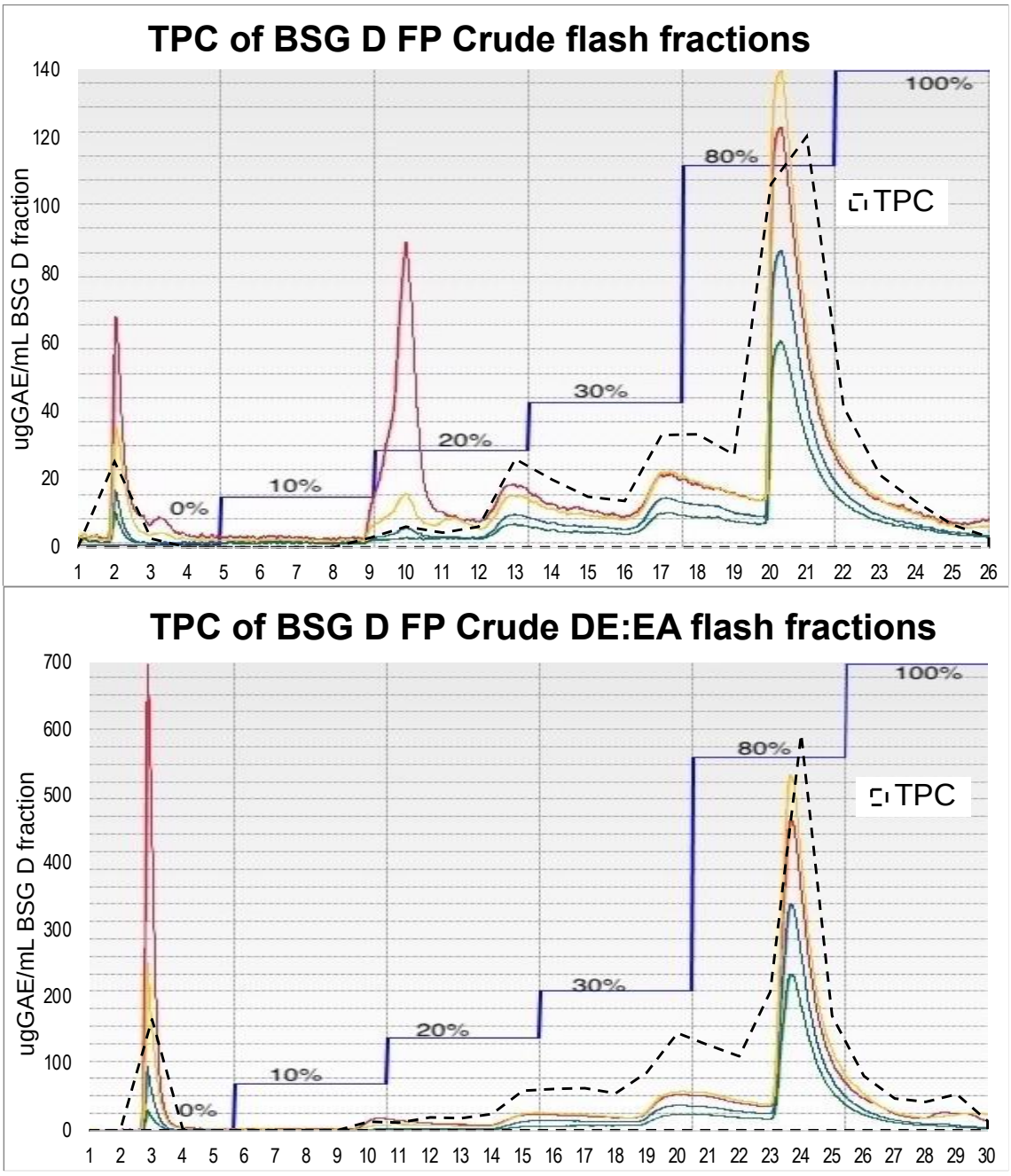
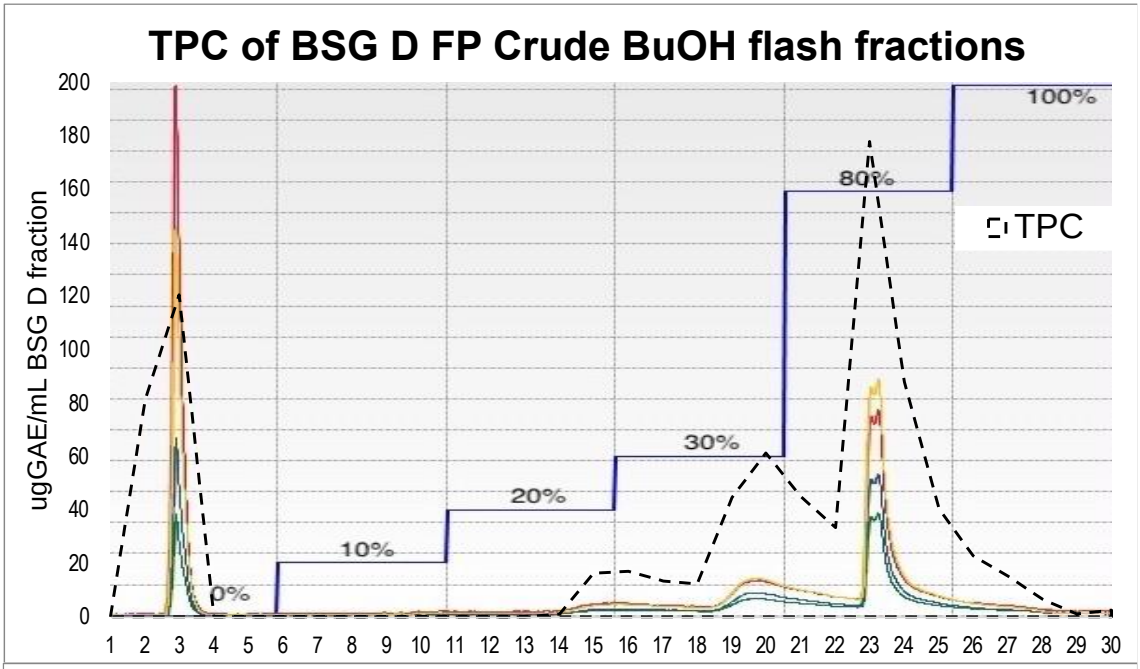
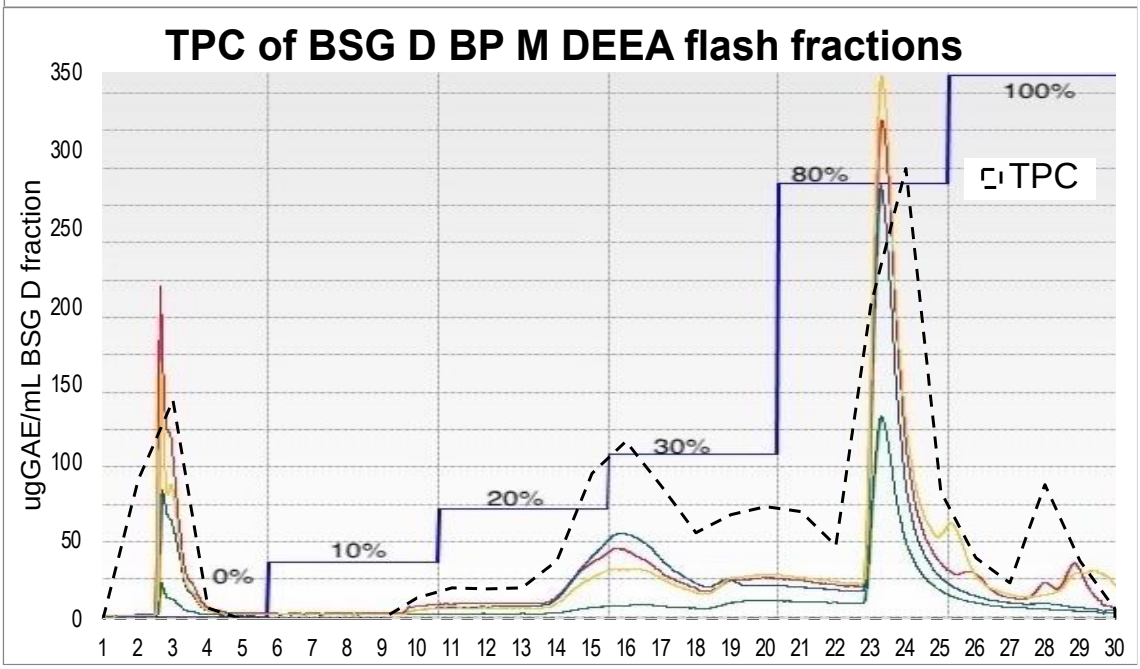
9. Appendices

Figure A6-1 TPC levels (ugGAE/mL fraction) in the BSG D FP and BP fractions with their individual flash chromatograms in the background




TPC of BSG D FP Crude BuOH flash fractions
ugGAE/mL BSG D fraction
0%
10%
20%
30%
80%
100%
TPC


TPC of BSG D BP M DEEA flash fractions
ugGAE/mL BSG D fraction
0%
10%
20%
30%
80%
100%
TPC

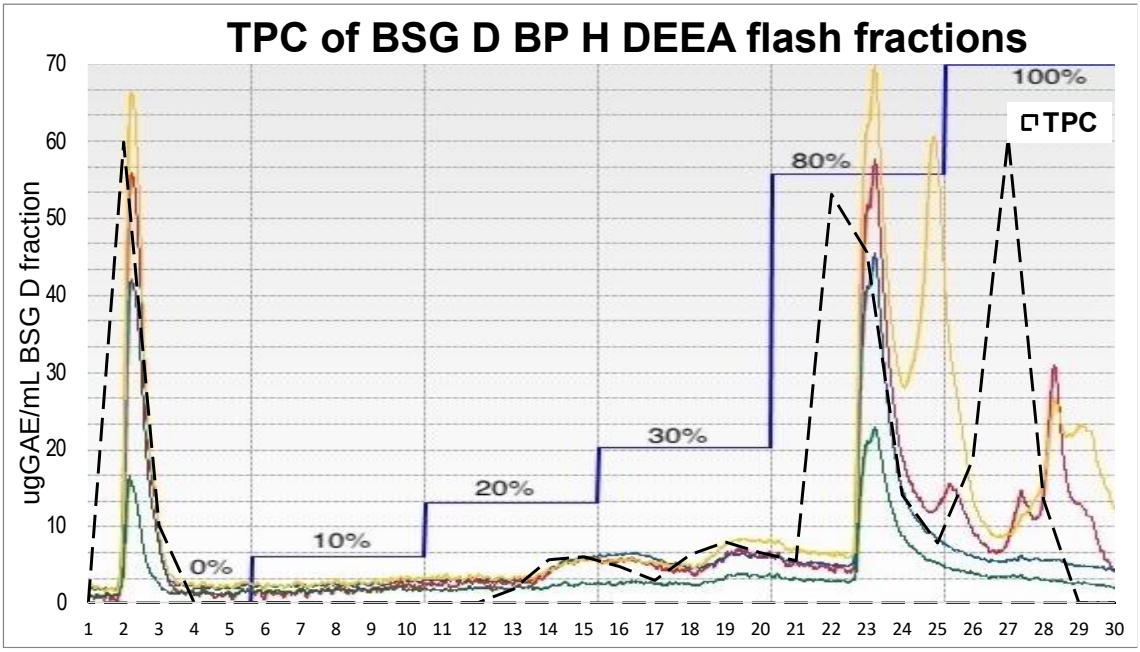


Figure A6-2 Inhibition of AChE activity by the generated BSG D H DE:EA extract

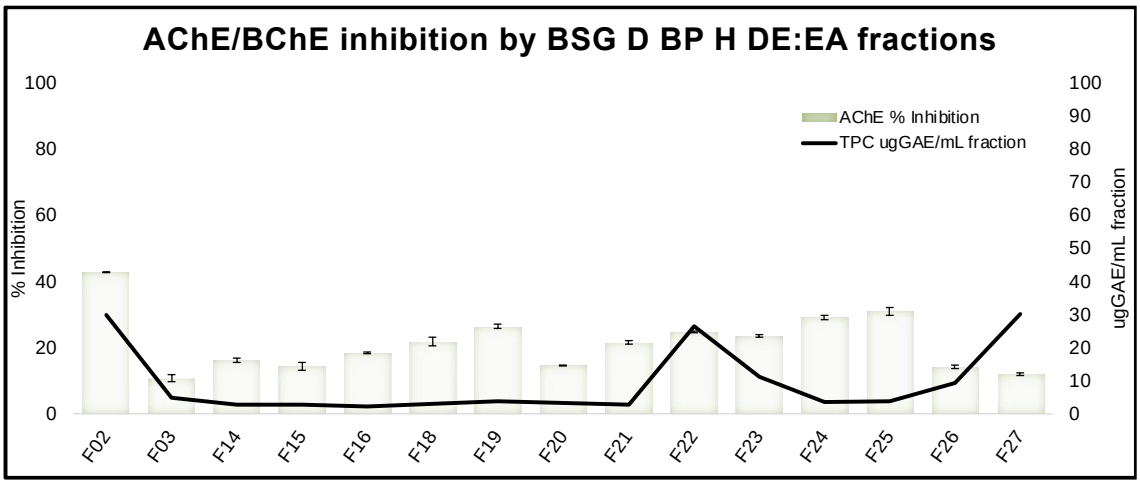
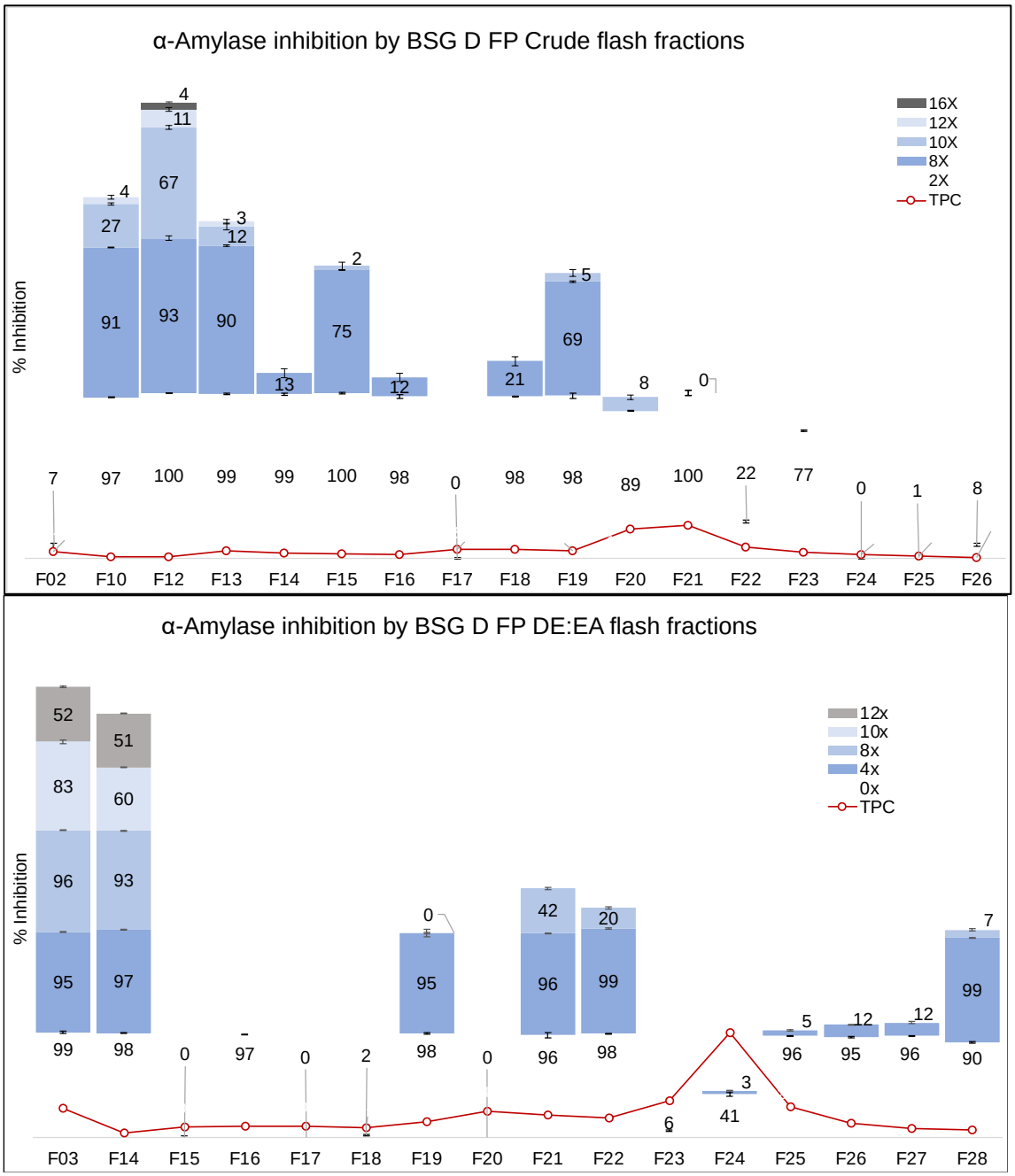
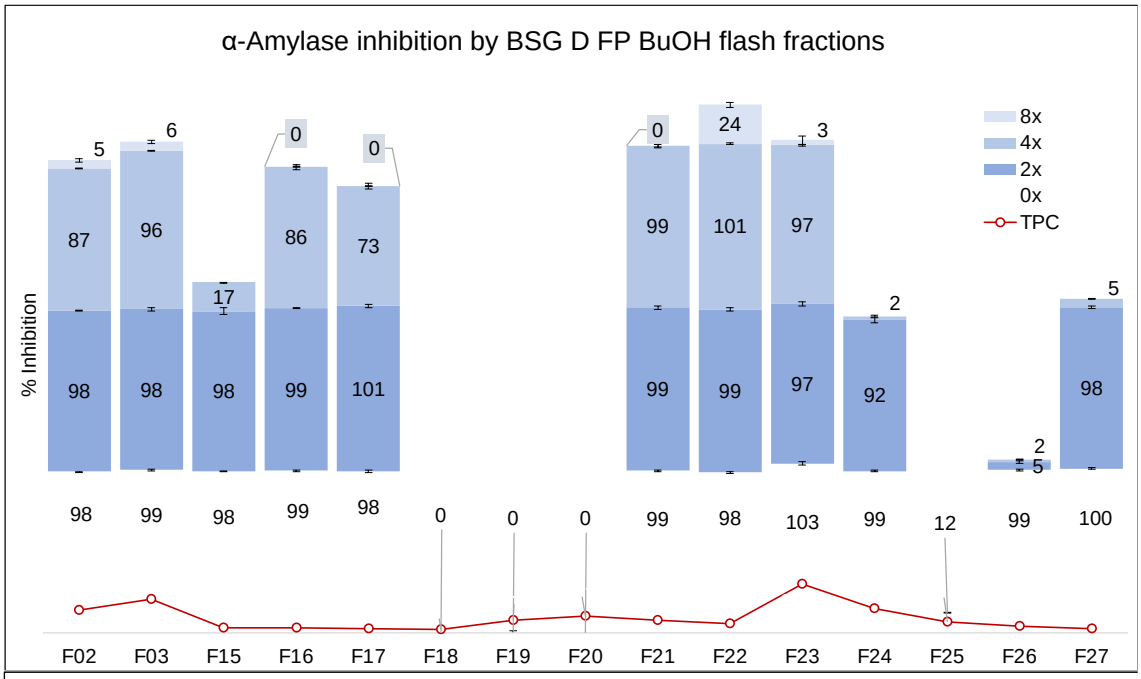
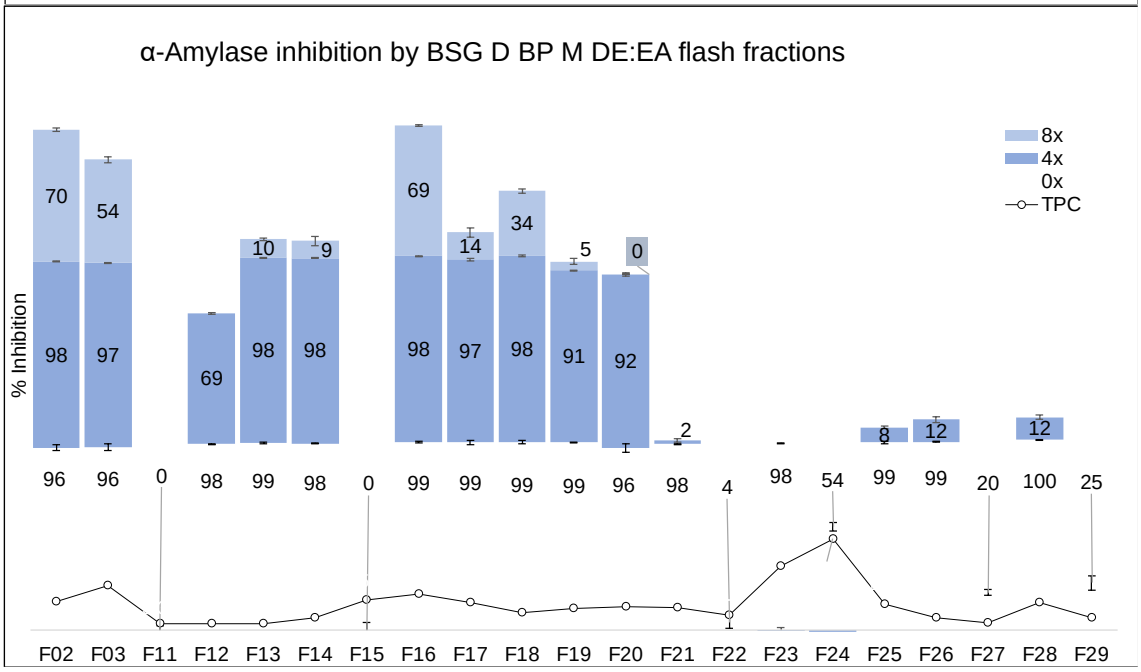


Figure A6-3 Inhibition of α-amylase activity by the generated flash fractions of the BSG D FP and BP extracts




α-Amylase inhibition by BSG D FP BuOH flash fractions
% Inhibition
8x
4x
2x
0x
TPC
F02 F03 F15 F16 F17 F18 F19 F20 F21 F22 F23 F24 F25 F26 F27


α-Amylase inhibition by BSG D BP M DE:EA flash fractions
% Inhibition
8x
4x
0x
TPC
F02 F03 F11 F12 F13 F14 F15 F16 F17 F18 F19 F20 F21 F22 F23 F24 F25 F26 F27 F28 F29

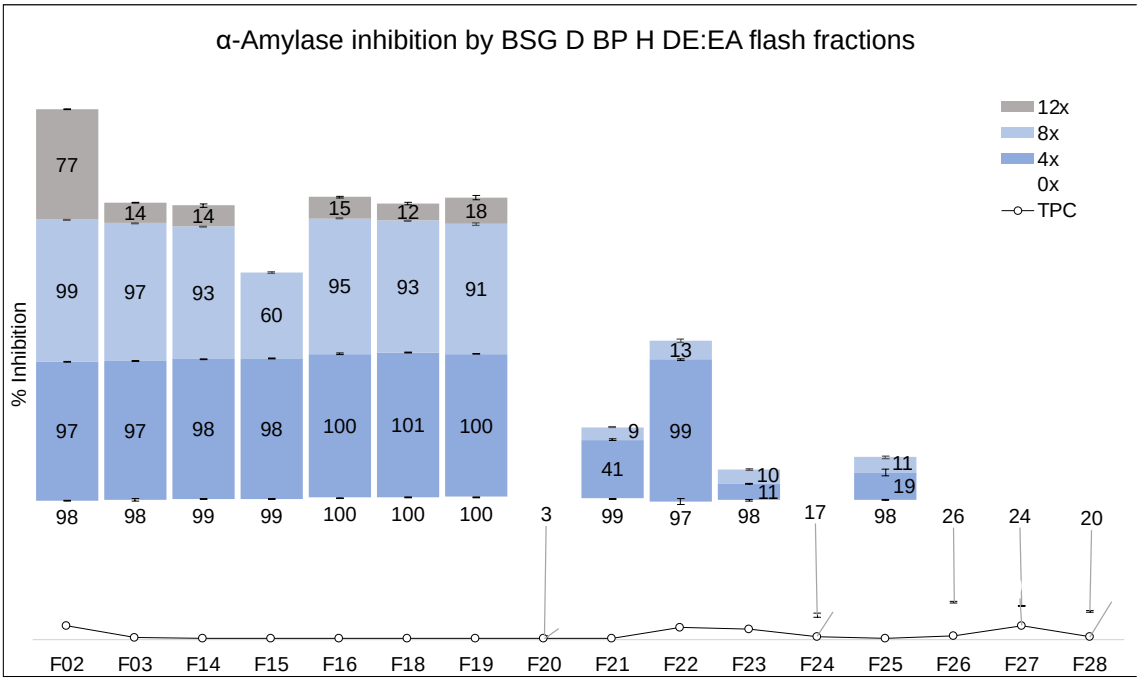
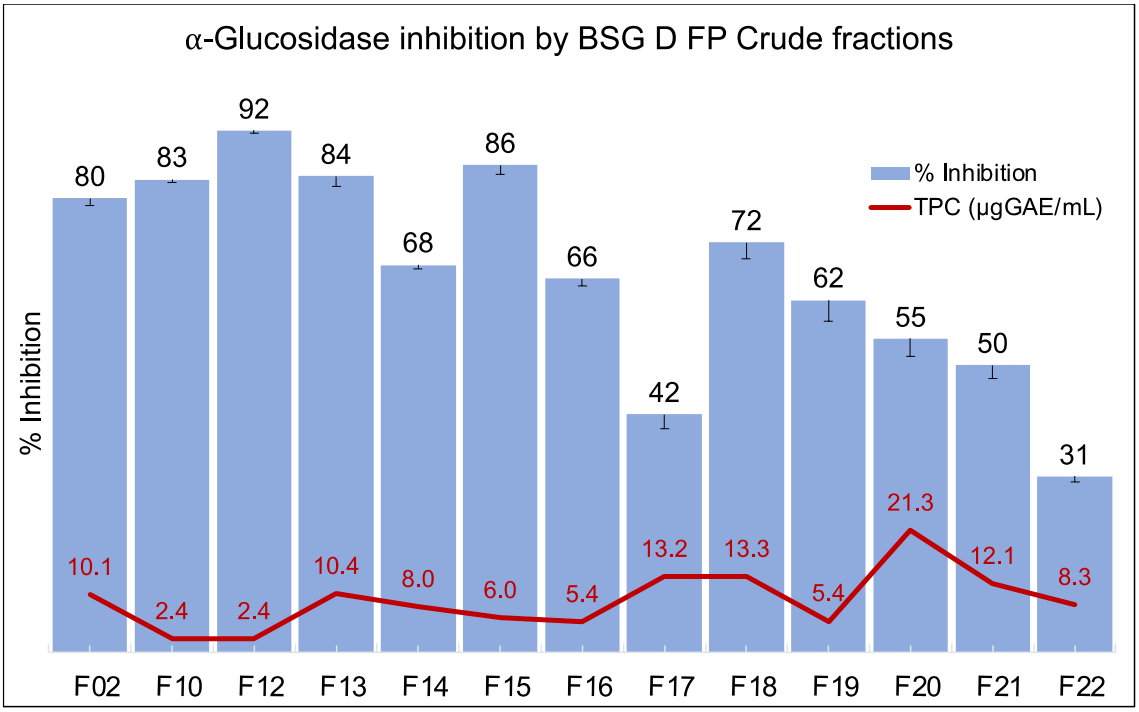
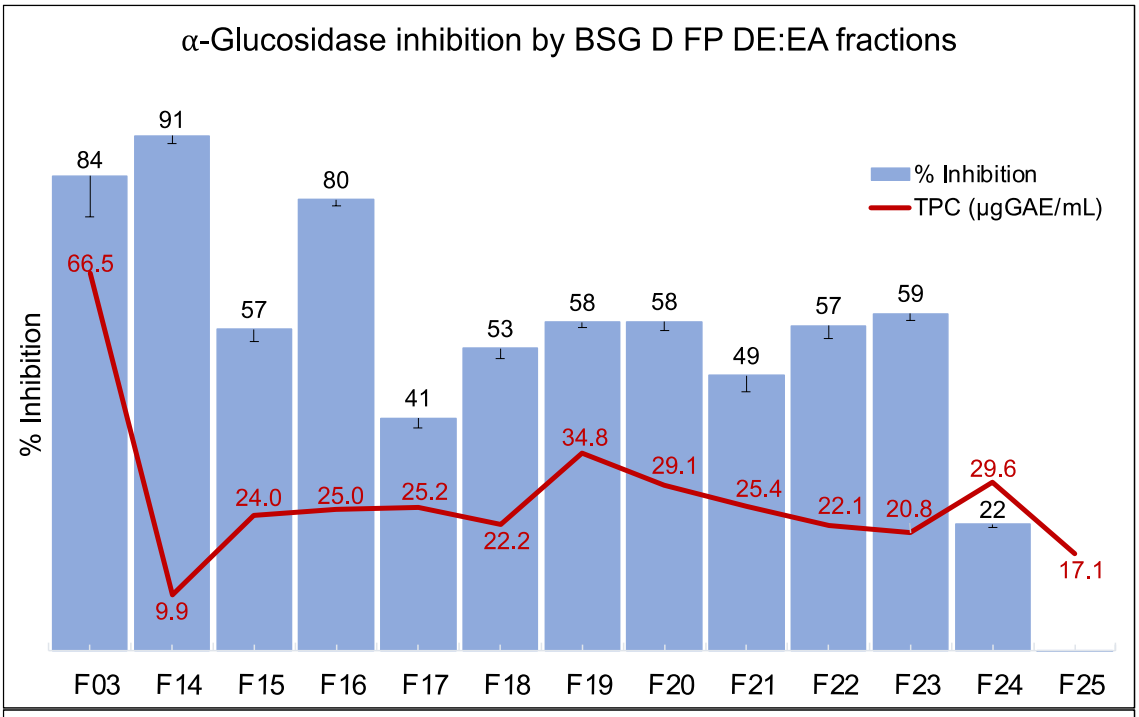
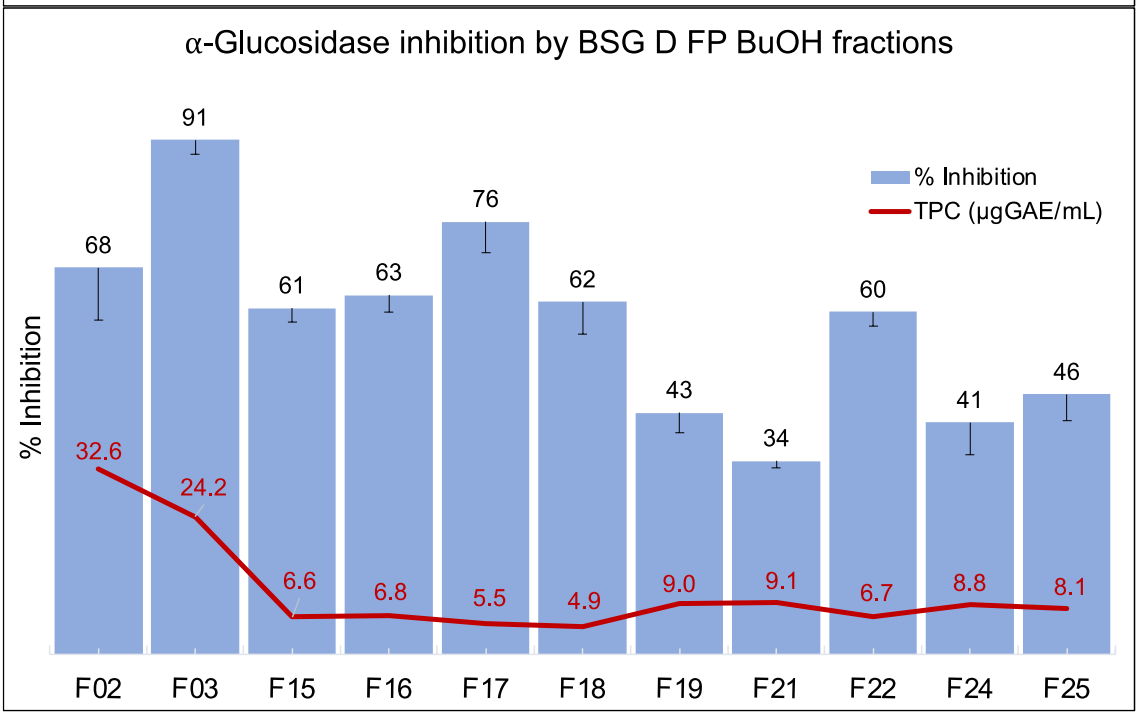
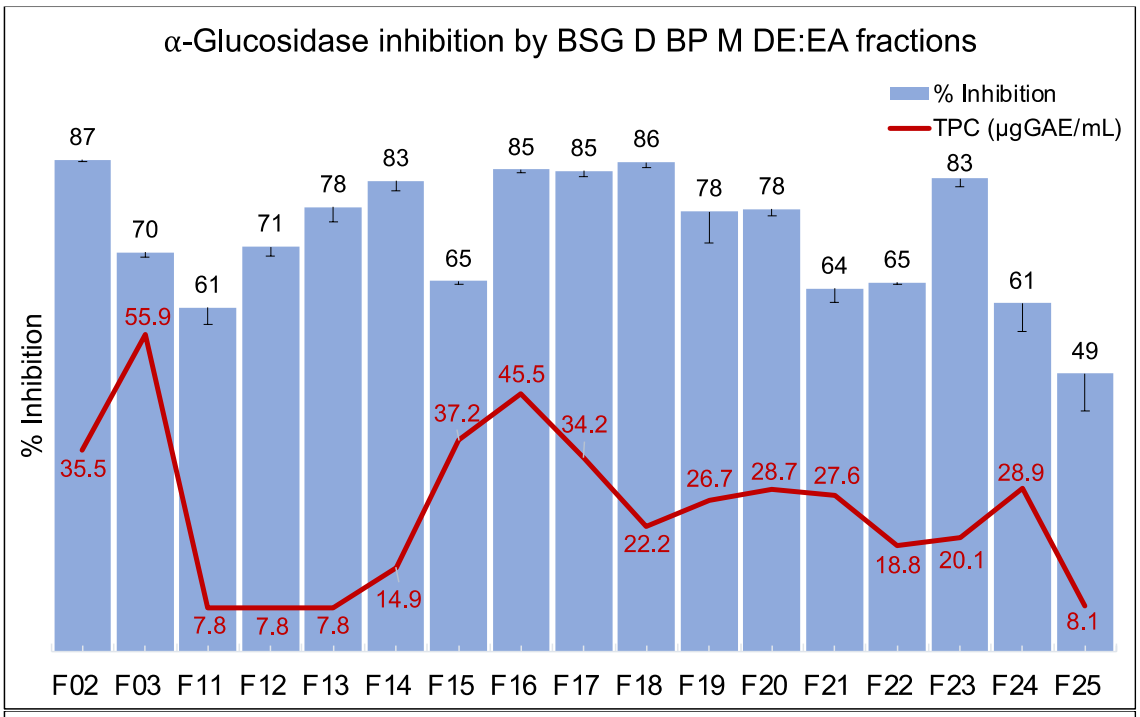


Figure A6-4 Inhibition of α-glucosidase activity by the generated flash fractions of the BSG D FP and BP extracts




α-Glucosidase inhibition by BSG D FP DE:EA fractions
% Inhibition
TPC (µgGAE/mL)
84
91
57
80
41
53
58
58
49
57
59
22
66.5
9.9
24.0
25.0
25.2
22.2
34.8
29.1
25.4
22.1
20.8
29.6
17.1
F03
F14
F15
F16
F17
F18
F19
F20
F21
F22
F23
F24
F25


α-Glucosidase inhibition by BSG D FP BuOH fractions
% Inhibition
TPC (µgGAE/mL)
68
91
61
63
76
62
43
34
60
41
46
32.6
24.2
6.6
6.8
5.5
4.9
9.0
9.1
6.7
8.8
8.1
F02
F03
F15
F16
F17
F18
F19
F21
F22
F24
F25


α-Glucosidase inhibition by BSG D BP M DE:EA fractions
% Inhibition
TPC (µgGAE/mL)
% Inhibition
87
70
61
71
78
83
65
85
85
86
78
78
64
65
83
61
49
35.5
55.9
7.8
7.8
7.8
14.9
37.2
45.5
34.2
22.2
26.7
28.7
27.6
18.8
20.1
28.9
8.1
F02 F03 F11 F12 F13 F14 F15 F16 F17 F18 F19 F20 F21 F22 F23 F24 F25

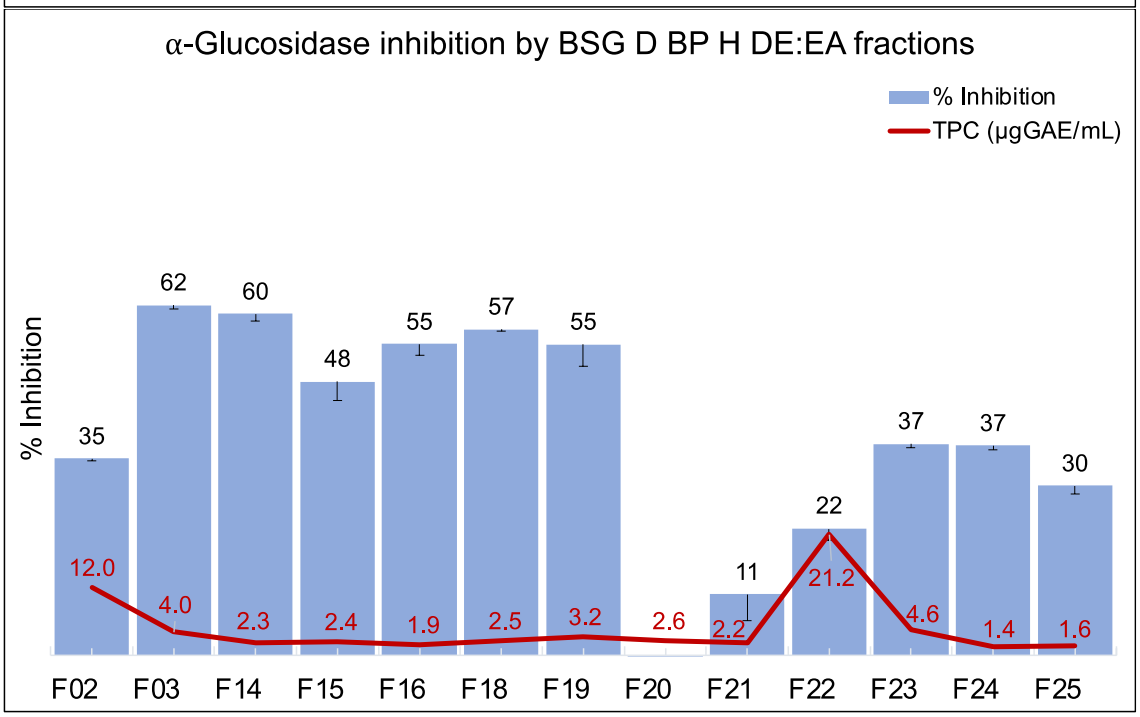
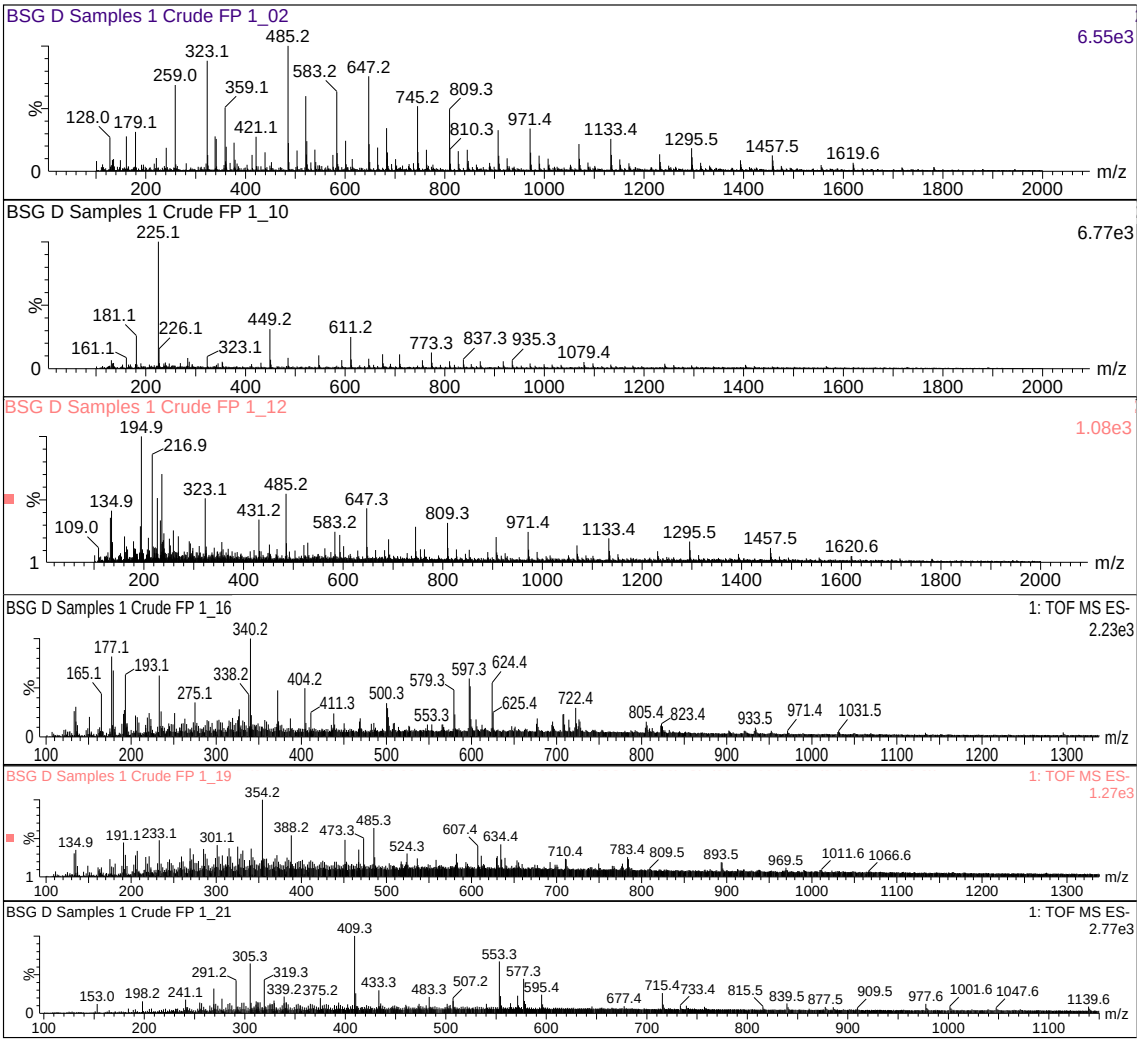
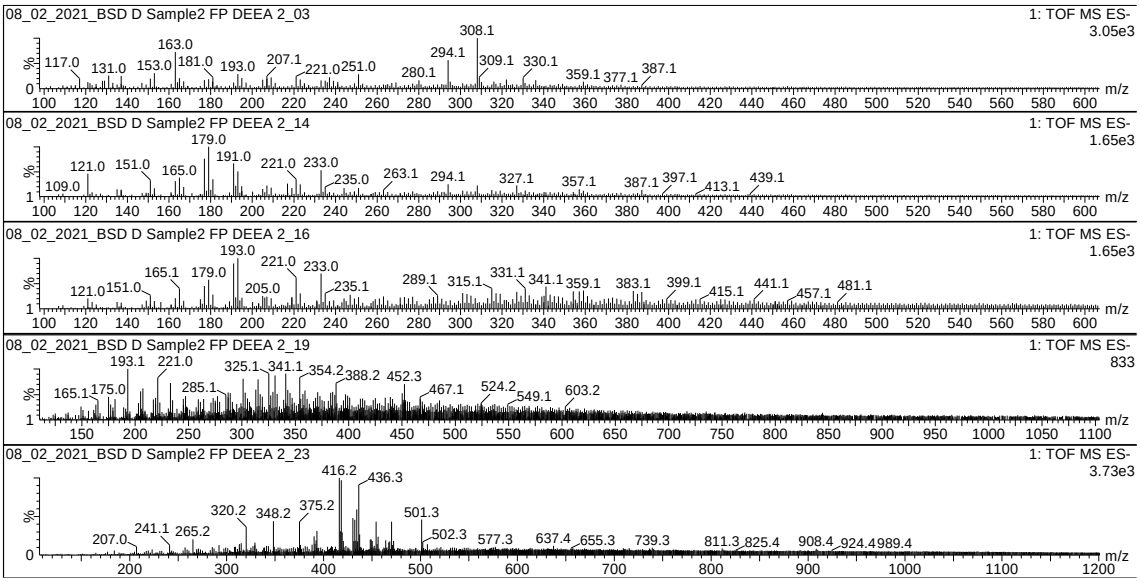

α-Glucosidase inhibition by BSG D BP H DE:EA fractions
% Inhibition
TPC (µgGAE/mL)
% Inhibition
35
62
60
48
55
57
55
11
22
37
37
30
12.0
4.0
2.3
2.4
1.9
2.5
3.2
2.6
2.2
21.2
4.6
1.4
1.6
F02 F03 F14 F15 F16 F18 F19 F20 F21 F22 F23 F24 F25

Figure A6-5 MS/MS spectrum generated by HPLC-Q-Tof-MS of the flash fractions of the BSG D FP and BP extracts

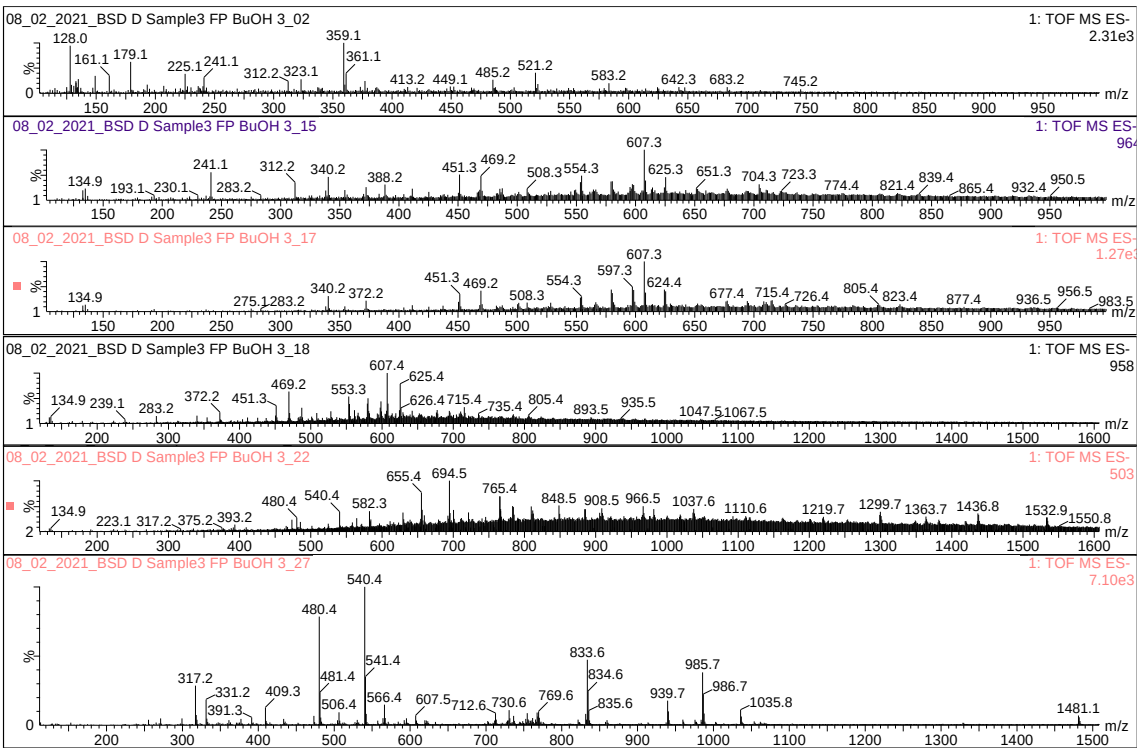
- BSG D FP Crude fractions F02, F10, F12, F16, F19, F21



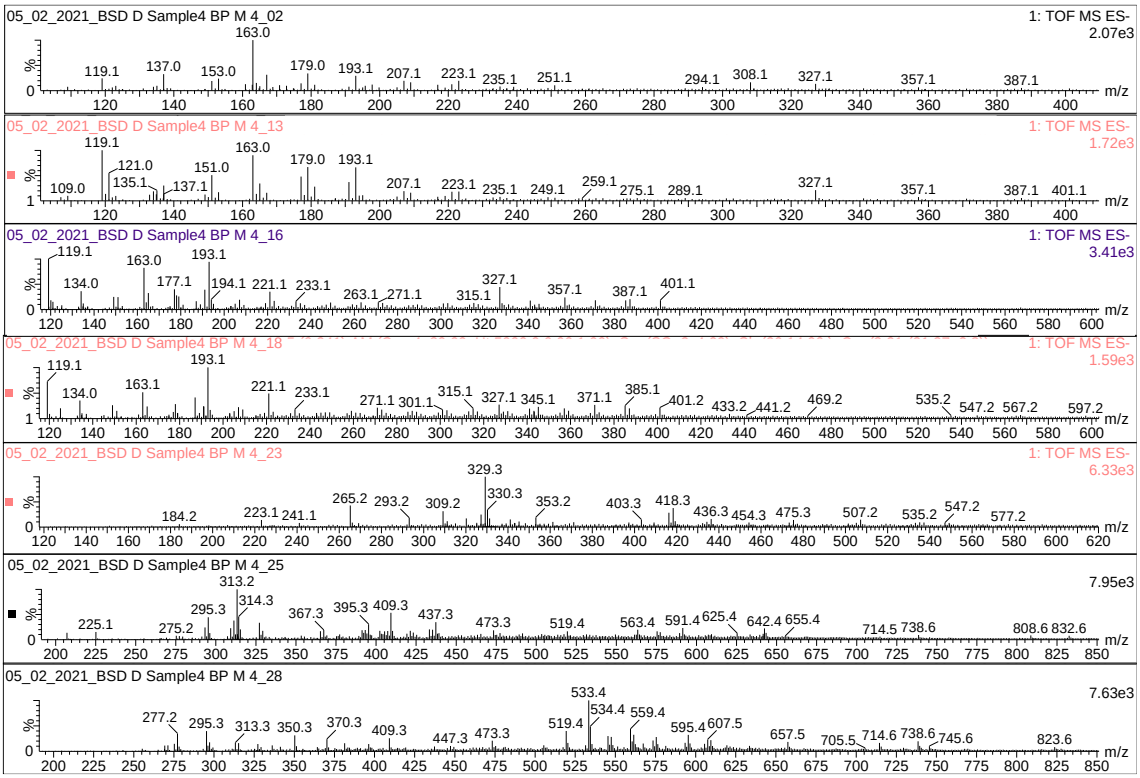
- BSG D FP DE:EA fractions F03, F14, F16, F19, F23



- BSG D FP BuOH fractions F02, F15, F17, F18, F22, F27



- BSG D BP M DE:EA fractions F02, F13, F16, F18, F23, F25, F28



- BSG D BP H DE:EA fractions F02, F03, F14, F16, F28, F22, F24

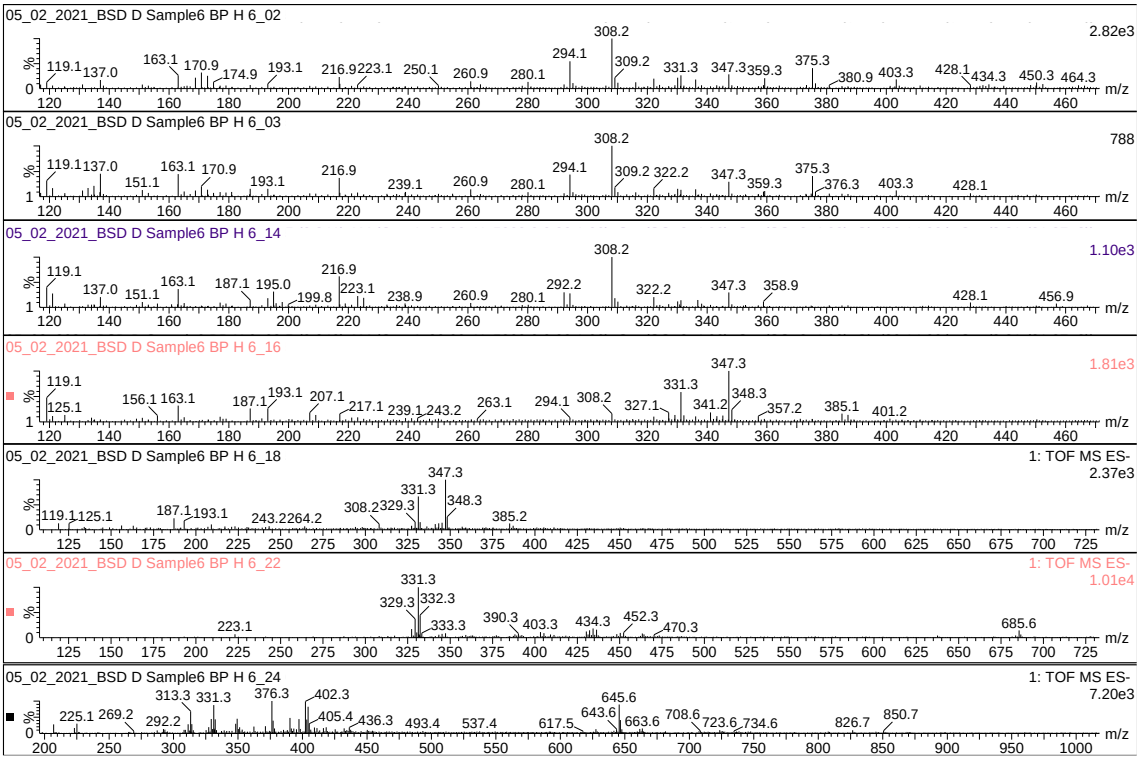
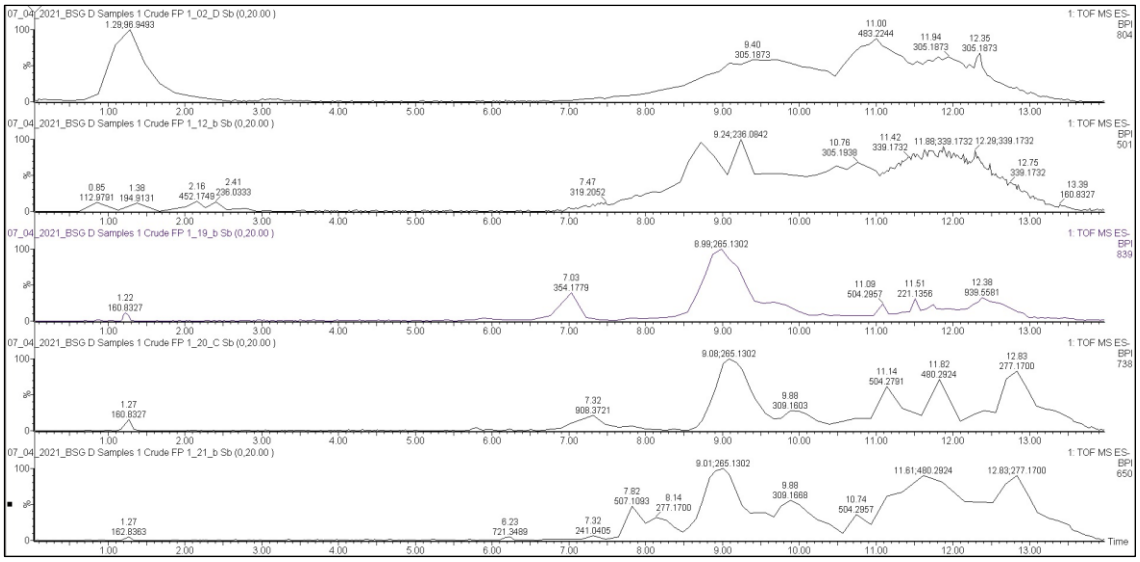
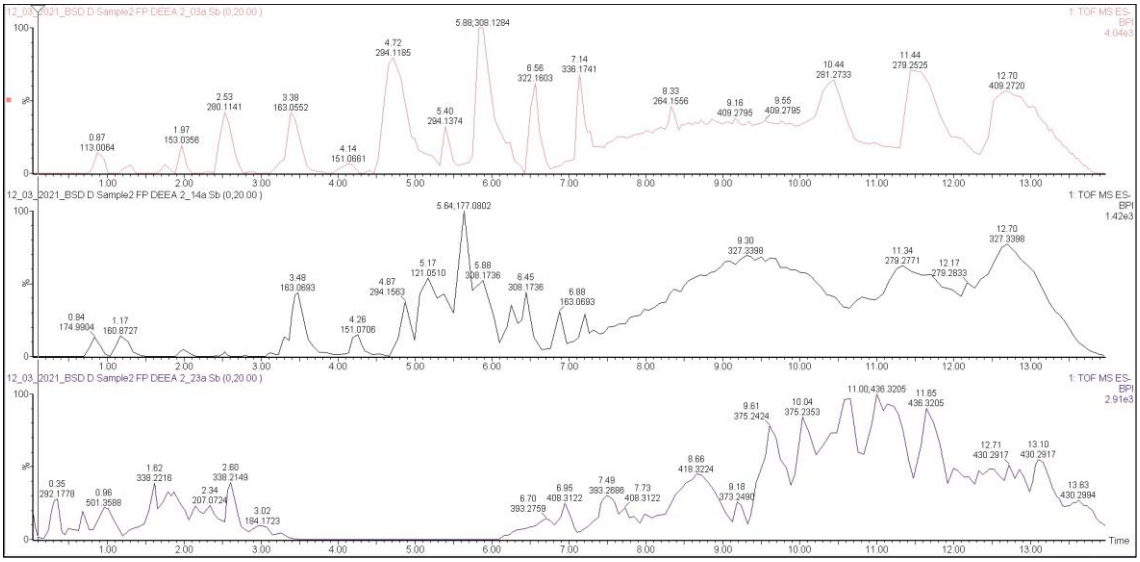


Figure A6-6 ESI- MS chromatogram generated by HPLC-Q-Tof-MS of the flash fractions of the BSG D FP and BP extracts

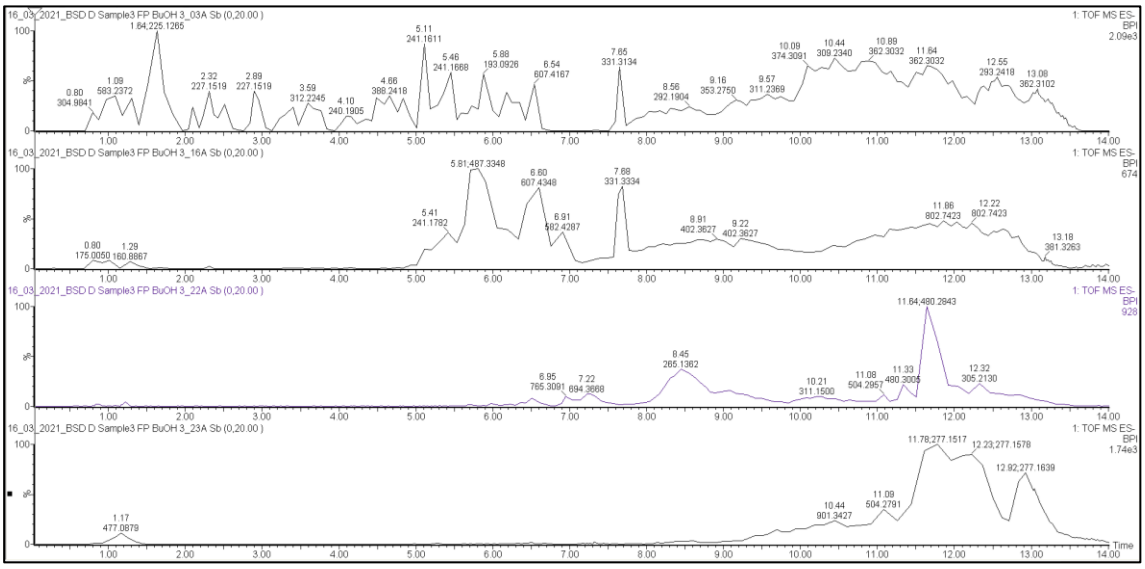
- BSG D FP Crude fractions F02, F12, F19, F22, F21



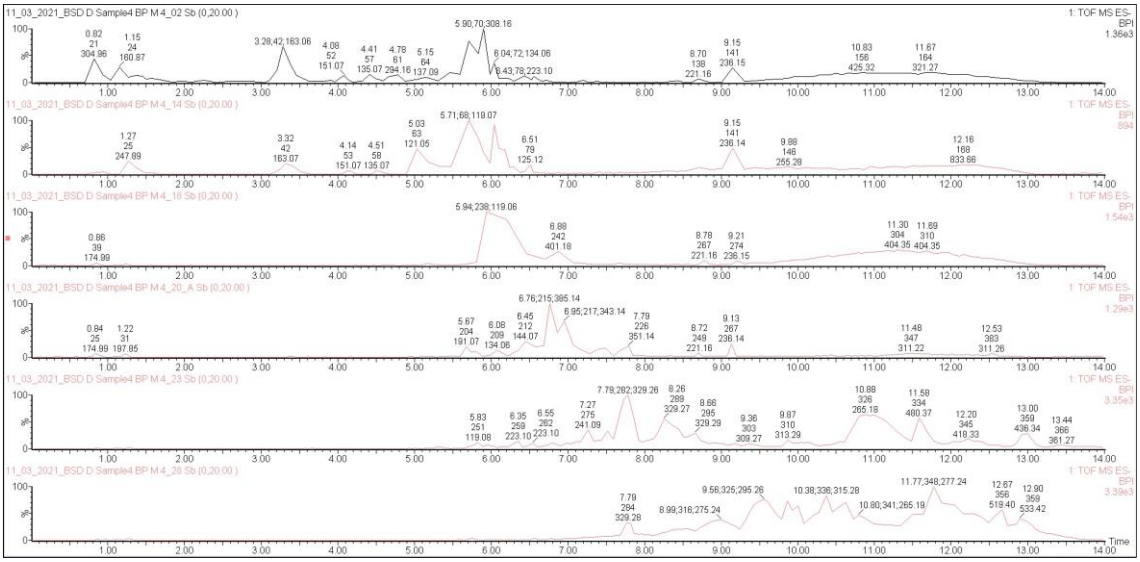
- BSG D FP DE:EA fractions F03, F14, F23



- BSG D FP BuOH fractions F03, F16, F22, F23



- BSG D BP M DE:EA fractions F02, F14, F18, F20, F23, F28



- BSG D BP H DE:EA fractions F02, F14, F19, F23

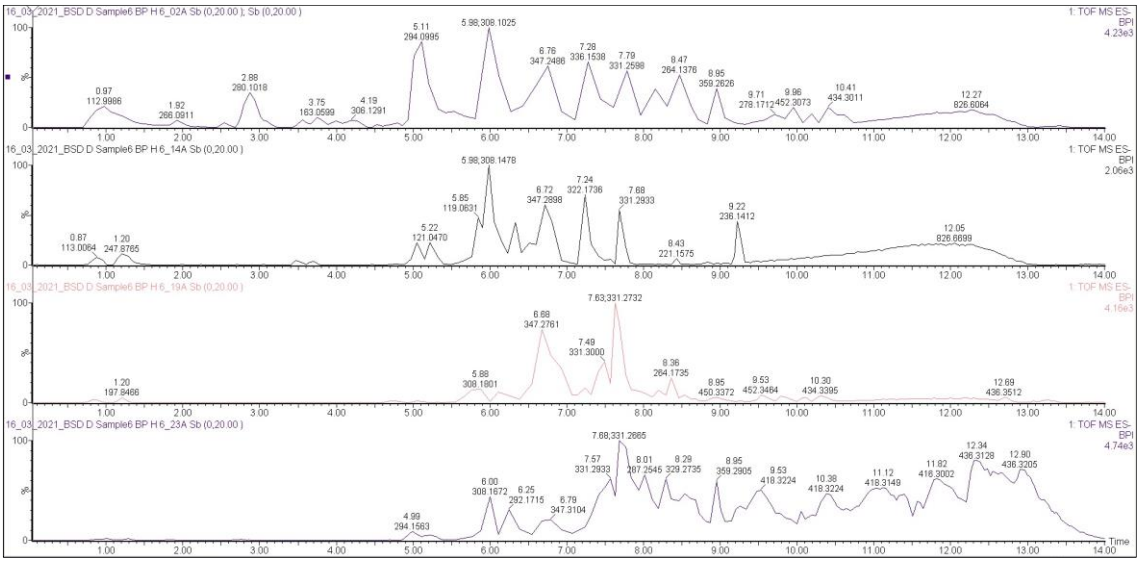


Table A6-7 MS/MS fragmentation of the peaks in the figure 6.6-5 chromatograms generated by HPLC-Q-Tof-MS, of the flash fractions of the BSG D FP and BP extracts

Peak No.	Sample	RT (min.)	Observed $[M-H]^-$(*m/z*)	Calculated $[M-H]^-$(*m/z*)	MS/MS fragment ions (*m/z)* calculated
Fractions of BSG D BP M DEEA					
1	F02, F20	0.8	174.98	174.95	118.9729(100%); 146.9693 (54.16%);
2	F02	0.82	304.96	304.91	118.9730 (17.00%); 146.9691/147 (100%); 174.9642 (18.99%);
3	F02	1.15	160.87	160.85	134.0825 (1%); 160.8474 (100%);
4	F20	1.22	197.85	197.82	162.8746 (100.0%);
5	F02	1.33	247.87	247.94	167.8921 (100%); 185.8969 (13.15%); 247.8514 (15.99%);
6	F02	1.85	167.05	167.04	122/123.0495 (100%); 139.0453 (16.77%); 149.0485; 167.0351 (11.66%);
7	F02	3.28	163.06	163.05	119.0588 (3.11%); 135.0533 (4.04%); 145.0371 (2.27%); 163.0468 (100%);
8	F02	4.43	135.07	135.05	107.0588 (4.3%);135.0533 (100%);
9	F02	5.24	179.06	179.04	107.0626 (9.33%); 135.0533 (100.0%);
10	F20	5.67	191.07	191.04	108.0272 (100.0%);119.0558 (16.65%); 121.0336 (10.60%); 163.0456 (61.86%); 191.0400 (79.33%);
11	F02, 20, 23	5.71	119.07	119.06	119.0581 (100.0%); 93.0432 (8.72%)
12	F20	6.08	134.06	134.04	53.0514 (5.44%); 93.0398 (5.44%); 106.0585 (5.49%); 123.0244 (5.43%); 135.0524 (100.0%);
13	F02, F23	6.43	223.10	223.07	79.0632 (26.6%); 96.9655 (100%); 122.9806 (13.5%); 151.0136 (9.07%); 223.0720 (15.44%);
14	F20	6.45	144.07	144.05	126.0618; 144.0702;
15	F20	6.76	385.14	385.09	(31.99%); 239.0775 (41.08%); 253.0938 (29.34%); 267.0717 (93.47%); 281.0868 (100%); 309.0819 (12.15%); 325.0759 (57.95%);

16	F20	6.92	371.17	371.11	109.0355 (10.78%); 145.0358 (100%); 159.0530 (6.56%); 267.0716 (23.76%); 282.0954 (19.15%); 283.0975 (5.11%);
17	F20	6.95	343.14	343.09	134.0441 (100%); 149.0275 (10.39%); 178.0333 (82.60%); 193.0540 (23.54%); 285.0491 (2514%); 313.0378 (40.02%);
18	F02, F23	6.99	193.09	193.06	134.0428 (100%); 149.0671 (7.70%); 179.0340 (13.76%);
19	F20	7.5	327.27	327.22	99.0867 (27.37%); 139.1187 (76.25%); 211.1397 (100%); 229.1495 (53.64%); 329.2620 (14.96%);
20	F23	7.79	329.26	329.28	99.1007 (19.67%); 127.1364 (21.49%); 139.1380 (52.10%); 171.1320/172 (100%); 183.1705 (14.40%); 211.1689/212 (46.24%); 229.1826 (14.97%); 329.2843 (7.64%).
21	F20	7.79	351.14	351.09	249.0607 (100.0%); 277.0561 (21.68%); 293.0503 (12.44%); 307/308.0727 (36.61%); 321.0432 (13.21%); 335/336.0691(19.37%);
22	F23	8.26	329.27	329.22	129.1207 (20.33); 169.1592 (24.40%); 181.1614 (15.96%); 199.1751 (100%); 201.1556 (53.15%);
23	F02, 20	8.7	221.16	223.04	74.9960 (39.82); 93.0032 (5.65%); 149.0147 (35.03%); 206.9983 (41.01%); 223.0374(100.0%);
24	F02, F20	9.15	236.15	236.10	136.0580 (1.74%); 148.0550 (14.76%); 177.0971 (100.0%); 192.1234 (33.45%);218.1025 (10.71%); 236.1276 (3.83%);
25	F23	9.36	309.27	309.22	87.0668 (16.51%); 107.1170 (85.96%); 119.1181 (14.82%); 127.12.94 (10.35%); 152.1264 (15.72%); 201.1656 (100%); 223.2236 (40.77%);
26	F20	10.41	434.32	434.25	79.9637 (52.61%); 106.9873 (29.41%); 138.0278 (52.41%); 153.0003 (9.20%); 434.2524 (100.0%);
27	F23	10.92	504.38	504.30	78.9790 (13.58%); 153.0310; 168.0786 (6.12); 224.1136 (8.33%); 279.2868/280 (100%);
28	F23	11.58	480.37	480.29	78.9649 (12.35%); 153.0011 (3.59%); 168.0494 (4.91%); 224.0746 (5.69%); 255.2368/256 (100%);
29	F23	12.01	506.41	506.33	78.9649 (8.63%); 153.00001 (3.02%);168.0468 (4.11); 224.0766 (6.24%); 281.2525/282/283 (100%);
30	F20	12.53	311.26	311.18	149.1024 (100.0%); 150.10.45 (12.56%); 183.0179 (33.49); 311.1761 (35.2%)
31	F23	13	436.34	436.28	79.9629/80 (21.88%); 106.9863 (27.48%); 138.0280 (35.86%); 184.1402 (4.16%); 436.2762/437/438 (100%);
32	F23	13.44	361.27	361.21	79.9631/80.9714/81 (96.53/100%); 279.2405 (13.69%); 361.2092/362/363 (41.78%);
Fractions of BSG D BP H DEEA					
1	F23	0.84	174.99	174.96	83.9491 (5.89%); 86.9991 (11.67%); 118.9985 (100%); 130.9995 (19.39%); 133.0039 (17.61%); 146.9998 (81.87%); 149.0061 (9.07%);
2	F02	0.97	113.00	112.98	113.0025 + ms/ms 68 => 180/248/316/384/452 etc - 1676
3	F02	1.13	216.92	216.94	96.9646 (100%); 98.0604 (5.36%); 136.9603 (0.46%); 152.0583 (3.12%);
4	F23	1.27	197.84	216.94	160.8818 (27.27%); 162.8792 (100%); 164.8739 (31.12%);
5	F02	2.88	280.10	280.09	138.0287/139/140 (100%); 79.9631/80 (24.3%); 106.9863 (19.38%); 128.1139 (3.61%); 172.1042 (3.77%); 280.0919 (11.17%)

6	F02	3.75	163.06	163.05	119.0564; 134/135.0518; 107.0563
7	F02	5.11	294.10	294.11	79.9626 (45.48%); 106/107.9879 (44.22%); 111.0864 (16.5%); 138.0278 (100%); 186.1183 (6.26%); 294.1057/295/296 (32.21%);
8	F02, F23	5.92	308.17	308.12	79.9790/80 (60.95%); 107.0077 (64.75%); 125.1280 (20.74%); 138.0563/139 (100%); 200.1750 (7.19%); 308.1832/309/310 (47.54%);
9	F02, F23	6.35	292.13	292.13	79.9628/80 (92.22%); 106.9865 (67.43%); 138.0285 (100%); 156.1459 (13.56%); 194.1404 (10.66%); 292.1276/293 (65.88%);
10	F02	6.44	364.19	364.15	79.9632 (33.16%); 106.9858 (43.71%); 125.1023 (7.93%); 138.0290 (100%); 304.1644 (9.81%); 306.1403 (9.33%); 346.1473 (7.85%).
11	F02	6.64	322.17	322.12	79.9630/80 (64.28%); 106.9861 (69.8%); 138.0281/139 (100%) 183.1087 (5.44%); 214.1504 (6.72%); 322.1368/323 (60.37%);
12	F02	6.76	347.25	347.25	127.1193 (32.59%); 143.1132 (14.72%); 155.1147 (22.09%); 171.1084 (44.64%); 201.1182 (75.22%); 215.1336 (42.07%); 311.2276 (15.31%); 329.2365/330 (52.30%); 347.2487 (100%);
13	F23	6.99	347.27	347.25	113.1046 (10.02%); 125.1040 (10.53%); 127.1186 (17.34%); 143.1154 (24.37%); 153.0998 (7.64%); 155.1143 (16.65%); 157.1324 (17.57%);171.1099/172/173 (100%); 185.1249 (17.73%); 201.1207 (45.76%); 329.2414 (20.09%); 347.2522/348 (51.04%);
14	F02	7.28	336.15	336.15	79.9631/80/81 (65.90%); 106.9867 (73.07%); 108.9823 (7.71%); 138.0294 (100%); 153.1340 (14.85%); 197.1222 (10.41%); 228.1631 (6.95%); 336.1518/337/338 (77.76%);
15	F02	7.34	250.11	250.12	79.9634/80/81 (100%); 106.9871 (47.94%); 138.0289 (41.36%); 142.1298 (21.66%); 250.1188/251 (40.49%).
16	F23	7.67	331.27	331.28	79.9634/80/81 (100%); 106.9871 (47.94%); 138.0289 (41.36%); 142.1298 (21.66%); 250.1188/251 (40.49%).
17	F23	7.73	327.27	327.22	79.9632 (33.16%); 106.9858 (43.71%); 125.1023 (7.93%); 138.0290 (100%); 304.1644 (9.81%); 306.1403 (9.33%); 346.1473 (7.85%).
18	F02	7.79	331.26	331.21	127.1307 (41.65%); 143.1268 (8.91%); 157.1439 (33.35%); 171.1243 (25.14%); 187.1579 (7.18%); 201.1375 (11.50%); 295.2608 (17.21%); 313.2727/314 (44.92%); 329.2689/330 (37.18%); 331.2835/332 (100%);
19	F02	7.91	350.17	350.12	79.9712/80 (50.15%); 106.9972 (63.36%); 138.0423/139 (100%); 167.1662 (14.07%); 211.1603 (16.81%); 242.2054 (7.02%); 350.2022/351 (76.15%).
20	F02	8.15	373.28	373.22	79.9716 (11.03%); 127.1299 (13.79%); 155.1275 (10.16%); 171.1251 (21.78%); 181.1466 (9.22%); 199.1589/201.1396 (27.78/13.60); 311.2579 (11.97%); 329.2682 (49.72%); 373.2975 (100%);
21	F23	8.33	264.17	264.30	79.9628/80 (92.22%); 106.9865 (67.43%); 138.0285 (100%); 156.1459 (13.56%); 194.1404 (10.66%); 292.1276/293 (65.88%);
22	F02	8.47	264.14	264.30	79.9709.80/81 (100%); 106.9967 (61.56%); 138.0427 (61.56%); 156.1597 (32.92%); 264.1577/265/266 (82.14%)

23	F02	8.95	359.26	359.24	125.1013 (7.31%); 127.1174 (35.85%); 143.1134 (7.21%); 155.1114 (15.46%); 157.1278 (30.06%); 171.1063 (21.35%); 187.1404 (7.34%); 201.1165 (9.28%); 295.2302 (17.30%); 313.2402 (48.90%); 329/330/331.2502/332(30.64/-/100/-%);
24	F02	9.22	236.12	236.11	148.0590 (18.55%); 177.0979/178 (100%); 192.1204/193 (41.80%); 217.0932/218 (9.99%);
25	F02, F23	9.96	452.31	452.27	79.9635/80 (15.36%); 106.9865 (23.80%); 138.0290 (30.43%); 452.2686/453/454 (100%).
26	F02	10.41	434.30	434.26	79.9629/80 (36.4%); 106.9868 (29.28%); 138.0288 (47.27%); 434.2584/435/436 (100%).
27	F23	11.63	826.65	826.53	255.2559 (9.01%);279.2590 (14.11%); 313.2684 (9.86%); 329/330/331.2776/332/333/334 (100%);
BSG D FP DEEA					
1	F03	1.34	128.05	128.03	85.0358 (15.90%); 128.0412/129/131 (100%);
2	F03	1.81	266.10	266.07	79.9634 (29.45%); 106.9866 (10.88%); 124.0127 (8.42%); 138.0288/139/140 (100%);
3	F03	1.9	128.05	128.03	87.0506 (100%); 113.0305 (7.74%); 131.0406 (14.58%);
4	F03	2.55	280.11	280.09	79.9632/80 (34.65%); 97.0720 (7.42%); 106.9867 (22.99%); 138.0284/139/140 (100%); 280.0914 (9.11%);
5	F03	2.91	193.08	193.06	124/125.0214 (8.47%); 137.0322 (5.58%); 150.0401 (10.68%); 162/162/163.0462/164 (100%); 175.0484 (10.63%); 193.0583 (10.50%);
6	F03	3.36	163.05	163.04	107.0562 (7.02%); 135.0507 (6.52%); 161/162/163.0439/164 (100%);
7	F03	4.02	207.10	207.07	107/108/109 (11.77%); 124.0227 (11.92%); 143.0567 (18.52%); 161.0676 (24.69%); 174.0385/175 (100%); 188.0547/189.0616/190 (75.79%); 207.0356 (31.01%);
8	F03	4.7	294.11	294.11	79.9628/80 (56.50%); 106.9861 (49.92%); 111.0872 (19.73%); 138.0272/139/140 (100%); 142.1298 (5.04%); 186.1196 (7.25%); 294.1057 (27.2%);
9	F03	5.86	308.12	308.23	79.9893/80 (75.05%); 107.0217 (74.78%); 125.1464 (26.74%); 138.0714/139/140 (100%); 200.2045 (8.92%); 308.2268/309 (56.05%);
10	F03	6.53	322.15	322.25	79.9922/81 (64.06%); 107.0250 (70.77%); 138.0783/139/140 (100%); 183.1753 (6.37%); 214.2284 (7.8%); 322.2533/323 (57.79%);
11	F03	7.16	336.17	336.17	79.9672/80 (67.27%); 106.9916 (73.82%); 138.0352/139/140 (100%); 153.1425 (19.81%); 197.1344 (15.84%); 228.1781 (8.04%); 336.1689/337/338 (63.03%);
12	F03	8.35	264.15	264.13	79.9630/80/81 (100%); 106.9863 (54.95%); 138.0285 (55.58%); 156.1449 (29.47%); 264.1321/265 (56.32%);
13	F03	10.54	299.28	299.26	225.2291 (3.22%); 249.2277 (2.66%); 251/252/253.2576/254/255 (100%); 281.2530 (2.29%); 299.2636/300 (7.52%);
14	F03	11.49	279.25	279.24	79.9635 (2.1%); 96.9641 (1.17%); 261.2289 (3.9%); 279.2377/280/281/282 (100%)
15	F03	11.61	409.27	409.21	78.9648 (33.50%); 96.9753 (6.93%); 150.9860 (3.37%); 153.0013/154/155 (100%); 171.0125 (2.08%); 255.2383/256 (5.19%);

16	F03	12.68	819.56	819.44	78.9650 (9.84%); 96.9763 (1.54%); 153.0018/154/155 (100%); 171.0122 (2.83%); 255.2386 (2.88%); 409.2364 (1.4%);
17	F14	0.84	174.99	174.96	76.9884 (3.48%); 84.9648/86.9822/88 (14.39%); 102.9788 (22.2%); 118.9729/119 (100%); 130.9739/132 (18.26%); 146.9675 42.08%);
18	F14	1.97	187.09	187.06	116.0562 (5.82%); 132.0515 (17.82%); 143.0666 (6.59%); 158/159.0628/160 (21.56%); 186/187.0573/188/189/190 (100%);
19	F14	3.46	163.06	163.04	107.0562 (7.02%); 135.0507 (6.52%); 161/162/163.0439/164 (100%);
20	F14	4.28	151.07	151.05	108.0275/109 (60.65%); 136.0217 (19.53%); 151.0455/152 (100%);
21	F14	4.94	294.14	294.11	79.9635/80 (66.62%); 106.9870 (50.91%); 111.0869 (18.68%); 138.0292/139/140 (100%); 142.1279 (4.84%); 186.1221 (4.43%); 294.1073 (18.17%);
22	F14	5.13	121.05	121.04	91/92.0324/93 (21.72%); 120/121.0350/122 (100%);
23	F14	5.69	191.06	191.04	80.0326 (1.22%); 91.0252/92 (9.27%); 103.0623 (4.39%); 108.0273/109 (100%); 119.0574 (9.09%); 135.0514/136 (25.16%); 163.0456 (64.56%); 191.0399 (84.33%);
24	F14	5.99	308.16	308.12	79.9640 (85.06%); 106.9872 (59.45%); 138.0296 (100%); 200.1353 5.35%); 308.1207 (25.20%);
25	F14	6.23	292.17	292.13	79.9635 (100%); 106.9866 67.74%); 138.0292 92.01%); 156.1434 (15.66%); 184.1402 (11.04%); 292.1280/293/294 (56.46%);
26	F14	12.68	327.33	327.29	253.2592 (4.14%); 279/280/281.2894 (100%); 327.2948 (5%);
27	F14	12.73	415.29	415.23	78.9666 (4.83%); 153.0026 (29.63%); 261.2283 (2.42%); 279.2386/280/281 (100%);
28	F23	0.99	207.07	207.05	106.0581 (1.52%); 135.0602/136 100%); 163.0576 (22.96%); 207.0488 (23.40%);
29	F23	2.24	257.10	257.08	143.0678 (5.77%); 171.0662 (7.48%); 185.0794 15.74%); 213.0794 (50.71%); 257.0762 (100%); and other
30	F23	7.47	393.28	392.33	78.9646 (100%); 96.9747 31.06%); 106.9856 2.58%); 138.0287 (6.01%); 392.2510 (14.27%);
31	F23	7.69	408.31	408.24	79.9631 (18.07%); 106.9863 (24.03%); 138.0291 (32.06%); 184.1413 (4.29%); 298.2370 (1.07%); 408.2446/409/410 (100%);
32	F23	8.5	449.30	449.08	78.9686 (100%); 96.9818 (5.51%); 171.0219 (1.84%); 336.1632 (3.22%);
33	F23	10.08	375.24	375.18	78.9649 (100%); 96.9753 (14.51%); 138.0289 (0.79%); 375.2105 (0.18%);
34	F23	10.99	436.30	436.36	79.9782 (100%); 107.0069 (80.43%); 138.0550 (96.13%); 156.1747 (8.54%); 184.1762 (12.33%); 292.1823 (3.33%); 306.2025 (2.57%); 436.3580/437 (81.94%);
35	F23	11.3	480.37	480.30	78.9786 (16.13%); 153.0285 (4.55%); 168.0781 (5.86%); 224.1135 (5.17%); 255.2809/256/257 (100%);

BSG D FP BuOH					
1	F03	0.82	304.98	304.93	86.9833 (1.54%); 94.0371 (3.67%); 102.9754 (2.86%); 118.9720 (13.31%); 130.9724 (26.54%); 146.9678 (100%); 174.9624 (17.81%); 217.0628 (18.49%); 261.0515 (4.15%);
2	F03	1.67	179.10	179.10	82.0421 (100%); 111/112.0554 (25.01%); 152.0776 (25.81%); 179/180/181.1179 (55.23%);
3	F03	1.36	128.06	128.05	85.0536 (10.57%); 128.0472/129.0668 (100%);
4	F03	2.27	227.15	227.14	82.0471 (94.42%); 112.0612 (40.91%); 116.0955 (20.72%); 127.1115 (85.64%); 183.1452 (100%); 227.1414 (14.47%);
5	F03	3.38	240.19	240.15	111.0257 (5.89%); 124.0355 (8.89%); 140.0743 (13.15%); 153.1190 (12.53%); 165.0774 (8.26%); 179/180.1190/181 (12.09%); 195.1105 (7.61%); 222.1373 (100%);
6	F03	5.3	312.22	312.17	82.0433 (6.99%); 88.0543 (100%);109.0559 (7.12%);127.0692 (21.67%); 136.1320 (7.73%);155.1047/157 (12.19%); 179/180.1263 (40.02%); 222/223.1367 (17.48%);
7	F03	5.05	241.05	241.15	82.0449 (100%); 112.0582 (29.28%); 130.1073 (33.52%); 141.1247 (85.65%); 168.1152/169 (12.78%); 197.1571/198 (83.45%); 241.1475 (9.61%);
8	F03	5.84	193.09	193.06	106.0510/107 (2.61%); 121.0753 (9.56%); 134.0468 (12.81%); 148/149.0698/150 (100%); 193.0615
9	F03	5.94	625.43	625.34	109.0486 (1.23%); 127.0606 (5.48%); 137.0813 (44.53%); 154.1092 (14.47%); 172.1208 (8.48%); 265.1429 (100%); 283.1522 (22.13%);
10	F03	6.51	607.42	607.33	112.0482 (10.83%); 116.0803 (59.75%); 141.0759 (49.70%); 167.0559 (100%); 183.1244 (31.43%); 222.1351 (26.92%); 240.1462 (80.75%); 284.1362 (87.39%);
11	F03	7.63	331.31	331.13	127.0712 (47.51%); 155/156157.0706 (36.78%); 171.0454 (25.76%); 187.0690 (7.63%); 201.0440 (10.75%); 295.1226 (18.70%); 313.1279 (50.53%); 331.1291 (100%);
12	F03	10.48	309.23	309.18	79.9637/80 (6.21%); 96.9657 (100%); 122.9818 (5%); 183.0185 (16.77%); 309.1779/310/311/312 (48.05%);
1	F22	6.44	721.30	721.35	93.0442 (6.26%); 110.0697 (19.62%); 154.0563 (100%); 228.0944 (10.74%); 245.1235 (8.8%); 260.1357 (9.34%); 339.1663 (32.65%); 497.2412 (8.88
2	F22	6.93	765.36	765.38	113.0775 (36.08%); 179.0544 (74.85%); 186.0649 (28.45%); 207.0872 (100%); 303.1624 (420.96%);
3	F22	8.91	265.11	265.14	79.9602 (1.44%); 96.9605 (100%); 265.144 (8.88%);
4	F22	9.53	277.14	277.18	79.9590 (100%); 96.9613 (6.09%); 277.1819/278/279 (19.44%);
5	F22	11.22	277.14	277.18	79.9590 (100%); 96.9613 (6.09%); 277.1819/278/279 (19.44%);
6	F22	11.61	291.15	291.27	79.9786 (100%); 96.9846 (22.15%); 291.2718 (25.33%);
7	F22	11.63	480.24	480.41	78.9896 (11.19%); 153.0306 (3.41%); 168.0826 (4.58%); 224.1219 (5.64%); 242.1376 (1.37%); 255.2926/255/256 (100%);
8	F22	13.1	353.16	353.20	79.9594 (9.02%);96.9611 (100%);122.9734 (4.91%); 182/183.1650 (1.64%); 353.1958/354/355 (30.27%);

9	F22	11.8	715.24	715.13	78.9789 (11.82%); 96.9896 (3.54%); 153.0284 (54.81%);
10	F22	13.1	153.01	153.00	78.9613 (100%); 96.9693 (7.73%); 136.9478 (10.63%); 150.9102 (4.95%); 153.0025/154 (8.17%);