Extraction and Characterization of Natural Antioxidants from Brewing Industry By-products

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À minha mãe,

que será sempre o meu exemplo de vida.

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Abstract

By-products from the processing of beverages, fruits and vegetables, traditionally considered as an environmental problem, are being increasingly recognized as sources for obtaining valuable products. To this regard, the recovery of phenolic compounds from brewing by-products is gaining considerable attention, especially ascribable to the antioxidant properties that these compounds exhibit. Food, pharmaceutical and cosmetic industries are nowadays claiming for natural solutions to some of the customers' needs such as the use of natural colorants, texturizers, functional ingredients or shelf life extenders. Polyphenols have also physiological functions which can additionally result in benefits for human health on the prevention of cardiovascular diseases, certain types of cancer and atherosclerosis. Therefore, the recovery of antioxidants from brewing industry by-products is of great importance, not only because of their aforementioned significant properties, but also because their valorization can constitute a contribution to the sustainable development of the agro-food sector by an exhaustive utilization of natural raw materials. This importance becomes even more relevant when a sector such as the brewing industry has a high influence in the economy of a country.

The research work presented in this thesis aimed at investigating the inexpensive residual products from brewing industry as sources of natural antioxidants. Amongst the by-products generated by the portuguese brewing industry, the most common are brewer's spent grains (BSG), surplus yeast and spent hops.

The first stage of the project consisted on an analytical screening of the phenolic composition of the brewing by-products in order to select that with a higher commercial potential and health interest. Different extraction techniques widely used in our laboratory to extract phenolic compounds from different matrices were evaluated. The antioxidant potential, evaluated by DPPH, ABTS, deoxyribose and FRAP assays, as well as the total phenolic content (TPC), were determined in different extracts. The results from this analytical first screening demonstrated that BSG exhibit highest antioxidant activity and total phenolic content (8.1 ± 0.2 mg GAE/g DW) than surplus yeast (6.73 ± 0.05 mg GAE/g DW) and spent hops (6.8 ± 0.5 mg GAE/g DW), showing to be a valuable source of natural antioxidant phenolic compounds.

Free, soluble esters and insoluble-bound phenolic fractions of BSG were also obtained. These results reveal that the contribution of bound phenolics (6.0 ± 0.3 mg

GAE/g DW) to the TPC was significantly higher than that of free (4.0 ± 0.2 mg GAE/g DW) and esterified (2.9 ± 0.5 mg GAE/g DW) fractions. These assays also show that 64% of phenolics present in insoluble bound fraction correspond to ferulic acid (FA), whereas 51% of esterified phenolics are *p*-coumaric acid based compounds. This data enables us to conclude that the majority of phenolics in BSG are associated with cell wall constituents, covalently cross-linked by ester and/or ether bonds to the phenolics.

To increase the commercial applicability of the BSG, efficient methods for the extraction of phenolics are needed. Traditional solvent extraction with organic solvents is both solvent and time consuming, leading to the consideration of alternative extraction methodologies such as microwave-assisted extraction (MAE). MAE of phenolic compounds from BSG was developed and optimized for various operational parameters (extraction time, temperature, solvent volume and stirring speed) using response surface methodology. The results showed that the optimal conditions were 15 min extraction time, 100 °C extraction temperature, 20 mL of solvent, and maximum stirring speed. Compared to the conventional extraction techniques (Soxhlet extraction, mechanical stirring and alkaline hydrolysis) the proposed MAE allows reaching approximately 5-fold higher FA yield (0.27 ± 0.02% for alkaline hydrolysis and 1.31 ± 0.04% for MAE). In addition, the developed new extraction method is an effective alternative to polyphenols recovery from BSG and a promising system for a potential large-scale extraction.

Parallel to this, TPC and antioxidant activity of BSG obtained from light (*pilsen*, *melano*, *melano 80* and *carared*) and dark (*chocolate* and *black*) malts were also investigated. The results obtained suggest that the type of malt used as well as the malting process, in particular the kilning regimes and roasting temperatures, may have important impact in terms of the phenolic composition and antioxidant features of BSG. Amongst the BSG studied, extracts from light types (malt KT ≤ 160 ºC) contained higher amounts of total and individual phenolic compounds, in particular *pilsen* BSG (20 ± 1 mg GAE/g DW BSG).

BSG extracts protection against the oxidative DNA damage in *Saccharomyces cerevisiae* cells was evaluated regarding to the antigenotoxic activity by using the yeast comet assay. The results obtained show that the *black* BSG extracts exhibit a 5-fold reduction in the genotoxic effects of H_2O_2, compared to the 2-fold reduction by the *pilsen* BSG extracts. These results are in contrast with previously reported data for DPPH, ABTS and deoxyribose assays, which demonstrated that *pilsen* BSG exhibited

the highest antioxidant activity. The observed difference can be attributed to the melanoidins compounds, mainly present in *black* malts, which possess reducing properties and can form iron chelates. Therefore, less OH radicals are produced and oxidative DNA damage occurs in a less extent in *black* BSG extracts.

The results of this thesis highlight the potential of MAE to extract higher FA amounts from BSG with a low environmental impact. Moreover, the obtained extracts exhibited biological activity by protecting against oxidant-induced DNA damage, offering the possibility to use the by-product BSG as a natural and inexpensive source of antioxidants.

Keywords

By-products; Brewing; Brewer's spent grains; Surplus yeast; Spent hops; Phenolic compounds; Ferulic acid; Microwave-assisted extraction; HPLC-DAD-MS/MS analysis; Antioxidant properties; Valorization

Resumo

Os subprodutos do processamento de frutas, legumes e bebidas, tradicionalmente considerados um problema ambiental, têm sido cada vez mais reconhecidos como potenciais fontes para a obtenção de produtos de valor acrescentado. Deste modo, a recuperação de compostos fenólicos a partir de subprodutos da indústria cervejeira tem-se revelado uma área bastante promissora, principalmente pelas propriedades antioxidantes que estes compostos exibem. Além disso, as indústrias alimentares, farmacêuticas e cosméticas têm exigido por soluções naturais para colmatar algumas das necessidades impostas pela sociedade, tais como o uso de corantes naturais, ingredientes funcionais e prolongadores do tempo de vida dos produtos. Adicionalmente, os polifenóis também possuem funções fisiológicas que podem ainda resultar em benefícios para a saúde humana na prevenção de doenças cardiovasculares, certos tipos de cancro e aterosclerose. Assim, a recuperação de compostos antioxidantes a partir dos subprodutos da indústria cervejeira é de grande importância, não só devido às propriedades acima mencionadas, mas também porque a sua valorização pode constituir um contributo para o desenvolvimento sustentável do sector agroalimentar através de um eficaz aproveitamento das matérias-primas. Esta importância torna-se ainda mais proeminente quando um sector como a indústria cervejeira tem um elevado peso na economia de um país.

O trabalho descrito na presente tese teve como objetivo investigar a possibilidade de utilizar subprodutos da indústria cervejeira como fonte de antioxidantes naturais. Dentro dos subprodutos gerados pela indústria cervejeira portuguesa podem-se destacar a drêche cervejeira (DC), o excedente de levedura e o excedente de lúpulo.

Numa primeira fase do projeto determinou-se a composição fenólica dos subprodutos da indústria cervejeira com o intuito de selecionar aquele com maior interesse quer a nível comercial quer a nível da saúde humana. Para isso, foram avaliadas várias técnicas de extração amplamente utilizadas no nosso laboratório para extrair compostos fenólicos a partir de diferentes matrizes. A atividade antioxidante, determinada pelos métodos DPPH, ABTS, desoxirribose e FRAP, bem como o teor de polifenóis totais (TPC), foram determinados nos diferentes extratos obtidos. Os resultados desta primeira análise demonstraram que a DC possui maior atividade antioxidante e TPC (8,1 ± 0,2 mg GAE / g DW) do que o excedente de levedura (6,73

± 0,05 mg GAE / g DW) e o excedente de lúpulo (6,8 ± 0,5 mg GAE / g DW), revelando-se uma valiosa fonte de compostos fenólicos naturais.

As frações fenólicas livre, esterificada e ligada da DC também foram obtidas. Os resultados demonstraram que a contribuição dos compostos fenólicos da fração ligada (6,0 ± 0,3 mg GAE /g de DW) para o TPC foi significativamente superior do que a das frações livre (4,0 ± 0,2 mg GAE/g de DW) e esterificada (2,9 ± 0,5 mg GAE/g de DW). Estes ensaios permitiram igualmente verificar que 64% dos compostos fenólicos na fração ligada corresponde ao ácido ferúlico, enquanto 51% dos compostos da fração esterificada é ácido *p*-cumárico. Estes dados permitem-nos concluir que a maioria dos compostos fenólicos na DC se encontram associados a componentes da parede celular, covalentemente ligados por ligações éster e/ou éter aos compostos fenólicos.

De forma a potenciar a aplicabilidade a nível comercial da DC são necessários métodos eficientes para a extração de compostos fenólicos. A extração com solventes orgânicos necessita de grandes quantidades de solvente e é bastante demorada, potenciando a procura de metodologias de extração alternativas, tais como a extração assistida por microondas (MAE). A MAE de compostos fenólicos da DC foi desenvolvida e otimizada para vários parâmetros (tempo de extração, temperatura, volume de solvente e velocidade de agitação) utilizando a metodologia de resposta de superfície. Os resultados obtidos demonstraram que as condições ótimas para a extração foram de 15 min, 100 ºC, 20 mL de solvente, e a velocidade máxima de agitação. Em comparação com as técnicas de extração convencionais (extração Soxhlet, agitação mecânica e hidrólise alcalina) o método MAE proposto permite obter aproximadamente 5 vezes maior rendimento de ácido ferúlico (0,27 ± 0,02% para a hidrólise alcalina e 1,31 ± 0,04% para o MAE). Além disso, o novo método de extração desenvolvido é uma alternativa ecológica e eficaz para a recuperação de compostos fenólicos da DC e um promissor sistema para uma potencial extração a grande escala.

Paralelamente, a atividade antioxidante e o TPC da DC obtida de variedades de malte claro (*pilsen*, *melano*, *melano 80* e *carared*) e escuro (*chocolate* e *black*) também foram investigadas. Os resultados obtidos sugerem que o tipo de malte e o processo de maltagem, nomeadamente os programas de secagem e as temperaturas de torrefação, podem influenciar a composição fenólica e as propriedades antioxidantes da DC. Das variedades de DC estudadas, os extratos obtidos a partir de maltes claros (malte KT ≤ 160 ºC) revelaram maior teor de compostos fenólicos totais e individuais, particularmente a DC de malte *pilsen* (20 ± 1 mg GAE/g DC).

A capacidade dos extratos de DC protegerem contra o dano oxidativo provocado no DNA em células de *Saccharomyces cerevisiae* foi avaliada em relação à atividade antigenotóxica utilizando o *yeast comet assay*. Os resultados obtidos demonstraram que o extrato *black* da DC permite uma redução de 5 vezes no efeito genotóxico do H_2O_2 em comparação com o extrato *pilsen* que permite uma redução de 2 vezes. Estes resultados são contraditórios com os previamente obtidos pelos métodos do DPPH, ABTS e desoxirribose, que demonstraram que a DC *pilsen* possuía maior atividade antioxidante. Esta diferença poderá ser atribuída às melanoidinas, presentes essencialmente nos maltes escuros, que possuem propriedades redutoras e podem formar quelatos com o ferro. Assim, menos radicais OH são produzidos e o dano oxidativo provocado no DNA ocorre em menor extensão no extrato *black* da DC.

Os resultados desta tese permitem destacar o potencial da MAE para extrair em maior quantidade o ácido ferúlico da DC com um baixo impacto ambiental. Além disso, os extratos obtidos exibiram atividade biológica através da proteção contra os danos oxidativos provocados no DNA, representando uma possibilidade para a reutilização do subproduto DC como fonte natural e de baixo custo de antioxidantes.

Palavras-chave

Subprodutos; Drêche cervejeira; Excedente de levedura; Excedente de lúpulo; Compostos fenólicos; Ácido ferúlico; Extração assistida por microondas; HPLC-DAD-MS/MS; Propriedades antioxidantes; Valorização

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Abbreviations and Symbols

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

ANOVA, Analysis of variance

ARP, Antiradical power

BER, Base excision repair

BSG, Brewer's spent grains

CFU, Colony forming units

CVD, Cardiovascular disease

DAD, Diode array detector

DiFA, Dehydrodiferulic acid

DNA, Deoxyribonucleic acid

DPPH, 1,1-diphenyl-2-picrylhydrazyl

DW, Dry weight

EC_{50}, Effect concentration for a 50% response

FA, Ferulic acid

FRAP, Ferric reducing antioxidant power

GA, Gallic acid

GAE, Gallic acid equivalents

HBAs, Hydroxybenzoic acids

HCAs, Hydroxycinnamic acids

HMW, High molecular weight

HPLC/UV, High performance liquid chromatography with UV detection

HPLC-ESI-MS/MS, High performance liquid chromatography-electrospray mass spectrometry

IXN, Isoxanthohumol

KT, Kilning temperature

LDL, Low density lipoproteins

LMA, Low melting agarose

LMW, Low molecular weight

LOD, Limit of detection

LOQ, Limit of quantification

MAE, Microwave-assisted extraction

MRP, Maillard reaction products

NER, Nucleotide excision repair

OD, Optical density

***p*-CA**, *p*-Coumaric acid

r, Pearson's correlation coefficient

RNA, Ribonucleic acid

ROS, Reactive oxygen species

RSM, Response surface methodology

SD, Standard deviation

SE, Soxhlet extraction

SC-CO_2, Supercritical carbon dioxide

SFE, Supercritical-fluid extraction

TE, Trolox equivalents

TPC, Total phenolic content

TriFA, Triferulic acid

XN, Xanthohumol

YPD, Yeast, peptone, glucose

I

Background

1. An overview of the malting and brewing process

The simplest definition of beer is a fermented and flavored drink derived from starch. This simple definition encompasses essentially the four essential ingredients, which are used in the brewing industry: barley (malt), hop, water and yeast. The advances in the scientific understanding of the brewing process over the past 150 years have led to a process that, while traditional, is under rigorous control. Brewing beer is a complex process that allows variation in multiple parameters which influence the type and quality of beer [1]. The fundamental processes of the malting and brewing operations are briefly explained in this chapter for a better understanding of by-products generation during the brewing process.

1.1. The malting process

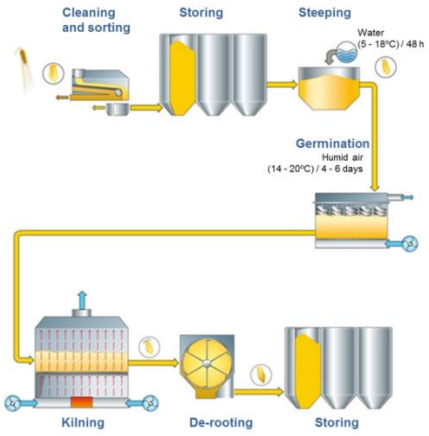

Fig. 1.1 - The malting process.

The main aim of malting is to transform the food reserves of grain, which are insoluble starch and protein, into a substrate capable of dissolution and extraction by hot water during the later mashing stage to produce wort. This wort is an aqueous solution, also called liquor, of fermentable carbohydrates and soluble proteins. The extent of the transformation during malting is referred to as modification and is controlled by management of the growth conditions to which the grain is exposed.

The essential changes which occur during the modification of grain are:

- a large increase in the amount, through release or synthesis, of some of the hydrolytic enzymes present in the grain;
- a partial enzymic hydrolytic degradation of cell wall material, gums, proteins and starch, and
- a reduction in the structural strength of the grain tissue.

There are three main process stages in a modern malting operation: steeping, germination and kilning (Fig. 1.1) [2-4].

Steeping

Steeping (Fig. 1.2) initiates malting and is a crucial step in producing quality malt. During steeping, barley takes up water and swells by one third. The purpose of this stage is to increase the moisture content of the grain from 11-12% to 42-47% within two days. Kernels will not germinate if their moisture content is below 32% [5].

Fig. 1.2 - The steeping stage consists of alternating periods when the grain is immersed in water, referred to as the “under water periods”, and periods with the water drained from the grain, referred to as the “air rest periods”. This combination is required to promote and maintain the efficiency of germination.

A typical steeping regime will consist of an initial water stage for 6-16 hours, to raise the moisture content to 33-37%. An air rest for 12 to 24 hours follows, during which air is sucked downward through the grain bed to disturb films of moisture on the grain, expose the embryos to oxygen, and remove carbon dioxide produced by respiration, all of which is designed to prevent the embryo from being “suffocated.” This will be followed by a second immersion of 10–20 hours [2, 3].

After the second immersion, the moisture content of the grain reached the required levels. There are no hard-and-fast rules for steeping regimes: they are determined on a barley-by-barley basis by small-scale trials. It must be also noticed that barley changes in its properties over time. It increases in so-called vigor (the speed of growth essential for malting), which is reflected in enhanced capability for synthesizing enzymes and, therefore, rate of modification of the endosperm. Barley, then, will need to be processed differently in the maltings as the year goes on. In some locations barley is graded before steeping according to size, because different sizes of barley take up moisture at different rates [3, 5].

Germination

The aim of germination is to develop the enzymes that can hydrolyze the cell walls, the protein, and the starch of the barley. This step is to ensure that the endosperm is softening by removing the cell walls and about half of the protein, while leaving the bulk

of the starch behind. In the germination stage, the steeped grain spends between 4 and 6 days in humic and ventilated conditions during which time modification occurs.

The germination grain has a controlled temperature between 14ºC and 20ºC by a flow of air through the bed and is humidified by atomizing water jets which may be cooled by refrigeration. Metabolism of the growing grain is an exothermic process, which causes a rise in temperature of the air. Since the air leaves the bed warmer and consequently with a higher moisture, some loss of moisture is inevitable. In fact, a loss of approximately 2% occurs during germination. This loss may be compensated by spraying water onto the green malt during the first and second days of germination. Germinating barley needs to be turned in order to separate the grains and their developing rootlets. Without turning, the barley would form a near solid mass which would restrict the airflow causing localized overheating. The interlocking of the rootlets would also make removal at the end of germination difficult. During germination the grain will develop an acrospires and rootlets. Normally, the rootlets can be expected to grow to a length of between 1.5 and 2 times the length of the grain, constituting a malting loss of between 4% and 7%.

Formation of α- and β-amylase, and degradation of proteins and β-glucans are essential features which the maltster should aim to control. Breakdown of β-glucan cell walls is essential to achieve fast lautering of the wort and improved filterability of the beer. Two main enzymes are involves. β-glucans solubililase converts the insoluble cell wall protein matrix to soluble β-glucans. These are broken down further to low molecular weight glucans and glucose by the enzyme endo-β-glucanase. Degradation of β-glucans is favored by an intense malting process, for example high moisture contents and a germination temperature of 19ºC maintained for extended periods [3-5].

Kilning

The final heat treatment, called kilning or roasting, has the characteristics of a typical industrial drying process. The primary aim of kilning is the reduction of the moisture content of green malt from the germination conditions of 45% to a condition which ensures stability for transportation and storage (typically in the range 4 to 5%). Regulated removal of water from green malt is essential to prevent further growth and modification, to achieve a stable product which can be stored, transported and to preserve enzymes. Moreover, this step is to develop and stabilize properties such as

flavor and color, remove undesirable flavors and dry the rootlets to permit their removal.

Drying occurs in two different stages. Initially, moisture is removed from the grain from approximately 44% to 12%. With an upward flow of air, this process takes approximately 12 hours to pass through the bed for a single-deck kiln and 24 hours for double-deck kiln, depending upon the airflow. This phase of drying is rapid and is referred to as the “free-drying”, “constant rate” or “withering” phase. The second phase of drying where the malt is dried from 12% to 4% occurs in a much slower process, commonly referred to as the “falling rate” phase. At the end of the drying process, the kiln temperature may be raised for one or two hours in the “curing” stage. This is followed by a cooling period to achieve a temperature suitable for discharge and storage. The kilning cycle should ensure that the maximum drying effect occurs at the lowest possible temperature. In this way, the final curing temperature can be achieved whilst the enzyme losses are kept to an acceptable level [3, 5].

Fig. 1.3 - Kilned malts are used for lager beer production. Brewers tend to use a mixture of mainly pale malts with a small amount of specialty malts, which provide flavor and color to the final product.

Beers which differ in taste, color, aroma, mellowness and head retention, need to use malts (Fig. 1.3) produced by different processes, in particular in the use of drying and roasting conditions. These later stages lead to the development of flavor and colors of different degrees of intensity for the preparation of a range of beers.

Compared to pilsner malt, dark specialty malts are subjected to higher kilning temperatures, leading to more intensive non-enzymatic browning reactions (Maillard reactions) [6]. Depending on the thermal treatment applied during kilning, a wide assortment of dark malts can be produced ranging in color from pale yellow through to amber to brown to nearly black, i.e. from 5 to 1600 EBC units. Dark malts can be further categorized into color malts, caramel malts and roasted malts. Color malts are produced on a kiln using elevated roasting temperatures, while caramel malts and roasted malts are obtained from roasting green malt and pilsner malt in a device similar to that used for coffee roasting [2].

1.2. The brewing process and by-products generation

The fundamental processes of brewing (mashing, boiling, fermentation, maturation, filtration and bottling) and the points where the main by-products are generated, which are illustrated in Fig. 1.4, are briefly explained in this subchapter.

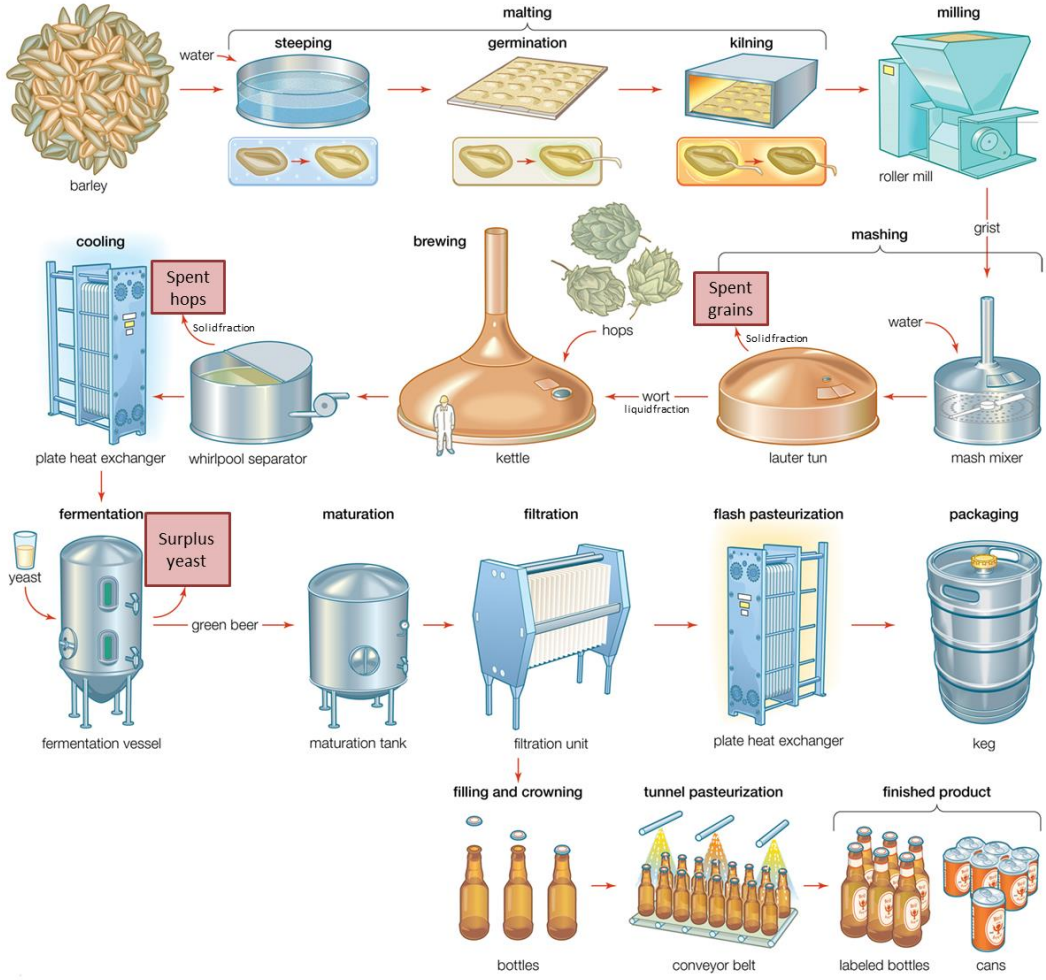

Fig. 1.4 - Flow diagram of the brewing process and points where the main by-products are generated.

Brewing begins with malted barley that is milled and mixed with hot water to form a mash. Mashing involves mixing milled malt and solid adjuncts with water at a set temperature and volume to continue the biochemical changes initiated during the malting process. The mashing process is conducted over a period of time at various temperatures in order to activate the enzymes responsible for the acidulation of the mash and the reduction in amount of proteins and carbohydrates. The most important change brought about in mashing is the conversion of starch molecules into fermentable sugars (mainly maltose and maltotriose) and unfermentable dextrins. The

principal enzymes responsible for starch conversion are α- and β-amylases but also proteases, leading to a mixture of sugars and peptides or amino acids, producing wort of the desired composition [2, 4, 7, 8].

After mashing, when all the starch has been broken down, it is necessary to separate the liquid extract (the wort) from the solids. Wort separation is important because the solids contain large amounts of protein, fatty material, silicates and polyphenols (tannins). This insoluble, undegraded part of the malted barley grain is allowed to settle to form a bed in the mash tun and the sweet wort is filtered through it (lautering). The filtered wort is used as the fermentation medium to produce beer. The residual solid fraction obtained after this stage is known as brewer's spent grains (BSG) [7, 8]. In Fig. 1.5 is a schematic representation of the process resulting in the production of BSG from barley grain.

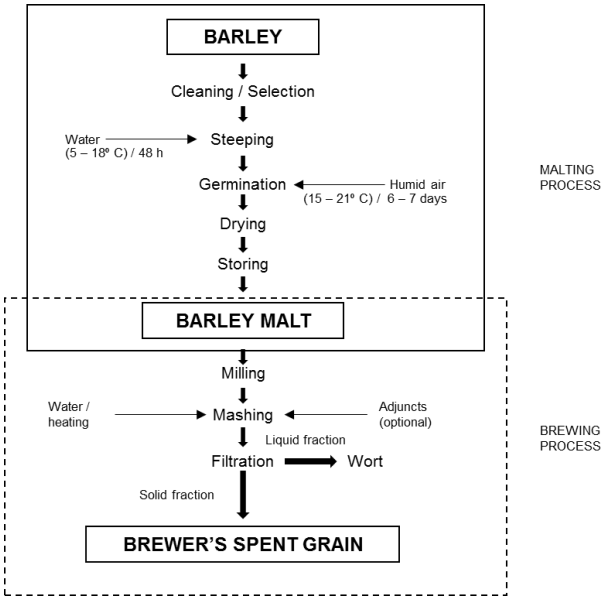

Fig. 1.5 - Schematic representation of the process to obtain BSG from barley (Source Mussatto *et al* [7]).

The brewing process is selective, removing only those nutrients from the malt necessary to produce the wort, leaving washed, water insoluble proteins and the cell wall residues of the husk, pericarp and seed coat within the spent grain [2, 4, 7]. In some cases, due to economic reasons or aiming to produce beers of distinct qualities, part of the barley malt – usually 15 to 20% – is replaced by unmalted cereal like corn, rice, wheat, oats, rye or sorghum, called adjuncts [2]. In these cases, at the end of the mashing process, the insoluble part of these grains is also separated with the undegraded part of the malted barley grain (BSG) [8].

Following extraction of the carbohydrates, proteins, and yeast nutrients from the mash, the clear wort must be conditioned by boiling in the kettle. After filtration, the wort is transferred to the brewing kettle, where it is boiled during at least one hour with the addition of hops. The purpose of wort boiling is to stabilize the wort and extract the

desirable components from the hops, which will confer typical beer qualities, such as bitter taste, flavor, and foam stability.

At the end of the boiling period, the hopped wort is transferred to a vessel known as a whirlpool, where further separation of hop residues (spent hops) and the trubaceous matter (hot break) takes place [2, 4, 8]. The hop residues, which are then useless, are dumped directly as being of no further value.

After boiling and clarification, the wort leaving the whirlpool has to be cooled in preparation for the addition of yeast and subsequent fermentation. Wort is usually cooled through plate heat exchangers. The principal changes that occur during wort cooling are as follow: cooling the wort to yeast pitching temperature, formation and separation of cold break and oxygenation of the wort to support yeast growth.

After removal of the precipitate produced during boiling, the cooled hopped wort is pitched with yeast in a fermentation vessel, where the yeast cells will convert the fermentable carbohydrates to alcohol and carbon dioxide. Fermentation is dependent upon the composition of the wort, the yeast, and fermentation conditions of each brewery. Wort constituents that may affect yeast growth include carbohydrates, amino acids, free fatty acids, and trace minerals such as zinc [9]. The type of yeast as well as the condition of the pitching yeast, such as yeast generation and glycogen content, can also affect yeast growth. At the end of this stage, most of the cells are collected as surplus yeast, at the top or at the bottom of the fermentor (according to the nature of yeast used) [2, 4, 8]. After beer aging has been completed and the yeast, along with other insoluble material, has settled, so-called tank bottoms are collected as by-product. The solids in surplus yeast include pure yeast solids, beer solids, and trub solids. Trub solids contain approximately the same amount of protein as yeast solids (40–50%) and their removal is not required if yeast is to be sold as animal feed. When yeast is sold for food uses, removal of both trub solids and beer solids is generally necessary [3, 9]. This brewing by-product has dry matter content close to 10% w/w and generates beer wastes between 1.5 and 3% of the total volume of produced beer [10].

After fermentation the green beer undergoes maturation. Traditionally, maturation involves secondary fermentation of the remaining fermentable extract at a reduced rate controlled by low temperatures and a low yeast count in the green beer. During secondary fermentation, the remaining yeast becomes re-suspended utilizing the fermentable carbohydrates in the beer. The carbohydrates can come from the residual

gravity in the green beer or by addition of priming sugar. Yeast activity achieves carbonation, purges undesirable volatiles, removes of all residual oxygen, and chemically reduces many compounds, thus leading to improved flavor and aroma [2, 9].

A final filtration is needed to remove residual yeast, other turbidity, and microorganisms in order to achieve colloidal and microbiological stability. To achieve beer stability it is also necessary to remove the proteins, polyphenols, or both from the beer. These haze precursors can be removed during the cold conditioning or filtration steps, i.e., during colloidal stabilization at the filter. During filtration, the most commonly used stabilizer for removing proteins is amorphous silica gel. Polyvinylpolypyrrolidone (PVPP) is typically used for removing polyphenols. The major process after filtration and prior to packaging is carbonation. Carbon dioxide not only contributes to perceived “fullness” or “body” and enhances foaming potential, but it also acts as a flavor enhancer and plays an important role in extending the shelf life of the product [2, 11].

Once the final quality of the beer has been achieved, it is ready for packaging. The packaging of beer is one of the most complex aspects of brewery operations and the most labor intensive of the entire production process. It is probably one of the most critical steps in beer flavor stability. The basis for pasteurization is the heating of the beer for a predetermined period of time at specific temperatures, thereby assuring the microbiological stability of the beer. The two main types of pasteurization techniques are flash and tunnel. Flash pasteurization is used for continuous treatment of bulk beer prior to filling the bottles, cans, or kegs. It is typically carried out in a plate heat-exchanger before transferring the beer to the bright-beer tank. Tunnel pasteurization is used mainly for in-pack treatment following the crowning of the bottles. The bottles are loaded at one end of the pasteurizer and passed under sprays of water as they move along the conveyor. The sprays are so arranged that the bottles are subjected to increasingly hot water until the pasteurization temperature (usually 60 ºC) is reached. The bottles are then gradually cooled with water until they are discharged from the end of the pasteurizer [2, 9, 11].

In Chapter 2 it will be discussed the chemical composition and market potential for the main by-products generated during the brewing process.

2. Brewery by-products

Nowadays, there is great political and social pressure to reduce the pollution arising from industrial activities. Therefore, waste disposal have become a significant cost factor and an important aspect in the running of a brewery operation. Despite the fact that waste disposal has become more stringent by the authorities [10], almost all developed and underdeveloped countries are trying to adapt to this reality by modifying their processes so that their residues can be recycled. Consequently, most large companies no longer consider residues as waste, but as a raw material for other processes [7, 9].

In the food industry, the brewing sector holds a strategic economic position with annual world beer production exceeding 1.34 billion hectolitres in 2002 [10, 11]. Beer is the fifth most consumed beverage in the world apart from tea, carbonates, milk, and coffee, and it continues to be a popular drink with an average consumption in 2007 of 9.9 L/capita by population aged above 15 [10-12].

Brewers are very concerned that the techniques they use are the best in terms of product quality and cost effectiveness. During beer production, several by-products are generated as shown in Fig. 1.4. The most common ones are BSG, spent hops and surplus yeast, which are generated from the main raw materials [8]. In 2005, Unicer SA reported that 60089 tonnes of by-products were generated, which 96% corresponds to BSG and excess yeast [13]. These by-products contain more than 20% protein and are generally sold as protein supplements for animal feeds [9]. In the case of Unicer, brewing by-products are fully used in cattle feed for local producers [13].

Due to the global intense pressure towards green environmental technology, both academic and industrial researchers are putting more efforts to reduce the amount of such wastes by finding alternative uses apart from the current general use as animal feed. As most of these are agricultural products, they can be readily recycled and reused, constituting real economic opportunities for improvements in brewing [14]. Furthermore, the position of beer as a natural product leads the brewers to pay attention to their marketing image and to take by-products treatment into account [10].

In this chapter it’s highlighted the main characteristics and potential applications of most common brewery by-products, namely BSG, spent hops and surplus yeast,

focussing on alternative uses as a raw material in foods, in energy production and in biotechnological processes.

2.1. Brewer's spent grains

BSG (Fig. 2.1) is the major by-product of the brewing industry, representing around 85% of the total by-products generated. Spent grains accounts, on average, for 31% of the original malt weight, representing approximately 20 kg per 100 L of beer produced [7, 10].

Fig. 2.1 - Appearance of brewer's spent grains (Source: Mussatto [8]).

BSG is available at low or no cost throughout the year, and is produced in large quantities not only by large, but also by small breweries. In 2005, Unicer, which has the major share of the beer market in Portugal, produced 405116 litres/year of beer generating 51504 tonnes of spent grains [13].

As reported, BSG has been used for the production of low value composts, livestock feed or disposed of in landfill as waste. In the case of Unicer, spent grains is fully used in cattle feed for local producers [13]. At present, BSG is sold as livestock feed with an average profit close to 5 €/ton (min, 1 €/ton; max, 6 €/ ton) [10]. However, due to legislative drivers, the cost for the disposal of BSG has increased, and with the decline in traditional disposal routes (as animal feed) alternative commercial uses for BSG are being sought [15-17].

Since BSG is the main by-product produced in our country, a more detailed description concerning the chemical composition and potential uses is presented in Chapter 0. Moreover, the main phenolic compounds present in BSG and their extraction techniques are also discussed.

2.2. Surplus yeast

2.2.1. Chemical composition

Fig. 2.2 - Surplus yeast.

In brewing, surplus yeast (Fig. 2.2) is another by-product that merits considerable attention, due to the large quantity produced (is the second largest by-product from breweries) [8, 18]. During fermentation, yeast cell mass increases three- to six- fold and after this step is collected as surplus yeast. After beer aging has been completed and the yeast, along with other insoluble material, has settled, so-called tank bottoms are collected as by-product [2, 9, 10].

Surplus yeasts are an inexpensive nitrogen source with good nutritional characteristics and a very bitter taste, generally recognized as safe (GRAS) [19]. However, it is generally sold as animal feed after inactivation by heat, and much of this by-product is considered industrial organic waste that causes a great deal of concerns [19]. This brewing by-product has rich chemical composition (Table 2.1) and attempts have been made to recover higher value compounds from surplus yeast [19-21].

Table 2.1 - Chemical composition of surplus yeast

Components / % dry weight basis	Reference		
	[9]	[18]	[19]
Protein	48	48	50
Lipid	1	1	NR
Ash	7	8	7
Crude fibre[b]	3	NR	NR
Carbohydrates	NR	36	42

[b]glucans, mannans, and polymeric hexosamines. NR = Non Reported

After the brewing process, the major constituent of yeast cell is carbon, which corresponds to about 50% of the dry weight. Other major elemental components are oxygen (30-35%), nitrogen (5%), hydrogen (5%) and phosphorus (1%). The most abundant classes of macromolecules are proteins and carbohydrates (Table 2.1) [2, 8]. The protein content presents several bound amino acids, such as arginine, cystine, among others, with tyrosine being the most abundant [9, 18, 19]. It is

also rich in vitamins, mainly niacin (50 mg/100 g), and minerals [8, 19]. The total mineral content of surplus yeast is approximately 5-10% of the cell dry weight. This fraction comprises a multitude of elements, specially potassium and phosphorus [8, 9]. Several compounds of industrial interest can be isolated from surplus yeast, such as β-glucan, enzymes, proteins, vitamins, amino acids, cytochromes, the purine components of DNA and ribonucleic acid (RNA) [19].

2.2.2. Potential uses

Compared to BSG, surplus yeast has a higher content of protein, and for this reason it has been currently utilized for animal feed and nutritional supplement [8, 9]. However, the inclusion of surplus yeast in food products is limited by the amount of nucleic acid, primarily RNA which may account for one third of the total cell protein [19], since in humans RNA is metabolized to uric acid, which can lead to gout [9]. Surplus yeast is not only a source of proteins but also an excellent source of B-complex vitamins, nucleic acids and minerals [8, 19]. Therefore, several attempts have been done aiming to reuse the surplus yeast in biotechnological processes.

Yeast extract, which is a mixture of amino acids, peptides, nucleotides and other soluble components of yeast cells, can be produced by the breaking down of surplus yeast using endogenous or exogenous enzymes [9]. These extracts are of particular interest for use in the food industry as a flavoring agent in soup, sauces, gravies, stews, snack food and canned food [8, 20]. Other application in food industry is the production of yeast protein concentrates (and isolates) while retaining the functional properties and nutritive values of surplus yeast [19, 20]. Protein and amino acids can be recovered from this by-product by employing various processes such as autolysis, plasmolysis in organic salt solution or non-polar organic solvent, acid or alkali catalyzed hydrolysis, enzymatic hydrolysis, or hydrothermal decomposition [18-20]. These yeast extracts produced are usually found in the form of powders, flakes or tablets, or in liquid form. They can be sprinkled on food, used as a seasoning or mixed with milk, juices, soups, and gravies [19]. Other application for surplus yeast is as vitamin supplements in health foods [19, 20].

β-glucan, a hydrocolloid of large interest for pharmaceutical and functional food industries, can also be extracted from surplus yeast. This compound is attracting increasing attention because of its positive effects on human and animal health, such as immune-stimulation, anti-inflammatory, antimicrobial, antitumoral, hepatoprotective,

cholesterol-lowering and antidiabetic [22]. β-glucan is also interesting due to the properties improved to food products, being used as a thickening, water-holding or oil-binding agent, and emulsifying or foaming stabilizer [21, 23]. It is expected that β-glucan from surplus yeast, with low cost of production, simple extraction technology, and potential infinite supply will be one of the major applications in the future [8, 21].

Another application of surplus yeast is the use in fermentative processes. Rakin *et al.* [24] produced a fermented bioproduct, which is a mixture of beetroot and carrot juices, enriched in brewer's yeast autolysate. These mixtures of juices were subjected to lactic-acid fermentation with *Lactobacillus acidophilus* NCDO1748. The use of surplus yeast autolysate during the fermentation of vegetable juices favorably affects the increase of the number of lactic acid bacteria, reduction of fermentation time and enrichment of vegetable juices with amino acids, vitamins, minerals and antioxidants. The use of surplus yeast in microbiological media is also well known and largely used as source of nutrients [8, 20].

2.3. Spent hops

2.3.1. Chemical composition

In 2005, the average hops and hop extracts usage for Unicer was 20 tonnes [13].

Fig. 2.3 - Female hop plant (*Humulus lupulus*).

As only 15% of the hops constituents end up in the beer, 85% will become spent hop material requiring disposal at the brewery or the hops processing plant [9]. Hop flowers (Fig. 2.3) are rich in phenolic compounds, such as xanthohumol (XN) and isoxanthohumol (IXN), and α-humulones and β-lupulones acids [25].

During the brewing process, phenolic acids, such as ferulic, gallic, *p*-hydroxycoumaric, protocatechuic and caffeic acids; catechins, flavones and anthocyanidines, among others, are precipitated with proteins during wort boiling. In turn, the lupulones, which are insoluble at the normal pH value of the wort and do not isomerize during boiling, are largely removed with the spent hops and the trub [2, 8]. Currently, spent hops are utilized as fertilizer [26]. However, as these compounds have been associated with several beneficial properties [1], hop processing industries have

been looking for alternative ways of their utilization, in order to increase the added value of spent hops.

As can be seen in Table 2.2, spent hop is a by-product with high amounts of nitrogen free extract, fibres and proteins [9].

Table 2.2 - Chemical composition of spent hops

Components / % dry weight basis	Reference	
	[9]	[2]
Protein	23	22.4
Lipid	4.5	NR
Ash	6.5	6
Crude fibre[b]	26	23.6
Nitrogen free extract	40	NR

[b]cellulose, hemicellulose and lignin. NR = Non Reported

The crude protein content of spent hops on a dry matter basis is 22–23%, which is higher than that of hops, probably because of trub inclusion [9]. Crude fibre is constituted by several sugars, such as rhamnose, arabinose, mannose, galactose, with glucose and xylose being the most abundant. Polysaccharides, namely pectic sugars, uronic acid, rhamnose, arabinose and galactose, account for 46% of the composition from spent hops [8, 26]. Fischer and Bipp [27] reported that mono- and multifunctional aliphatic carboxylic acids, such as oxalic, glucaric, gluconic, threonic, glyceric, glycolic, lactic and acetic acid, could also be detected in spent hops.

2.3.2. Potential uses

Despite the chemical composition rich in nitrogen, carbon and protein (Table 2.2), potential applications for spent hops have been few explored [8, 26, 27].

Traditionally, spent hops have been used as a fertilizer and soil conditioner, due to the high nitrogen content [9, 28]. Besides this application, one of the most prevalent methods for disposal of this by-product at the brewery site is the addition of spent hops to BSG. In opposite to BSG, the direct use of spent hops as feed supplement is not desirable due to the presence of bitter substances in this residue [9]. As spent hops are rich in protein (up to 23% of crude protein; [9]), their employment as a feed supplement is reasonable. However, it is firstly necessary to remove or degrade the spent hops

bitter acids, which can be made by selected fungi or yeasts like *Candida parapsilosis* [8, 28].

Hop-processing industries have been looking for alternative ways to reuse spent hops. Some studies described that spent hops can be used as a source of value-added compounds. Huige [9] reported that this brewery by-product can be used in chipboard and paper making, and that the residual hop resins can be utilized as a binder or extracted with acetone to obtain an unsaturated drying oil for paints. Flavors, saccharides or organic acids, which can be obtained after oxidation or hydrolysis of this material, can be recovered from this brewery by-product [26, 27]. Ruckle and Senn [29] have reported that hop acids, detected in spent hops, can act as natural antibacterial in distillery mashes for alcoholic fermentation. This represents a safe alternative to control bacteria in ethanol fermentations, being able to efficiently replace antibiotics in ethanol production. Pectins, compounds widely used in food industry as gelling and thickening agents, represent a large part of the polysaccharides in spent hops and can be recovered by acid extraction conditions. Spent hops pectins include homogalacturonans, and arabinogalactan-proteins with a protein part rich in cystein, threonin, serinin, alanin, and hydroxyprolin [26].

3. Brewer's spent grains as a natural source of antioxidant compounds

According to the previously mentioned, BSG is the main by-product produced by portuguese brewing industry, and it will be the focus of our investigation. This solid residue contains water insoluble proteins in addition to the husk, pericarp and seed coat of the original barley grain [7, 8]. Protein and fibre account for 20 and 70% of BSG dry matter, respectively, while the starch content of BSG is insignificant (due to the absence of starchy endosperm). BSG is also rich in phenolic compounds, particularly ferulic and *p*-coumaric acids [17, 30-32], along with oligosaccharides and polysaccharides [7, 33]. However, until now BSG has been widely used as an animal feed [9, 34], but with the increased cost of disposal of the solid fraction, alternative uses are highly sought-after [8, 35, 36].

The chemical composition and potential applications for BSG will be reviewed in this chapter. Taking into account the work already developed by our research group in the recovery of antioxidant compounds from the main raw materials of the brewing process [25, 37-39], namely malt, barley and hop, a specific focus is placed on the potential bioactivities of phenolic compounds (particularly ferulic and *p*-coumaric acids) present in BSG, as well as on their extraction techniques.

3.1. Brewer's spent grains

3.1.1. Chemical composition

BSG is basically composed by the husk, pericarp and seed coat layers that covered the original barley grain. Depending of the brewing regime used, some residual starch endosperm, walls of empty aleurone cells and some residues of hops introduced during mashing may be present [7]. It can also include some residues from other cereals eventually used during mashing together with barley, such as wheat, rice, corn or others [8, 9]. Moreover, the barley variety and harvest time may affect the composition of BSG [9, 40, 41]. However, as shown in Table 3.1 there is relatively good consistency with regard to the chemical composition of BSG.

Table 3.1 - Chemical composition of brewers' spent grains

Components / % dry weight basis	Reference			
	[7]	[33]	[42]	[32][a]
Protein	15.3	24.6	19-23	24.69 ± 1.04
Ash	4.6	1.2	4-4.5	4.18 ± 0.03
Cellulose	16.8	21.9	23-25	21.73 ± 1.36
Hemicellulose	28.4	29.6	30-35	19.27 ± 1.18
Lignin	27.8	21.7	7.0-8	19.40 ± 0.34

[a]Values correspond to mean ± SD

A microscopic examination of BSG (Fig. 3.1) reveals the presence of numerous fibrous tissues from the surface layers of the original barley grain [7]. Since the barley malt husk is a lignocellulosic material, BSG is a residue rich in cellulose (a linear homopolymer of glucose units), hemicellulose and lignin (a polyphenolic macromolecule), and also contain high protein content and some lipids [7, 43]. According to Mussatto and Roberto [16], cellulose and hemicellulose comprise almost 50% (w/w) of the BSG composition (Table 3.1), revealing the presence of a large amount of sugars in this material, with xylose, glucose and arabinose being the most abundant. Lignin contains numerous phenolic components, mainly acids such as ferulic, *p*-coumaric, syringic, vanillic and *p*-hydroxybenzoic [17, 31]. The husk also contains considerable amounts of silica of the barley grain [7]. The scanning electron microscopy in Fig. 3.1 reveals that the bright points in the external portion of a BSG particle are silicates [7].

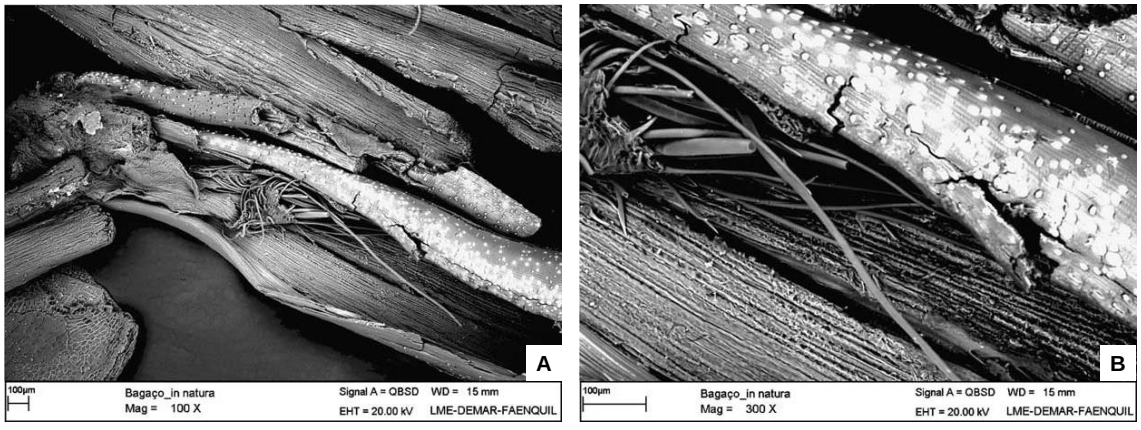

Fig. 3.1 - Scanning electron microscopy of BSG particles. (A) magnification 100 fold; (B) magnification 300 fold (Source: Mussatto *et al.* [7]).

BSG is rich in protein, which accounts for around 20% of its composition [7]. Santos *et al.* [40] found, besides fibre, 24.2% protein, 3.9% lipid and 3.4% ash in oven-dried BSG. Protein and fibre are highly concentrated in spent grains because most of the barley starch is removed during mashing [7]. BSG also contains extractives, a fraction consisting of waxes, lipids, gums, resin, tannins, essential oils and various other cytoplasmatic constituents. Lipids include triacylglycerols, diacylglycerols, fatty acids (palmitic, oleic and linoleic acids), sterols, sterol esters and sterol glycosides, plus various hydrocarbons (including alkanes and carotenoids) [3].

In addition to the components detailed in Table 3.1, it has been shown that BSG is also a valuable source of minerals, vitamins and amino acids [7, 9]. High amounts of phosphorus, calcium, sulphur and magnesium were reported to be 5186, 3515, 1980 and 1958 mg L^{-1}, respectively, while other minerals (such as sodium, potassium, iron, zinc, manganese, aluminum and copper) detected in BSG were present in concentrations lower than 309 mg L^{-1} [8, 9]. Vitamins, such as biotin (0.1), choline (1800), folic acid (0.2), niacin (44), pantothenic acid (8.5), riboflavin (1.5), thiamine (0.7) and pyridoxine (0.7), are also present in this material (amounts expressed in mg L^{-1}). Protein bound amino acids present in the largest amounts include leucine, valine, alanine, serine, glycine, glutamic acid and aspartic acid, whereas in smaller amounts were detected tyrosine, proline, threonine, arginine, and lysine. Cystine, histidine, isoleucine, methionine, phenylalanine, and tryptophan can also be present [7-9, 44].

3.1.2. Techniques for BSG preservation and storage

Several methods have been proposed to prolong BSG storage time as a result of its high moisture content. In average BSG has a water content above 70% (w/w), but the moisture level of different portions of the grain may vary considerably [9, 40, 42]. Due to its high moisture and fermentable sugar contents, BSG is a very unstable material and is liable to deteriorate rapidly. Drying is a possible alternative for BSG preservation with the advantage of reducing the product volume and, therefore, decreasing transport and storage costs [7, 40, 45]. Nowadays, many breweries have plants for drying BSG. The process consists of two steps: pressing (to get a material with <65% humidity) and drying (to get a material with <10% humidity) [7, 45]. However, this traditional process is based on the use of direct rotary-drum dryers (Fig. 3.2), a procedure considered to be very energy-intensive [45].

Fig. 3.2 - Rotary-drum dryer equipment.

Bartolomé *et al.* [30] evaluated the main differences between three methods (freeze-drying, oven-drying and freezing) for preserving BSG. They have concluded that freezing is not a good method because large volumes must be stored and alterations in arabinose content may occur. Preservation by oven-drying or freeze-drying reduces the volume of the product and do not alter its composition. But overall, freeze-drying is economically not feasible at the large scale. Nevertheless, oven-drying also presents some disadvantages. At higher temperatures (above 60 ºC) unpleasant flavors can be generated. Further, there could be the risk that the grain temperature is near to the dryer exit leading to toasting or burning of the dried grains [7, 9].

Manufactures of drying equipment are looking for most effective techniques, and Tang *et al.* [46] proposed thin-layer drying using superheated steam as an alternative method. The circulation of superheated steam occurred in a closed-loop system, reducing the energy wastage. Also, the exhaust steam produced from the evaporation of moisture from the BSG can be used in other operations. Other advantages include reduction in environmental impact, improvement in drying efficiency, elimination of fire or explosion risk, and recovery of valuable volatile organic compounds [45, 46]. Another method is the use of membrane filter press proposed by El-Shafey *et al.* [47]. In this process, BSG is mixed with water and filtered at a feed pressure of 3 to 5 bar, washed with hot water (65 ºC), membrane-filtered and vacuum-dried to reach moisture levels between 20 and 30%. No bacterial activity was observed after storing the cake in the open air for 6 months.

3.1.3. Potential uses

Although BSG is the main by-product of the brewing process, it has received little attention as a marketable commodity, and its disposal is often an environmental issue. Nevertheless, due to its chemical composition (Table 3.1), several attempts have been made to use them in biotechnological processes. Some possible applications are described below.

3.1.3.1. Food ingredient

As aforementioned, the main application of BSG has been as animal feed (mainly for cattle). The presence of cellulose, hemicellulose and lignin, and also the amount of readily available substances, such as sugars and amino acids, aid in its utilization as feed for ruminants. BSG consumption has been investigated for a range of animals, including poultry, pigs, fish and chicken [7, 48, 49]. Kaur and Saxena [50] reported the incorporation of BSG in supplementary fish feed, replacing rice bran. It was observed that fish fed with a diet containing rice bran and 30% BSG had a superior body weight gain when compared with fish fed with rice bran only. According to these authors, the better growth performance was due to the increased content of proteins and essential amino acids provided by the BSG. In addition to its high nutritional value, BSG is reported to promote increased milk production [43, 48]. Belibasakis and Tsirgogianni [48] found that BSG supplementation (45% w/w) increased actual milk yield, milk total solids content and milk fat yield when compared to control containing maize silage (45% w/w) [48, 49]. Blood plasma concentrations of glucose, total protein, albumin, urea, triglycerides, cholesterol, phospholipids, sodium, potassium, calcium, phosphorus and magnesium were not affected [7, 48].

Since BSG is derived from materials utilized for humans, it can be incorporated into human diets, such as breads and snacks, especially where there is need to boost the fibre contents, decrease the calorific content and increase the protein content of food products [7, 9]. High protein flour prepared from BSG can be successfully incorporated into a number of bakery products, such as breads, muffins, cookies, among others [9]. Stojceska and Ainsworth [51] observed an increase in fibre content (from 2.3 to 11.5%) when 30% BSG was incorporated into wheat flour for the production of high-fibre enriched breads. However, the degree of softening and loaf volume were lower than the one containing only wheat flour. Öztürk *et al.* [52] also studied the incorporation of BSG of different particle size at 5 to 25% level into wheat flour for the production of wire-cut cookies. The results indicated a proportional increment between the particle size of the BSG and the dietary fibre content. The ingestion of BSG or its derived products provides health benefits, which are associated with increased fecal weight, accelerated transit time, increased cholesterol and fat excretion and decrease in gallstones [7]. Vietor *et al.* [53] reported that the addition of spent grains to rat diets was beneficial to intestinal digestion, alleviating both constipation and diarrhoea. They attributed those effects to the content of glutamine-rich protein, and to the high content

of noncellulosic polysaccharides (arabinoxylan, 20–47%) and smaller amounts (less than 1%) of (1→3, 1→4)-β-glucans. These results demonstrate that BSG is a cheap source of protein and fibre that may provide benefits when incorporated in human diets, showing to be a potentially important food ingredient [7].

3.1.3.2. Bioethanol production

Another proposed use for BSG is in bioethanol production. Bioethanol is an alcohol produced by fermenting the sugar components of plant materials (renewable biomass). It is produced mostly from starch and sugar-based crops as well as lignocellulosic biomass. Most of the starch and sugar-based crop (molasses, maize starch, sugarcane, rice, wheat, etc) compete with human food production and have high production prices that restrict their industrial production. With the growing demand for ethanol, the search for a cheaper and abundant substrate, as well as the development of an efficient and less expensive technology increased the number of investigations in this reuse [36, 54-56]. The composition of BSG, described in the Section 3.1.1, containing primarily grain husks and other residual compounds such as hemicelluloses, cellulose and lignin, makes it a good low-cost feedstock for ethanol production. However, the structural complexity of lignin, its high molecular weight, chemical stability and insolubility make the biodegradation of this lignocellulosic substrate quite difficult. BSG can be used in energy production, either by direct combustion or by fermentation to biogas (a mixture of 60–70% CH_4, 40–30% CO_2 and small fractions of H_2, N_2, H2S and CO) [7].

Current advances for the conversion of residues like BSG to ethanol require chemical or enzymatic hydrolysis to produce fermentable sugars, followed by microbial fermentation. Thus, large amounts of enzymes required for enzymatic conversion of cellulose to fermentable sugars impacts severely on the cost effectiveness of this technology [55]. Xiros *et al.* [55] and Xiros and Christakopoulos [56] found that *Neurospora crassa* and *Fusarium oxysporum* have an exceptional ability of converting cellulose and hemicellulose directly to ethanol through the consecutive steps of hydrolysis of the polysaccharides and fermentation of the resulting oligosaccharides by secreting all the necessary enzyme systems. They reported an ethanol yield of 74 and 109 g/kg of dry BSG by *N. crassa* and *F. oxysporum*, respectively, under microaerobic conditions [55, 56].

Recently, the Carlsberg Research Centre in Copenhagen has developed a new pilot biogas plant with the purpose of generating energy from BSG based in the anaerobic fermentation (Fig. 3.3). For that, they have used a Continuous Stirred Tank Reactor (CSTR), which is the most used reactor for biogas production, where occurs the anaerobic biodegradability of BSG. This process is divided into the hydrolysis of the fibre material in BSG, the acidogenic and methanogenic steps. In the acidogenic phase, carbohydrates, amino acids and fat are converted to the organic acids, and subsequently, methanogenic bacteria convert these organic acids to methane. In 2012, they have reported that around 3.5% of their thermal energy consumption came from renewable sources such as biogas captured from wastewater treatment and biomass [57]. Thus, BSG can be used to generate a wide range of feedstock materials to supplement current bioethanol production from starchy feedstock.

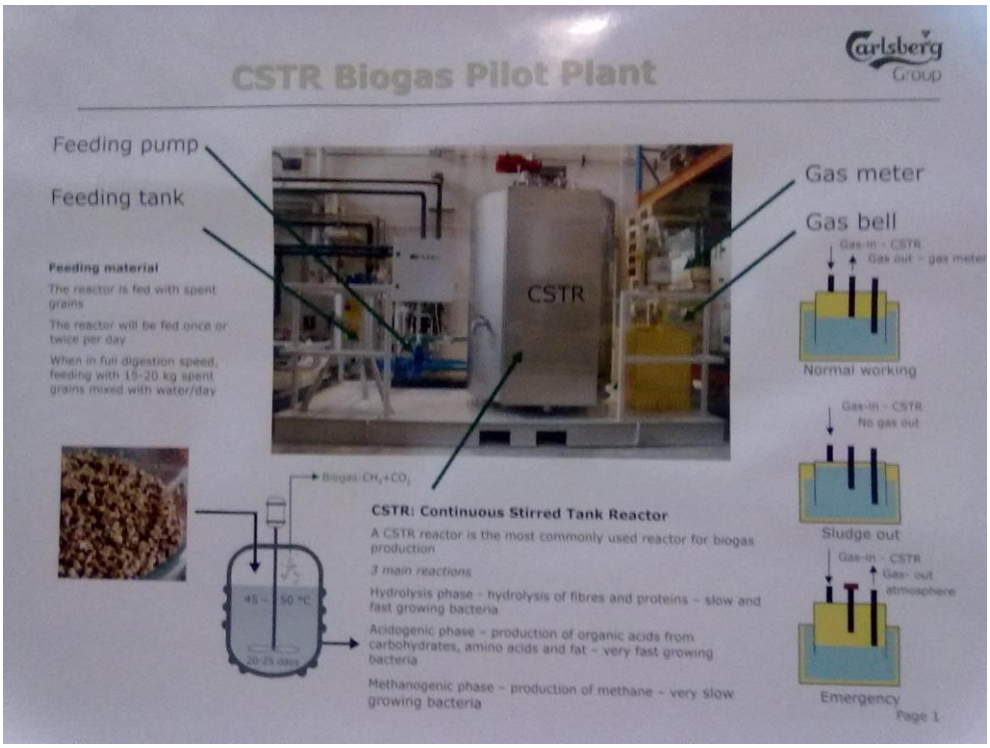

Fig. 3.3 - Biogas pilot plant proposed by the Carlsberg Research group.

3.1.3.3. Substrate for microorganisms and enzyme production

The polysaccharide, protein and high moisture contents of BSG make it susceptible for cultivation of microorganisms. Several studies have demonstrated that BSG is a suitable medium for isolation and maintenance of unknown strains and highly suitable for screening and production of new biologically active substances [7, 8, 41, 58]. Wang *et al.* [58] proposed that BSG favors the growth of *Pleurotus ostreatus* not only due to

its high protein content, but also to its high moisture content and physical properties, such as particle size, volume weight, specific density, porosity and waterholding capacity.

BSG, which has composition and physical structure similar to other cereal brans, has also been evaluated as an alternative substrate for the production of commercial enzymes in so called solid-state fermentations [7]. The substrate composition as well as the strain used will determine the enzyme type and activity produced [59]. BSG has digestible and non-digestible organic residues in its composition becoming a potential substrate on which amylolytic organisms could be cultured for the production of β-amylase and amyloglucosidase. Other enzymes of interest include xylanases, feruloyl esterases and α-L-arabinofuranosidases. As previously mentioned, BSG is rich in hemicellulose and its components constitute 1,4-β linked xylose backbone with heterogeneous substituents such as L-arabinose, O-acetyl, FA, *p*-CA acid and 4-O-methylglucuronic acid [7, 8]. Complete breakdown of these components by microorganisms requires the action of several enzymes which are recognized as a xylanolytic system/complex [60]. Feruloyl esterases act synergistically with xylanases and other cell wall degrading enzymes to digest the plant cell walls and facilitate the access of hydrolases to the backbone of the wall polymers. Thus, BSG has been effectively used as a carbon source for feruloyl esterase and xylanolytic enzyme production by *Talaromyces stipitatus* and *Penicillium janczewskii*, respectively [59, 60]. *Streptomyces avermitilis* CECT 3339 also produces feruloyl esterase and (1→4)-β-D-xylan xilanohydrolase (xylanase) while growing on BSG [61].

3.1.3.4. Additive or carrier in brewing

The reuse of BSG in the brewing process can be attractive from the point of view of brewery economics. In 1976, Roberts [62] showed that a BSG extract (concentrate from the pressings of BSG) was effective as an antifoaming agent in the fermentor. Additionally, the hop utilization was improved and the properties of the final beer were not adversely affected. Another possibility is the addition of a neutralized extract of BSG to wort which resulted in increased yeast performance and produced beer of quality equal to that of beer fermented without spent grains [7, 8].

Brányik *et al.* [63] proposed another alternative for BSG reuse in breweries. These authors developed a simple method to obtain a cellulose based carrier from BSG, sequentially pre-treated with HCl and NaOH solutions, to be used for brewing yeast

immobilization in a continuously operating bubble-column reactor. The results obtained suggest that BSG was very efficient, due to its high yeast loading capacity. BSG is irregular in shape and non-homogeneous in chemical composition, providing 'active sites' that are readily colonized by yeasts [8, 64, 65]. It also presents advantages from an economic viewpoint, due to its ease of preparation, stability and the fact of being a brewery by-product not requiring investments. Further, BSG meets the requirements of food grade, its inert under fermentation conditions, and it's possible to regenerate by simple washing in caustic [63-65].

3.1.3.5. Source of added-value products

Arabino-oligoxylosides production

As described in Section 3.1.1, the cell walls of the BSG are rich in cellulose and non-cellulosic polysaccharides, which can be degraded into their corresponding constituents by hydrolytic procedures (hydrothermal, enzymatic or acidic) [7, 8]. On the hydrolysis treatment of BSG, cellulose yields glucose and the non-cellulosic polysaccharides produce xylose, mannose, galactose and arabinose, as well as acetic and hydroxycinnamic acids [15, 66, 67]. A wide variety of arabino-oligoxylosides with different structural features can be obtained according to the hydrolysis process used [8]. Hydrothermal hydrolysis (autohydrolysis by acetic acid released from its esterified form on the arabinoxylans) treatment of BSG with water produced a wide variety of arabino-oligoxylosides with different molecular weight distributions, which depend on the temperature and reaction time employed [33]. The higher thermal sensitivity of the arabinose components compared to xylose, leads to release of large amounts of free arabinose when the temperature of the process is increased [7, 33]. These compounds are of industrial interest, mainly in the food industry. They can be purified for immediate use, or can be applied as precursors of food grade chemicals or as energy sources in microbial fermentations.

Hydroxycinnamic acids (ferulic and p-coumaric acids) extraction

Ferulic (4-hydroxy-3-methoxycinnamic acid; FA) and *p*-coumaric acids (4-hydroxycinnamic acid; *p*-CA) are the most abundant hydroxycinnamic acids present in BSG, with potential uses in the food industry [17, 30-32, 68]. FA and *p*-CA exhibit a number of potential applications and health benefits [69-72], such as natural antioxidant, food preservative/antimicrobial agent, anti-inflammatory agent,

chemoprotectant and antioxidant properties, which will be discussed in more detail in Section 3.2.2. Furthermore, the extractions methods for the recovery of these hydroxycinnamic acids from BSG will be discussed in Section 3.3 [61, 68, 73-75]. Thus, there is a strong interest in the utilization of hydroxycinnamic acids as feedstock for bioconversion into other value added products such as styrenes, polymers, epoxides alkylbenzenes, vanillic acid derivatives, guaiacol, catechol and vanillin. This opens up new possibilities for the use of this brewery by-product [7].

Xylitol production

Xylitol is a rare sugar that exists in low amounts in nature. It is an alternative to sucrose as a sweetener with many health benefits, such as the ability to combat dental caries, to treat illnesses (such as diabetes, disorders in lipid metabolism and parenteral and renal lesions) and to prevent lung infection [15]. Xylitol can be produced by fermentation from xylose in hydrolysates of BSG. When compared with other lignocellulosic materials, BSG appears to be a more economical alternative to produce xylitol, because it requires no preliminary detoxification steps. In addition, the overall production is favoured by high initial xylose concentrations, oxygen limitation, high inoculum density and appropriate medium supplementation [76]. BSG has been reported to be easily and readily utilized as a fermentation medium by the yeasts *Debaryomyces hansenii* [77, 78] and *Candida guilliermondii* where they grow and produce xylitol [76]. Thus, production of xylitol from BSG by yeasts is a potential option to upgrade this residue.

Lactic acid production

Lactic acid (2-hydroxypropanoic acid) has found many applications in food industries as an acidulant, flavor and preservative, also in the pharmaceutical, leather and textile industries. Recently, there has been an increasing interest in lactic acid production because it can be used as a starting material for manufacture of biodegradable poly-lactate polymers. However, the growth rate of this market increases the demand for lactic acid production at the lowest cost possible [67]. One of the major challenges in the large-scale production of lactic acid is the cost of the raw material. The use of glucose, sucrose or starch as carbon sources is not economical sustainable because lactic acid is a relatively cheap product. Thus, the exploitation of less expensive sources would be beneficial. The agroindustrial residues are attractive alternatives to substitute these costly raw materials [67, 79]. Mussatto *et al.* [67, 79]

evaluated the use of BSG as a raw material for lactic acid production. Enzymatic saccharification of pre-treated BSG was applied to produce the hydrolysate used as fermentation medium. This hydrolysate was used without any nutrient supplementation by *Lactobacillus delbrueckii* which produced 5.4 g/L of lactic acid at 0.73 g/g glucose consumed (73% efficiency) [67].

3.1.3.6. Other applications

A range of other uses for BSG has also been proposed. Recently, Okamoto *et al.* [80] developed a process for producing charcoal bricks from BSG, and evaluated their physical and chemical properties. BSG (67% water content) was dried, pressed at high temperature and pressure to make bricks without any binder and carbonized in a low oxygen atmosphere. The charcoal bricks produced contained various minerals, had a constant quality and a high yield and calorific value (27 MJ/kg), which compared favorably with the calorific value of charcoals produced from other raw materials.

Another possibility for the disposal of BSG is to use them to increase the porosity in brick [42]. The low amount of ash coupled with the high amount of fibrous material (lignin, hemicellulose and cellulose) makes BSG suitable for use in building materials. Russ *et al.* [42] observed that BSG increase the porosity of bricks, improved their dry characteristics, but did not influence color or compromise brick quality, making BSG suitable for use in building materials.

Adsorption processes must be fast, efficient, and use cheap adsorbents to compete with other techniques. Due to its low cost and easy availability, BSG has been tested as an adsorbent for several types of compounds [7]. Pyrolysed spent grains were used as an adsorbent for removing volatile organic compounds from waste gases [7]. BSG have also been utilized as adsorbents for heavy metals from aqueous solutions. Low *et al.* [81] investigated the adsorption of cadmium and lead by BSG and found maximum adsorption capacities of 17.3 and 35.5 mg/g, respectively. BSG was also tested as an adsorbent of acid orange 7 dye (AO7), a monoazo acid dye currently used in paper and textile industries, whose presence in effluents causes environmental problems [82].

3.2. Brewer's spent grains phenolic compounds

Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups. They are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants [71, 83]. These compounds are widely distributed in the plant kingdom, with more than 8000 phenolic structures currently known, ranging from simple molecules, such as phenolic acids, to highly polymerized substances, such as tannins. They play an important role in growth and reproduction, providing protection against pathogens, parasites and predators, as well as contributing to overall organoleptic properties of plant foods. In addition, polyphenols also have several industrial applications, such as in the production of paints, paper, and cosmetics, as tanning agents, and in the food industry as additives (as natural colorants and preservatives) [83]. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects [83-85]. The beneficial effects derived from these compounds have been attributed to their antioxidant activity [71, 86]. Dietary phenolic compounds exert their health benefits by various biological effects such as free radical scavenging, metal chelation, reducing potential, chain breaking, and modulation of enzymatic activity [71].

As previously mentioned, BSG consists predominantly of the husk, pericarp and seed coat and is largely made up of cell walls [7, 8]. Since most of the phenolic compounds of the barley grain are contained in the husk [38] and hydroxycinnamic acids accumulate in the cell walls, BSG is a potentially valuable source of these compounds [17, 30, 32, 87, 88]. These constituents, which have been considered to be the most important source of antioxidants in cereals, exist in free as well as in the bound form. The majority of free phenolics in barley are flavanols, whereas the bound phenolics are mainly phenolic acids [37, 38, 41, 87, 88]. In BSG, phenolic acids are the major phenylpropanoid components reported [17, 30, 32, 41, 87, 88]. In this section the chemistry of main phenolic compounds present in BSG, as well as the biological activity associated to them, will be discussed.

3.2.1. Chemistry

In nature, phenolics are usually found in conjugated form, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist [83]. The associated sugars can be present as

monosaccharides, disaccharides, or even as oligosaccharides, with glucose being the most common sugar residue. Associations with other compounds, such as carboxylic and organic acids, amines, and lipids, and linkages with other phenols are also common [83, 89]. Depending on the number of phenol rings that they contain and on the structural elements that bind these rings to another, polyphenols can be divided into at least 12 different classes [83, 89, 90]. Table 3.2 illustrates the basic chemical structure of the main phenolic compounds.

Table 3.2 - Main classes of phenolic compounds

Class	**Structure**
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6–C_1
Acethophenones, phenylacetic acids	C_6–C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6–C_3
Napthoquinones	C_6–C_4
Xanthones	C_6–C_1–C_6
Stilbenes, anthraquinones	C_6–C_2–C_6
Flavonoids, isoflavonoids	C_6–C_3–C_6
Lignans, neolignans	$(C_6–C_3)_2$
Biflavonoids	$(C_6–C_3–C_6)_2$
Lignins	$(C_6–C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6–C_3–C_6)_n$

Further, flavonoids (Fig. 3.4), which share a common structure consisting of 2 aromatic rings (A and B) that are bound together by 3 carbon atoms that form an oxygenated heterocycle (ring C), may themselves be divided into 6 subclasses as a function of the type of heterocycle involved: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins). Despite this structural diversity, this group of compounds is often referred to as 'polyphenols'.

Of these polyphenols, phenolic acids and flavonoids are regarded as the main dietary phenolic compounds [89].

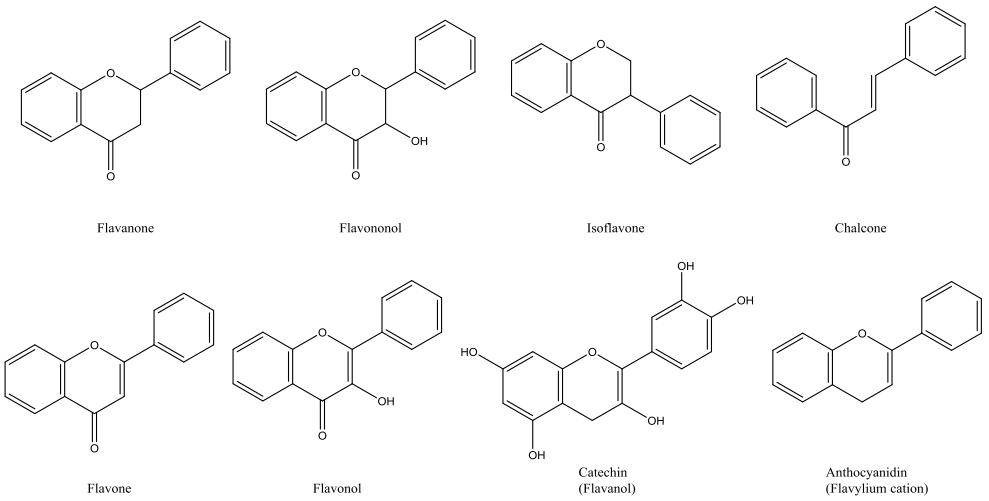

Fig. 3.4 - Chemical structures of flavonoids.

3.2.1.1. Hydroxybenzoic acids

Phenolic acids can be distinguished in two subgroups, i.e., the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids (HBAs) are phenolic metabolites with a general C_6–C_1 structure. Variations in the basic structure of these acids include hydroxylations and methoxylations of the aromatic ring, leading to the formation of gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids. The chemical structures of these HBAs are represented in Fig. 3.5. Although HBAs can be detected as free acids in barley and malts, as a general rule they are present in the insoluble-bound form [37, 38, 91]. They are often the component of a complex structure like lignins and hydrolyzable tannins. HBAs are also found in the form of organic acids and as sugar derivatives [90, 91].

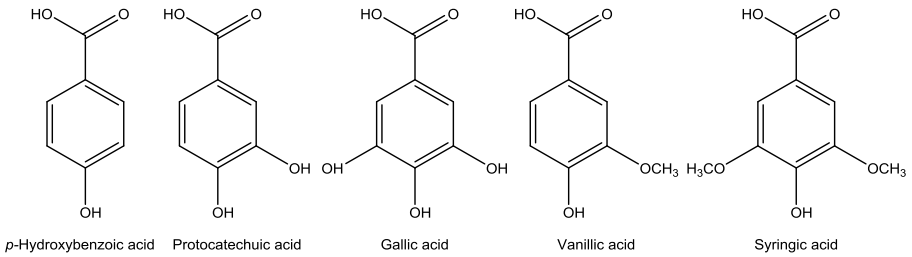

Fig. 3.5 - Chemical structure of hydroxybenzoic acids.

The HBA content of BSG is generally very low when comparing to the amount of hydroxycinnamic acids [87, 88, 92]. The levels of HBAs present in BSG are reported in

Table 3.3. Several authors found different levels of these phenolics in BSG, but all reported that the most abundant HBA is syringic acid. *p*-Hydroxybenzoic, vanillic and protocatechuic acids were also found, but in lesser amounts. These differences in phenolic acids content between the Athanasios *et al.* [92] and Szwajgier *et al.* [87] can be explained by the variety, time of harvest and the characteristics of the growing region from BSG.

Table 3.3 - Content of hydroxybenzoic acids in BSG

Hydroxybenzoic acids / mg/100 g of dry matter	**Reference**	
	[87]	[92]
Syringic acid	6.5 ± 0.1	6.84 ± 0.07
p-Hydroxybenzoic acid	1.2 ± 0.6	1.15 ± 0.02
Vanillic acid	NR	3.49 ± 0.01
Protocatechuic acid	0.5 ± 0.1	NR

Mean values of three replicates ±SD; NR: Not Reported

3.2.1.2. Hydroxycinnamic acids

Hydroxycinnamic acids (HCAs) are aromatic compounds with a phenyl ring (C6) and a C3 side chain which serve as precursors for the synthesis of lignins and many other compounds [91]. The structures of HCAs are shown in Fig. 3.6 . As for the HBAs, hydroxycinnamates are intermediates in the phenylpropanoid synthetic pathway and occur in the free, soluble esterified/etherified and insoluble-bound forms [37, 93, 94]. HCAs found in the insoluble-bound form are attached to the plant cell wall structural components such as cellulose, lignin, and proteins through ester bonds, whereas soluble forms are in the cytoplasm. The common types of reported conjugates are esters and amides, whereas glycosides rarely occur [91].

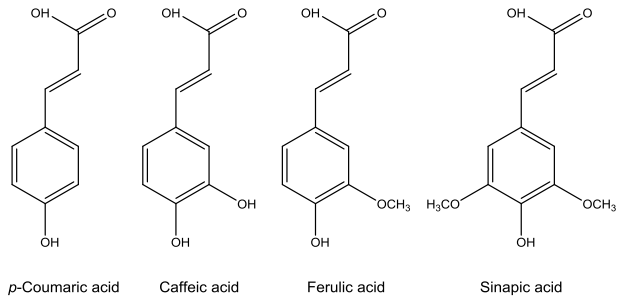

Fig. 3.6 - Chemical structure of hydroxycinnamic acids.

The occurrence of hydroxycinnamates in BSG is given in Table 3.4 There is evidence that FA and *p*-CA are the most abundant members among HCAs present in BSG [17, 30, 32, 41, 87, 88, 92].

Table 3.4 - Content of hydroxycinnamic acids in BSG

Hydroxycinnamic acids / mg/100 g of dry matter	Reference			
	[87]	[92]	[95]	[41]
Ferulic acid	336.3 ± 16.0	271.6 ± 20.2	195 ± 14	490 ± 30
p-Coumaric acid	64.4 ± 4.6	137.3 ± 11.5	74 ± 6	180 ± 60
Sinapic acid	42.0 ± 1.1	NR	NR	NR
Caffeic acid	9.9 ± 0.7	NR	NR	NR

Mean values of three replicates ±SD; NR: Not Reported

Recent studies show that BSG consists of 1.16% mono and dimeric phenolic acids, with 53% of the monomeric phenolic acids accounted for FA. Also, the vast majority of phenolic acids were found to be in the bound form [37]. It has been reported that following ferulic and *p*-coumaric acids, the next most abundant HCA in BSG was found to be sinapic, followed by caffeic acid [87]. Until now, all reports are consistent with the information that FA is the main phenolic compound from BSG. Thus, more detailed data concerning the structure, as well as the beneficial properties associated to FA will be discussed.

FA was first isolated from a commercial resin in 1866 and chemically synthesized in 1925 [72, 96]. FA results from the phenylpropanoid metabolism and is, in general, uniformly distributed across the pant cell wall, and is particularly abundant in epidermis, xylem vessels and sclerenchyma [96]. Its synthesis, represented in Fig. 3.7, can be accomplished by two routes, via a phenylalanine or tyrosine [91, 96]. Through phenylalanine route (Fig. 3.7A), the enzyme phenylalanine ammonia lyase (PAL) catalyzes the release of ammonia from phenylalanine and leads to the formation of a carbon-carbon double bond, yielding *trans*-cinnamic acid. Introduction of a hydroxyl group into the *para* position of the phenyl ring of cinnamic acid proceeds via catalysis by monooxygenase utilizing cytochrome P_{450} as the oxygen binding site. The *p*-CA formed may be hydroxylated further in positions 3 and 5 by hydroxylases and possibly methylated via O-methyl transferase with S-adenosylmethionine as methyl donor; this leads to the formation of caffeic and ferulic acids. In some plants and grasses tyrosine is converted into *p*-CA via the action of tyrosine ammonia lyase (TAL) (Fig. 3.7B). After this step, *p*-CA undergoes the remaining steps as those used in phenylalanine pathway yielding FA [91, 96].

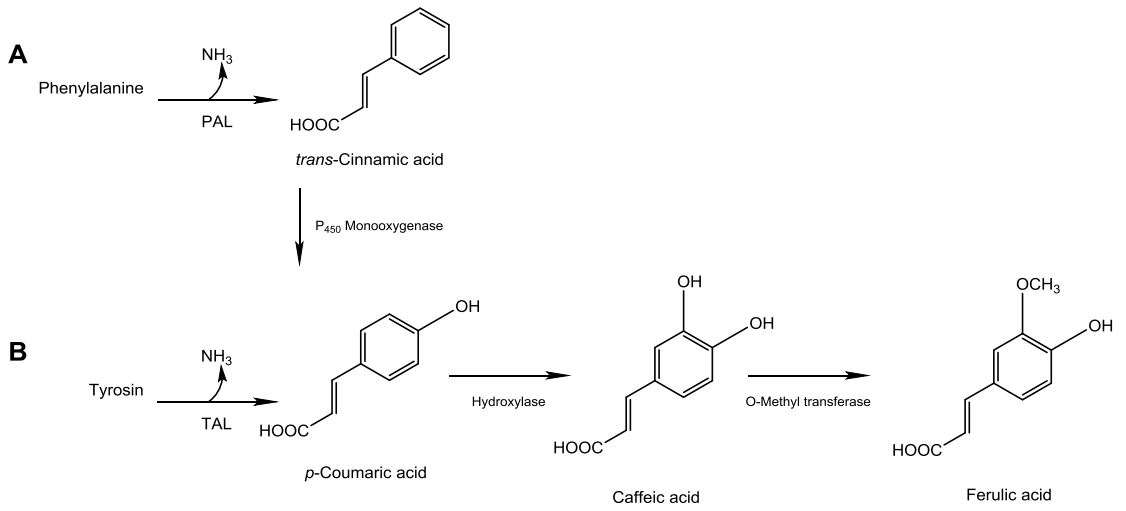

Fig. 3.7 - Formation of FA from phenylalanine (A) and tyrosine (B). PAL denotes phenylalanine ammonia lyase and TAL denotes tyrosine ammonia lyase (Source: Shahidi & Naczk [91] and Zhao & Moghadasian [96]).

The antioxidant potential of FA can usually be attributed to its structural characteristics (Fig. 3.8). Because of its phenolic nucleus and unsaturated side chain FA can readily form a resonance stabilized phenoxy radical, which accounts for its potent antioxidant activity. Any reactive radical colliding with FA easily abstracts a hydrogen atom to form phenoxy radical. This radical is highly resonance stabilized since the unpaired electron may be present not only on the oxygen but it can be delocalized across the entire molecule. Additional stabilization of the phenoxy radical is provided by the extended conjugation in the unsaturated side chain. This resonance stabilization accounts for the effective antioxidant potential of FA. Moreover, this phenoxy radical is unable to initiate or propagate a radical chain reaction, and its most probable fate is a collision and condensation with another ferulate radical to yield the dimer curcumin. Such coupling may lead to a host of products, all of which still contain phenolic hydroxyl groups capable of radical scavenging. The presence of a second phenolic hydroxyl group substantially enhances the radical scavenging activity due to additional resonance stabilization and o-quinone formation [94, 96].

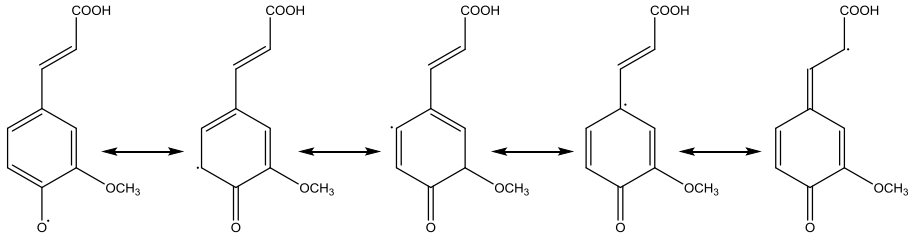

Fig. 3.8 - Resonance stabilization of ferulic acid radical (Source: Kylli *et al.* [94]).

As aforementioned, FA is the major phenylpropanoid component in BSG, and different levels can be found in different fractions of cereals. In cereals, the starchy endosperm contains low levels, whereas the outer layers of the grain (pericarp, aleurone layer, and germ) contain the highest. FA is mostly concentrated in the aleurone layer and in the pericarp [37, 87]. As can be seen in Fig. 3.9, FA is covalently cross-linked to cell wall polysaccharides by ester bonds and to components of lignin by ether or ester bonds [71, 97]. According to Max *et al.* [97], as ethers bonds are resistant to mild alkaline hydrolysis but acid-labile, this difference in stability of the ester and ether bonds is useful for the separation of ester- and ether-bounded FA. These authors recommended a prehydrolysis step for the cleavage of ether bond to release the ether-bonded FA (Fig. 3.9b). On the contrary, mild alkaline hydrolysis with NH_4OH, KOH or NaOH serves to release ester-bonded FA (Fig. 3.9a).

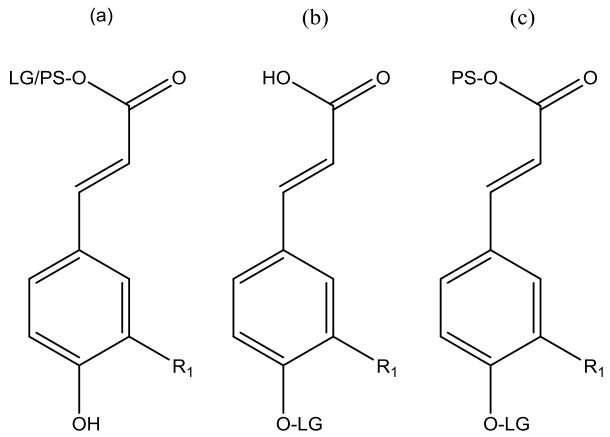

Fig. 3.9 - Schematic representation of linkages of ferulic acid to lignin (LG) and/or polysaccharides (PS): (a) ester bonds, (b) ether bonds, and (c) ester-ether bridges. $R_1 = OCH_3$ (Source: Max *et al.* [97]).

Arabinoxylans possess a β-(1→4)-linked xylopyranosyl backbone substituted with α-L-arabinofuranosyl residues. These arabinose molecules may be esterified with HCAs, monomeric or dimeric FA and *p*-CA [30, 41, 73]. FA is covalently linked to arabinose residues in arabinoxylans by ester bonds between the carboxyl group from FA and hydroxyl group of C_5 from side chain from arabinose (Fig. 3.10A). This also occurs for the following polysaccharides: xyloglucans and pectins (Fig. 3.10B). Robertson *et al.* [41] have estimated that 1 in 12 arabinose units is esterified to a ferulate-based component in BSG. They reported that around 60% of the esterified ferulate is present as FA, with the remaining 40% involved in dehydrodimer crosslinking. These results confirm that BSG is a highly crosslinked material.

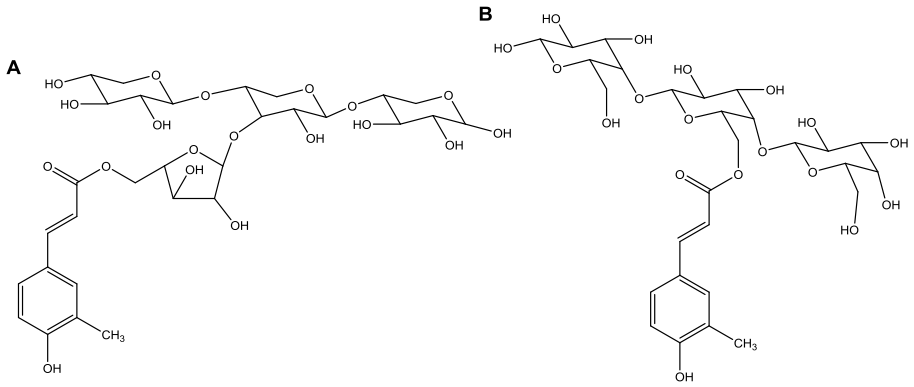

Fig. 3.10 - Ferulic acid ester bond to arabinose (A) and to the side chain of a galactose residue in the structure of pectin (B) (Source: Robertson *et al.* [41]).

FA has also been shown to occur in dimer- and trimerized forms through oxidative (peroxidase-catalyzed) coupling between esterified and/or etherified FA residues [68, 98]. Recently, dimers and trimers of FA have been found in BSG by Hernanz *et al.* [95] and Faulds *et al.* [73]. These cross-links may contribute to the control of cell wall extensibility and cell-cell adhesion, having significant effects on the texture of plant-derived foods. However, such compounds may also limit cell wall degradability by rumen microorganisms, thus decreasing the digestibility of plant cell walls by ruminants.

As previously mentioned, FA and *p*-CA are the phenolic acids at highest concentrations in BSG (Table 3.3 and Table 3.4). In recent years, extensive research has been conducted looking at the antioxidant activity of HCAs, particularly FA and *p*-CA [71]. Some of the existing literature regarding the beneficial effects of HCAs will be discussed in Section 3.2.2.

3.2.2. Biological activity

Oxidative stress (Fig. 3.11), the consequence of an imbalance of prooxidants and antioxidants in the organism, is considered as the leading cause in the development of degenerative conditions [71, 99]. A particularly destructive aspect of oxidative stress is the production of reactive oxygen species (ROS), which include free radicals and peroxides that damage all components of the cell, including proteins, lipids, and DNA [99]. To protect against this oxidative damage the antioxidant defenses available within the cell and extracellular should be adequate. However, the balance can be lost because of overproduction of free radicals by exposure to sources that overwhelm the antioxidant defenses, or by inadequate intake of nutrients that contribute to the defense

system [99]. Several investigations have led to the conclusion that diet is the key environmental factor and the potential tool for the control of chronic diseases. Fruits and vegetables have been shown to exert a protective effect. These effects have been attributed to the high content of antioxidants, vitamins C and E, α-tocopherol, β-carotene and phenolic compounds present in fruits and vegetables [69, 89]. Therefore, phenolic compounds, mainly HBAs, present in BSG can help limit the oxidative damage and acting directly on ROS or by stimulating endogenous defense systems [71].

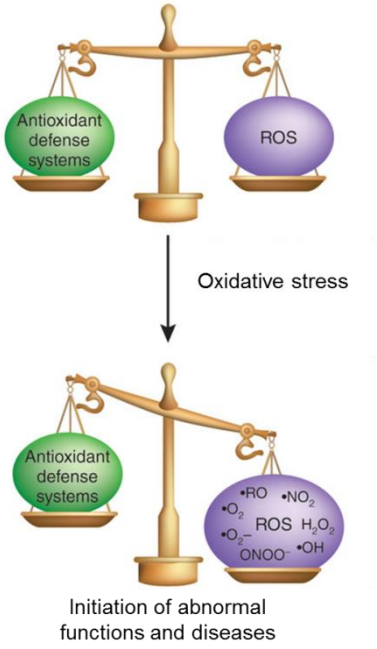

Fig. 3.11 - Oxidative stress balance.

Several authors study the relationship between the presence of HCAs and the beneficial health effects exercised through their antioxidative activities. Chen and Ho [100] have shown that FA act as an antioxidant *in vitro* and scavenged radicals including DPPH (1,1-diphenyl-2-picrylhydrazyl) and the superoxide anion. However, FA was a less potent antioxidant than caffeic acid and α-tocopherol [70]. Similarly, but using the β-carotene linoleic acid model system, Kim *et al.* [101] investigated the phenolic compounds in wheat bran extract and their antioxidant activity, confirming that FA was one of the strongest antioxidants using. They concluded that the wheat bran extracts with highest FA concentrations exhibited higher antioxidant activity.

FA and caffeic acid have been reported to have excellent antioxidant potential at low concentrations, with the ability to scavenge a range of free radicals. Both phenolic acids scavenge the ROS and reactive nitrogen species, with concentration-dependent scavenging of NO, superoxide and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical [102]. In a recent study on beers, a direct correlation was found between ferric-reducing antioxidant power and a number of phenolic acids including ferulic, *p*-coumaric, caffeic, sinapic and vanillic acids [103]. A second study also showed that some of the phenolic acids present in beer correlate with the antioxidant activity measured by the DPPH radical and superoxide anion scavenging, metal chelation and reducing power [104]. Since FA is so well recognized as an antioxidant, it is approved for use as a food additive in some countries to prevent oxidation in foods [71, 72]. In addition, it is important to highlight that while phenolic compounds can have an antioxidant effect, they have also been shown to act as pro-oxidants under certain

conditions, thus inducing oxidative stress. Recent literature suggests that at low concentrations, many phenolics exhibit pro-oxidant behavior, whereas the synthetic antioxidants, including α-tocopherol, do not [105]. For FA to act as pro-oxidant, higher concentration is required [102]. It has also been found by using the comet assay that at high concentrations *p*-CA enhanced DNA breakage induced by H_2O_2. This may be due to the production of ROS by *p*-CA as a result of its pro-oxidant activity [106]. It has been suggested that this pro-oxidant effect is related to the presence of metal ions in the body (for example due to tissue injury releasing Fe and Cu) and is of relevance for the bioactivity of phenolic compounds *in vi*vo [107]. However, a small number of *in vivo* studies report that the HCAs have antioxidant properties. Therefore, more studies are needed to understand the biological role of these phenolic acids [71].

In addition to their antioxidant potential, there is increasing evidence suggesting that phenolic acids can have an anti-carcinogenic effect. The cyclooxygenase-isoform 2 (COX-2) assay has been used for determination of the anti-cancer potential of these compounds. Overexpression of COX-2 increases the conversion of arachidonic acid to prostaglandins, which are important mediators of inflammation, and are associated with cancer. Phenolic acids, including FA, have been shown to inhibit the expression of COX-2, possibly reducing cancer risk [108]. Apoptosis in cancer cell lines is also an indicator of anti-carcinogenic potential and can be assessed by a number of methods including DNA fragmentation and the Hoechst staining assay. Cinnamic acid derivatives induced apoptosis in human leukaemia (HL60) and colon cancer (SW480) cell lines, as measured by the aforementioned apoptosis methods [109]. In addition, the anti-apoptotic effect of phenolic compounds, including FA and caffeic acid, on human peripheral blood mononuclear cells was investigated and it was shown that pre-treating cells with caffeic acid, FA or ellagic acid before exposure to H_2O_2 inhibited DNA fragmentation [85]. In the inflammatory process, NF-*k*B is a transcription factor, whose increased activation has been reported in several human cancers. Hole *et al.* [110] measured the ability of phenolic compounds to modulate NF-*k*B activity. They reported that free phenolic acids that can be found in cereal grains (including FA and *p*-CA) significantly modulate NF-*k*B activity in U9373*xkB*-LUC cells, with a desired level of modulation being achieved by the synergistic action of phenolic acids and other phenolic compounds.

Animal studies (Fig. 3.12) have also been carried out to determine the anti-carcinogenic potential of phenolic acids. The effect of chlorogenic acid, FA and caffeic acid on tumour promotion in the skin of mice showed that these compounds prevented the number of 12-O-tetradecanoylphorbol-13-acetate-induced tumours per mouse by 60, 28 and 35%, respectively [111].

Fig. 3.12 - The phenolic compounds of beer appear to be the main agents responsible for the inhibition of certain types of diseases.

Cytokines are small cell signaling molecules involved in the inflammatory response, which include interleukins and interferons (for example interferon-γ). The ability of a compound to alter the production of a stimulated cytokine or NO indicates the compound potential to act as an immune-modulator. Sakai *et al.* [112] have shown that FA could inhibit macrophage inflammatory protein-2 and TNFα production induced by lipopolysaccharide in a macrophage cell line. The effect, although dose-dependent, was very weak compared with the effect of dexamethasone (a well-known inhibitor of interleukins). In Japanese Oriental medicines, *Cimicifuga heracleifolia* is often used as an anti-inflammatory drug. FA has been shown to be among the main phenolic acids in *C. heracleifolia*. Sakai *et al*. [113] showed that FA and isoferulic acid could reduce macrophage inflammatory protein-2 production in a dose-dependent manner in RAW264·7 cells. It was suggested that FA and isoferulic acid are responsible, at least in part, for the anti-inflammatory properties of the *C. heracleifolia* drug. Recently published data have shown that FA and *p*-CA inhibited lipopolysaccharide-induced NO production and inducible NO synthase in macrophages [84]. This supports earlier evidence suggesting that these phenolic acids can act as anti-inflammatory agents, by reducing TNFα induced IL-6 production in adipocytes.

Oxidized low density lipoproteins (LDL) are a well-recognized risk marker of cardiovascular disease (CVD) which is principally caused by atherosclerosis. Evidence exists for the effect of HCAs on the inhibition of LDL oxidation. Nardini *et al.* [114] demonstrated the antioxidant effect of HCA derivatives such as caffeic, ferulic and *p*-coumaric acids on LDL oxidation *in vitro*, with the use of Cu^{2+} as a catalyst. At a concentration of 100 μM, all phenolic acids except *p*-CA inhibited LDL oxidation; at 20 μM, FA inhibited about 92% of Cu-catalysed human LDL oxidation; at 5 μM only caffeic acid strongly inhibited the oxidation of LDL. A second study using similar methodology

also found that both FA and *p*-CA showed a dose-dependent inhibition of human LDL oxidation *in vitro* when tested at 5, 10 and 20 μM [115].

Recently, McCarthy *et al.* [116] investigated the ability of phenolic rich BSG extracts to protect against the genotoxic effects of oxidants, H_2O_2, 3-morpholinosydnonimine hydrochloride, 4-nitroquinoline 1-oxide and *tert*-butylhydroperoxide in human lymphocytic U937 cells. U937 cells were pre-incubated with four pale and four black BSG extracts, exposed to the oxidants and the DNA damage was measured by the comet assay. They observed that black BSG extracts and FA significantly protected against H_2O_2-induced DNA damage, demonstrating the antioxidant activity of BSG extracts.

In summary, there is increasing evidence suggesting that phenolic acids, including those found at highest concentrations in BSG, possess health benefits including anti-inflammatory, antioxidant, anti-carcinogenic and anti-atherogenic effects. Therefore, further research on the extraction and characterization of phenolic compounds from BSG is warranted.

3.3. Extraction techniques of phenolic compounds

The analysis and determination of bioactive compounds in plant materials can be divided into different steps, namely sample pretreatment, extraction, isolation, and purification [117, 118]. The first step can include maceration, homogenization, grinding and milling, which may be preceded by air-drying or freeze-drying. Drying can be useful for increasing yield per unit weight of raw material, increasing storage life and decreasing space requirement [117]. However, some drying processes, including freeze-drying, can cause undesirable effects on the constituent profiles of samples; therefore, caution should be taken when planning the sample pretreatment [69]. The maceration, grinding, milling, and homogenization steps can increase the contact surface area between the solvent and the sample containing the solute. These pretreatment steps lead to the breakdown of cellular structures which enhances further the yield of the bioactive compounds. From various extraction studies [7, 9, 30, 45, 46], it is observed that the choice of pretreatment is not coherent. Some of the properties that influence the pretreatment process are polarity, acidity, presence of hydroxyl

groups and aromatic rings, concentration levels and the complexity of the matrix [117, 118].

It is evident that the choice of the proper extraction technique represents the most important step in the recovery and isolation of bioactive phytochemicals from plant materials. The extraction process is influenced by the source and the type of compounds to be extracted, the extraction method and the type of solvent employed, sample particle size, as well as the presence of interfering substances. Additional, a clean-up step may be required for the removal of unwanted phenolics and non-phenolic substances such as sugars, fats, organic acids, and terpenes [118], or in some cases, to pre-concentrate the phenolic compounds [119]. Recently, several research articles have been published on the advancement of different extraction techniques with comparative discussions between them for the recovery of bioactive compounds from cereals samples, such as ultrasound assisted extraction [120], supercritical fluid extraction [121], microwave assisted extraction [117] and pressurized liquid extraction [122, 123]. In this section the main extraction techniques applied for the recovery of phenolic compounds from cereals, especially from BSG, will be discussed.

3.3.1. Solid-liquid extraction

A large number of phenolic compounds have been traditionally extracted from natural sources with organic solvents. Solid-liquid extractions are the most commonly used procedures prior to analysis of phenolics in plant materials, due to their ease of use, efficiency, and wide applicability [86, 122, 124-126].

One of the most common used for extraction of bioactive compounds is hot water bath extraction. Recently, Meneses *et al.* [32] evaluated the efficiency of different solvent compositions (methanol, ethanol, acetone, hexane, ethyl acetate, water, methanol:water mixtures, ethanol:water mixtures, and acetone:water mixtures) for extracting antioxidant phenolic compounds from BSG by using a hot water bath extraction with magnetic agitation. They reported that all the produced extracts showed antioxidant activity, but the extract produced with 60% (v/v) acetone had the most elevated content of total phenols (9.90 ± 0.41 mg GAE/g dry matter).

Alkaline Hydrolysis

Another solid-liquid extraction technique widely used for phenolic recovery from BSG is alkaline hydrolysis [17, 30, 40, 95, 116]. Table 3.5 detail alkaline extraction conditions used to extract phenolic compounds, mainly FA and *p*-CA, from BSG.

Table 3.5 - Efficiency of and conditions used to extract phenolic compounds from BSG using alkaline hydrolysis

Reference	Extraction conditions	FA content	*p*-CA content
Hernanz *et al.* [95]	2 M NaOH, 20 ºC, 16 h under N_2	1948 ± 143 μg/g	794 ± 58 μg/g
Bartolomé *et al.* [30]	1 M NaOH, 20 ºC, 16 h under N_2	0.24% dry weight	0.121% dry weight
Mussatto *et al.* [17]	pre-treatment with H_2SO_4 + 2% (0.5 M) NaOH, 120 ºC, 90 min	9.65 mg/g solubilized lignin	9.22 mg/g solubilized lignin
McCarthy *et al.* [116]	1 M NaOH, RT[2], 16 h	27.3 ± 0.7 mg/mL	NR

[1]NR: not reported; [2]RT: room temperature

Hernanz *et al.* [95] analysed the HCA and FA dehydrodimer content in BSG after an alkali hydrolysis using 2 M NaOH followed by incubation at 20 ºC for 16 h under N_2. They observed that BSG exhibits 5-fold higher levels of FA, *p*-CA and FA dehydrodimers than the unprocessed barley grains, with *p*-CA content ranging from 565 ± 39 to 794 ± 58 μg/g and FA from 1860 ± 190 to 1948 ± 143 μg/g. In 2002, Bartolomé *et al.* [30] evaluated the total pentose (xylose and arabinose) and HCAs (FA and *p*-CA) conten in eight lots of BSG preserved by different methods (freeze-drying, oven drying and freezing). HCA content was determined using the same conditions as Hernanz *et al.* [95], but the NaOH concentration was 1 M. The total alkali-extractable HCA content of the samples varied between 0.17 and 0.24% dry weight for FA, and between 0.068 and 0.121% dry weight for *p*-CA. Mussatto *et al.* [17] investigated the simultaneous effects of the variables NaOH concentration (1.0, 1.5 and 2.0%, w/v), temperature (80, 100 and 120 ºC), and reaction time (30, 60 and 90 min), on the alkaline hydrolysis of BSG, using a solid-liquid ratio of 1:20 (w/w). Under the best alkaline hydrolysis conditions (2% NaOH, 120 ºC, 90 min), 0.286% of FA was obtained. Despite the fact that this alkaline hydrolysis enables to obtain a higher FA recovery and uses less drastic conditions than the other processes described [30, 40, 95], it requires a pre-treatment with dilute sulfuric acid making this methodology more time consuming

and laborious. Recently, McCarthy *et al.* [116] investigated the ability of phenolic rich BSG extracts to protect against the genotoxic effects of oxidants in human lymphocytic U937 cells. The BSG phenolic extract which provides the highest FA (27.31 ± 0.69 μg/mL) and total phenol content (0.732 ± 0.020 mg GAE/ mL) was obtained after an alkaline hydrolysis using 1 M NaOH at room temperature for 16 h. The differences found in FA, *p*-CA and total phenolic content among the applied alkaline hydrolysis processes could be explained not only by the conditions used in the assay, but also by the differences in BSG, namely time of harvest and the characteristics of the growing region.

Enzymatic Hydrolysis

Enzymatic hydrolysis is also a solid-liquid technique that has been commonly used for release of phenolic compounds from BSG. An overview of the results obtained of enzymatic hydrolysis of HCAs, namely FA and *p*-CA, from BSG is shown in Table 3.6.

Table 3.6 - Efficiency of and enzyme preparations used to extract phenolic compounds from BSG using enzymatic hydrolysis

Reference	Enzyme preparation	FA released	*p*-CA released
Bartolomé *et al.* [68]	FAE[1] from *Aspergillus niger*	3.3%	NR[2]
	FAE from *Aspergillus niger* + xylanase from *Trichoderma viride*	30%	NR
Bartolomé *et al.* [61]	FAE + xylanase from *Streptomyces avermitilis* CECT 3339	43%	< 9%
Bartolomé *et al.* [31]	Ultraflo L commercial enzyme	70%	8%
	Viscozyme L commercial enzyme	33%	0.8%
	Lallzyme commercial enzyme	55%	1.6%
Faulds *et al.* [73, 74]	Ultraflo L, a β-glucanase preparation from *Humicola insolens*	65%	9%

Xiros *et al.* [75]	Crude enzyme extract of *Fusarium oxysporum*	49%	NR
	Monoenzymes feruloyl esterase (FAE, FoFaeC-12213) and xylanase (*Trichoderma longibrachiatum* M3)	15%	NR
Szwajgier *et al.* [87]	FAE from *Lactobacillus acidophilus* K1	7.00 ± 0.07 mg_{FA}/100 g	1.9 ± 0.1 mg_{CA}/100 g

[1]FAE: ferulic acid esterase; [2]NR: not reported

Bartolomé *et al.* [68] reported that an esterase from *Aspergillus niger* (FAE-III) was able to release 3.3% of the total FA from BSG. Moreover, they observed that the presence of a xylanase from *Trichoderma viride* increased this release up to 30%. Probably the action of the xylanase on BSG solubilises low-molecular-mass feruloylated material, which is further degraded by the esterase to produce FA. Further, due to the high cost of pure enzymes, the same authors tested the use of three different commercial enzyme preparations (Viscozyme L, Ultraflo L and Lallzyme) that also exhibit cinnamic acid esterase activity [31]. Incubation of Ultraflo L (1.3 U g^{-1} substrate, 24 h) with BSG released 70% of the total alkali-extractable FA and 8% of the total alkali-extractable *p*-CA. Under the same conditions, Viscozyme L and Lallzyme released, respectively, 33 and 55% of the total alkali-extractable FA, and 0.8 and 1.6% of the total alkali-extractable *p*-CA. Faulds *et al.* [73, 74] also investigated the ability of Ultraflo L, a β-glucanase preparation from *Humicola insolens* sold for reducing viscosity problems in the brewing industry, exhibited activity against the methyl esters of ferulic, caffeic, *p*-coumaric and sinapic acids, displaying mainly type-B feruloyl esterase activity. They observed a release of 65% of the available FA together with three forms of diferulate, especially the 8,5' benzofuran form, and a 9% release of *p*-CA from BSG. These results demonstrate that FA and its dimeric forms present in BSG require the addition of more than a xylanase. This suggests either that FA is not solely attached to arabinoxylan in the barley cell wall, or that the cell wall polysaccharides in BSG hinder the accessibility of enzymes to the ferulates, due to processing treatments. Recently, Xiros *et al.* [75] studied the factors affecting FA release from BSG by the crude enzyme extract of *Fusarium oxysporum*. More than double amount of FA release (1 mg/g dry BSG, nearly 49% of the alkali extractable FA) was observed during hydrolytic reactions by the crude enzyme extract compared to hydrolysis by the monoenzymes feruloyl esterase (FAE, FoFaeC-12213) and xylanase (*Trichoderma longibrachiatum* M3) (0.32 mg/g dry BSG corresponding to 15% of alkali extractable FA content). These results are in agreement with the earlier studies which demonstrated the significance of the

presence of many different auxiliary hydrolytic activities for the efficient FA release from BSG. Szwajgier *et al.* [87] reported the use of a novel ferulic acid esterase from *Lactobacillus acidophilus* K1 for the release of phenolic acids from BSG. The enzyme released 7.00 ± 0.07 mg FA from 100 g of BSG after 3 h of incubation (approx. 2.1% of the total alkali extractable FA present in BSG). The maximal content of the released *p*-CA was 1.88 ± 0.10 mg/100 g of BSG.

According to the previously exposed, FA extraction from BSG utilizing enzymes as extraction agents yielded less phenolics than in the alkaline hydrolysis reported by Mussatto *et al.* [17]. Besides lower yields, these enzymes require a longer reaction time (up to 48 h). Therefore, from the above mentioned extraction processes for phenolic acids recovery, the method proposed by Mussatto *et al*. [17] appears to be more efficiently as low NaOH concentrations (2%) and short reaction times (90 min) are used.

Soxhlet extraction

Soxhlet method is still used as a standard technique in almost all cases for comparison of advanced methods in terms of their yield [127]. Solvents, such as methanol, ethanol, acetone, diethyl ether, and ethyl acetate, have been used for soxhlet extraction of phenolics [118, 127, 128]. However, very polar phenolic acids (benzoic and cinnamic acids) could not be extracted completely with pure organic solvents, and mixtures of alcohol-water or acetone-water are recommended [32, 118]. Most of these solvents must be used with care as they are toxic for humans and dangerous for the environment. Moreover, the solvent must be separated from the final extract, especially if the product is to be used in food applications, requiring an evaporation/concentration step for recovery. Luque de Castro and Garcia-Ayuso [129] outlined the importance of soxhlet extraction as a model to which the performance of other conventional and new extraction techniques is referred. The main drawbacks and advantages of Soxlet extraction are listed in Table 3.7.

Table 3.7 - Main advantages and disadvantages of Soxhlet extraction

Advantages	Disadvantages
Simple to perform	Long extraction times
Cheap	High extraction temperatures
Not require specialized personnel	Large amounts of solvents
No filtration procedure	Agitation can not be used
Repeatedly brings fresh solvent	Expensive and hazardous solvents

SE is a generally well-established technique. Wide industrial applications, better reproducibility and efficiency has favored significantly their widespread use over other novel extraction methods such as ultrasound-assisted, microwave-assisted, supercritical fluid, or accelerated solvent extractions. However, the main drawbacks mentioned lead to criticism of the conventional SE method, placing problems in terms of sustainability and protection of the environment [118, 130]. Meanwhile, auxiliary features such as a vacuum pump, a membrane separation unit, a source of ultrasound and microwave, and supercritical fluids can be incorporated into the conventional Soxhlet method to improve its performance. Use of non-toxic extracting solvents such as supercritical CO_2 and water can be further investigated.

According to our knowledge, there are no reports concerning the SE of phenolic compounds from BSG. However, some studies reporting the extraction of phenolics from barley was found [131-133].A comparison of different SE conditions and the phenolic content recovered from barley are given in Table 3.8.

3.3.2. Solid-phase extraction

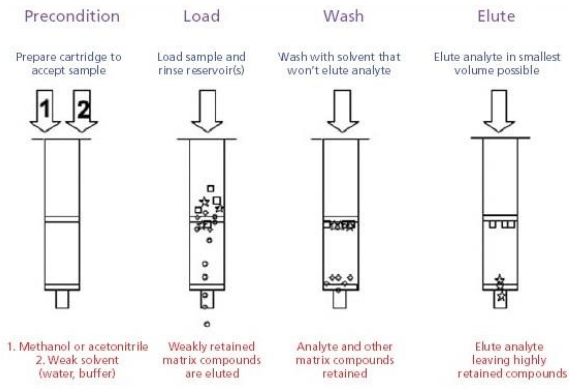

Fig. 3.13 - Steps in the solid phase extraction process.

Many of the problems associated with SLE, such as less-than-quantitative recoveries, use and disposal of large and expensive quantities of organic solvents can be overcomed with solid-phase extraction (SPE, Fig. 3.13). With the use of SPE there is an increase in sample throughout and minimal matrix interferences.

SPE approaches using various adsorbents and elution solvents of varying pH has been applied for the the recovery of phenolics in barley grains [134-136]. Irakli *et al.* [135] compared four different solid phases cartridges: silica-based C_{18} (LiChrolut EN and BakerBond) and polymeric (Oasis HLB and Abselut Nexus) for the recovery of standard phenolic compounds in barley. Polymeric cartridges provided good results, while C_{18}-based phases afforded less satisfactory results for extracting more polar compounds. These authors also show that Abselut Nexus, which is a hydrophilic commercial sorbent based on the copolymer of methacrylate-divinylbenzene, had similar recovery rates to Oasis HLB cartridges; thus, Oasis HLB was selected for extraction of phenolics since this sorbent is better for extracting more polar and offers a high specific surface area (800 m^2 g^{-1}). Some applications of SPE are listed in Table 3.8.

In order to seek for more environmentally friendly methods, decrease the solvent consumption, shorten the extraction time, increase the extraction yield, and enhance the quality of extracts, several novel extraction techniques have been developed such as microwave and ultrasound-assisted extractions. Techniques based on the use of compressed fluids as extracting agents, such as supercritical fluid extraction (SFE), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE), which are less labour intensive and more environmentally friendly, were also applied in the extraction of phenolic compounds from plant materials [121, 122, 137-139]. Moreover, these techniques have the possibility of working at elevated temperatures and/or pressures, greatly decreasing the time of extraction. In any case, these conventional techniques are still widely used as a comparison when an advanced extraction technique is applied.

3.3.3. Supercritical fluid extraction

SFE is another popular method applied for extraction of many bioactive compounds [126, 140, 141]. The supercritical state is reached when both the temperature and pressure are raised above their critical value. The supercritical fluid has characteristics of both gases and liquids. Compared with liquid solvents, supercritical fluids have

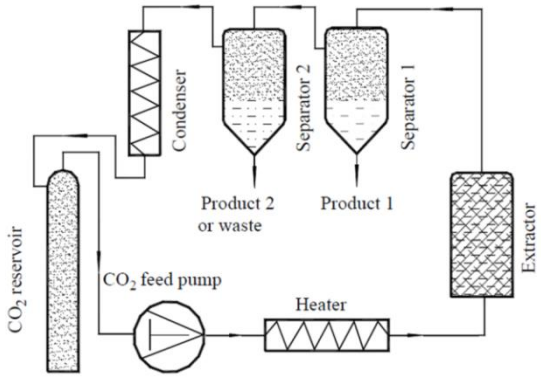

Fig. 3.14 - Schematic diagram of a process-scale supercritical fluid extraction system (Source: Wang and Weller [130]).

several major advantages. Dissolving power of a supercritical fluid solvent depends on its density, which can be highly adjustable by changing the pressure and/or temperature. Furthermore, the supercritical fluid has a higher diffusion coefficient, lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer [69, 142, 143]. A SFE system is shown in Fig. 3.14.

During SFE, material is loaded into an extraction vessel, which is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The extraction vessel is pressurized with the fluid by a pump. The fluid and the dissolved compounds are transported to separators, where the salvation power of the fluid is decreased by decreasing the pressure or increasing the temperature of the fluid. The product is then collected via a valve located in the lower part of the separators. The fluid is further regenerated and cycled [130, 142].

SFE is a clean method, considered environmental friendly as CO_2 is generally used as the solvent. It is a very efficient method for extraction of non-polar compounds, as CO_2 is a non-polar solvent. However, to increase the solubility of polar compounds, solvent such as methanol, ethanol, acetonitrile, acetone, water, ethyl ether, and dichloromethane can be added increasing the extraction selectivity of this technique [130]. As evidenced from the literature, the modification of the solvent composition has lead to the successful extraction of phenolic compounds from biological matrices [142]. However, for some very polar phenolics, the extraction efficiency of supercritical extraction is quite low, because the content of the organic modifier is not sufficient for their complete isolation [118]. Additionally, the economics and onerous operating conditions of the SFE processes has restricted the applications to some very specialized fields such as essential oil extraction, coffee decaffeination and to university research [130].

Fernández *et al.* [121] evaluated the use of supercritical fluid technology coupled with pretreatment processes for the recovery of tocopherol from BSG. The SC-CO_2 extraction of BSG was performed at temperatures of 313, 333, and 353 K and at pressures from 10 to 35 MPa using raw and milled BSG. The operating conditions

selected to obtain the maximum yield were a temperature of 313 K, a milling step to obtain a particle size of 0.85 mm, and a pressure of 35 MPa. Under these conditions, BSG contained a tocopherol concentration in the extract of 2 mg L^{-1}. Other applications for SFE for phenolics extraction from other cereals are reported in Table 3.8.

3.3.4. Microwave assisted extraction

Microwave-assisted extraction (MAE) is another advanced method of extraction which has become popular for the separation of bioactive compounds from plant matrices. Microwaves are electromagnetic waves, which are usually operated at a frequency of 2.45 GHz. Microwaves can access biological matrices and interact with polar molecules, such as water, and generate heat. The temperature will rise, which generally leads to enhanced extraction efficiency [130]. By using MAE the extraction time can be reduced in comparison to conventional extraction methods [141, 144] . MAE extraction efficiency principally depends on microwave energy, treatment time and temperature used. It should be noted that in general temperature increase enhances extraction rates of solutes due to an increase in diffusion rates and in the capacity of solvents to solubilise solutes, a better disruption of solute-matrix bonds, and the decrease in surface tension and in viscosity of the solvent [143]. But very high extraction temperatures can generate unwanted compounds. For example, Tsubaki *et al.* [145] reported that the proportion of polyphenol in the extract of tea residue increased from 25.3% to 74.4% when the temperature was increased from 110 ºC to 230 ºC using MAE. However, 5-(hydroxymethyl)furfural, a potentially harmful compound, was also formed at 230 ºC. Therefore, caution should be taken while increasing the temperature in MAE. Liazid *et al.* [137] evaluated the stability of 22 phenolic compounds of different families (benzoic acids, benzoic aldehydes, cinnamic acids, catechins, coumarins, stilbens and flavonols) under working temperatures ranging from 50 to 175 ºC. They concluded that all the compounds studied are stable up to 100 ºC, whereas at 125 ºC there is significant degradation of epicatechin,

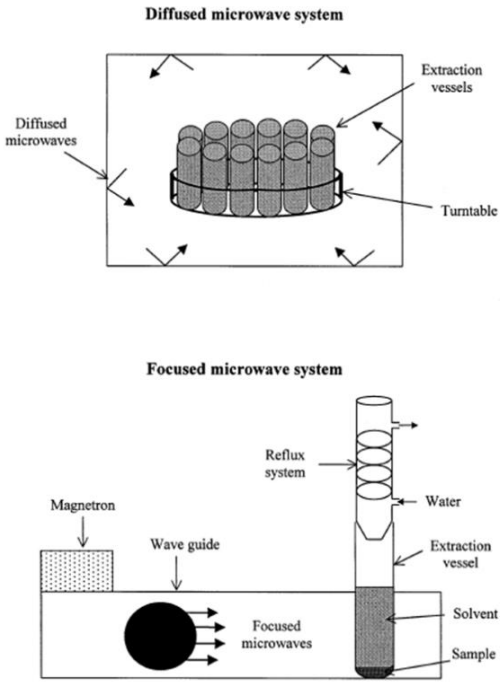

Fig. 3.15 - Scheme of the two microwave systems using diffused or focused microwaves (Source: Camel [146]).

resveratrol and myricetin. They observed that compounds with a greater number of hydroxyl-type substituents are more easily degraded with increased MAE temperatures.

There are two types of commercially available MAE systems: closed extraction vessels under controlled pressure and temperature, or open vessels under atmospheric pressure [146, 147]. They are commonly named either pressurized MAE (PMAE) and focused MAE (FMAE), respectively. Both systems are schematized in Fig. 3.15. The closed MAE system is generally used for extraction under drastic conditions such as high extraction temperature. The pressure in the vessel essentially depends on the volume and the boiling point of the solvents. The FMAE system can be operated at a maximum temperature determined by the boiling point of the solvents at atmospheric pressure.

MAE has been considered as a potential alternative to traditional SLE for the extraction of metabolites from plants. It has been used to extract phenolic compounds for several reasons: (1) reduced extraction time (2) reduced solvent usage and (3) improved extraction yield. MAE is also comparable to other modern extraction techniques such as SFE due to its process simplicity and low cost. By considering economical and practical aspects, MAE is a strong novel extraction technique for the extraction of bioactive compounds. However, compared to SFE, an additional filtration or centrifugation is necessary to remove the solid residue during MAE. Furthermore, the efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar, or when they are volatile [117, 146].

Several classes of phenolic compounds have been efficiently extracted from a variety of matrices, such as apple pomace [148], red raspberries [149], green tea leaves [150], wheat bran [151], rice bran oil [152] and distillers dried grains [138]. However, only one report was found concerning the determination of phenolic acids in

BSG using some kind of microwave-based technique. Athanasios *et al.* [92] used microwave irradiation for the trimethylsilyl derivatization of phenolic acids in a closed vial. These authors suggest that this derivatization procedure is a very efficient method comparatively to the conventional heating method. MAE conditions and phenolic content for other cereals is reported in Table 3.8.

3.3.5. Accelerated solvent extraction

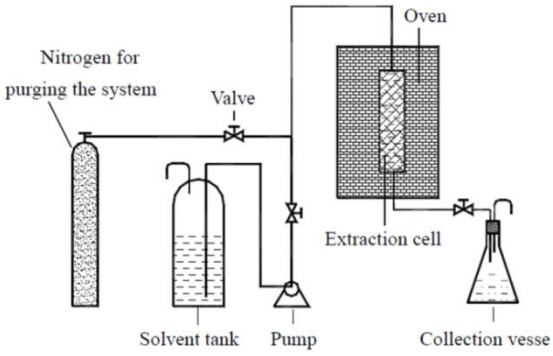

Fig. 3.16 - Schematic diagram of an accelerated solvent extraction system (Source: Wang and Weller [130]).

Accelerated solvent extraction (ASE) is a type of pressurized solvent extraction where the temperature range is between 50 to 200 ºC, pressure generally varies from 10 to 15 MPa and the solvent remains below critical condition, in its liquid state [130, 146]. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus achieving safe and rapid extraction. Also pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. Elevated temperatures enhance diffusivity of the solvent resulting in increased extraction kinetics [130]. A typical schematic diagram of an ASE system is given in Fig. 3.16.

The solvent, in the case of ASE, is usually an organic solvent. Water can also be used as the extraction medium; where application of high pressure allows the solvent to be used above its boiling point. As mentioned by Raynie [153], water loses its effective polarity at these high temperatures which allows it to act as a solvent for both polar and non-polar compounds. This method is also known as subcritical water extraction or pressurized hot water extraction.

ASE is considered as a potential alternative technique to SFE for the extraction of polar compounds, because supercritical CO_2 extraction uses a considerable quantity of polar modifier to extract polar compounds [130]. Compared with traditional SE, there is a dramatic decrease in the amount of solvent and the extraction time for ASE. Particular attention should be paid to the ASE performed with high extraction temperature, which may lead to degradation of thermolabile compounds. Finally, while MAE gives extraction efficiencies comparable to SFE and ASE, with similar extraction

times, it must be pointed out that the use of the latter two techniques requires a higher investment cost (for more expensive equipment) [146].

Very few applications of ASE have been published in the field of phenolic extraction [134, 139]. In Table 3.8 it's reported the main extraction conditions and the phenolic content obtained for barley samples.

3.3.6. Pressurized liquid extraction

Pressurized liquid extraction (PLE) has been widely applied for the extraction of phenolic compounds in recent years [119, 122, 123]. During PLE, pressure is applied, allowing the use of temperatures above the boiling point of solvents. Extracting at elevated temperatures can be advantageous due to changes in mass transfer and surface equilibrium. Higher extraction temperatures will increase the mass transfer rate and extraction rates of solutes due to the same reasons as mentioned with MAE [154]. The applied high pressure, usually ranging from 4 to 20 MPa, ensures the solvent maintains in the liquid state at the applied temperature [154]. This is the main reason for the use of pressure, although pressure has also been reported to help driving the solvent into the pores of the matrix and enhance analyte solubility [142, 154]. This process needs less solvent and the oxidative degradation of the phenolic compounds is lower, as the process is free of oxygen and light [118, 119, 130]. PLE has been mainly used to optimize analytical extractions [155], although a few commercial applications exist in the field of extraction of flavorings from natural products as an alternative to steam distillation. PLE generally requires less time and a lower consumption of organic solvents than conventional techniques. This is the reason why PLE may have commercial interest as alternative extraction method to obtain bioactive compounds from byproducts.

The extraction of polyphenolic compounds by PLE has been demonstrated in numerous studies which have presented several approaches to optimize the extraction conditions or evaluated their efficiency compared with other methods [119, 122, 123]. However, as already mentioned for other advanced techniques such as MAE and ASE, no reports were found concerning the extraction of polyphenols from BSG. Bonoli *et al.* [122, 123] evaluated the recovery of phenolic compounds from barley by PLE. The extraction recovery of phenolics was compared to conventional solid-liquid extractions. PLE extracted less simple phenols and hydrolysable tannins compared to the conventional extraction method. Moreover, increasing the extraction temperature

decreased the amount of phenolic compounds detected, probably due to their degradation at higher PLE temperatures. The highest amount of total phenolic compounds recovered was obtained when ethanol was used as the extraction solvent (2.13 ± 0.36 mg phenols/g barley). Other applications of PLE technique for other cereals are reported in Table 3.8.

3.3.7. Ultrasound assisted extraction

The development of ultrasound technology is not new; however, it is only recently that the main advances in the exploitation of power ultrasound have been achieved [156]. Ultrasound-assisted extraction (UAE) has been proposed as an alternative to conventional extraction techniques, providing higher recovery of targeted compounds with lower solvent consumption and/or faster analysis and bioactivity properties. The technique uses high frequency sound waves (higher than 20 kHz). When the ultrasound waves are strong enough, the expansion cycle can create cavities or microbubbles in the liquid. Eventually the formed bubbles cannot absorb the energy any longer and will collapse: "cavitation" takes place. This collapse causes a change in temperature and pressure within the bubble and hence energy for chemical reactions is generated. Extremely high temperatures of 5000 ºC and pressures of 1000 bar have been measured [156]. Thus, the implosion of cavitation bubbles can hit the surface of the solid matrix and disintegrate the cells causing the release of the desired compounds [142].

Various studies have tested ultrasound and its effect on the extraction of polyphenols. Ultrasound has been shown to enhance the recovery of bioactive compounds, such as polyphenols, from different plant by-products [157, 158]. Some aspects related to the stability of the compounds extracted has not fully addressed, however, recent studies revealed that UAE of phenolic compounds were less degraded than others [159]. Barley grain was treated with UAE to extract polyphenols. Extraction temperature, ethanol concentration, solvent to material ratio and extraction time were optimized using response surface methodology (RSM). A temperature of 50 ºC, an ethanol concentration of 100%, a solvent to material ratio of 60 mg/L and an extraction time of 18 min were determined as optimal conditions. These conditions resulted in an enhanced total polyphenol yield of 19.68 mg_{GAE}/g barley grain [120].

A comparison of different extraction methods for BSG and other cereals samples is given in Table 3.8. To obtain the most effective and potential extract, it is necessary to

take into account the characteristics of sample, the nature of solvents used for extraction and the extraction procedure employed. Sometimes, the high yield of extract will not ensure a high yield of bioactive components in the extract. For example, some bioactive components are very sensitive to oxygen and heat. In this case, more care should be taken to prevent the oxidation and thermal degradation of those components. Therefore, the yield and quality of bioactive components should also be considered when an extraction method is selected.

Table 3.8 - Comparison of different extraction techniques for BSG and other cereals

Technique	Sample	Extraction conditions	TPC	Individual phenolic content	Reference
Solid-liquid extraction					
Hot water bath extraction with magnetic agitation	BSG	60% acetone	9.90 mg_{GAE}/dry BSG	NR	Meneses *et al.*[32]
Alkaline hydrolysis	BSG	pre-treatment with H_2SO_4 + 2% (0.5 M) NaOH, 120 ºC, 90 min	NR	FA (9.65 mg/g solubilized lignin), *p*-CA (9.22 mg/g solubilized lignin)	Mussatto *et al.* [17]
Soxhlet extraction	Barley	ethyl acetate; 9 h	0.495 mg_{GAE}/g extract	3,4-dihydroxybenzaldyde (3.07 mg/g) Vanillin (2.23 mg/g) *p*-CA (1.36 mg/g)	Conde *et al.* [131]
	Barley roots	0.3 g sample; 80% ethanol; 20 h	3.31 mg/g dry sample	NR	Dudjak *et al.* [132]
	Barley straw	50 g sample, 1800 mL hexane-acetone (2:1, vol/vol), 12 h	2.05% (wt/wt)	Protocatechuic acid (0.12%) *p*-CA (0.20%) FA (0.066%)	Sun & Sun [133]
Solid-phase extraction	Barley	Oasis HLB cartridges	Free fraction (11.53 μg/g) Bound fraction (485.06 μg/g)	Free *p*-CA (0.97 μg/g); Bound *p*-CA (75.9 6 μg/g) Free FA (3.00 μg/g); Bound FA (320.82 μg/g)	Irakli *et al.* [135]
	Out layers of barley	Polyamide column	3618 μg/g	Catechin fraction (158 μg/g); dimeric fraction (1339 μg/g); trimeric fraction (1455 μg/g)	Quinde-Axtell & Baik [136]
Supercritical-fluid extraction	BSG	313 K, 35 Mpa	NR	Tocopherol (2 mg/L)	Fernández *et al.* [121]
	Roasted wheat germ	2 g sample, 240 bar, 56 ºC, 20 min	6.63 mg_{GAE}/g extract	α-tocopherol (4.8 mg/g), β-tocopherol (2.4 mg/g), γ-tocopherol (11.7 mg/g)	Gelmez *et al.* [160]

Microwave-assisted extraction	Distillers Dried Grains	50% EtOH, 150 ºC, 15 min	12.02 mg/g	NR	Inglett *et al.* [138]
	Wheat bran	10 g sample, 40 mL MeOH, 120 ºC, 20 min	Phenolic content (> 467.5 μgCE/g); Tocopherol content (19.5 μg/g)	NR	Oufnac *et al.* [151]
	Rice bran	20 g sample, isopropanol, 120 ºC, 15 min	NR	α-tocopherol (47.49 g/g), γ-tocopherol (7.29 g/g)	Zigoneanu *et al.* [152]
	Oat bran	10 g sample, 40 mL Hexane, 60 ºC, 3.5min, 2450 MHz	1.98 mg_{GAE}/g	NR	Dar & Sharma [161]
Accelerated solvent extraction	Barley	1 g, 90 °C, 14.2 MPa, 10 min	NR	7-Hydroxymatairesinol (541 μg/100 g)	Smeds *et al.* [139]
		4 g sample, 60% acetone, 60 ºC, 200 bar	Phenolic content (1346 μg/g), Total DiFA (247 μg/g)	*p*-CA (374 μg/g), FA (696 μg/g), 5-5'-DiFA (64 μg/g), 8-O-4'-DiFA (79 μg/g), 8-5'-DiFA (104 μg/g), TriFA (28 μg/g)	Holtekjolen *et al.* [134]
Pressurized liquid extraction	barley	5 g sample, EtOH, 60º C, 20 Mpa, 10 min	2.13 mg_{GAE}/g	NR	Bonoli *et al.* [122]
	brown black glutinous rice	2.5 g, 70% MeOH, RT, 1500 psi, 15 min		p-CA (1.06 mg/100 g), FA (5.27 mg/100 g)	Vichapong *et al*. [119]
	wheat bran	5 g, 0.5 M NaOH, 180 ºC, 5.2 Mpa, 40 min	NR	p-CA (20 mg/100 g), FA (391 mg/100 g)	Buranov & Mazza [162]
	corn bran		NR	p-CA (350 mg/100 g), FA (2510 mg/100 g)	
Ultrasound assisted extraction	barley	EtOH, 50 ºC, 18 min	19.68 mg_{GAE}/g	NR	Wang *et al.* [120]

II

Research work

Scope of the thesis

The main purpose of this research was to investigate if by-products of the brewing industry could be used as new sources of natural antioxidants, especially phenolic compounds. The recovery of antioxidants from brewing industry by-products is of great importance, not only due to the health promoting properties of phenolics, but also because their valorization can contribute to the sustainable development of the agro-food sector by an exhaustive utilization of natural raw materials. This importance becomes even more relevant when a sector such as brewing industry has a high influence in the economy of a country. To achieve this main goal, some specific aims of this thesis were:

- To investigate the phenolic composition of the brewing by-products, namely spent grains, surplus yeast and spent hops, to select that with a potential higher commercial and health interest (Chapter 4).

- To develop and optimize an efficient method for the extraction of phenolics in order to increase the commercial applicability of the by-product selected in the previous step. Furthermore, our investigation was focused on the characterization of the phenolic composition of this by-product by HPLC-DAD and HPLC-ESI-MS/MS analysis (Chapter 5).

- To study the influence that the type of malt used in the brewing process may have on the phenolic composition and antioxidant properties of by-product. With this purpose, six types of malts (*pilsen*, *melano*, *melano 80*, *carared*, *chocolate* and *black*) were chemically characterized with chromatographic techniques (Chapter 6).

- To evaluate the antigenotoxic activity of the previous obtained extracts by investigating the inhibition of oxidative DNA damage in *Saccharomyces cerevisiae* cells, using the yeast comet assay (Chapter 7).

4. Brewery by-products as a source of phenolic compounds: analytical first screening

Growing knowledge about the health promoting impact of antioxidants in everyday foods, combined with the assumption that a number of common synthetic preservatives may have hazardous effects, has led to multiple investigations in the field of natural antioxidants. By-products from the processing of beverages, fruits and vegetables, traditionally considered as an environmental problem, are being increasingly recognized as sources for obtaining valuable products. To this regard, the recovery of phenolic compounds from industrial wastes is gaining considerable attention [17, 30, 32], due to the physiological functions which can result in benefits for human health on the prevention of cardiovascular diseases, certain types of cancer and atherosclerosis that these compounds may exert [84]. Furthermore, food, pharmaceutical and cosmetic industries are also claiming for natural solutions to some of the customers' needs such as the use of natural colorants, texturizers, functional ingredients or shelf life extenders. Therefore, the recovery of antioxidants from brewing by-products is of great importance, not only because of their aforementioned significant properties, but also because their valorization can constitute a contribution to the sustainable development of the brewing sector by an exhaustive utilization of natural raw materials. This importance becomes even more relevant when a sector like brewing industry has high influence in the economy of a country. Thus, it is very important to know the contribution and composition of by-products from brewing industry. Within several by-products generated in portuguese beer production, the most common ones are BSG, spent hops and surplus yeast [8]. As previously mentioned, Unicer SA generated 60089 tonnes of by-products in 2005, of which 96% corresponds to BSG and excess yeast [13].

Although the antioxidant potential of brewing by-products, namely BSG, is being investigated [30, 32, 73], as yet little effort to utilize their by-products for phenolics recovery has been reported. This might be caused by a limiting factor often overlooked in scientific studies: the effectiveness of recovery and extraction, the marketability of resulting extracts and the practical suitability for the food, cosmetic or pharmaceutical products. As reported in Chapter 3, solid-to-liquid extraction is the most common

method used to recover natural antioxidants from BSG. However, the efficiency of extraction is affected by several factors, among them, the type of solvent has been considered one of the most important [32, 163]. Meneses *et al.* [32] have recently evaluated the efficiency of different solvent compositions (methanol, ethanol, acetone, hexane, ethyl acetate, water, methanol:water mixtures, ethanol:water mixtures, and acetone:water mixtures) for extracting antioxidant phenolics from BSG using a hot water bath extraction with magnetic agitation. They reported that all the produced extracts showed antioxidant activity, however the extract produced with 60% v/v acetone had the most elevated content of total phenols (9.90 ± 0.41 mg GAE/g dry matter). Nevertheless, studies on the extraction of these compounds from surplus yeasts and spent hops are scarce. Only one report was found concerning the quantification of xanthohumol (XN) losses to spent hops during beer production [164]. To the best of our knowledge, there are no studies concerning the recovery of phenolics from surplus yeast.

In the first part of this work, the principal aim was to investigate the potential of using inexpensive residual products from brewing industry, namely BGS, spent hops and surplus yeast, as sources of natural antioxidants. By-products were subjected to different extraction techniques, widely used in our research group, in order to study their effect on the extraction yield of phenolics and the corresponding antioxidant activity of extracts. This first screening will contribute to the selection of extraction techniques for further studies on the phenolic compounds recovery.

4.1. Material and Methods

4.1.1. Chemicals

The solvents employed for the extraction of the samples were high-purity water from a Millipore Simplicity 185 water purification system (Millipore Iberian S.A.), methanol (VWR, Darmstadt, Germany), diethyl ether (Panreac, Barcelona, Spain) and sodium hydroxide (Sigma-Aldrich, Madrid, Spain).

Folin-Ciocalteu's reagent (Merck, Darmstadt, Germany) and sodium carbonate (Sigma-Aldrich) were employed for the measurement of the total phenolic content (TPC). The calibration curve was constructed with gallic acid (GA, Sigma-Aldrich).

For the antiradical activity assessment, DPPH (Sigma-Aldrich) was used. The ABTS methodology employed 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and potassium persulfate ($K_2S_2O_8$) purchased from Sigma-Aldrich. 2-deoxyribose assay was performed using L(+)ascorbic acid (Sigma-Aldrich), trichloroacetic acid (Riedel-de-Haën), 2-thiobarbituric acid, 2-deoxy-D-ribose, hydrogen peroxide 35% and $FeCl_3$ (Fluka). The determination of the reducing power was carried out by using potassium ferricyanide (Sigma-Aldrich), iron (III) chloride hexahydrate (Sigma- Aldrich), and trichloroacetic acid (TCA, Sigma-Aldrich).

The solvents employed for HPLC analyses were prepared with methanol and formic acid of HPLC quality (VWR) and high-purity water. All eluents used were filtered through a nylon filter of 0.45 μm pore size (Whatman, Clifton, USA) and degassed for 10 min in an ultrasound bath. Calibration curves were constructed for the following phenolic compounds: (+)-catechin, (-)-epicatechin, gallic, protocatechuic, caffeic, *p*-coumaric, ferulic, sinapic and cinnamic acids, quercetin, kaempferol and xanthohumol (Sigma-Aldrich). Stock standard solutions (500 mg L^{-1}) of these compounds were prepared by rigorous dissolution of the analyte in methanol. Standard solutions were stored at -20 ºC and used for further dilutions.

4.1.2. Samples

The materials investigated were residues from the brewing process: BSG, surplus yeast and spent hops. The starting materials were frozen and lyophilized. The samples were then finely ground in a laboratory EBC mill (Casela, London, UK) and sieved trough a 35-mesh (≤0.5 mm) sieve. BSG and surplus yeast used throughout this work were kindly supplied by Unicer-Bebidas de Portugal (S. Mamede de Infesta, Portugal). Spent hops were supplied by Institute of Chemical Technology, Prague. The samples were stored at -20 ºC until further use.

4.1.3. Mechanical stirring

Taking into account our knowledge in the extraction of phenolic compounds from raw materials for beer production [25, 37, 38], phenolic extraction was carried out following the procedure of Markéta *et al.* [38]. Two grams of ground by-product were extracted three times (for periods of 20 min) with 20 mL of methanol/water (70/30, v/v)

or acetone/water (70/30, v/v) on gyratory shaker (Gravimeta, V. N. Gaia, Portugal) at 250 rpm. After centrifugation (5000 rpm, 5 min), the supernatant was collected and evaporated under vacuum at 35 ºC. Then the obtained residue was dissolved in 1 mL of mobile phase (for the chromatographic analyses) or 5 mL of methanol or water (depending on the spectrophotometric method used). Prior to the chromatographic analysis, the extract was filtered through a 0.45 µm cellulose filter. All experiments were performed, at least, in triplicate.

4.1.4. Soxhlet extraction

An exhaustive Soxhlet extraction was also performed following the procedure developed by Kalia *et al.* [127], with slight modifications. A classical Soxhlet apparatus was used with accurately weighed 5 g of the freeze dried by-product. Extraction was performed for 4 h with 150 mL of methanol 70%, acetone 70% and water as the extracting solvents. After extraction, the supernatant was allowed to cool at room temperature and evaporated to dryness under vacuum at 35 °C. The obtained residue was dissolved in 1 mL of mobile phase (for the chromatographic analyses) or 5 mL of methanol or water (depending on the spectrophotometric method used). Prior to the chromatographic analysis, the extract was filtered through a 0.45 µm cellulose filter. All experiments were performed, at least, in duplicate.

4.1.5. Extraction of free, soluble ester and insoluble-bound phenolics

Another extraction process, well established in our research group, which separates three different fractions of phenolic compounds (namely free, soluble-ester and insoluble-bound phenolics), was also performed according to the procedure described by Markéta *et al.* [37].

Two grams of ground by-product were extracted twice (for periods of 20 min) with 20 mL of methanol/water (70/30, v/v) on gyratory shaker (Gravimeta, V. N. Gaia, Portugal) at 250 rpm. After centrifugation (5000 rpm, 5 min), the supernatant was collected and the organic solvent was evaporated under vacuum at 35 ºC. The extract was acidified with HCl 6 M to pH 2 and then extracted with diethylether (3 × 10 mL). The ether extracts were collected and evaporated to the dryness under vacuum at 35°C. Phenolic acids so extracted were labelled as free phenolics. The aquous phase

was treated with 30 mL of 2 M NaOH for 2 h at room temperature. The resultant hydrolyzate was acidified to pH 2 using 6 M HCl and extracted three times with diethyl ether. The ether extracts were combined and evaporated to dryness at 35 ºC under vacuum. The phenolic acids extracted were those liberated from their esters and labelled as esterified phenolic acids.

The residue obtained after extraction with methanol 70% was treated with 30 mL of 2 M NaOH for 2 h at room temperature. The samples were then centrifuged (5000 rpm, 5 min) and the supernatant acidified to pH 2 with 6 M HCl. The mixture was extracted twice with diethylether (2 × 10 mL). The ether extracts were combined and evaporated to dryness under vacuum at 35 ºC. The phenolic acids so extracted were labelled as bound phenolics.

Free, esterified and insoluble-bound phenolics were dissolved separately in 1 mL of mobile phase (for HPLC analysis) or 5 mL of methanol or water (for spectrophotometric analysis). Prior to the chromatographic analysis, the extract was filtered through a 0.45 µm cellulose filter. All experiments were performed, at least, in triplicate.

4.1.6. Determination of total phenolic content

The TPC of the extracts was determined by the Folin–Ciocalteu method as described by Dvorakova *et al.* [38]. In a test tube, 1 mL of diluted sample or standard solution and 5 mL of 10-fold diluted Folin-Ciocalteu's phenol reagent were mixed. After 5 min of incubation, 4 mL of sodium carbonate solution (7.5%, w/v) was added and mixed well. After 2 h of incubation at room temperature in the dark, the absorbance was measured at 740 nm in a Shimadzu UV-3101 spectrophotometer (Kyoto, Japan). The total phenolics concentration was calculated from a calibration curve, using GA as standard (5–150 mg L^{-1}). The results were expressed as mg GA equivalents (GAE) per dry weight (DW) of sample. All measurements were conducted in triplicate.

4.1.7. Measurement of the antioxidant activity

Several methods are constantly being developed for measuring the total antioxidant activity of food and beverage. These assays differ in their chemistry generation of different radicals/or target molecules and in the way the end products are measured.

Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the total antioxidant activity of foods.

4.1.7.1. DPPH radical scavenging activity assay

DPPH is a stable free radical and has been commonly used to screen phenolic compounds containing high free radical scavenging ability, mainly due to its simplicity and high sensitivity. Antioxidants react with DPPH, scavenging a number of DPPH molecules equal to the number of their available hydroxyl groups (Fig. 4.1). Additionally, DPPH has the advantage of being unaffected by certain side reactions of polyphenols, such as metal ion chelation and enzyme inhibition [165]. The DPPH radical scavenging activity of each extract was measured according to the method of Brand-Williams [166] modified by Goupy *et al.* [167]. DPPH radicals have an absorption maximum at 515 nm, which disappears with reduction by an antioxidant compound. The DPPH solution in methanol (6.6×10^{-5} M) was daily prepared and 2.85 mL of this solution was mixed with 150 μL of the by-product extracts and vortexed. The absorbance decrease at 515 nm was measured after 120 min at room temperature in the dark. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Results were expressed as antiradical power (ARP, %) which is defined as ($1/EC_{50} \times 100$), where EC_{50} is the quantity of extract capable of reducing the initial concentration of DPPH to one half. Data were reported as mean ± SD for three replicates.

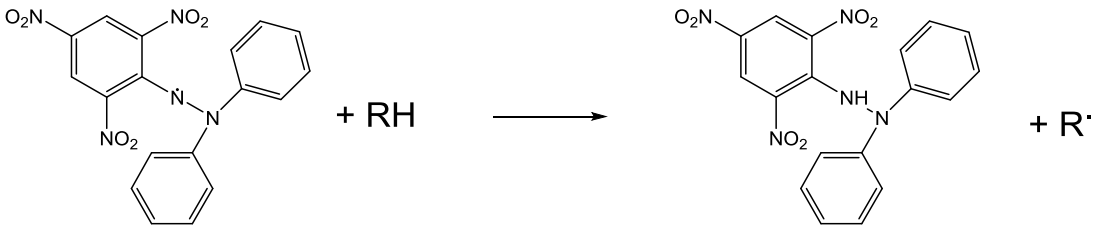

Fig. 4.1 - Reaction of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical with radical scavengers.

4.1.7.2. ABTS radical scavenging activity assay

The free radical scavenging activity of extracts was also evaluated using the ABTS radical cation decolorization assay [168], which is based on the scavenging of ABTS

radicals by antioxidants (Fig. 4.2). The stock solutions included 7 mM ABTS solution and 2.45 mM $K_2S_2O_8$ solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react in the dark for 16 h at room temperature to produce a dark green solution. Prior to the assay, the ABTS solution was diluted in deionized water to obtain an absorbance of 0.70 ± 0.02 units at 734 nm. Then phenolic extracts or standards (150 μL) were allowed to react with 2850 μL of the ABTS solution, and the absorbance reading was taken after 2 h at room temperature in the dark. A calibration curve was obtained by using trolox standard solutions at various concentrations (0–100 mg L^{-1}). The degree of ABTS radical-scavenging activity of sample extracts was calculated, based on the trolox standard curve, and was expressed in terms of mg trolox equivalents (TE)/g DW of sample. All solutions were prepared on the day and all measurements were carried out in triplicate.

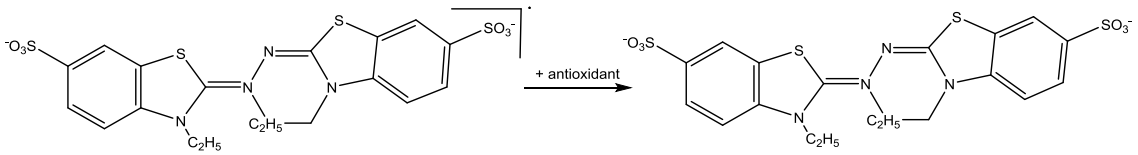

Fig. 4.2 - Reaction of the ABTS radical in the presence of the antioxidant compound.

4.1.7.3. Ferric reducing antioxidant power (FRAP) assay

The reducing power of a compound is related to the electron transfer ability of that compound and may serve as a significant indicator of its potential antioxidant activity. FRAP assay was performed according to the method described by Dudonné and collaborators [168] with some modifications. This method is based on the reduction, at low pH, of a colorless ferric complex (Fe^{3+}-tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+}-tripyridyltriazine) by the action of electron-donating antioxidants. The reduction is monitored by measuring the change of absorbance at 593 nm. Briefly, the FRAP reagent was prepared from acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was prepared fresh daily and was warmed to 37 ºC in a water bath prior to use. 2850 µL of this solution was allowed to react with 150 µL of extracts or standards for 30 min at room temperature in dark condition. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm and the calibration curve was performed with trolox (5 – 150 mg L^{-1}). Results were expressed as mg TE/g DW of sample. All measurements conducted done in triplicate.

4.1.8. HPLC-DAD analysis

For the by-products extracts with higher TPC and antioxidant activity, the phenolic composition was analyzed by the HPLC method described by Rubilar *et al.* [169] with minor modification. The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of a low pressure quaternary gradient unit (model LG-1580-04) with an in-line DG-1580-54 degasser and a model AS-950 auto-sampler. The system is equipped with a photodiode array detector (model MD-1510 UV/Vis multiwavelength detector).

Separation of polyphenols was achieved on a *Phenomenex* Synergi Hydro-RP C_{18} column (150 mm x 4.6 mm, 4 μm) and a guard column with the same characteristics kept at room temperature. The chromatographic conditions were the following: flow rate 0.3 mL min^{-1}, sample injection volume of 20 μL and mobile phase A (100% methanol) and mobile phase B (0.1% aqueous formic acid). A gradient program was used as follows: 90% B in 0 min, from 90% to 0% B in 110 min, followed by 100% A for 20 min and back to 90% B in 10 min and 10 min of reconditioning before the next injection. The photodiode array detection was conducted by scanning between 190 and 600 nm, and the quantification was conducted at 280 nm for monomeric flavan-3-ols ((+)-catechin and (-)- epicatechin) and GA, at 320 nm for the derivatives of cinnamic acid (caffeic, *p*-CA, FA and sinapic acid), at 250 nm for protocatechuic acid and at 370 nm for quercetin, kaempferol and XN. The data acquisition and treatment were conducted using the Jasco ChromPass chromatography data system. Analytes in each sample were identified by comparing their retention times and UV-Vis spectra with those of standard compounds. Peak purity was checked to exclude any contribution from interfering peaks.

The concentrations of individual phenolic compounds in extracts were determined using external standard calibration curves in the concentration range of 5 to 200 mg L^{-1} using a mixture of 12 standards. The analytical parameters of the calibration curves were calculated with the Excel program. Coefficient of correlation, limit of detection (LOD) and limit of quantification (LOQ, mg L^{-1}) are shown in Table 4.1. Results in samples were expressed in mg/g of dry sample (mg/g of DW).

Table 4.1 - Analytical parameters of the calibration curves used for HPLC-UV quantification of BSG extracts

Compound	r^2	LOD / mg L^{-1}	LOQ / mg L^{-1}
Gallic acid	0.9988	0.05	0.15
Protocatechuic acid	0.9997	0.03	0.10
Catechin	0.9999	0.09	0.31
Caffeic acid	0.9998	0.01	0.04
Epicatechin	0,9997	0.07	0.23
p-Coumaric acid	0.998	0.02	0.05
Ferulic acid	0.9997	0.02	0.06
Sinapic acid	0.9998	0.02	0.05
Cinnamic acid	0.9991	0.04	0.09
Quercetin	0.9989	0.05	0.12
Kaempferol	0.9976	0.03	0.14
Xanthohumol	0.9995	0.05	0.16

4.1.9. HPLC-ESI-MS analysis

The qualitative study of the phenolic composition in samples was performed by HPLC coupled on-line with electrospray ionization (ESI) mass spectrometry. The HPLC system (Finnigan, Thermo Electron Corporation, San Jose, USA) consisted of a low-pressure quaternary pump (Finnigan Surveyor Plus) and an auto-sampler (Finnigan Surveyor Plus with 200-vial capacity sample). Separations were achieved in the same conditions as for HPLC-DAD analysis but the injection volume was 25 μL. A quadrupole ion trap mass spectrometer (Finnigan LCQ Deca XP Plus) equipped with an ESI source in the positive ion mode and Xcalibur software Version 1.4 (Finnigan) were used for data acquisition and processing. The interface conditions were applied as follows: capillary temperature, 325 ºC; source voltage, 5.0 kV; capillary voltage, 4.0 V; sheath gas (N_2) flow at 90 arbitrary units and auxiliary gas (N_2) flow rate at 25 arbitrary units. Data acquisition was performed between *m/z* 100 and 1000. For the MS^2 analyses an activation energy of 45% was applied. The positive ion mode was

used in this study due to a better signal-to-noise ratio in comparison with negative ion mode.

4.1.10.Statistical analysis

All data points are the mean and standard error values of at least three independent experiments. Differences in means were detected using two-way ANOVA and Bonferroni-Dunn test. The software employed for statistical analysis was Graphpad Prism, version 5 for Windows. The *p*-value less than 0.05 ($p < 0.05$) was considered as statistically significant.

4.2. Results and Discussion

4.2.1. Total phenolic content

Extraction of phenolic compounds from plant materials is influenced by several important factors such as solvent, temperature, solid-liquid ratio, extraction time, particle size, extraction method, sample, and presence of interfering substances [170]. Despite being probably the most investigated parameter, solvent selection is still a complicated issue because extract yields and resulting antioxidant activities of the sample are strongly dependent on the nature of the extracting solvent. This is due to the presence of different antioxidant compounds of various chemical characteristics and polarities that may or may not be soluble in a particular solvent [32, 37]. In our case, the content of total phenols for by-products extracts obtained from mechanical stirring extraction is in all cases higher for 70% acetone, as shown by the data presented in Table 4.2. Under these conditions, the extracts obtained from the three by-products in 70% acetone contained a phenolic concentration significantly different ($p < 0.05$) compared to the extracts obtained with 70% methanol. Our results are in agreement with those of Meneses *et al.* [32] who evaluated methanol, ethanol, acetone, hexane, ethyl acetate, water, and their mixtures with water as extraction solvents for BSG. These authors reported that BSG extracts obtained with 60% v/v acetone had the most elevated content of total phenols (9.90 mg GAE/g). A lower phenolic content was found in the BSG used in this work (6.6 ± 0.3 mg GAE/g DW) when compared with their results. The samples type and origin as well as the extraction temperature used by Meneses *et al.* [32] (extractions performed at 60 ºC) can be pointed as the main reasons for the verified differences. Further, several

authors reported that the polarity of extracting solvent and the solubility of chemical constituents in the extracting solvent are the main factors responsible for the differences in the TPC of the extracts [165, 171, 172]. Roby *et al.* [171] studied the influence of four solvents (methanol, acetone, diethyl ether and hexane) in the TPC from three aromatic plants and they concluded that TPC varied with polarity of the solvent with methanolic extract showing relatively higher TPC. These results are in contrast with our findings, where a less polar solvent (acetone) enables to recover higher amounts of phenolics. This could be explained by the fact that the diverse chemical structures of the phenolic compounds, ranging from simple to polymerized forms present in by-products extracts, present different solubility behaviors [172]. Marketa *et al*. [38] have determined the TPC for the 70% methanol and 70% acetone extracts obtained for 10 samples of barley and the corresponding malts. These authors also reported that the extraction solvent has impact on the extraction capacity and selectivity of individual phenolic compounds, with 70% acetone showing the highest capacity for the phenolic compounds extraction either for barley or malt. They concluded that aqueous acetone selectively enhanced the catechin and proanthocyanidins extraction yield in comparison to 70% methanol. Thus, the difference observed between solvents can be explained not only by differences in the extraction procedures and nature of the analyzed sample, but also on the polymeric nature of phenolics to be extracted.

Comparing the TPC values obtained for spent hops and surplus yeast, for the same extraction solvent, no statistically differences ($p > 0.05$) were found (Table 4.2). Despite the fact that the TPC for these two by-products is lower than for BSG, 70% acetone revealed to be, again, more efficient for the extraction of phenolic compounds. As yet little practical effort to utilize spent hops and surplus yeast for phenolics recovery has been reported. This might be caused by three limiting factors often overlooked in scientific studies: the effectiveness of recovery and extraction, the marketability of resulting extracts and the practical suitability for the food, cosmetic or pharmaceutical products. According to our knowledge, only one report concerning the quantification of xanthohumol (XN), the main prenylflavonoid of hops (0.2-1.1%, w/w), in spent hops was found. Magalhães *et al.* [164] investigated the content losses of XN to the waste materials, namely spent hops. They reported that 21% and 17% of the hops' XN was found in the spent hops from pale and dark beers, respectively. According to these authors, the main reason for this loss is the isomeric conversion of XN into its flavanone, isoxanthohumol (IXN), during wort boiling. However, large quantities of XN

from hops are also removed during wort production together with the spent hops. Losses can be explained by the hydrophobic character of XN and the insufficient extraction of XN in wort.

Table 4.2 - Total phenolic content for by-products extracts obtained from mechanical stirring and soxhlet extraction. Values are expressed as mean ± SD (*n* = 3)

	TPC (mg GAE/g DW)		
	BSG	**Surplus Yeast**	**Spent hops**
Mechanical stirring			
Methanol 70%	2.9 ± 0.2[a]	2.4 ± 0.1[a]	2.4 ± 0.2[a]
Acetone 70%	6.6 ± 0.3[b]	5.6 ± 0.5[c]	5.4 ± 0.5[c]
Soxhlet extraction			
Methanol 70%	6.6 ± 0.4[a]	5.5 ± 0.3[d]	5.16 ± 0.08[d]
Acetone 70%	8.1 ± 0.2[b]	6.73 ± 0.05[e]	6.8 ± 0.5[e]
Water	4.3 ± 0.3[c]	4.43 ± 0.04[c]	3.9 ± 0.4[c]

Means within the same extraction technique with different superscript letter are statistically significant ($p < 0.05$). GAE – gallic acid equivalents.

Concerning the results obtained for soxhlet extraction, Table 4.2 shows that by using this technique higher TPC values were obtained (2.4 ± 0.2 to 6.6 ± 0.3 mg GAE/g DW for mechanical stirring and 5.16 ± 0.08 to 8.1 ± 0.2 mg GAE/g DW for soxhlet extraction). This could partially be explained by the release of bound phenolic compounds due to the higher extraction temperature used, which is in accordance to what has been reported [37]. However, other authors have found that the use of higher temperatures is not encouraged since elevated temperatures could affect the antioxidant activity of the extracts as well as to decrease the stability of the phenolic compounds [163]. Amongst the three by-products studied, the TPC was higher for the BSG extract in 70% acetone (8.1 ± 0.2 mg GAE/g DW), however lower than the value reported by Meneses *et al.* [32]. For comparison, assays using only distilled water as extraction solvent were also performed; however, the use of 70% acetone as solvent gave better extraction results. This could be explained by the fact that phenolic compounds are often more soluble in organic solvents less polar than water [32, 170, 172]. Meneses *et al.* [32] also reported that 60% acetone was more efficient than water in extracting phenolics from BSG. Furthermore, these results were in accordance with previous reports suggesting that a binary solvent system (acetone/water) is more

efficient than a mono-solvent system (water or pure acetone) in the extraction of phenolic compounds in regard to their relative polarity [170].

A wide array of phenolic compounds is reported to be present in food and beverages, and these include derivatives of benzoic and cinnamic acids, flavonoids, proanthocyanidins, tannins, and amino phenolic compounds [38, 172]. These constituents, which have been considered to be the most important source of antioxidants in cereals, exist in free as well as in the bound form. The majority of free phenolics are flavanols, whereas the bound phenolics are mainly phenolic acids [37]. Nevertheless the free forms of phenolic compounds are very rarely present in comparison with esters, glycosides and bound complexes [173]. Even though some of the chemical components of brewing by-products have been reported in the literature, the chemical composition of free, soluble ester and insoluble-bound phenolic acids have not been established. TPC of the separated fractions is represented in Fig. 4.3.

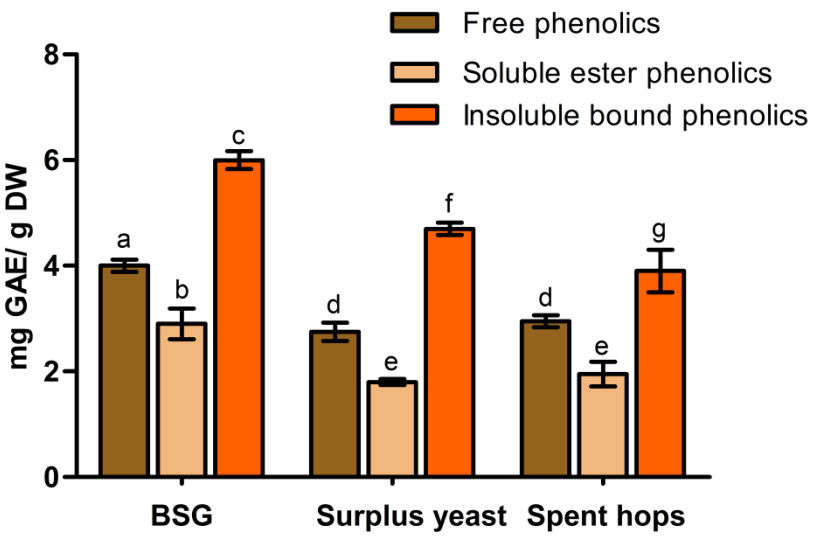

Fig. 4.3 - Total phenolic content in free, soluble ester and insoluble-bound fractions from by-products extracts. Bars represent mean ± SD (n = 3). Bar means with different superscript letter are statistically significant (p < 0.05). GAE – gallic acid equivalents.

According to the results obtained, the contribution of bound phenolics to the TPC was significantly higher than that of free and esterified fractions from the three by-products investigated. Free phenolics for the samples analyzed ranged from 2.8 ± 0.3 (surplus yeast) to 4.0 ± 0.2 (BSG) mg GAE/g DW. For ester and bound fractions, the phenolic content ranged from 1.8 ± 0.1 to 2.9 ± 0.5 and between 3.9 ± 0.7 and 6.0 ± 0.3 mg GAE/g DW, respectively. Most of the studies reported in the literature have

ignored insoluble bound phenolic compounds, hence underestimating the content of phenolic compounds present. Our results reported that 52%, 51% and 44% of total phenolics present in BSG, surplus yeast and spent hops, respectively, are in the insoluble-bound form.

The total sum of the phenolic compounds present in each fraction for BSG (11.6 ± 0.3 mg GAE/g DW), surplus yeast (9.3 ± 0.4 mg GAE/g DW) and spent hops (8.7 ± 0.6 mg GAE/g DW) is higher than that obtained for the aqueous methanolic or acetonic extracts, which is due to the fact that mechanical or soxhlet extractions do not consider the contribution of the bound phenolics. Several authors [8, 30, 32] have reported that BSG is a lignocellulosic material which contains significant amounts of phenolic acids esterified to the cell wall components, making their recovery harder. Therefore, these results enable us to conclude that the majority of phenolics, in the analyzed by-products, are in the bound form, probably associated with cell wall constituents. Having both carboxylic acid and hydroxyl groups in their structures, phenolic acids are capable of forming both ester and ether bonds with cell wall constituents, especially to arabinoxylans and lignin. Phenolic acids can also be esterified with small molecules such as alcohols, other phenolic acids, phenols, and alkaloids, among others [173]. Non-starch polysaccharides such as xylose and arabinose units may easily esterify with phenolic acids. Phenolic acids can bind either starch or other polysaccharides through hydrogen bonds, chelation, or covalent bonds [174].

4.2.2. Antioxidant properties

Phenolic compounds present in by-products extracts are complex, and their antioxidant activities and mechanisms will largely depend on the composition and conditions of the tested radical system. Many authors had stressed the need to perform more than one type of radical systems to evaluate the antioxidant activity of a selected sample [172, 173]. In the present work, DPPH, ABTS and FRAP assays were used to evaluate antioxidant activity of the extracts obtained by the different extraction techniques applied.

Table 4.3 - Radical scavenging activity for by-products extracts obtained by mechanical stirring and soxhlet extraction assessed by DPPH and ABTS assays. Values are expressed as mean ± SD (*n* = 3).

	DPPH (%)			ABTS (mgTE/ g DW)		
	BSG	**Surplus Yeast**	**Spent hops**	**BSG**	**Surplus Yeast**	**Spent hops**
Mechanical stirring						
Methanol 70%	4.7 ± 0.4^{a}	2.4 ± 0.2^{c}	3.7 ± 0.2^{e}	5.0 ± 0.3^{a}	4.2 ± 0.2^{a}	4.3 ± 0.3^{a}
Acetone 70%	7.5 ± 0.3^{b}	4.6 ± 0.5^{d}	5.4 ± 0.1^{f}	11.1 ± 0.4^{b}	9.7 ± 0.7^{c}	9.4 ± 0.6^{c}
Soxhlet extraction						
Methanol 70%	5.6 ± 0.4^{a}	3.4 ± 0.3^{d}	4.0 ± 0.2^{d}	11.1 ± 0.6^{a}	9.5 ± 0.4^{d}	9.06 ± 0.03^{d}
Acetone 70%	8.2 ± 0.3^{b}	5.7 ± 0.4^{e}	6.6 ± 0.3^{g}	13.7 ± 0.3^{b}	11.3 ± 0.1^{e}	11.4 ± 0.6^{e}
Water	3.1 ± 0.2^{c}	1.7 ± 0.2^{f}	2.2 ± 0.1^{f}	8.5 ± 0.3^{c}	8.11 ± 0.06^{c}	7.3 ± 0.6^{c}

Means within the same extraction technique with different superscript letter are statistically significant ($p < 0.05$). TE – trolox equivalents

The results shown in Table 4.3 indicate that BSG extracts obtained by mechanical and soxhlet extractions using 70% acetone have the highest scavenging activities towards the radicals DPPH and ABTS. This is in accordance with the higher TPC previously obtained (r^2 = 0.7114 and 0.8951 for DPPH and ABTS, respectively), confirming that phenolics are likely to contribute to the radical scavenging activity of these extracts.

Regarding the DPPH radical-scavenging activity, Table 4.3 shows that the best results in the DPPH assay (8.2 ± 0.3 % for BSG) were achieved when 70% acetone is used as the solvent. This indicates that extraction solvent had a significant ($p < 0.05$) influence on DPPH scavenging activity evaluation, which is in accordance with TPC results. Among the three by-products extracts analyzed, 70% acetone extract exhibited the highest DPPH scavenging activity, followed by 70% methanol, and water extracts. Meneses *et al.* [32] also reported that the highest values of DPPH inhibition were obtained for the BSG extracts obtained with acetone at 60% and 40% v/v. Zhao *et al.* [172] also performed a study similar to ours and report that the aqueous acetone extract from barley had the highest DPPH scavenging activity, followed by 80% methanol and water extracts. These authors also report that the 80% acetone extracts were found to contain the highest levels of (+)-catechin and ferulic, caffeic, vanillic, and *p*-coumaric acids, whereas the 80% methanol extract contained the highest (-)-

epicatechin and syringic acid contents and the water extract had the highest levels of protocatechuic and gallic acids. These phenolic compounds had various DPPH scavenging activities because of different chemical structures. A relationship between structure and activity of different compounds by the DPPH method has been established. Generally, monophenols are less efficient as antioxidants than polyphenols. Methoxy substitution also can increase the antioxidant activity of monophenols, but for phenolic acids, this fact is less important than the addition of a hydroxyl group [166]. Therefore, the differences in DPPH scavenging activities of the three kinds of solvent extracts from brewing by-products might be due to the difference in solvent selectivity for extracting certain phenolic groups with diverse DPPH scavenging activities. These data indicated that 70% acetone might be a better antioxidant extraction solvent from BSG, surplus yeast and spent hops against DPPH scavenging activity evaluation.

The ABTS assay was also used for *in vitro* assessment of free radical activity and the relative ability to scavenge the ABTS radical has been compared with the standard trolox (Table 4.3). ANOVA showed that different solvent extracts from the same by-product exhibited significantly ($p < 0.05$) different ABTS scavenging activities for the same extraction technique. Similar to the DPPH scavenging activity mentioned above, the 70% acetone extract from BSG showed the highest ABTS cation radical-scavenging activity. This finding was in agreement with the report that an 80% acetone extract from barley had the greatest ABTS scavenging activity among all barley extracts [172]. These data also verified considerable effects of extraction solvent mixtures on ABTS scavenging activity evaluation. Thus, 70% acetone might be the appropriate solvent for extracting phenolics from by-products with higher ABTS scavenging activity.

The antiradical activity, assessed by DPPH and ABTS assays, of free, soluble esters and insoluble-bound phenolics fractions is shown in Fig. 4.4.

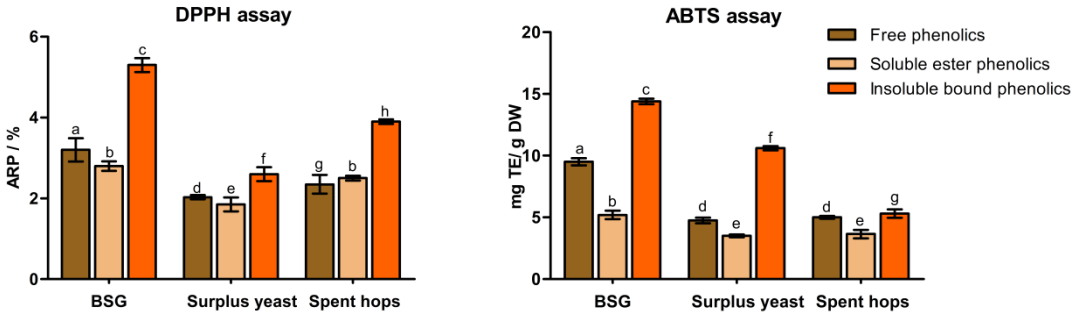

Fig. 4.4 - Radical scavenging activity assessed by DPPH and ABTS assays for free, soluble ester and insoluble-bound phenolics fractions from by-products. Bars represent mean ± SD (n = 3). Bar means with different superscript letter are statistically significant (p < 0.05). TE – trolox equivalents.

Results for the three by-products were in agreement with those obtained for the TPC, with insoluble-bound extracts exhibiting higher antioxidant activity than the other two phenolic fractions. For both assays, more than 40% of the antioxidant properties are owed to the phenolics from insoluble-bound fraction. Madhujith and Shahidi [173] also reported that more than 50% of total phenolics present in rice and corn are in the insoluble-bound form. As mentioned before, the high radical scavenging activity assessed by DPPH and ABTS assays reported in insoluble-bound phenolic fraction of by-products extracts can be attributed to the ability of this process to release phenolic compounds ether- or ester-linked with cell wall constituents, especially to arabinoxylans and lignin [134]. According to Markéta *et al* [37], phenolic acids are the major phenylpropanoid components in cereals, and different levels of these phenolics are found in different fractions of cereals. In cereals, the starchy endosperm contains low levels, whereas the outer layers of the grain (pericarp, aleurone layer, and germ) contain the highest. Naczk and Shahidi [93] also found high concentrations of phenolic acids in the outer layers of the grains predominantly existing in insoluble bound form. Therefore, the higher antioxidant activity of insoluble bound phenolic fraction can be attributed not only to the presence of different phenolics with different antioxidant potential, but also to the release of phenolics from the bound form.

BSG, surplus yeast and spent hops extracts were also assayed for their reducing capacity using the FRAP method (Table 4.4). Various solvent extracts from the same by-product showed significant (p < 0.05) differences in their reducing power, indicating that extraction solvent also influenced significantly by-products reducing power. Regardless of by-product analyzed, the reducing power of different solvent extracts decreased in the following order: 70% acetone extract > 70% methanol extract > water

extract. This is in accordance with the trend observed for DPPH and ABTS scavenging activities. These observations suggested that 70% acetone was more efficient solvent than other solvent mixtures concerning the by-products reducing power. Meneses *et al* [32] also evaluated the antioxidant activity of BSG extracts by the FRAP assay and the highest value was obtained for the extract produced with 60% acetone. According to these authors, acetone is able to provide high antioxidant yield due to its hydrogen-bonding ability, which is crucial for the extraction of antioxidant phenolic compounds. Other authors reported that the reducing power of a sample might be due to the presence of phenolic acids, which had been found to contribute to antioxidant activity by various reaction mechanisms [165, 170]. Indeed, some phenolic compounds such as catechin and ferulic, caffeic, and *p*-coumaric acids exhibited antioxidant activity through their reductive capacity in a Fe^{3+}–Fe^{2+} system [86]. In the current study, these phenolic compounds were found in by-products extracts (study reported in Section 4.2.3) and made contributions to the reducing power. However, the different levels and varieties of phenolic compounds can justify the differences of reducing power for different solvent extracts from BSG, surplus yeast and spent hops.

Table 4.4 - Ferric reducing capacity for by-products extracts obtained by mechanical stirring and soxhlet extraction. Values are expressed as mean ± SD (*n* = 3).

	FRAP (mgTE/ g DW)		
	BSG	**Surplus Yeast**	**Spent hops**
Mechanical stirring			
Methanol 70%	3.7 ± 0.2[a]	3.1 ± 0.1[a]	3.1 ± 0.2[a]
Acetone 70%	8.1 ± 0.3[b]	7.1 ± 0.5[c]	6.8 ± 0.5[c]
Soxhlet extraction			
Methanol 70%	8.1 ± 0.5[a]	6.9 ± 0.3[d]	6.60 ± 0.02[d]
Acetone 70%	10.0 ± 0.2[b]	8.2 ± 0.1[e]	8.3 ± 0.5[e]
Water	5.8 ± 0.3[c]	5.90 ± 0.05[c]	5.3 ± 0.5[c]

Means within the same extraction technique with different superscript letter are statistically significant ($p < 0.05$). TE – trolox equivalents

FRAP values of free, soluble ester and insoluble-bound phenolic fractions ranged from 3.5 ± 0.3 to 6.9 ± 0.4, 2.6 ± 0.2 to 3.8 ± 0.5, and 6.9 ± 0.2 and 9.6 ± 0.3 mg TE/g DW, respectively (Fig. 4.5). In agreement with DPPH and ABTS radical scavenging data, the contribution of insoluble-bound fraction to reducing capacity was 2-fold higher

than free and soluble ester fractions. As stated above, these differences in the reducing power between the phenolic fractions could be attributed to their phenolic acids constituents. Therefore, phenolics that reduce Fe^{3+} to Fe^{2+} are mainly found in the insoluble-bound fraction.

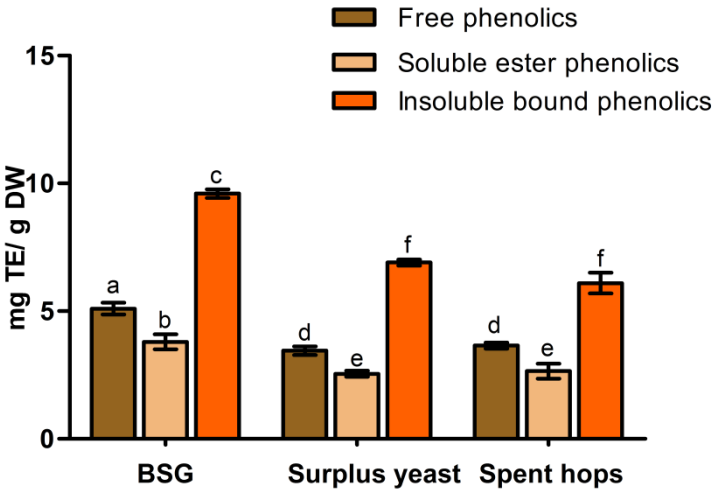

Fig. 4.5 - Ferric reducing capacity for free, soluble ester and insoluble-bound phenolics from by-products. Bars represent mean ± SD (n = 3). Bar means with different superscript letter are statistically significant (p < 0.05). TE – trolox equivalents.

Briefly, the reported results show that 70% acetone was more efficient solvent than other solvent mixtures concerning the phenolics extraction from brewing by-products. The structure of the phenolic compounds present in each extracts is also a key determinant of their differences in radical scavenging activity. Therefore, to explore the individual phenolic constituents present in BSG, surplus yeast and spent hops extracts HPLC analyzes were performed.

4.2.3. Determination of phenolic compounds in by-products extracts by HPLC-DAD analysis

In order to study the influence of the extraction technique on the phenolic composition, the HPLC chromatograms for BSG extracts obtained by mechanical stirring and soxhlet extraction using 70% acetone as extractant are represented in Fig. 4.6. The chromatograms with phenolic composition are only reported for BSG, since this by-product presented the highest TPC and antioxidant activity. Additionally, amongst the by-products studied, BSG is the one produced in larger quantities by the brewing industry.

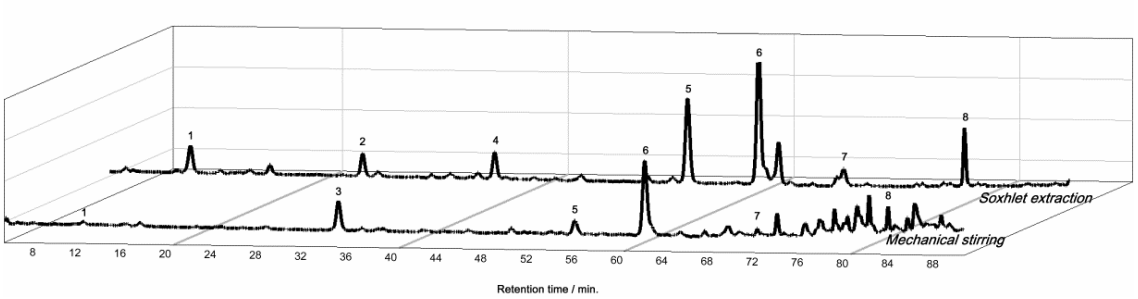

Fig. 4.6 - HPLC chromatogram at 280 nm for BSG acetonic extracts obtained by mechanical or soxhlet extractions; numbered peaks represent identified compounds listed in Table 4.5.

The compounds present in BSG extracts were identified by comparisons with the retention time and UV spectra of authentic standards, while the quantitative data were calculated from the calibration curves. The content of individual phenolic compounds in BSG extracts is summarized in Table 4.5. The results indicated that FA was the dominant phenolic compound in BSG acetonic extracts, which accounted for 37% and 43% of the total extracted phenolics obtained by mechanical stirring and soxhlet extraction, respectively. *p*-CA was the second compound present in higher concentration in BSG representing 25% (for mechanical stirring) and 20% (for soxhlet extraction) of the total identified phenolics. These results are in agreement with previous works [17, 30, 87], which reported that the major compounds contributing to the phenolic content of BSG are FA and *p*-CA. Szwajgier *et al.* [87] reported that FA and *p*-CA content was 3.36 and 0.644 mg/g, respectively. The same authors also reported that following FA and *p*-CA, the next most abundant phenolic acids in BSG were found to be sinapic (0.42 mg/g), caffeic (0.099 mg/g) and syringic (0.065 mg/g) acids. In the present study, lower levels of sinapic acid were detected for both extraction techniques, while caffeic acid was only reported for soxhlet extracts (0.61 mg/g). Additionally, it was also observed that the sum of individual phenolic content for soxhlet extraction was 1.6-fold higher than the value obtained for mechanical stirring, indicating that this extraction technique was more efficient. Soxhlet extraction showed the highest extraction capacity for gallic (3-fold), ferulic (1.9-fold), *p*-coumaric (1.3-fold) and sinapic acids (2-fold), whereas mechanical stirring had the strongest extraction capacity only for cinnamic acid (1.1-fold). Moreover, (+)-catechin and caffeic acid were only extracted by soxhlet extraction whereas epicatechin by mechanical stirring. These results confirm that different extraction techniques had significantly different extraction capacity and selectivity for phenolic compounds. The higher soxhlet extraction temperature is probably the major factor contributing to this difference. In fact,

temperature increase has been reported to improve the efficiency of extraction due to enhanced diffusion rate and solubility of the compounds in solvents [32]. Moreover, BSG is a lignocellulosic material which contains significant amounts of phenolic acids esterified to the cell wall components, making their recovery harder. Therefore, the higher temperature employed by soxhlet extraction seems to be more effective in the release of these bound phenolics.

Table 4.5 - Content of specific phenolic compounds in BSG acetonic extracts obtained by mechanical and soxhlet extraction (mg/g DW)

Compounds	Peak label	t_R /min	Mechanical stirring	Soxhlet Extraction
Gallic acid	1	12,18	0.09	0.27
Catechin	2	26,44	ND[a]	0.21
Epicatechin	3	34,21	0.27	ND
Caffeic acid	4	38,14	ND	0.61
***p*-Coumaric acid**	5	56,24	0.88	1.1
Ferulic acid	6	59,34	1.29	2.40
Sinapic acid	7	70,96	0.15	0.32
Cinnamic acid	8	79,07	0.80	0.70
		∑ TPA	**3.48**	**5.61**

[a]ND: not detected; ∑TPA – Total phenolic acid content = sum of individual phenolic compounds from mechanical and soxhlet extractions

Table 4.6 shows the content of major phenolics for free, soluble ester and insoluble bound fractions from BSG, surplus yeast and spent hops.

Hydroxycinnamic acids (caffeic, sinapic, *p*-coumaric and ferulic) were the major phenylpropanoid components in BSG, surplus yeast and spent hops, and different levels of these phenolics were found in different fractions. FA and *p*-CA were clearly the most abundant phenolic compounds found in BSG, as reported in the literature [30, 32]. These results are also in agreement with those obtained for mechanical and soxhlet extractions, representing more than 90% of phenolics extracted from BSG in the free, soluble-ester and insoluble-bound fractions (Table 4.6). As can be seen in Fig. 4.7, 64% of phenolics present in insoluble bound fraction correspond to FA, whereas 51% of esterified phenolics are *p*-CA based compounds. This finding was in agreement

with Faulds *et al.* [73], who pointed out that 65% of the available FA together with three forms of diferulate were released from BSG by the action of an “esterase-free” Ultraflo preparation that enhanced the ability of a feruloyl esterase from *Aspergillus niger*, AnFAEA, to release FA from BSG. This data enable us to conclude that the majority of phenolics in BSG are associated with cell wall components, covalently cross-linked by ester and/or ether bonds.

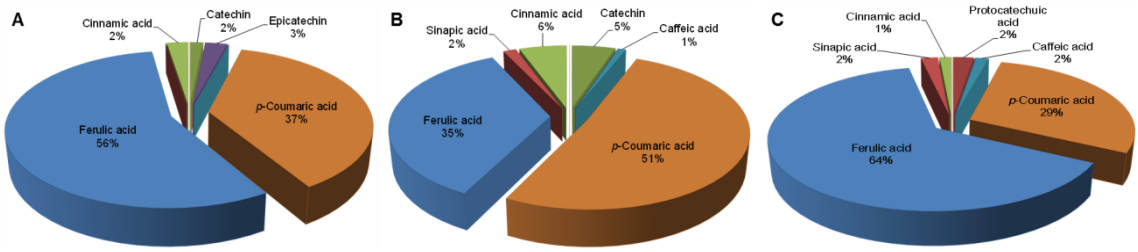

Fig. 4.7 - Contribution of each phenolic to the total phenolic content for free (A), soluble ester (B) and insoluble bound (C) fractions from BSG.

Regarding the phenolic composition from surplus yeast, FA and *p*-CA also appears as the main contributors to the phenolic fractions. As can be seen in Fig. 4.8, these compounds were essentially found in the free or soluble ester phenolic fraction. In addition to these two compounds, another one was identified which had not been detected for BSG fractions. Although present in the three phenolic fractions, XN detected in three phenolic fractions only represents 6% of the total extracted compounds for surplus yeast. Magalhães *et al.* [164] reported that during fermentation process an additional 18% of XN was lost, mainly due to the adsorption of this compound to yeast cells. They reported that the combined losses (spent hops, hot trub and surplus yeast) totalled 57%, leaving 24% of the amount of XN initially present in the hops unaccounted for.

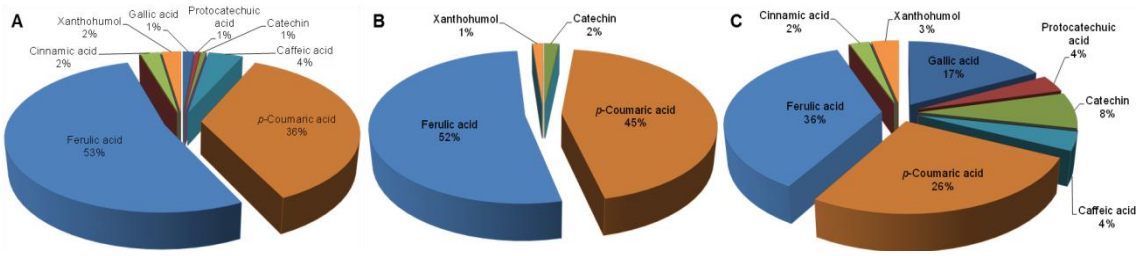

Fig. 4.8 - Contribution of each phenolic to the total phenolic content for free (A), soluble ester (B) and insoluble bound (C) fractions from surplus yeast.

For spent hops, the dominant compound was XN, which accounted for 87%, 77% and 68% of the total phenolic content for soluble ester, free and insoluble bound phenolic fractions, respectively (Fig. 4.9). Besides FA and *p*-CA were also detected in

all fractions, however their content was at least 5-fold lower in comparison with the reported for BSG. Furthermore, another flavonoid compound, namely quercetin, was also identified and quantified only for spent hop extracts. This compound was mainly found in the insoluble bound phenolic fraction representing 7% of total extracted phenolics.

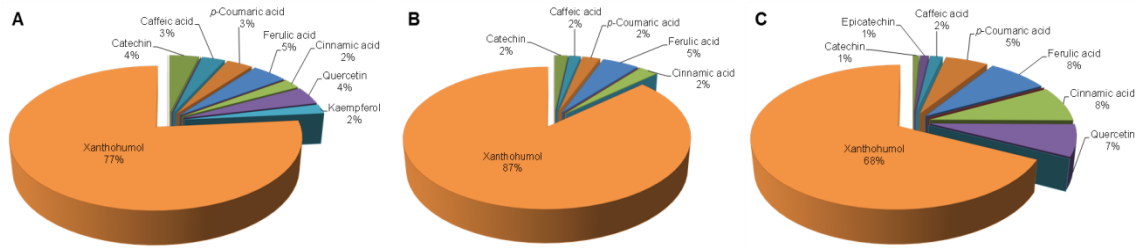

Fig. 4.9 - Contribution of each phenolic to the total phenolic content for free (A), soluble ester (B) and insoluble bound (C) fractions from spent hops.

For the three by-products analyzed, the results of this study indicated that at least 48% of total phenolics identified were in the insoluble bound form (Fig. 4.10). The carboxylic acid and hydroxyl groups present in the majority of the structures of these phenolics are capable of forming ester and/or ether bonds with cell wall polysaccharides, which are difficult to dissociate [173]. Generally, alkaline hydrolysis are the methods mostly adopted to release insoluble-bound phenolics from these type of samples [17, 30, 174]. This type of extraction will be discussed in more detail in Chapter 5.

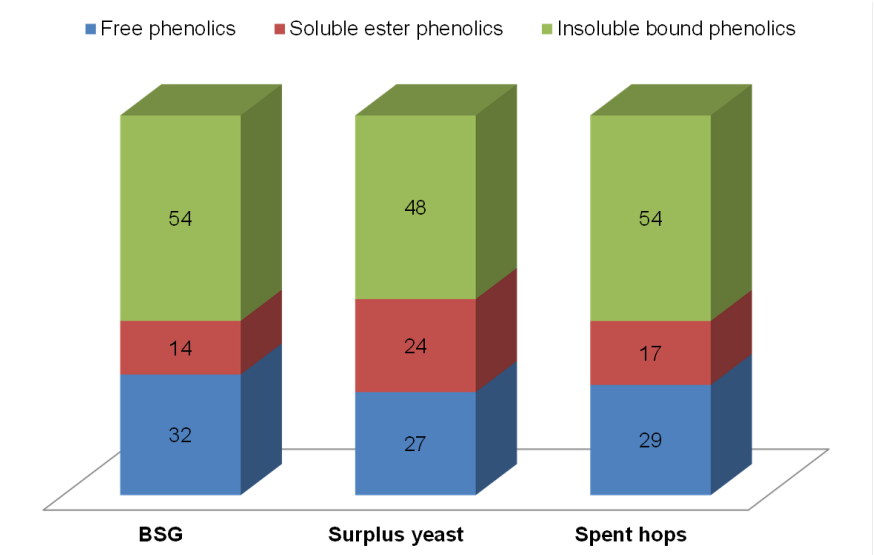

Fig. 4.10 - Contribution of free, soluble ester and insoluble bound phenolic fractions to the total extracted phenolics for BSG, surplus yeast and spent hops.

Since phenolic compounds provide health benefits, by-products extracts could be of great interest for application in food and pharmaceutical industries. However, it merits emphasizing that although the reported techniques are the most commonly used, they are timing and solvent consuming and the solvents used are usually toxic, arising serious issues when the purpose of the compounds extracted is the application in food and pharmaceutical products. To overcome this problem, the next step of our research work will be focused on finding environmentally acceptable extraction methods. The techniques used in the present study were useful to verify the possibility of obtaining antioxidant phenolic extracts from these brewing by-products. However, a more efficient and friendly extraction technique which fulfills the desirable features of green extraction methods, namely low solvent consumption, short extraction time and high extraction yield, is needed.

Table 4.6 - Content of specific phenolic compounds in free, soluble-ester and insoluble-bound fractions from BSG, surplus yeast and spent hops (mg/g DW)

Compounds	Peak label	BSG			Surplus yeast			Spent hops		
		Free	Soluble-ester	Insoluble-bound	Free	Soluble-ester	Insoluble-bound	Free	Soluble-ester	Insoluble-bound
Gallic acid	1	ND[a]	ND	ND	0.025	ND	0.580	ND	ND	ND
Protocatechuic acid	1'	ND	ND	0.120	0.012	ND	0.120	ND	ND	ND
Catechin	2	0.045	0.068	ND	0.012	0.030	0.250	0.061	0.016	0.022
Epicatechin	3	0.078	ND	ND	ND	ND	ND	ND	ND	0.034
Caffeic acid	4	ND	0.012	0.075	0.078	ND	0.130	0.048	0.014	0.048
***p*-Coumaric acid**	5	1.110	0.680	1.46	0.680	0.760	0.880	0.054	0.022	0.164
Ferulic acid	6	1.670	0.470	3.250	0.990	0.890	1.210	0.073	0.045	0.233
Sinapic acid	7	ND	0.020	0.098	ND	ND	ND	ND	ND	ND
Cinnamic acid	8	0.074	0.074	0.067	0.043	ND	0.078	0.035	0.023	0.235
Quercetin	9	ND	ND	ND	ND	ND	ND	0.064	ND	0.210
Kaempferol	10	ND	ND	ND	ND	ND	ND	0.034	ND	ND
Xanthohumol	11	ND	ND	ND	0.044	0.021	0.110	1.20	0.790	1.980
	∑ TPA	**2.977**	**1.324**	**5.07**	**1.884**	**1.701**	**3.358**	**1.569**	**0.910**	**2.926**

[a]ND: not detected; ∑TPA – Total phenolic acid content = sum of individual phenolic compounds from isolated fractions

4.3. Supercritical fluid extraction

In this section, SFE, a greener extraction technique more indicated for the production of extracts to be used in food, pharmaceutical or cosmetic industries will be applied in an attempt to increase the amount of phenolics released from BSG. According to our previous results, BSG exhibited the highest antioxidant activity and phenolic content compared to the surplus yeast and spent hops. Additionally, from these three by-products investigated, BSG is the major by-product generated by the brewing industry.

As stated before, the extraction of phenolic compounds is frequently done by conventional solvent extraction followed by fractionation methods. This procedure consumes large amounts of organic solvents and requires a long period of extraction; additionally, the final product may have undesirable residues [142, 147]. The increasing interest in both high-quality products and environmentally friendly technologies indicates that SFE could be a suitable process for the extraction of these compounds. The SFE is a relatively recent technique which presents various advantages over traditional methods, like the use of low temperatures and reduced energy consumption and high product quality due to the absence of solvent in solute phase [126, 147]. Supercritical carbon dioxide (SC-CO_2) is the most used solvent for SFE due to its particular characteristics such as moderate critical conditions (31.1 ºC and 7.4 MPa) and of easy availability. A compound is in its supercritical state when it is heated and compressed above its critical temperature and critical pressure. In the supercritical state, the substance exists as a single fluid phase with properties intermediate between those of liquids and gases: the densities are liquid-like, whereas the diffusivities and viscosities are gas-like [143]. Moreover, supercritical CO_2 has zero surface tension, which allows easy penetration into most matrices. In the supercritical state, supercritical CO_2 is extremely sensitive to small changes in temperature and pressure such that a compound may be extracted from a matrix at one set of conditions and then separated from supercritical CO_2 in a downstream operation under a slightly different set of conditions [142, 143]. Some of the other advantages of supercritical CO_2 extraction include the CO_2 properties as non-toxic, inflammable, chemically stable, environment-friendly and easily separated from the extract. In the areas of cosmetics, essences, foods and agricultural products, the applications of SFE are multiple, from decaffeination of coffee and tea, to extraction of flavors, aromas, pigments, antioxidants and spices, among others [142, 147]. Though, SC-CO_2 dissolves

preferably nonpolar and low-polarity substances of small and medium molecular size. Phenolics are polar compounds; hence, they are difficult to extract using pure carbon dioxide. The introduction of modifiers, such as ethanol, methanol or water, enhances the solvating power of CO_2, which may increase the selectivity and extraction yield of target compounds [175]. Fernández *et al.* [121] evaluated the use of supercritical fluid technology coupled with pretreatment processes for the valorization of BSG to design a global management process producing extracts containing valuable compounds, such as tocopherols. The preliminary technical analyses set the best operating conditions of the SC-CO_2 extraction at 35 MPa and 313 K using a milled vacuum-dried BSG sample. Under these conditions, BSG contained a tocopherol concentration in the extract of 2 mg L^{-1}.

The present investigation was undertaken to determine the application of the supercritical technology for the extraction of phenolic fractions with antioxidant activity from BSG, using SC-CO_2 and SC-CO_2 with ethanol as co-solvent. This alternative method was compared to results previous obtained with traditional extraction methods, such as Soxhlet and mechanical stirring, in terms of process yield evaluated by the HPLC-ESI-MS analysis of the extracts.

4.3.1. Materials and Methods

Chemicals

Carbon dioxide was purchased from Praxair (Porto, Portugal) with a purity of 99.95%. Hexane and ethanol were supplied by VWR (Darmstadt, Germany).

Samples

Dried BSG sieved trough a 35-mesh (≤0.5 mm) sieve was used for these studies. The dried BSG submitted to a previous Soxhlet extraction with hexane for 6 h, to remove the lipophilic components, was also used in these experiments.

Supercritical fluid extraction

Supercritical fluid extractions were performed using a homemade SFE system, which is schematically represented in Fig. 4.11. In this system, the liquid CO_2 taken from a cylinder is compressed to the desired extraction pressure by means of a cooled liquid pump after which it goes through a Coriolis mass flow meter used to measure the

CO_2 flow rate. The liquid stream is then heated to the operating temperature in a vessel placed before the extractor column. The solvent in the supercritical state then flows through the extractor where the sample was previously loaded. Afterwards, the effluent from the extractor is depressurized in a heated backpressure regulator valve and bubbled in ethanol to capture the extract. Then, ethanol extract is evaporated to dryness at 35 ºC under vacuum and resuspended in 500 μL ethanol for subsequent LC-MS analysis. The spent CO_2 is vented to the atmosphere. The addition of co-solvent to the system is accomplished by a liquid pump (LabAlliance Model 1500) coupled to the gas line between the mass flow meter and the heating vessel placed before the extractor column, in order to mix both CO_2 and co-solvent before feeding the extraction vessel. The co-solvent flow rate is controlled by the liquid pump.

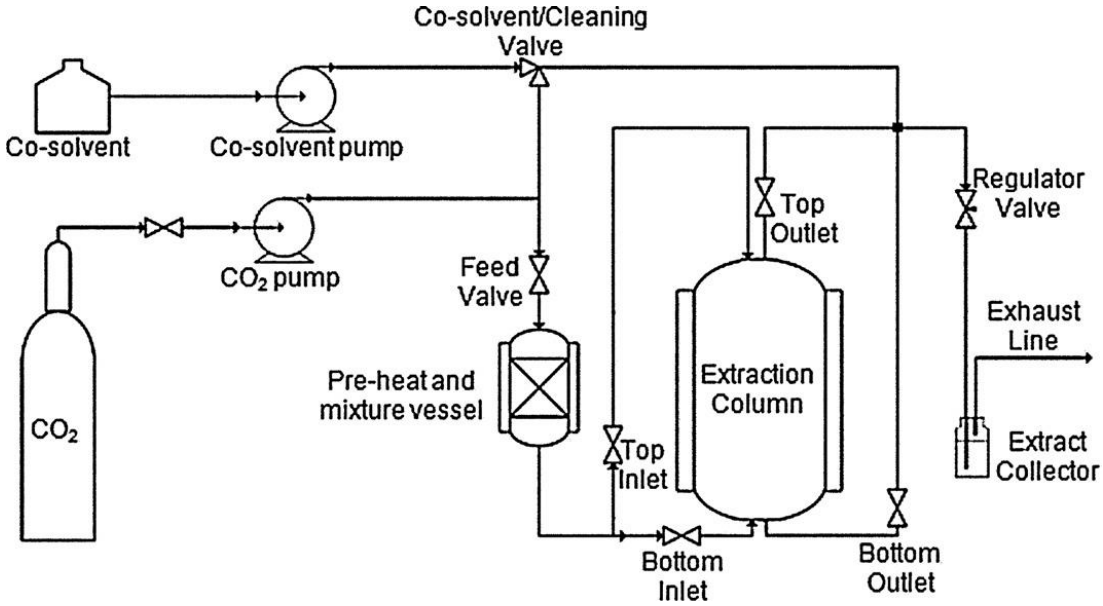

Fig. 4.11 - Supercritical fluid extraction unit (Source: Santos *et al*. [175]).

For each experiment the extraction column was fed with 40 g of dried BSG. The experimental conditions were 100 bar, 60 ºC, and CO_2 flow rate of 8 g min^{-1}, during 4 hours. The carbon dioxide was used pure and modified with 10% of ethanol (CO_2/EtOH). The co-solvent and its content were fixed according to literature data for the critical properties of these binary mixtures [141, 175]. The extracts were collected in ethanol and evaporated under nitrogen. To evaluate the influence of pressure in the removal of our target compounds an SFE experiment was also carried out using a pressure of 150 bar.

HPLC-ESI-MS analysis

Analyses were carried out according to the previously reported procedure in (Section 4.1.9).

4.3.2. Results and Discussion

The first step in the SFE of phenolic compounds is to optimize the conditions of extraction. Pressure, temperature, solvent flow rate and type of solvent are considered as the most important factors as they play a critical role in the extraction yields, phenolic concentration and antiradical activity of extracts obtained. The origin of the plant material, chemical composition and particle size of the material, different types of tissues (leaves, stems, seeds, etc.) and other variables, like pretreatment and storage conditions also affect yield and the composition of the extract in SFE. Therefore, optimization of the process and material parameters are important to provide maximum yields with highest quality and making the final product suitable for use in foods, cosmetic or pharmaceutical industries. Several researchers studied the supercritical extraction process of phenolic phytochemicals in a wide group of representative samples from the food sources. Experimental data showed that temperatures for SFE of phenolic compounds range from 35 to 120 ºC, pressures from 50 to 600 bar, time from 5 to 300 minutes and co-solvent from 0 to 20% [126, 143, 176]. Therefore, the selection of the conditions to be used for the SC-CO_2 extraction of phenolics from BSG was made according to the literature information and taking into consideration the operating limits of the supercritical equipment.

Fig. 4.12 displays the HPLC chromatograms of the BSG extracts obtained after the SFE under the tested conditions. According to our previous results, as ferulic and *p*-coumaric acids were the major phenolics in the BSG extracts, we used them to evaluate the efficiency of the SC-CO_2 extraction.

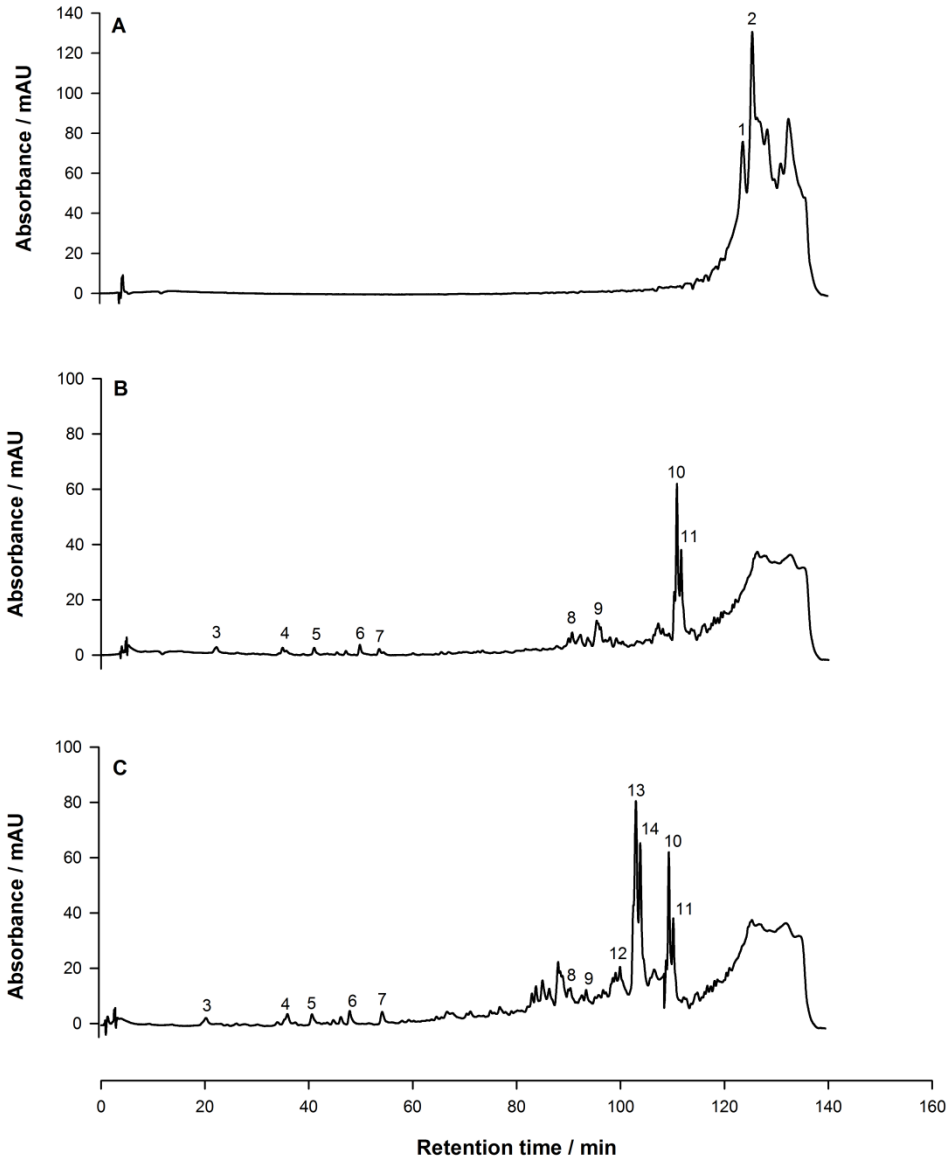

Fig. 4.12 - HPLC chromatograms at 280 nm of BSG supercritical extracts at 100 bar, 60 ºC and without the previous soxhlet extraction with hexane (A), 100 bar, 60 ºC and after the soxhlet extraction with hexane (B) and 150 bar, 60 ºC, after soxhlet extraction and with 10% ethanol as co-solvent (C): numbered peaks represent identified compounds listed in Table 4.7.

For the SC-CO_2 extraction without the previous soxhlet extraction with hexane (Fig. 4.12A), FA, *p*-CA or any other phenolic compounds were not identified. HPLC-ESI-MS analyses (Table 4.7) enabled us to identify two compounds that correspond to tocopherol (peak 2) and to an unsaturated fatty acid, linoleic acid (peak 1). Therefore, the compounds detected in the HPLC chromatogram probably correspond to the essential oil fraction of BSG, which accounts for 13% of the composition and could be easily extracted with SC-CO_2 [55, 177]. Panfili *et al.* [177] studied the working conditions for the extraction of wheat germ oil in a supercritical CO_2 pilot plant of 1 L

extraction capacity. They reported that under the best extraction conditions (38 MPa; temperature, 55 ºC; wheat germ particle size, about 0.35 mm; CO_2 flow rate, 1.5 L min^{-1}) a yield of about 92% of total oil after 3 h of processing was recovered. Further, Fernández *et al.* [121] also used SC-CO_2 extraction for the valorization of BSG and they reported that under the best extraction conditions (35 MPa and 313 K) a tocopherol concentration of 2 mg L^{-1} was recovered from the BSG extract. According to our knowledge, there are no studies concerning the SC-CO_2 extraction of essential oils from BSG. However, the compounds present in essential oil fraction are also valuable owing to its nutritional and pharmaceutical properties. Despite being out of the main goal for this thesis, these results could be a starting point for another way to valorize BSG.

It was previously observed, that pre-treatment with non-polar solvents (e.g. hexane) to remove essential oils improves the extraction of polyphenols from grape [144]. Therefore, a previous soxhlet extraction of BSG with hexane was performed in order to remove the essentials oils. The comparison of the HPLC chromatograms from Fig. 4.12A and Fig. 4.12B enables us to conclude that the previous extraction with hexane enhances the detection of other compounds in BSG extracts after SC-CO_2 extraction. The compounds identified in SC-CO_2 BSG extract by HPLC-ESI-MS analysis were sugars, such as arabinose, xylose and glucose, and some vitamins of the B group, like B_3 and B_6. Further, some fatty acids were also identified in the SC-CO_2 BSG extracts, namely palmitic (C_{10}), stearic (C_{18}), oleic ($C_{18:1}$) and linolenic ($C_{18:3}$) acids (Table 4.7). None of the phenolics identified before for the BSG extracts obtained by conventional extractions, such soxhlet and mechanical stirring, were detected. This behavior could be explained by the non-polar characteristic of the carbon dioxide, which increases the extraction of low polarity compounds compared with polar ones (particularly found in the phenolic fraction). As previously observed, BSG contain more polar phenolics, such as FA and *p*-CA, thus the non-polar SC-CO_2 is not an effective extractant. Our results are in agreement with those reported by Castro-Vargas *et al* [141], who reported that the yield of the SFE process in terms of phenolic fraction was lower than the value obtained by soxhlet extraction with ethanol. Nevertheless, it should be mentioned that the identified sugars can be purified or alternatively fermented by microorganisms to produce value-added products such as xylitol, organic acids, amino acids, vitamins, ethanol or butanediol as suggested by Mussatto and Roberto [16]. These authors have reported that BSG contains sugars polymerized to cellulose (glucose) and hemicellulose (basically xylose and arabinose), which can be liberated by a hydrolysis

process (liquid-to-solid ratio of 10 g g^{-1} and 120 mg H_2SO_4 g^{-1} dry matter). Under these reaction conditions 76.2% of the hemicellulose was hydrolyzed and the xylose and arabinose sugars were recovered with 67 and 97.8% efficiency, respectively.

Table 4.7 - HPLC-ESI-MS/MS of compounds identified in supercritical fluid extracts of BSG. Main MS fragment and typical fragmentations of the compounds are given

Peak label	$[M+H]^+$	MS/MS fragments	Attribution
1	280	----	Linoleic acid
2	431	----	Tocopherol
3	124	106	Vitamin B_3
4	151	133	Xylose
5	151	133	Arabinose
6	170	152; 134	Vitamin B_6
7	181	145; 127	Glucose
8	256	239; 115	Palmitic acid
9	278	260	Linolenic acid
10	282	264	Oleic acid
11	284	267	Stearic acid
12	287	251	Kaempferol
13	303	285	Quercetin
14	317	153	Isorhamnetin

Generally, the studies reporting the use of SC-CO_2 extraction for the recovery of phenolics use higher pressures and a co-solvent. Castro-Vargas *et al* [141] reported that the best extraction conditions to obtain the phenolic fraction from guava seeds (*Psidium guajava L.*) were 50 ºC, 300 bar and ethanol as co-solvent in a concentration of 10%. Santos *et al* [175] have also studied the influence of SC-CO_2 extraction conditions on phenolics recovery from *Eucalyptus globulus* bark and reported that an extraction performed at 300 bar, 70 ºC and 20% of ethanol as co-solvent gives the highest phenolic content (119.46 mg g^{-1} of extract). Therefore, another experiment using higher pressure (150 bar) and a co-solvent (10% ethanol) was performed (Fig.

4.12C). The SC-CO_2 extraction could not be performed at a pressure higher than 150 bar, due to the equipment restrictions.

As can be seen by the comparison of HPLC chromatograms from Fig. 4.12B and Fig. 4.12C, the change in pressure from 100 to 150 bar with constant temperature of 60 °C did not cause any significant change in the extracted compounds from BSG. It was observed that the absorbance intensity was higher when the SC-CO_2 extraction was performed at 150 bar. At constant temperature (60 ºC), the increase in pressure increases the yield due to the density enhancement. An increase on pressure can result in an increase in the fluid density, which alters solute solubility. Therefore, it is possible to modify the composition of supercritical extracts altering extraction pressure [143, 176]. A common practice in SFE is to change the polarity of the supercritical fluid and increase on the solvating power towards the target compounds by addition of small amounts of organic co-solvents like ethanol and methanol. The co-solvents interact strongly with analytes improving significantly the extraction yields. Ethanol and methanol are capable of hydrogen-bonding, dipole-dipole and others polarity interactions with phenolics, which make them interesting for the recovery of these compounds [142, 143, 176]. This could explain the enhanced extraction yield of the identified compounds when 10% ethanol was used as co-solvent. Furthermore, the use of a co-solvent also enables us to identify three more compounds, namely kaempferol, quercetin and isorhamnetin, which antioxidant properties are widely recognized (Table 4.7). However, the co-solvent addition is still not enough for efficient BSG phenolics extraction by SC-CO_2, probably because the content of the organic modifier is not sufficient for their complete isolation [118]. Vatai *et al*. [126] also reported a positive effect of pressure on total phenolic content of grape marc. Their results also agree that higher percent of co-solvent (up to 20% methanol or ethanol) and extraction at subcritical conditions is more efficient for the extraction of polyphenols from this sample. However, they concluded that the yield of polyphenols in such extraction is still lower when compared to conventional solvent extraction.

Additionally, the solubility of the phenolics in the solvent is another important consideration for the extraction efficiency. For example, when using SFE, solubility is a strong function of supercritical CO_2 density and the properties of the solute such as molecular weight, polarity and vapor pressure [176]. Antioxidants are soluble in supercritical CO_2 to different extents depending on the temperature and pressure conditions. Solubility behavior of phenolics, namely hydroxycinnamic acids, in

supercritical CO_2 has been previously reviewed [178, 179]. Murga *et al* [179] determined the solubility of three *trans*-HCA, namely *p*-CA, caffeic acid and FA, in SC-CO_2 at different pressures up to 50 MPa and temperatures from 313 to 333 K. Under the conditions used in this assay, 333 K and 100 bar, the solubility of *p*-CA and FA expressed as solute mole fraction was 0.032×10^{7} and 0.653×10^{7}, respectively. When the pressure increased to 150 bar, the solubility of these compounds increased to 1.886×10^{7} (*p*-CA) and 38.310×10^{7} (FA). In fact, they reported that the solubility increased with pressure, at constant temperature, for all tested conditions. They also concluded that under the same conditions of temperature and pressure, FA shows a higher solubility in SC-CO_2 than *p*-CA whose solubility is higher than that of caffeic acid. This behavior could be explained by the differences in chemical structures, since the addition of a hydroxyl group generally results in a decrease of the solubility of the new molecule (caffeic acid is formed from *p*-CA by addition of a hydroxyl group), and partial etherification (FA is formed by etherification of caffeic acid) generally causes a large increase in solubility. As these HCAs are some of the major phenolic compounds present in BSG, the low solubility of them under the tested conditions can explain the absence of phenolics in the obtained SC-CO_2 extracts.

Despite the results obtained, data presented in this work is valuable to know the possibility of phenolics separation from BSG by extraction with SC-CO_2. Taking into account what was previously presented, one of most important factors for SC-CO_2 extraction is the solubility of phenolics in CO_2. Therefore, further studies at pressures higher than 150 bar could be performed for the extraction of phenolics from BSG. Moreover, a two-step extraction could also be an effective approach in the SC-CO_2 extraction of phenolics from BSG. SC-CO_2 can be employed in the pre-treatment of BSG, mainly for the purification of the primary extract in order to remove the non-polar compounds. After this treatment, the polar polyphenols became more accessible for further extraction process. This approach has been used to extract some phenolics fractions from cocoa hulls, grape seeds, elder berry, and wine by-product [126, 140]. In general, these investigations showed that the TPC obtained in the two-step extraction was significantly higher compared to the single-step extraction procedure. They reported that the non-polar components were removed by CO_2, thus in the second-step extraction, 2 to 3 times more total phenols were obtained than in the single-step extraction. Possibly, the extraction efficiency was improved also due to the ‘‘cell-opening” of the plant, caused by high pressure.

According to the aforementioned, the use of SC-CO_2, under the tested conditions, has not proven to be an efficient process to extract phenolics of BSG. The main targets of our research are to provide maximum yields, preserving highest quality with antioxidant activity, making the final product suitable for use in food, cosmetic or pharmaceutical industries. The selection of an appropriate extraction method mainly depends on the advantages and disadvantages of the processes, such as extraction yield, complexity, production cost, environmental friendliness and safety. In general, SFE is a clean method, considered an environmental friendly technology. The drawbacks of this extraction technique are the economics and onerous operating conditions needed, which has restricted the applications to some very specialized fields, such as essential oil extraction and coffee decaffeination. Therefore, the next step of this research will be focused on finding other extraction methods that considerably reduces extraction time, energy and solvent consumption, while generating higher amounts of phenolics extracted from BSG. This should be combined with technologies and methods convenient for commercial applications, with low-cost process and easy to scale-up.

5. Application of microwave-assisted extraction of polyphenols from brewer's spent grains with HPLC-DAD-MS analysis

The low-cost and large availability of BSG, associated with the current interest in the health benefits of phenolic acids, have stimulated the development of new processes for the extraction of the polyphenolic fraction from BSG [8]. Generally, the conventional extraction techniques for FA extraction are mechanical stirring [38], Soxhlet extraction [126], enzymatic [31, 87] and alkaline extractions [17, 30]. These extraction techniques are timing and solvent consuming, submitting polyphenols to thermal degradation and decreasing the antioxidant activity of the extracts due to the long extraction time used.

In recent years, several faster and more automatic extraction techniques for solid samples have been replacing conventional techniques. Among the modern techniques are extraction by supercritical fluids (SFE) and extraction assisted by microwave (MAE). These alternatives techniques considerably reduce the consumption of solvents, increase the speed of the extraction process, and simplify it.

As demonstrated in Section 4.3, SFE was not effective for the extraction of phenolics from BSG under the tested conditions. The principal advantage of SFE is that practically no organic solvents are utilised, although this technique is limited to compounds of low or medium polarity. The literature contains descriptions of extraction methods for polyphenols by SFE, the main characteristics of which are the need for high percentages of organic modifier; this usually means that the process takes place under subcritical conditions.

Recently, microwave-assisted extraction (MAE) has been successfully applied to the extraction of natural products that typically needed hours to reach completion with conventional methods [180]. This technique uses the energy of microwaves to cause molecular movement and rotation of liquids with a permanent dipole leading to a very fast heating of the solvent and the sample, offering advantages like improved efficiency, reduced extraction time, low solvent consumption and high level of automation compared to conventional extraction techniques. In addition, by using closed vessels, the extraction can be performed at elevated temperatures accelerating

the mass transfer of target compounds from the sample matrix [149]. Several classes of phenolic compounds have been efficiently extracted from a variety of matrices, such as apple pomace [148], red raspberries [149], green tea leaves [150], grape seed [137], wheat bran [151], and distillers dried grains [138]. However, only one report was found concerning the determination of phenolic acids in BSG using some kind of microwave-based technique. Athanasios *et al.* [92] used microwave irradiation for the trimethylsilyl derivatization of phenolic acids in a closed vial. These authors suggest that this derivatization procedure is a very efficient method comparatively to the conventional heating method.

The purpose of this work was to develop a new extraction process of polyphenols from BSG based on MAE. As many factors can influence the polyphenol yield, response surface methodology (RSM) was applied to fit and exploit mathematical models representing the relationship between the response (FA extraction yield) and input variables (extraction time, temperature, solvent volume, and stirring speed) [181]. To our knowledge, this is the first report describing the application and optimization of the MAE of polyphenols from BSG using RSM. The phenolic composition of the obtained BSG extract was investigated by HPLC-DAD-MS analysis. The developed MAE procedure was compared in terms of polyphenol extraction yield with the conventional extraction techniques usually applied to this matrix.

5.1. Materials and Methods

5.1.1. Chemicals

FA (99%) standard was purchased from Sigma-Aldrich Chemical. A stock standard solution (500 mg L^{-1}) of this compound was prepared by rigorous dissolution of 0.0125 g of the analyte in methanol (25.0 mL). The standard solution was stored at -20 °C and used for further dilutions. All other reagents used were of analytical grade and were purchased from Merck and Sigma-Aldrich. High-purity water from a Millipore Simplicity 185 water purification system (Millipore Iberian S.A., Madrid, Spain) was used for all chemical analyses and glassware washing. The solvents employed for HPLC analyses were filtered through a nylon filter of 0.45 μm pore size (Whatman, Clifton, NJ) and degasified for 10 min in an ultrasound bath.

5.1.2. Samples

BSG used throughout this work was kindly supplied by Unicer−Bebidas de Portugal (S. Mamede de Infesta, Portugal), which was frozen and lyophilized. The sample was then finely ground in a laboratory EBC mill (Casela, London, UK) and sieved trough a 35-mesh (≤0.5 mm) sieve. The samples were stored at -20 °C until further use.

5.1.3. Microwave-assisted extraction

MAE was performed with a MARS-X 1500 W (Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA) configured with a 14 position carousel. One gram of dried sample was transferred to the teflon extraction vessels with the tested volume of 0.75% NaOH concentration; then the vessels were closed. During operation, both temperature and pressure were monitored in a single vessel (control vessel). Magnetic stirring in each extraction vessel and a sensor registering the solvent leaks in the interior of the microwave oven were also utilized. After extraction, vessels were allowed to cool at room temperature before opening and the extracts were then centrifuged for 15 min at 4,000 rpm. After filtration of the supernatant through cellulose filter (0.45 μm), the samples were preserved at −20 °C until further analysis.

5.1.4. Optimization strategy of ferulic acid MAE

The optimization of FA was carried out using RSM, according to Montgomery [181]. It is a combination of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is affected by several factors with complex interactions. The main objective of RSM is to optimize this response or determine the region that satisfies the operating specifications. This procedure involves fitting a function to the experimental data and then using optimization techniques to obtain the optimum parameters [180]. In most of the cases, the real relation between the response and the independent variables is unknown and, usually, polynomial models are used as they give a good approximation to the true relationship of the considered variables.

The experimental domain was defined taking into account the results obtained in preliminary tests, as well as the operative limits of the instrument and all significant parameters in a typical MAE process were chosen: extraction time (X_1; in minutes),

temperature (X_2; in degrees Celsius), solvent volume (X_3; in milliliters), and stirring speed (X_4; four positions are available in modern apparatus: turned off, minimum, medium, and maximum speed). The response variable studied was FA extraction yield (Y_1; in percent, w/w), which was determined by HPLC-DAD analysis. An orthogonal central composite design with four parameters, 2^4, was the approach made to the optimization problem. This design included 36 experiments to estimate the models coefficients: 16 points of a factorial design at levels α=±1.000, 8 axial points at a distance α=±2.000 from the center, and a center point with 12 replications (Table 5.1). The 12 replicates at center point allowed estimating experimental error and checking the fit. Additionally, double replications were performed for each of remaining experimental runs minimizing the error associated with measurements made under the same conditions. Mean and standard deviation (SD) values of response are presented in Table 5.1.

Table 5.1 - Real values and coded levels for the experimental design 2^4 (X_1 – extraction time (min); X_2 – temperature (ºC); X_3 – solvent volume (mL); X_4 – stirring speed); results (mean of two replicates for each run except for the center point that corresponds to twelve experiments) for the observed FA extraction yield (Y_1,exp; %, w/w, mean ± SD) and the values predicted by the model (Y_1,mod,%)

Exp.	X_1 (min)	X_2 (ºC)	X_3 (mL)	X_4[a]	$Y_{1,exp}$ ± SD (%)	$Y_{1,mod}$ (%)
1	10 (-)	80 (-)	30 (-)	min. (-)	0.93 ± 0.02	1.12
2	10 (-)	80 (-)	30 (-)	max. (+)	0.93 ± 0.02	1.12
3	10 (-)	80 (-)	50 (+)	min. (-)	0.609 ± 0.009	0.46
4	10 (-)	80 (-)	50 (+)	max. (+)	0.60 ± 0.01	0.44
5	10 (-)	100 (+)	30 (-)	min. (-)	0.949 ± 0.002	1.14
6	10 (-)	100 (+)	30 (-)	max. (+)	0.9480 ± 0.0007	1.17
7	10 (-)	100 (+)	50 (+)	min. (-)	0.61 ± 0.02	0.49
8	10 (-)	100 (+)	50 (+)	max. (+)	0.620 ± 0.006	0.50
9	20 (+)	80 (-)	30 (-)	min. (-)	0.953 ± 0.006	1.17
10	20 (+)	80 (-)	30 (-)	max. (+)	0.98 ± 0.02	1.22
11	20 (+)	80 (-)	50 (+)	min. (-)	0.595 ± 0.002	0.43
12	20 (+)	80 (-)	50 (+)	max. (+)	0.594 ± 0.003	0.46
13	20 (+)	100 (+)	30 (-)	min. (-)	0.94 ± 0.02	1.17
14	20 (+)	100 (+)	30 (-)	max. (+)	0.981 ± 0.006	1.24
15	20 (+)	100 (+)	50 (+)	min. (-)	0.59 ± 0.03	0.44

16	20 (+)	100 (+)	50 (+)	max. (+)	0.616 ± 0.004	0.49
17 – 28 (CP[b])	15 (0)	90 (0)	40 (0)	med. (0)	0.7380	0.73
Additional runs - model expansion						
29	5($-2^{4/4}$)	90 (0)	40 (0)	med. (0)	0.694 ± 0.008	0.68
30	25($+2^{4/4}$)	90 (0)	40 (0)	med. (0)	0.730 ± 0.002	0.73
31	15 (0)	70($-2^{4/4}$)	40 (0)	med. (0)	0.71 ± 0.02	0.72
32	15 (0)	110($+2^{4/4}$)	40 (0)	med. (0)	0.761 ± 0.004	0.78
33	15 (0)	90 (0)	20($-2^{4/4}$)	med. (0)	1.25 ± 0.02	1.76
34	15 (0)	90 (0)	60($+2^{4/4}$)	med. (0)	0.523 ± 0.003	0.35
35	15 (0)	90 (0)	40 (0)	t.o.[c]($-2^{4/4}$)	0.710 ± 0.004	0.72
36	15 (0)	90 (0)	40(0)	n.a.[d]($+2^{4/4}$)	n.d.[e]	n.d.[e]

[a]X_4 (stirring speed) 4 positions available: turned off (0), minimum (1), medium (2) and maximum speed (3); [b]center point; [c]turned off; [d]not available, the equipment does not have a stirring speed higher than the maximum one; [e]not determined

The results in the initial set of experiments (runs 1 – 16 in Table 5.1) were fitted to a first-order model [180, 181] and its adequacy was checked. If the lack of fit was not significant, steepest ascent method should be applied in order to move rapidly to the optimum region. On the contrary, if the first-order model lack of fit reached significance, probably due to a quadratic effect, additional runs were performed to improve model adjustment. Then, experimental data were fitted to the following second-order model [180, 181],

$$Y_i = b_0 + \sum_i b_i X_i + \sum_{ij} b_{ij} X_i X_j + \sum_i b_{ii} x_i^2 + \varepsilon$$

where Y_i is the experimental response, X_i are the studied factors, b_0 is the average response, b_i are the average effects of the different factors, b_{ij} are the average effects of second interaction factors and b_{ii} are the quadratic components and ε is the experimental error. The lack of fit in the second-order model is desired to be not significant and if it persists steepest ascent method should be used in order to move rapidly to the optimum region [180, 181].

All statistical analyses were made using the software Statistica version 6.0 (StatSoft, Inc., Tulsa, UK), namely, multifactor variance analysis (ANOVA) and

response surface 3D plots. The two factors not represented by the horizontal axes were fixed at their 0 level values.

In order to validate a model, appropriate ANOVA must be carried out . The total sum of squares of the mathematical model is divided into the sum of squares due to the regression (SS model in Table 5.2) and the residual sum of squares (SS residual in Table 5.2). The latter can be divided in two parts, the first part is due to pure experimental error and is computed as the sum of squared deviations (SS pure error in Table 5.2) in the center point and remain experiments, and the second part corresponds to the lack of fit (SS lack of fit in Table 5.2). The fitted models are considered adequate if they reach significance (p value < 0.05 for a 95% confidence level) and their lack of fit is not significant (p value > 0.05 for the same confidence level).

Table 5.2 - Analysis of variance (ANOVA) for regression models

Source	SS[a]	DF[b]	MS[c]	*F*-value	*p*
Model	0.803828	8	0.100479	374.4845	0
Residual	0.006976	26	0.000268		
Lack of fit	0.004491	15	0.000299	1.325033	0.323348
Pure error	0.002485	11	0.000226	444.7074	0
Total (Model + residual)	0.810804	34			
R^2	0.9914				

[a]Sum of squares; [b]Degree of freedom; [c]Mean square

Significance of each coefficient present in regression equations as well as, studied factors and their interactions effects, was determined by the student's t test and p values (a 95% confidence level was used). Factors and/or interactions with an experimental error greater than the effect (p value > 0.05) were not influential. If the model did not predict a satisfactory solution, optimum extraction conditions were obtained by surface 3D plots inspection and based on statistical information. All experiments were performed in randomized order to minimize bias effect.

5.1.5. Other extraction techniques tested

Three conventional extraction techniques namely, Soxhlet, mechanical stirring, and alkaline hydrolysis were further used for comparison with MAE technique.

5.1.5.1. Soxhlet extraction

Exhaustive Soxhlet extraction (SE) was performed following the procedure developed by Kalia *et al.* [127], with slight modifications. A classical Soxhlet apparatus was used with accurately weighed 3 g of the dried BSG (screened through sieve 35-mesh). Extraction was performed for 4 h with 150 mL of ethanol as the extracting solvent. After extraction, the supernatant was allowed to cool at room temperature and evaporated to dryness at 35 °C using a rotary evaporator. Before chromatographic analysis, the residue was re-dissolved in 5.0 mL of ethanol and filtered through a 0.45 μm cellulose filter. All experiments were performed, at least, in duplicate.

5.1.5.2. Mechanical stirring extraction

The extraction procedure of phenolic compounds from BSG was performed according to Dvorakova *et al.* [38], with slight modifications. Briefly, 2 g of dried sample was extracted three times (for periods of 20 min) with 25 mL of methanol/water (70/30, v/v) on a gyratory shaker (Gravimeta, V. N. Gaia, Portugal) at 250 rpm. The suspension after extraction was centrifuged (4,000 rpm, 10 min) and the supernatant evaporated to dryness under vacuum at 35 °C. The residue was re-dissolved in 5.0 mL of methanol/water (70/30, v/v) and filtered through a 0.45 μm cellulose filter for the chromatographic analysis. All experiments were performed, at least, in duplicate.

5.1.5.3. Alkaline hydrolysis

Before the alkaline hydrolysis extraction, performed accordingly to Mussatto *et al.* [17], BSG was subjected to a pretreatment with dilute sulfuric acid, under conditions previously optimized by Mussatto and Roberto [15] with slight modifications. A mixture of 1 g of dried sample with 8 mL of sulfuric acid 0.1 M was heated at 100 °C for 30 min. At the end of the reaction, the solid residue was separated by centrifugation (4,000 rpm, 10 min), washed with water until neutral pH and dried at 50±5 °C to attain approximately 50% moisture content.

One gram of the acid pre-treated BSG was extracted with 20 mL of 2% NaOH in a bath at 110 °C for 90 min. At the end of the reaction the sample was immediately cooled in an ice bath, and the mixture separated by centrifugation (4,000 rpm, 10 min). The supernatant was filtered through a 0.45 μm cellulose filter for the chromatographic analysis. All experiments were performed, at least, in duplicate.

5.1.6. HPLC-DAD analysis

The phenolic composition of the BSG extracts was analyzed by the HPLC method described by Rubilar *et al.* [169] with minor modification. The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of a low-pressure quaternary gradient unit (model LG-1580-04) with an in-line DG-1580-54 degasser and a model AS-950 auto-sampler. The system is equipped with a photodiode array detector (model MD-1510 UV/Vis multiwavelength detector).

Separation of polyphenols was achieved on a Phenomenex Synergi Hydro-RP C_{18} column (150 mm × 4.6 mm, 4 μm) and a guard column with the same characteristics kept at room temperature. The chromatographic conditions were the following: flow rate 0.3 mL min^{-1}, sample injection volume of 20 μL and mobile phase A (100% methanol) and mobile phase B (0.1% formic acid). A gradient program was used as follows: 90% B in 0 min, from 90% to 0% B in 110 min, followed by 100% A for 20 min and back to 90% B in 10 min and 10 min of reconditioning before the next injection. The photodiode array detection was conducted by scanning between 190 and 600 nm. Analytes in each sample were identified by comparing their retention times and UV-Vis spectra with those of standard compounds or from literature. Peak purity was checked to exclude any contribution from interfering peaks.

The yield of FA extracted from dried BSG (%, w/w) was used to evaluate the effect of each MAE variable. Quantitative determination of the target compound in the extracts was performed using external calibration curves in the concentration range of 5 to 200 mg L^{-1} (detection at 320 nm, $n = 7$, $R^2 = 0.9997$).

5.1.7. HPLC-ESI-MS analysis

The qualitative study of the phenolic composition in all samples was performed by HPLC coupled on-line with electrospray ionization (ESI) mass spectrometry. The HPLC system (Finnigan, Thermo Electron Corporation, San Jose, USA) consisted of a low-pressure quaternary pump (Finnigan Surveyor Plus) and an auto-sampler (Finnigan Surveyor Plus with 200-vial capacity sample). Separations were achieved in the same conditions as for HPLC-UV analysis but the injection volume was 25 μL. A quadrupole ion trap mass spectrometer (Finnigan LCQ Deca XP Plus) equipped with an ESI source in the positive ion mode and Xcalibur software Version 1.4 (Finnigan) were used for data acquisition and processing. The interface conditions were applied as follows: capillary temperature, 325 °C; source voltage, 5.0 kV; capillary voltage, 4.0 V;

sheath gas (N_2) flow at 90 arbitrary units and auxiliary gas (N_2) flow rate at 25 arbitrary units. Acquisition of the mass data was performed between *m/z* 100 and 1000. For the LC-MS-MS study energy of activation of 45% was applied. The positive ion mode was used in this study due to a better signal-to-noise ratio in comparison with negative ion mode.

5.2. Results and Discussion

BSG has been receiving considerable attention for its possible use in several fields, such as in bioethanol and lactic acid production, animal nutrition and hydroxycinnamic acids extraction [8, 30, 43]. This last utilization of BSG is a promising area to explore [17, 30], because of the chemoprotectant and antioxidant properties associated with ferulic and *p*-coumaric acids [43]. The present study is aimed at developing an efficient MAE method for the extraction of BSG hydroxycinnamic acids. For this purpose, microwave-assisted and conventional extraction techniques were compared in terms of FA extraction yield. According to our knowledge, this is the first application of MAE of polyphenols from BSG.

5.2.1. MAE

5.2.1.1. Preliminary testes

Before using RSM, preliminary experiments were carried out in order to choose the relevant variables in FA recovery as well as the experimental range for the factors.

One of the most important parameters in MAE is the extraction solvent. Several solvents were considered in the preliminary studies, namely NaOH, which is the most common used solvent for the extraction of phenolic acids from BSG [17, 30, 95]. Methanol, acetonitrile, acetone, and water were also investigated, as they were used for MAE extraction of polyphenols from wheat bran [151] and distillers dried grains [138]. The best results were obtained using NaOH 0.5% (at 90 °C for 15 min), with FA extraction yield of 0.33 ± 0.02% (w/w; Fig. 5.1). The other solventes tested gave considerably lower yields for FA, with water giving the second highest extraction yield (0.013 ± 0.001%, at 150 °C for 25 min), however clearly below that obtained with NaOH 0.5%. Therefore, NaOH was the selected solvent.

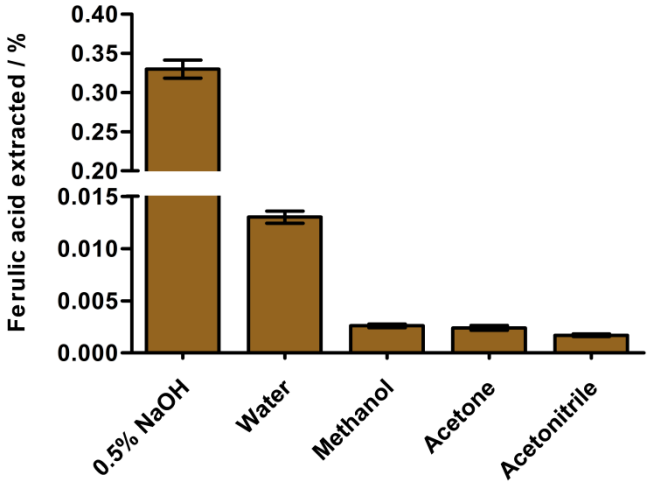

Fig. 5.1 - Influence of solvent on the FA extraction yield. MAE conditions used were: 15 min, 90 ºC, 40 mL and medium stirring speed for 1 g of BSG. The vertical bars represent the standard deviation (*n* = 3).

Another parameter studied before RSM optimization was the NaOH concentration, which was tested in the range of 0.25% to 1% (w/v; 40 mL at 90 °C with constant medium stirring for 15 min and 1 g of dried BSG). As can be seen in Fig. 5.2, the extraction yield increases with NaOH concentration up to 0.75%, with a FA extraction yield of 0.808 ± 0.008%. Mussatto *et al.* [17] also found that increasing NaOH concentration resulted in higher extraction yields. However, the best extraction yield was considerably lower (0.285%, for 2% NaOH at 120 °C for 90 min) than the one obtained with MAE.

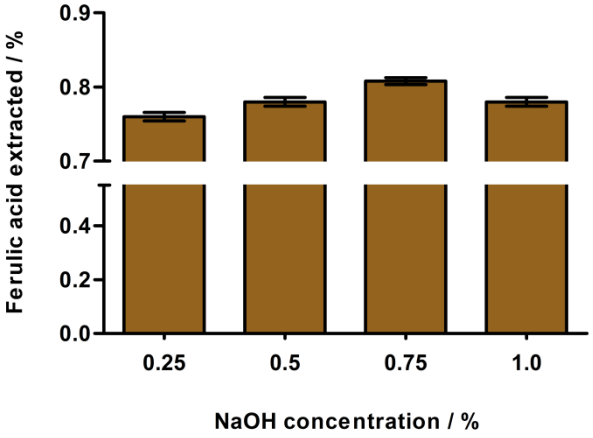

Fig. 5.2 - Influence of the NaOH concentration on the FA extraction yield. MAE conditions used were: 15 min, 90 ºC, 40 mL and medium stirring speed for 1 g of BSG. The vertical bars represent the standard deviation (*n* = 3).

Concerning the extraction temperature, previous data from the literature were considered. In fact, the stability of polyphenols was evaluated by several researchers

[137, 155] and, accordingly to the results obtained, FA can be extracted without degradation at temperatures up to 100‑150 °C for extraction times of 20 min [137]. Thus, the influence of the extraction temperature was studied between 90 and 200 °C. Significant degradation of BSG was only detected for temperatures higher than 150 °C. Therefore, for optimization by RSM, the extraction temperature range was selected as 70-110 ºC.

5.2.1.2. ANOVA and surface response methodology analysis

The experimental domain and the selection of solvent were established based on the results obtained in the preliminary tests. All important parameters in a typical MAE process were selected (extraction time, temperature, solvent volume and stirring speed; Table 5.1).

A total of 36 runs were performed for optimizing the four individual parameters in the current model. The response values, expressed as yield of FA extracted, at different experimental combination for coded variables are listed in Table 5.1.

In the initial set of experiments (runs 1–16 in Table 5.1), the lack of fit of the first-order model was not significant (p>0.05) suggesting steepest ascent method application in order to move more rapidly to the optimum vicinity. However, it suggested operational parameters values impossible to apply, namely in run 36 (the equipment does not have a stirring speed higher than the maximum one), which was not performed nor statistically considered by the software; so, remaining experiments were performed (runs 29–36 in Table 5.1). The lack of fit of the second-order model was non-significant (p>0.05) and the model reached high statistical significance (p<0.05; Table 5.2). The quadratic correlation coefficient, r^2=0.9914, can be considered acceptable (>0.8; [180]), advocating a good correlation between observed and predicted values (Fig. 5.3). This means that regression model provides an excellent explanation of the relationship between the variables (factors) and the response (yield).

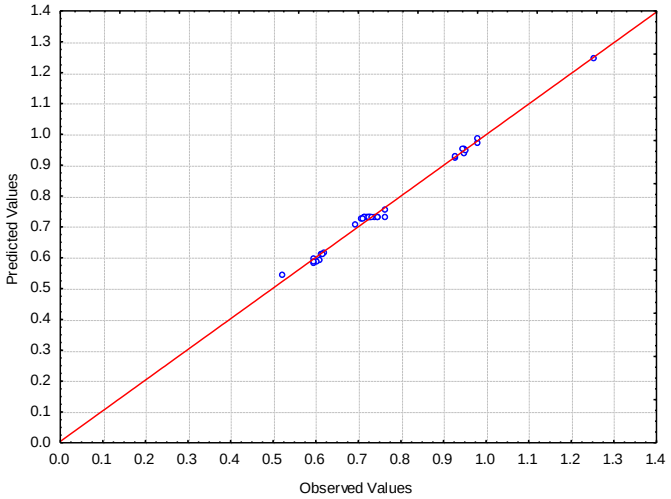

Fig. 5.3 - Correlation between the predicted and observed values.

By eliminating the non-significant parameters (p>0.05), response surface regression gave the following model equation:

$$Y = 2.1 - 0.048X_3 + 0.00041X_3^2 - 0.00018X_1X_3$$

The software predicted a saddle point: Y=0.535% at critical values X_1=13.9 min, X_2=67.8 ºC, X_3=61.7 mL, and X_4=maximum stirring speed. Therefore, optimum conditions were obtained through close observation of experimental data, 3D surface plots observation and based on ANOVA results.

According to ANOVA results, X_3 and X_3^2 effects were the most influential parameters in FA extraction yield (p< 0.0001), with solvent volume in the range 20 to 22 mL giving the best response (Fig. 5.4A and Table 5.1). Extraction time (X_1) didn't have a significant influence in FA yield (p> 0.05). However, X_1X_3 reached statistical significance (p=0.03) for low solvent volumes (20–22 mL) coupled with extraction times higher than 8 min producing highest recoveries (Fig. 5.4A). Also, Table 5.1 revealed that enhanced yields were achieved with lower solvent volume (20 to 30 mL) coupled with shorter extraction time (10 to 15 min). Considering solvent and energy savings, wastes generation concerns, and that the highest FA extraction yield (1.25 ± 0.02%, run 33) was obtained for 20 mL of solvent and 15 min of extraction, these operational values were chosen as possible optimum conditions.

Extraction temperature (X_2) did not reach positive significance in FA extraction yield (p>0.05). However, the 3D surface plots analysis (Fig. 5.4B) showed that temperatures

above 100 °C produced higher yield for an extraction time higher than 14 min. Taking into account energy savings, 100 °C was admitted as an optimum possibility.

Regarding the stirring speed (X_4), the linear effects were verified to be statistically no-significant (p>0.05). 3D surface plots analysis (Fig. 5.4C) revealed that high stirring speed rate (maximum speed) coupled with extraction time longer than 14 min increased the FA extraction yields.

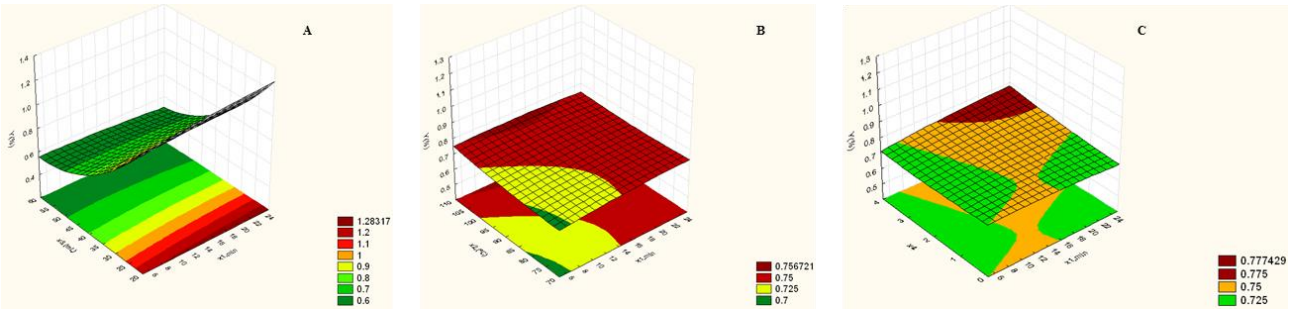

Fig. 5.4 - Response surface of FA yield (Y, %, w/w) as a function of extraction time (X_1) and the other variables studied: A – solvent volume (X_3); B – temperature (X_2); C – stirring speed (X_4).

Four independent experiments were carried out using the optimal conditions recommended by the software and the above referred parameters. In Table 5.3 are reported the experimental run conditions used in each set. Three replicates were performed for each one of the sets performed. A Student's *t* test was applied and significant differences (p<0.05) between the experimental sets were detected. Therefore, the choice between one of the tested sets was made considering the highest FA extraction yield. Thus, optimum FA yield conditions were considered to be the operational parameters used in set 3 (15 min of extraction, 100 °C, 20 mL of solvent and maximum stirring speed).

Table 5.3 - Comparison of the FA extraction yield (%, w/w) for optimum conditions established by 3D surface plots analysis (sets 1, 2 and 3) and experimental run with the highest FA yield (set 4).

MAE conditions	**Experimental sets**			
	1	**2**	**3**	4[a]
Extraction time / min	15	10	15	15
Temperature / ºC	68	100	100	90
Solvent volume / mL	62	20	20	20
Stirring speed	maximum	maximum	maximum	medium
Yield / %, w/w	0.480 ± 0.006	1.23 ± 0.01	1.31 ± 0.04	1.25 ± 0.02

5.2.1.3. Comparison with other extraction techniques

The selection of an appropriate extraction method mainly depends on the advantages and disadvantages of the processes, such as extraction yield, complexity, production cost, environmental friendliness and safety. In general, sonication, mechanical stirring and SE are the most frequently used extraction procedures [127, 140]. However, alkaline hydrolysis is also one of the most common used methods for the extraction of hydroxycinnamic acids, such as FA, from cereal samples [17, 30]. The drawbacks of these extraction methods are the large amount of solvent and long extraction time needed.

In the present study, SE, mechanical stirring and conventional alkaline hydrolysis were carried out in order to compare MAE efficiency with other extraction methods for the recovery of polyphenols from BSG. The conditions of diferente techniques and their results are summarized in Table 5.4.

Table 5.4 - Comparison of conventional applied extraction techiniques (SE, mechanical stirring and alkaline hydrolysis) with MAE

Parameter	Extraction technique			
	MAE	**SE**	**Mechanical stirring**	**Alkaline hydrolysis**
Ratio of solvent to raw material (mL g^{-1})	20	30	15	20
Extraction time	15 min	4 h	30 min	90 min
Temperature (ºC)	100	b.p. of solvent	25	110
Solvent	NaOH 0.75%	Ethanol	Methanol 70%	NaOH 2%
Number of samples extracted per assay	14	2	4	4
Yield of ferulic acid (% w/w, mean ± SD)	1.31 ± 0.04	0.0014 ± 0.0001	0.0018 ± 0.0001	0.27 ± 0.02
RSD (%)	3.0 (n = 3)	9.4 (n = 3)	7.0 (n = 3)	7.4 (n = 3)

Overall MAE affords several advantages over classical extraction methods, as shown in Table 5.4 (lower solvent consumption, extraction time and the potential to recover tightly bound residues not easily released by conventional techniques). In

addition, the obtained results showed that in terms of yield of the target analyte, the extraction heating assisted by microwaves allows the possibility of recovering at least approximately fivefold higher FA (1.31 ± 0.04%) than that obtained with traditional applied methods (0.27 ± 0.02% for alkaline hydrolysis; 0.0014 ± 0.0001% for SE; 0.0018 ± 0.0001% for mechanical stirring). Moreover, the developed methodology requires less energy and NaOH concentration and does not involve labor intensive pre-treatment procedures as the conventional alkaline hydrolysis [17, 30], while generating fewer wastes. These features position MAE as a valuable and cost effective technology suitable for today's highly competitive industries with growing demand for increased productivity, improved efficiency and reduced cycle time.

5.2.1.4. Methods performance

In order to study the performance of the developed method, recovery experiments were conducted. Thus, known amounts of FA were added to the BSG samples, and the resulting spiked samples were subjected to extraction under the optimum MAE conditions previously described. Samples were spiked at three different levels (6.6, 13.1, and 19.6 mg of FA), and recoveries were calculated. All analyses were carried out in triplicate. The average recoveries obtained were all higher than 83% (ranging from 83 ± 3% to 90 ± 4%), testifying the accuracy and selectivity of the proposed methodology.

The precision was evaluated as the coefficient of variation (CV) for five repeated analyses of the sample in the same day (intra-day precision). The intermediate precision (inter-day variability) was performed on three validation days and repeated five times within each day. The results showed that the intra-day CV (2%) and the inter-day CV (4%) were lower, showing that the method is precise.

5.2.2. Phenolic profiles of BSG extracts

An HPLC chromatogram at 280 nm of BSG extract after MAE is shown in Fig. 5.5.

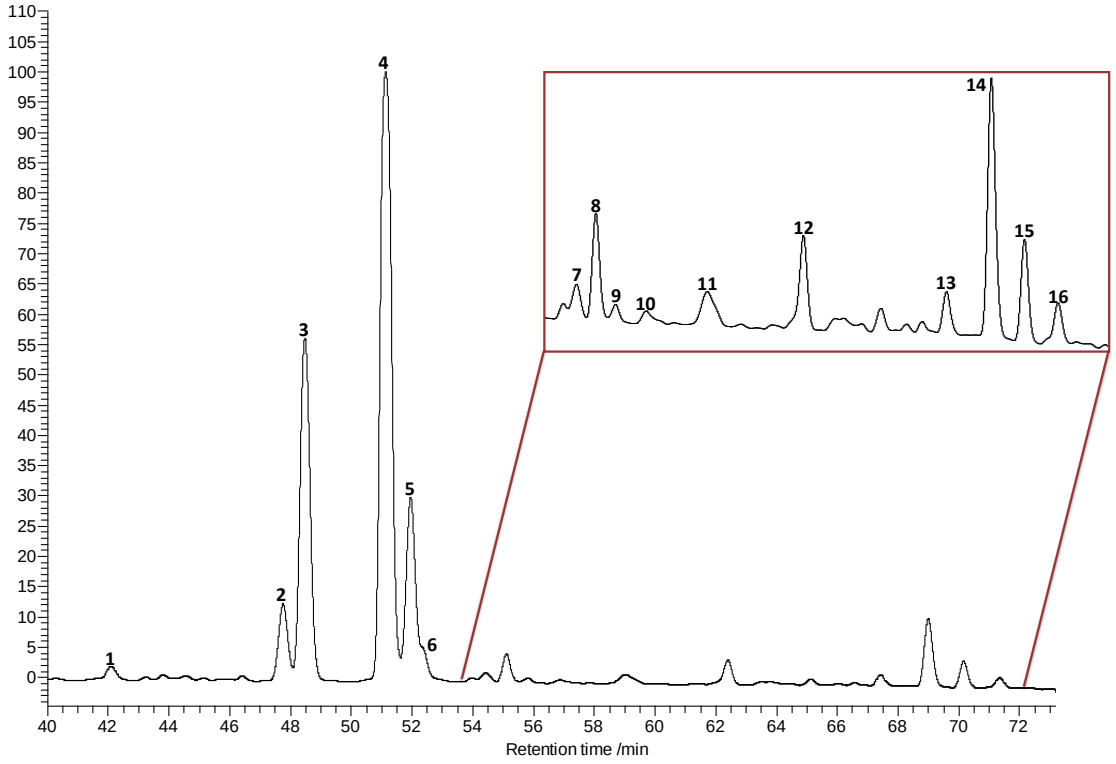

Fig. 5.5 - HPLC chromatogram at 280 nm of BSG extract after application of MAE; numbered peaks represent identified compounds listed in Table 5.5.

HCAs (*p*-coumaric (peak 2), ferulic (peak 4) and sinapic (peak 13)) were unambiguously identified by comparing retention times, UV and MS data with those of the reference standards. As expected [17, 30, 95], FA is the main phenolic compound in the BSG extract, with a concentration of 1.31±0.04 g/100 g of dried BSG at the optimum MAE conditions. The possible structures of the other peaks in the chromatogram were tentatively characterized on the basis of literature data, due to the lack of standard compounds. Table 5.5 summarizes the list of compounds and their main fragments observed during HPLC-DAD-ESI-MS analyses.

Table 5.5 - Identification of phenolic compounds from BSG extract by HPLC-ESI-MS/MS. Retention time, main MS fragment and typical fragmentations of the compounds are given

Peak label	t_R /min	λ_{max} /nm	$[M+H]^+$	MS/MS fragments	Attribution
1	41.79	335	387	369, 341, 323	8-8'-DiFA[a] (aryltetralin form)
2	45.75	308	165	121	*trans p*-coumaric acid
3	48.41	320	387	369, 341, 323, 245	8-5'-DiFA (benzofuran form)
4	51.02	317	195	177, 145	*trans* FA[b]
5	51.91	280	245	199, 123	syringic acid + HCOOH

6	52.10	314	387	369, 341, 193	8-8'-DiFA
7	54.64	320	387	371, 369, 341	8-5'-DiFA
8	55.11	323	387	369, 341, 351, 309	8-8'-DiFA (cyclic form)
9	55.78	315	402	387, 341	5-5'-DiFA (methylated form)
10	56.87	316	387	341, 369	DiFA
11	59.42	320	387	341, 369	DiFA
12	62.53	319	343	325, 297	8-5'-DiFA (decarboxylated form)
13	67.70	320	225	207, 193, 175	sinapic acid
14	68.98	326	387	369, 263, 351, 325	8-O-4'-DiFA
15	70.14	317	579	561, 533, 357	5-5',8'-O-4''-TriFA[c]
16	71.34	320	405	387, 369, 351	DiFA (hydrated form)

[a]DiFA: dehydrodiferulic acid; [b]FA: ferulic acid; [c]TriFA: triferulic acid

The chemical structures for some of these compounds are presented in Fig. 5.6.

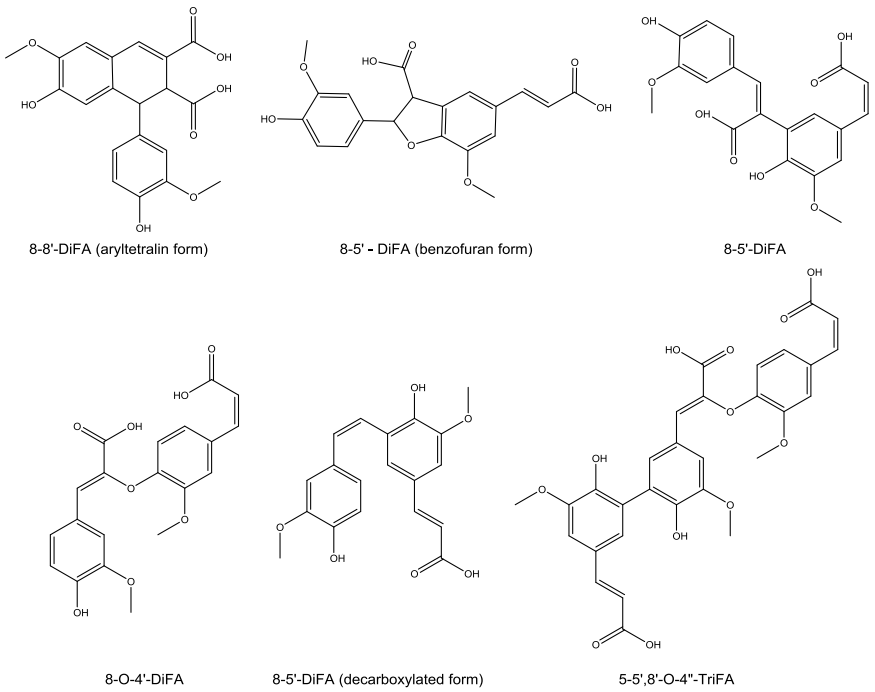

Fig. 5.6 - Structures of some ferulic acid dehydrodimers and trimer identified in the obtained BSG extract.

An intense molecular ion $[M+H]^+$ with *m/z* value of 387 was detected for several chromatographic peaks, namely (1), (3), (6-8), (10), (11) and (14). The fragmentation, in positive ion mode, of this peak resulted in a major fragment with *m/z* 369 (Table 5.5),

by the loss of 18 Da corresponding to the loss of a water molecule. An *m/z* value of 341 is produced by the loss of a carbonyl group from the ion with *m/z* 369. According to this fragmentation pattern, these peaks correspond to FA dehydrodimers, which have already been detected in BSG [30, 95].

The identified dehydrodiferulic acids (DiFA) can be distinguished by their elution pattern and UV-spectrum in comparison with literature, as well as by their fragments generated [95, 98]. Thus, peaks 3, 6, 7, 8 and 14 correspond to the diferulate isomers 8-5-DiFA (benzofuran form), 8-8'-DiFA, 8-5'-DiFA, 8-8'-DiFA (cyclic form), and 8-O-4-DiFA, respectively. Peak 1 corresponds to 8-8'-DiFA (aryltetralin form, Fig. 5.6), which has the same fragmentation pattern than the others DiFA, but the UV maximum absorption is 335 nm enabling to distinguish it from the others isomers. Dobberstein and Bunzel [98] also detected this compound in the cell wall of several cereals. Two other minor peaks (10 and 11) were also attributed to FA dehydrodimer-type compounds, although final structures were not confirmed.

Peak 5 was identified as an adduct of syringic acid. The $[M+H]^+$ with *m/z* 245 produced a major MS^2 ion at *m/z* 199 (syringic acid) with the loss of *m/z* 46 corresponding to the molecular weight of formic acid, which is used in the mobile phase. In addition, the fragmentation of ion at *m/z* 199 resulted in an ion at *m/z* 123, characteristic from the fragmentation of syringic acid, confirming its presence.

The fragmentation, in positive mode, of the ion $[M+H]^+$ with *m/z* 402 (peak 9) resulted in a major fragment with *m/z* 387 (Table 5.5). The 15-Da difference can correspond to the loss of a methyl group leading us to deduce that the polyphenol detected is a methylated DiFA.

Peak 12 (*m/z* 343) also corresponds to a DiFA (*m/z* 387), but in the decarboxylated form (Fig. 5.6). The fragmentation pattern of this ion is similar to the DiFA aforementioned. *m/z* value of 325 and 297 correspond to the loss of a water and a carbonyl group, respectively. Recently, Dobberstein and Bunzel [98] identified this compound in the cell wall of several cereals, such as corn, wheat and rye grains.

The fragmentation of the ion $[M+H]^+$ with *m/z* 579 (peak 15) resulted in two major fragments with *m/z* 561 and 533 (Table 5.5). The fragmentation pattern of this compound is fully consistent with that observed for DiFA, leading us to deduce that the detected compound (*m/z* 579) is TriFA, whose structure is shown in Fig. 5.6.

The peak 16 was tentatively identified as DiFA in hydrated form. The $[M+H+H_2O]^+$ with *m/z* 405 produced a major MS^2 ion at *m/z* 387 (DiFA) corresponding to the loss of the water molecule (Table 5.5).

As observed, the developed new extraction method is an effective alternative to phenolic compounds recovery from BSG and may be a promising system for a potential large-scale extraction. Therefore, as the type and origin of the raw-materials used in the brewing process can affect the content of phenolic compounds extracted from BSG, in Chapter 6 will be discussed the influence of different malt types in the polyphenols extracted. This study may be useful not only for the selection of malt types for brewing production in order to optimize the antioxidant properties of the final product as well as the BSG by-products obtained for its further industrial and pharmaceutical applications.

6. Evaluation of antioxidant activity and identification of major phenolics of brewer's spent grains from different types of malt

In the last few years, numerous research studies have associated the consumption of foods rich in bioactive compounds with the ability to promote a number of benefits for human health. The most common bioactive compounds include secondary metabolites such as antibiotics, mycotoxins, alkaloids, food grade pigments, plant growth factors, and phenolic compounds [32, 117]. Particularly, phenolic compounds are of considerable interest to scientists, manufacturers and consumers due to their importance in food quality, with protective and preventive roles in certain types of cancer and several other chronic diseases [93]. Hydroxycinnamic acids, which are the predominant phenols in BSG [17, 30], have shown antioxidant properties and the *in vitro* antioxidant effect of these compounds was reported to be similar to that exhibited by the well-known antioxidants α-tocopherol and ascorbic acid [116].

Although there is a large amount of information available relating to the health effects of polyphenol-rich foods, such as tea (catechins), coffee (chlorogenic acid), wine (resveratrol) and fruits, the information regarding the antioxidant potential of BSG is scarce. To the best of our knowledge, there are no published reports relating the phenolic composition and antioxidant activity of BSG obtained from different malt types. Recently, McCarthy *et al.* [116] have investigated the ability of phenolic rich BSG extracts to protect against DNA damage in human lymphocytic U937 cells measured by the Comet assay. Their results showed that *black* BSG extracts, which had the highest phenol content, provided the greatest protection against H_2O_2-induced DNA damage.

The present study aims at evaluating the phenolic composition and antioxidant activity of BSG extracts obtained by MAE. Light (*pilsen*, *melano*, *melano 80* and *carared*) and dark (*chocolate* and *black*) malts were assessed for the TPC by the Folin–Ciocalteu assay, and for the antioxidant activity by the DPPH, ABTS and 2-deoxyribose degradation assays. The phenolic compounds present in the BSG extracts were identified by HPLC-DAD/ESI-MS/MS.

6.1. Materials and Methods

6.1.1. Chemicals

Folin-Ciocalteu's reagent (Merck, Darmstadt, Germany) and sodium carbonate (Sigma-Aldrich) were employed for the measurement of the total phenolic content (TPC). The calibration curve was constructed with gallic acid (GA, Sigma-Aldrich).

For the antiradical activity assessment, DPPH (Sigma-Aldrich) was used. The ABTS methodology employed ABTS and potassium persulfate ($K_2S_2O_8$) purchased from Sigma-Aldrich. 2-deoxyribose assay was performed using L(+)ascorbic acid (Sigma-Aldrich), trichloroacetic acid (Riedel-de-Haën), 2-thiobarbituric acid, 2-deoxy-D-ribose, hydrogen peroxide 35% and $FeCl_3$ (Fluka).

FA (99%) and *p*-CA (98%) standards were purchased from Sigma-Aldrich. Stock standard solutions (500 mg L^{-1}) of these compounds were prepared by rigorous dissolution of the analyte in methanol. Standard solutions were stored at -20 ºC and used for further dilutions. High-purity water from a Millipore Simplicity 185 water purification system (Millipore Iberian S.A.) was used for all chemical analyses and glassware washing. The solvents employed for HPLC analyses were filtered through a nylon filter of 0.45 μm pore size (Whatman) and degassed for 10 min in an ultrasound bath.

6.1.2. Samples

BSG samples used throughout this work were kindly supplied by *Unicer* – Bebidas de Portugal, S.A. (S. Mamede de Infesta, Portugal) and *Os Três Cervejeiros*, Lda (Porto, Portugal), and were obtained from six malt types. Different malt types result from special malting procedures applied to a range of raw materials. These are usually grouped into three types based on the material used, namely, raw cereals, pale malt, or green malt (germinating cereal) [5]. For a matter of facilitation, the different BSG extracts from the six malt types were divided into two main families, named here light malts (*pilsen*, *melano*, *melano 80* and *carared*) and dark malts (*chocolate* and *black*). *Pilsen* malt is an exceptionally light colored 2-row base malt that produces a very light colored, clean, crisp wort and is used as a base malt for all beer styles. *Caramel* malt is made from green malt that is produced by drying the wet germinated barley at controlled temperatures, causing the starches to convert to sugars and caramelize. *Melano* malts are specialty malts slowly dried as the temperature is raised, allowing

melanoidins to form as part of the kilning process. *Chocolate* malt shares many of the characteristics of *black* malt but, because it is roasted for a slightly shorter period and end temperatures are not so high, some of the harsher flavors of *black* malt are not so pronounced and EBC (European Brewing Convention) color is 200 units lighter. The specifications of each malt type, in particular color range and kilning temperature (KT) are listed in Table 6.1.

Table 6.1 - Color and kilning temperatures for the six malt types used in the wort production

	Malt type	Color range / EBC units	Kilning temperatures / ºC
Light malts	*Pilsen*	3.5 - 5.7	80 - 85
	Melano	37 - 43	130
	Melano 80	75 - 85	130
	Carared	90 - 360	120 - 160
Dark malts	*Chocolate*	800 -1000	220
	Black	1350 - 1500	230

The BSG samples used for phenolic extraction correspond to the remaining solid fraction obtained following the removal of wort during the pilot scale production of beer in the brewing process. For wort production 25 g of *pilsen* malt was mixed with the same quantity of the colored malt and milled in a Bühler Miag disk mill. 200 mL of distilled water, at 45 ºC, was added. After 30 min, temperature was increased at 1 ºC/min for 25 min until it reached 70 ºC. The temperature was maintained at 70 ºC for 1 h. The mash was cooled, made up to 450 g with water and filtered. The obtained solid residue was frozen, lyophilized and then finely ground in a laboratory mill (Casella, London, UK) and sieved through a 35-mesh (≤ 0.5 mm) sieve. The dried BSG samples were stored at -20 ºC until further use. *Pilsen* wort production was obtained using 50 g of *pilsen* malt, and following the same procedure.

6.1.3. Microwave-assisted extraction of phenolic compounds

BSG phenolics were extracted according to the method previously optimized and reported by in Chapter 5. MAE was performed with a MARS-X 1500 W (Microwave

Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA) configured with a 14 position carousel. Dried BSG sample (1 g) was transferred to the PTFE extraction vessels with 20 mL of 0.75% NaOH concentration; then the vessels were closed. Extraction was performed during 15 min at 100 ºC at maximum stirring speed. During operation, both temperature and pressure were monitored in a single vessel (control vessel). Magnetic stirring in each extraction vessel and a sensor registering the solvent leaks in the interior of the microwave oven were also used. After extraction, vessels were allowed to cool at room temperature before opening and the extracts were then centrifuged for 15 min at 4000 rpm. The pH of the supernatant was adjusted to pH 6.5 with HCl 6 M, and after filtration through a cellulose filter (0.45 μm), the extracts were preserved at -20 ºC until further analysis.

6.1.4. Determination of total phenolic content

The TPC of the BSG extracts was determined by the Folin–Ciocalteu method as described by Dvorakova *et al.* [38]. In a test tube, 1 mL of diluted sample or standard solution and 5 mL of 10-fold diluted Folin-Ciocalteu's phenol reagent were mixed. After 5 min of incubation, 4 mL of sodium carbonate solution (7.5%, w/v) was added and mixed well. After 2 h of incubation at room temperature in the dark, the absorbance was measured at 740 nm in a Shimadzu UV-3101 spectrophotometer (Kyoto, Japan). The total polyphenol concentration was calculated from a calibration curve, using GA as standard (5–150 mg L^{-1}). The results were expressed as mg GA equivalents (GAE) per dry weight (DW) of BSG. All measurements were done in triplicate.

6.1.5. Measurement of the antioxidant activity

6.1.5.1. DPPH radical scavenging activity assay

DPPH is a stable free radical and has been commonly used to screen phenolic compounds containing high free radical scavenging ability. The DPPH radical scavenging activity of each extract was measured according to the method of Brand-Williams [166] modified by Goupy *et al.* [167]. DPPH radicals have an absorption maximum at 515 nm, which disappears with reduction by an antioxidant compound. The DPPH solution in methanol (6.6×10^{-5} M) was prepared daily and 2.85 mL of this solution was mixed with 150 μL of the BSG extracts and vortexed. The absorbance decrease at 515 nm was measured after 120 min at room temperature in the dark. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Results were expressed as antiradical power (ARP, %) which is defined as

($1/EC_{50} \times 100$), where EC_{50} is the quantity of extract capable of reducing the initial concentration of DPPH to one half. Data were reported as mean ± SD for three replicates.

6.1.5.2. ABTS radical scavenging activity assay

The free radical scavenging activity of BSG extracts was also evaluated using the ABTS radical cation decolorization assay [168], which is based on the reduction of ABTS radicals by antioxidants of the BSG extracts. The stock solutions included 7 mM ABTS solution and 2.45 mM $K_2S_2O_8$ solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react in the dark for 16 h at room temperature to produce a dark green solution. Prior to the assay, the ABTS solution was diluted in deionized water to obtain an absorbance of 0.70 ± 0.02 units at 734 nm. Then BSG extracts or standards (150 μL) were allowed to react with 2850 μL of the ABTS solution, and the absorbance reading was taken after 2 h at room temperature in the dark. A calibration curve was obtained by using trolox standard solutions at various concentrations (0–100 mg L^{-1}). The degree of ABTS radical-scavenging activity of BSG extracts was calculated, based on the trolox standard curve, and was expressed in terms of mg trolox equivalents (TE)/g DW of BSG. All solutions were prepared on the day that they were used and all measurements were carried out in triplicate.

6.1.5.3. 2-Deoxyribose assay

The scavenging activity of BSG extracts towards hydroxyl radical was measured by the deoxyribose method with some modifications [172]. 100 μL of BSG extract, 100 μL of 1 mM EDTA, 100 μL of 1 mM $FeCl_3.6H_2O$, 100 μL of 36 mM 2-deoxy-D-ribose, 100 μL of 10 mM H_2O_2 and 100 μL of 1 mM L(+)-ascorbic acid were mixed and the final volume was adjusted to 1.0 mL with 25 mM phosphate buffer (pH 7.4). After incubation at 37 °C for 1 h, the reaction was stopped by adding 1.0 mL of 10% TCA (w/v) and 1.0 mL of 1.0% TBA (w/v) in 0.05 M NaOH. The mixture was heated in a water bath at 85 ºC for 30 min. Once samples were cooled, the final volume was adjusted to 5.0 mL with deionized water. Absorbances were measured at 532 nm against water, and compared to control containing 100 μL of deionized water instead of sample. Results were expressed as antiradical power (ARP, %) which is defined as ($1/EC_{50} \times 100$), where

EC_{50} is the quantity of extract capable of reducing the initial concentration of hydroxyl radicals to one half. Data were reported as mean ± SD for three replicates.

6.1.6. HPLC-DAD analysis

The phenolic composition of the BSG extracts was analyzed by the HPLC method described by Rubilar *et al.* [169] with minor modification. The HPLC system (Jasco Corporation, Tokyo, Japan) consisted of a low pressure quaternary gradient unit (model LG-1580-04) with an in-line DG-1580-54 degasser and a model AS-950 auto-sampler. The system is equipped with a photodiode array detector (model MD-1510 UV/Vis multiwavelength detector).

Separation of polyphenols was achieved on a Phenomenex Synergi Hydro-RP C_{18} column (150 mm × 4.6 mm, 4 μm) and a guard column with the same characteristics kept at room temperature. The chromatographic conditions were the following: flow rate 0.3 mL min^{-1}, sample injection volume of 20 μL and mobile phase A (100% methanol) and mobile phase B (0.1% formic acid). A gradient program was used as follows: 90% B in 0 min, from 90% to 0% B in 110 min, followed by 100% A for 20 min and back to 90% B in 10 min and 10 min of reconditioning before the next injection. The photodiode array detection was conducted by scanning between 190 and 600 nm. Analytes in each sample were identified by comparing their retention times and UV-Vis spectra with those of standard compounds. Peak purity was checked to exclude any contribution from interfering peaks.

The concentrations of individual phenolic compounds, in particular FA and *p*-CA, in BSG extracts were determined using external standard calibration curves in the concentration range of 5 to 200 mg L^{-1} (detection at 320 nm, n = 7, r^2 = 0.9997 for FA; detection at 316 nm, n = 7, r^2 = 0.998 for *p*-CA). Results were expressed in milligrams per 100 g of dry BSG (mg/100 g of DW).

6.1.7. HPLC-ESI-MS analysis

The qualitative study of the phenolic composition in all samples was performed by HPLC coupled on-line with electrospray ionization (ESI) mass spectrometry. The HPLC system (Finnigan, Thermo Electron Corporation, San Jose, USA) consisted of a low-pressure quaternary pump (Finnigan Surveyor Plus) and an auto-sampler (Finnigan Surveyor Plus with 200-vial capacity sample). Separations were achieved in the same conditions as for HPLC-DAD analysis but the injection volume was 25 μL. A

quadrupole ion trap mass spectrometer (Finnigan LCQ Deca XP Plus) equipped with an ESI source in the positive ion mode and Xcalibur software Version 1.4 (Finnigan) were used for data acquisition and processing. The interface conditions were applied as follows: capillary temperature, 325 ºC; source voltage, 5.0 kV; capillary voltage, 4.0 V; sheath gas (N_2) flow at 90 arbitrary units and auxiliary gas (N_2) flow rate at 25 arbitrary units. Data acquisition was performed between *m/z* 100 and 1000. For the MS^2 analyses an activation energy of 45% was applied. The positive ion mode was used in this study due to a better signal-to-noise ratio in comparison with the negative ion mode.

6.1.8. Statistical analysis

All data points are the mean and standard error values of at least three independent experiments. Differences in means were detected using one-way ANOVA and Tukey's test. The software employed for statistical analysis was Graphpad Prism, version 5 for Windows. The *p*-value less than 0.05 ($p < 0.05$) was considered statistically significant.

6.2. Results and Discussion

6.2.1. Total phenolic content

After application of MAE method, the obtained BSG phenolic extracts were analyzed in terms of TPC by using the Folin–Ciocalteu method. TPC from BSG extracts decreased with the increasing KT applied in the preparation of the different malt types (Table 6.1 and Fig. 6.1).

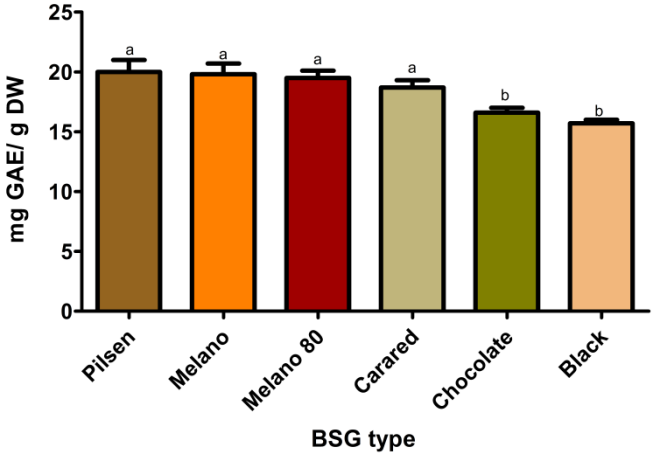

Fig. 6.1 - Total phenolic content of BSG extracts. Bars represent mean ± SD. Bar means with different superscript letter are statistically significant ($p < 0.05$). GAE – gallic acid equivalents.

BSG from the light malt types (*pilsen*, *melano*, *melano 80* and *carared*; KT ≤ 160 ºC, Table 6.1) were found to contain higher amounts of phenolic compounds when compared to the darker BSG's (*chocolate* and *black*; KT ≥ 200 ºC), 19.5 ± 0.6 and 16.2 ± 0.6 mg GAE/g DW BSG for light and dark types, respectively (Fig. 6.1). In contrast to our results, McCarthy and co-workers [116] recently reported that *black* BSG extracts exhibited the highest phenolic content. This difference can partially be explained by the type of malt used in the brewing process which has been shown to interfere with the content of individual phenolic compounds extracted [37, 39]. In addition, this difference can also be due to the melanoidins generated during kilning by the Maillard reaction, whose content increases with KT. These compounds are widely reported to possess antioxidant properties [182], however other studies reported that melanoidins can trap polyphenols within its structure lowering the content of free phenolic compounds [183].

Among the BSG analyzed, *pilsen* type presents the highest phenolic content, 20 ± 1 mg GAE/g DW (Fig. 6.1). Kähkönen *et al.* [184] studied the antioxidant capacity of 92 phenolic extracts from edible and nonedible plant materials (berries, fruits, vegetables, herbs, cereals, tree materials, plant sprouts, and seeds) and determined low phenolic content levels in cereals (0.2-1.3 mg/g GAE). Those results were considerably lower than those obtained in the present work for BSG extracts. Recently, Meneses *et al.* [32] also evaluated the efficiency of different solvent compositions (methanol, ethanol, acetone, hexane, ethyl acetate, water, methanol:water mixtures, ethanol:water mixtures, and acetone:water mixtures) for extracting antioxidant phenolic compounds from BSG and reported that the extract produced with 60% v/v acetone had the highest content of total phenols (9.90 mg GAE/g). Again a higher phenolic content was found in

the *pilsen* BSG used in this work when compared with the results of Meneses *et al.* [32]. The higher values of phenolic content determined in the present work when compared with those from others works can be extensively discussed. The samples type and origin and the extraction technique used can be pointed as the main reasons for the verified differences. Although, it is possible to conclude that the *pilsen* BSG sample studied in this work and the extraction procedure are responsible for the highest values of phenolic content determined in this type of samples. In fact, the extraction technique is probably the most influential factor since BSG is a lignocellulosic material which contains significant amount of phenolic acids esterified to the cell-wall, making their recovery harder [32]. Generally, the conventional extraction techniques, such as mechanical agitation solvent extraction, Soxhlet extraction, enzymatic and alkaline hydrolysis extractions [17, 38, 87], applied for the recovery of these compounds are time and solvent consuming, submitting polyphenols to thermal degradation and decreasing the phenolic content of the extracts. Furthermore, BSG phenolic extraction by MAE procedure may be more efficient in breaking ether and ester bonds of lignin to phenolic compounds, allowing a higher amount of these compounds, which explains the main difference between our results and those reported in the literature.

6.2.2. Ferulic and *p*-coumaric acids content

The major compounds contributing to the phenolic content of BSG were FA and *p*-CA. For the six BSG analyzed it was found that FA occurs in higher amounts than *p*-CA (Fig. 6.2), which is in accord with previous works [17, 30]. In the current study, the average of total FA and *p*-CA amounts of the light extracts was approximately 2-fold higher as compared to the dark extracts. The highest levels obtained for the light extracts were 149 ± 7 mg FA/100 g DW and 47 ± 7 mg *p*-CA/100 g DW (*pilsen* type) while those for the dark extracts were 77 ± 3 mg FA/100 g DW and 21 ± 1 mg *p*-CA/100 g DW (*black* type). McCarthy and co-workers [116] have also reported that the content of FA is significantly reduced for *black* extracts. As mentioned before, this can be attributed to the formation of melanoidins during kilning, mainly present in dark malts, which can trap polyphenols within its structure lowering the content of these phenolic compounds [183]. Also, the thermal degradation with increasing KT can promote an overall decrease in the level of these hydroxycinnamic acids in dark BSG

samples [182]. Moreover, the higher content of these two acids in light BSG can also be due to the release of bound phenolics from cell walls by the action of ferulic acid esterase (FAE) and associated enzymes, which are more active at lower KT. As the temperature increases, the FAE becomes less active and the FA release is slowed. Samaras *et al.* [185] also reported that neither catechin nor FA were identified in *black* or *chocolate* malt or roasted barley (highly roasted malts).

The correlation analysis between the TPC and FA and *p*-CA contents in the BSG extracts (r^2 = 0.9493 and 0.8038, respectively) suggests that these two cinnamic acids are the major contributors for the higher TPC of *pilsen* BSG.

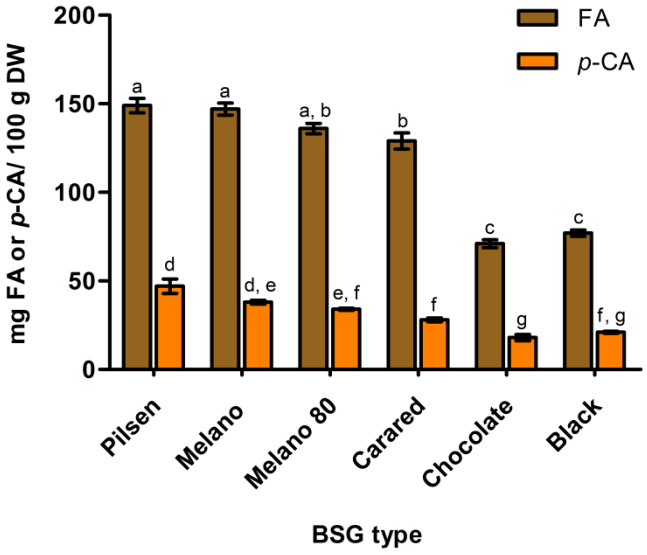

Fig. 6.2 - Ferulic and *p*-coumaric acids content of BSG extracts. Bars represent mean ± SD. Bar means with different superscript letter are statistically significant ($p < 0.05$).

6.2.3. Antioxidant properties of BSG extracts

It is well known that the radical system used for antioxidant evaluation may influence the experimental results, and two or more radical systems are required to investigate the radical-scavenging capacities of a selected sample. Thus, in the present work, DPPH, ABTS and deoxyribose assays were used for the evaluation of the antioxidant activity of the extracts obtained from BSG samples. In this study, the extracts obtained from the six BSG displayed radical scavenging activity as shown in Fig. 6.3.

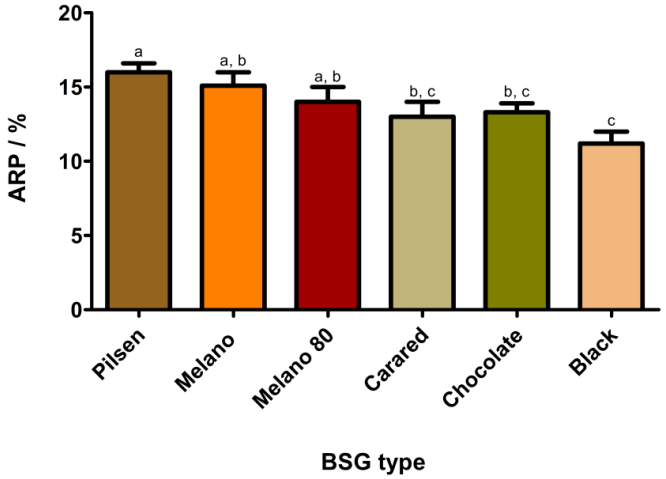

Fig. 6.3 - Radical scavenging activity of BSG extracts assessed by DPPH. Bars represent mean ± SD. Bar means with different superscript letter are statistically significant ($p < 0.05$).

The light extracts showed higher scavenging activities towards the radicals DPPH and ABTS than the dark extracts, which is in accordance with the higher TPC previously obtained (r^2 = 0.7540 and 0.8129 for DPPH and ABTS, respectively). The DPPH radical-scavenging activity decreases with increasing degree of malt color and KT (Table 6.1, Fig. 6.3), with light extracts exhibiting higher DPPH radical-scavenging activity (15 ± 1 and 12 ± 1% for light and dark types, respectively). It has been reported that free radical-scavenging activity is greatly influenced by the phenolic composition of the sample. Therefore, this result could be anticipated because of the higher amounts of total phenolics and FA and *p*-CA contents present in the light BSG extracts.

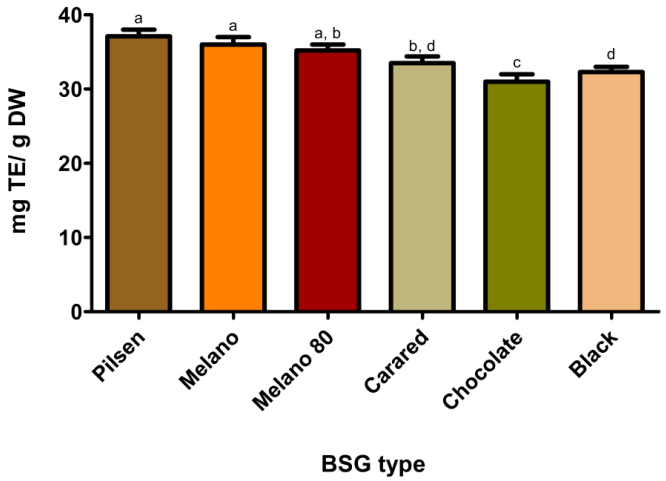

Fig. 6.4 - Radical scavenging activity of BSG extracts assessed by ABTS. Bars represent mean ± SD. Bar means with different superscript letter are statistically significant ($p < 0.05$). TE – trolox equivalents.

The ABTS assay was also used for *in vitro* assessment of free radical activity and the relative ability to scavenge the ABTS radical has been compared with the standard trolox. Results were similar to those obtained for DPPH assay, with light BSG exhibiting a stronger ABTS cation radical-scavenging activity than dark BSG (35 ± 2 and 31.7 ± 0.9 mg TE/g DW for light and dark types, respectively) as shown in Fig. 6.4.

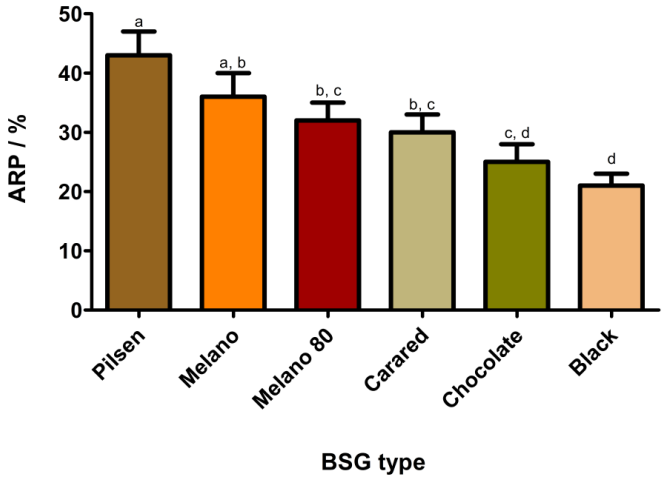

Fig. 6.5 - Radical scavenging activity of BSG extracts assessed by deoxyribose assay. Bars represent mean ± SD. Bar means with different superscript letter are statistically significant ($p < 0.05$).

The scavenging effect of hydroxyl radical was investigated using the 2-deoxyribose assay and the results are shown in Fig. 6.5. The results were in agreement with those obtained for the DPPH and ABTS methods, with light BSG extracts having an antioxidant activity higher than dark BSG extracts (35 ± 6 and 23 ± 3% for light and dark types, respectively). BSG extract from *pilsen* type exhibited the greatest scavenging effect of hydroxyl radicals among the BSG samples (43 ± 4). Vijayabaskar & Shiyamala [186] determined the ARP by DPPH assay for the standard GA which is recognized for its antiradical properties. The value obtained (55%) was closer to the ARP from *pilsen* BSG, confirming that this by-product exhibited good antiradical activity. Additionally, the results shown in Fig. 6.5 demonstrate that the difference in the ARP between light and dark BSG extracts assessed by the deoxyribose assay is higher than in the ARP measured by DPPH method. The average percentage for the difference was 20% for deoxyribose assay and only 5% for DPPH method. This variation may be caused by phenols, such as hydroxycinnamic acids, which are present in higher amount in light BSG extracts and are more prone to scavenge hydroxyl radicals than DPPH radicals. Significant positive correlations, $r^2 = 0.7851$ and 0.9299, were observed between FA and *p*-CA and antiradical efficiency determined

using deoxyribose method, indicating that these phenolic compounds are the major contributors to the antiradical activity of these BSG extracts.

Briefly, the reported results suggest that BSG extracts from light malts (Table 6.1) were found to contain higher amounts of total and individual phenolics, such us FA and *p*-CA, which might be responsible for the strong antioxidant activity measured in several chemical assays. It was observed that the BSG containing higher levels of TPC also exhibited a strong antioxidant effect, which was statistically confirmed by correlation analysis (r^2 = 0.7540, 0.8129 and 0.8375 for DPPH, ABTS and deoxyribose assays, respectively).

6.2.4. Phenolic characterization

The characterization of the MAE extract of BSG from industrial production by HPLC-DAD/ESI-MS/MS has previously revealed that matrix was mainly composed of phenolic acids and several isomeric ferulate dehydrodimers and one dehydrotrimer (Chapter 5). In the present work, the same study was performed over the BSG extracts for *pilsen*, *chocolate* and *black* malts in order to examine the main differences in their phenolic composition, mainly in FA dimers. Comparing the MS results obtained for the three BSG samples analyzed in this study and BSG from industrial production major differences were not detected in the compounds identified. As expected, hydroxycinnamic acids (*p*-CA (peak 4), FA (peak 5) and sinapic (peak 13)) were the main phenolic compounds in the BSG extracts. The structures of the other peaks identified in the chromatograms shown in Fig. 6.6 were the same as those already characterized in Chapter 5. Several FA dehydrodimers (DiFA) with a [M+H]+ ion at *m/z* 387 were identified, namely 8-8'-DiFA aryltetralin form (peak 2), 8-5'-DiFA benzofuran form (peak 3), 8-8'- DiFA (peak 7), 8-5'- DiFA (peak 8), 8-8'- DiFA cyclic form (peak 9), 5-5'- DiFA methylated form (peak 10), 8-5'- DiFA decarboxylated form (peak 11) and 8-O-4'- DiFA (peak 14). These compounds were identified based on collision induced dissociation (CID) fragmentation (MS^2 and MS^3 spectra) in which subsequent loss of water (*m/z* 369) and a carbonyl group (*m/z* 341) were observed. This DiFA can be distinguished by their retention times and UV-spectra. Two other peaks (12 and 16) were also attributed to FA dehydrodimer-type compounds, although final structure for peak 12 was not confirmed, while peak 16 corresponds to DiFA in hydrated form. The

fragmentation pattern of the compound identified as peak 15 with a protonated molecule at *m/z* 579 corresponds to TriFA (5-5',8'-O-4''-TriFA). This parent ion resulted in two major fragments with *m/z* 561 and 533, which is fully consistent with that previously observed for DiFA.

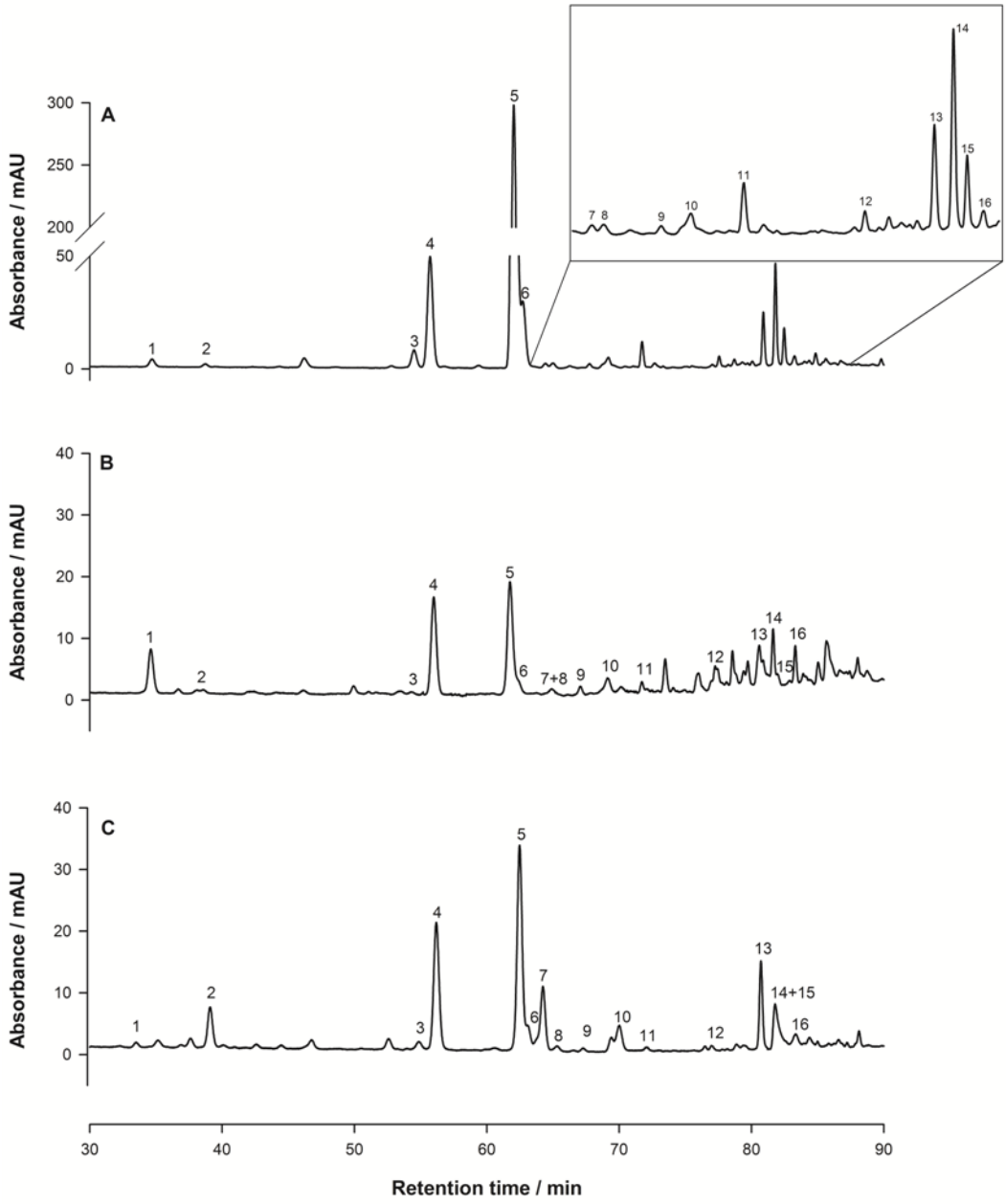

Fig. 6.6 - HPLC chromatograms at 280 nm obtained for BSG extracts of *pilsen* (A), *chocolate* (B) and *black* (C); (1) caffeic acid, (2) 8-8'-DiFA aryltetralin form, (3) 8-5'-DiFA benzofuran form, (4) *trans p*-CA, (5) *trans* FA, (6) syringic acid+HCOOH, (7) 8-8'-DiFA, (8) 8-5'-DiFA, (9) 8-8'-DiFA cyclic form, (10) 5-5'-DiFA methylated form, (11) 8-5'-DiFA decarboxylated form, (12) unknown FA dehydrodimer, (13) sinapic acid, (14) 8-O-4'-DiFA, (15) 5-5',8'-O-4''-TriFA and (16) DiFA hydrated form.

Although no differences were detected in the phenolic profile from *pilsen*, *chocolate* and *black* BSG, the content of some DiFA compounds was different. The peak areas obtained for the main DiFA identified in the three samples are reported in Table 6.2.

Table 6.2 - Peak areas (expressed in mAu min) obtained at 280 nm of ferulic acid dehydrodimers in BSG extracts from *pilsen*, *chocolate* and *black* types

BSG type	8-5'-DiFA[1] benzofuran form	8-8'-DiFA cyclic form	5-5'-DiFA methylated form	8-5'-DiFA decarboxylated form
Pilsen	4.4 ± 0.4^{a}	1.5 ± 0.4^{c}	4.0 ± 0.3^{d}	3.7 ± 0.3^{f}
Chocolate	1.3 ± 0.3^{b}	1.5 ± 0.4^{c}	2.2 ± 0.3^{e}	1.6 ± 0.2^{g}
Black	1.6 ± 0.3^{b}	1.2 ± 0.4^{c}	4.0 ± 0.2^{d}	1.2 ± 0.3^{g}

[1]DiFA: dehydrodiferulic acid; Values are expressed as mean ± SD (n = 3)
Means within a column with the same superscript letter are not significantly different (p>0.05)

Among these compounds, 5-5'-DiFA in methylated form was the most abundant in the three studied BSG. Moreover, the pilsen BSG exhibited the highest total DiFA content, followed by the black and chocolate BSG. In fact, BSG types with higher FA content also showed higher levels of DiFA. ANOVA analysis showed significant differences ($p < 0.05$) among the three studied BSG types for the compounds that were studied except for 8-8'-DiFA in cyclic form (Table 6.2). This clearly indicates that the concentration of DiFA in BSG was influenced by the kilning regimes used in malting process.

These results may be the starting point for further study of the influence of the malting process, in particular the kilning regimes and roasting temperatures, on the BSG phenolic composition and antioxidant potential. This knowledge can surely be valuable for exploring new applications for different types of BSG.

7. Evaluation of the ability of brewer's spent grains to protect against DNA damage in *Saccharomyces cerevisiae*

Reactive oxygen species (ROS), such as the superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), pose a significant threat to cellular integrity. In the presence of redox-active metal ions, such as iron, $O_2^{-\bullet}$ and H_2O_2 can undergo the Fenton reaction generating the extremely reactive $^{\bullet}OH$, which attacks almost all cell components, including DNA [187]. High levels of ROS, formed through both endogenous and exogenous routes, and the DNA damage produced contribute to genetic instability. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain that diffuse out freely through membranes and attack other cellular and mitochondrial components. Exogenous ROS occur through exposure to numerous agents including ultraviolet radiations, chemotherapeutic drugs, environmental toxins and hyperthermia [188, 189]. The incapacity of the cellular defenses to cope with the oxidative challenge generates oxidative stress, which can cause oxidative damage in macromolecules, including genomic DNA [190]. DNA lesions such as DNA base modifications, single- and double-strand breaks, and the formation of apurinic/apyrimidinic sites may be formed [188]. Once DNA damage is sensed, cell cycle is arrested so that repair mechanisms can operate, or induction of programmed cell death may take place if damage cannot be repaired. The major DNA repair pathways are base excision repair (BER) in the removal of damage of single bases caused by oxidation and nucleotide excision repair (NER), which is involved in repairing bulky DNA lesions caused by ultraviolet light [189].

The DNA damage can be assessed by single cell gel electrophoresis, or comet assay, where the chromosomal DNA migration distance correlates with the extent of DNA damage. The comet assay was firstly described by Östling and Johanson in 1984 [191]. Cells are embedded in low-melting agarose on a microscope slide, lysed and an electric field is applied, leading to electrophoresis of genomic DNA, which is then visualized by staining with a fluorescent dye and observed by microscopy. DNA will migrate towards the anode, producing a shape resembling a comet with a tail. DNA strand breaks introduce a relaxation in the normally supercoiled chromosomal DNA,

resulting in more mobile DNA [191]. This is observable as pronounced migration of DNA towards the anode after electrophoresis (or longer comet tails). Determination of tail length, or the percentage of DNA in the tail, is a simple way of quantifying the degree of DNA damage in an individual cell. The comet assay is, in principle, simple and inexpensive to perform but is also sensitive and has been used in a number of different applications, such as testing for genotoxicity, ecological monitoring and human biomonitoring [192].

The comet assay has been used for a variety of applications with several organisms [193], and the range of applications for this method was extended to include yeast [194] by applying it to *Saccharomyces cerevisiae* cells. Despite the great potential of this model organism, application of this method has only been reported a few times [193, 194]. A possible reason for this absence is that in yeasts, the amount of DNA per cell is considerably lower than for higher eukaryotes, which has been suggested to pose a problem for the application of the comet assay [193]. Azevedo *et al.* [189] reported an optimization of the comet assay protocol for yeast cells that is robust and sensitive enough to reproducibly detect background DNA damage and oxidative damage caused by H_2O_2. The performance improvements have been possibly due to the generally milder conditions of this comet protocol. In addition, increasing low-melting agarose concentration provides a denser electrophoresis matrix for migration of damaged DNA from cells with low chromatin content. The combination of *S. cerevisiae* cells and the comet assay offers great advantage, since it may be one of the most economically accessible techniques for genotoxicity testing in terms of consumables.

The antigenotoxic activity of many phytochemicals and plant extracts can be attributed to their antioxidant properties, which can be determined by assessing the ability of a compound to protect against oxidant-induced DNA damage [192]. As aforementioned, BSG is a unique source of bioactive ingredients including phenolic acids, particularly the hydroxycinnamic acids, ferulic and *p*-coumaric acids. Several studies have shown that these compounds protect against H_2O_2-induced oxidative damage [85, 106].

Despite the fact that some studies concern the antioxidant properties of BSG [30, 32], only one report has associated these extracts with antigenotoxic activity [116]. McCarthy *et al.* [116] have investigated the ability of phenolic BSG extracts to protect against DNA damage in human lymphocytic U937 cells measured by the comet assay. However, in our study the use of yeast cells enable us to use mutant strains in the

study of DNA repair pathways allowing us to understand the mechanism of antioxidant and antigenotoxic properties of BSG extracts. The aim of the present study was to assess the ability of phenolic rich BSG extracts to protect against DNA damage in *S. cerevisiae*. In Chapter 6, BSG extracts were prepared from six malt types (*pilsen*, *melano*, *melano 80*, *carared*, *chocolate* and *black*) and the total phenolic content, antioxidant activity (assessed by DPPH, ABTS and deoxyribose assays) and ferulic and *p*-coumaric acids content of the extracts were determined. As the main differences were more pronounced between the BSG from *pilsen* and *black* types, in these chapter we investigated if these extracts could inhibit oxidative DNA damage induced by H_2O_2 using a recently developed protocol of comet assay applied to yeast [189]. According to our knowledge, the present report is the first application of the yeast comet assay to the study of the protective capacity of BSG against DNA oxidative damage.

7.1. Materials and Methods

7.1.1. Chemicals and samples

All reagents were of analytical grade and were purchased from Sigma‑Aldrich, unless otherwise stated. BSG extracts studied in this chapter were those whose differences were more pronounced, and correspond to extracts from *pilsen* and *black* malts. As previously described in Section 6.1.2, *pilsen* malt produces a very light colored, clean and crisp wort and is used as base malt for all beer styles. The color range from 3.5 to 5.7 EBC untis and KT between 80 to 85 ºC. *Black* malt is roasted for a higher period and the end temperature is very high (230 ºC), forming harsher flavors and EBC color ranges from 1350 to 1500 units. The remaining solid fraction obtained following the removal of wort during the pilot scale production of beer was frozen, lyophilized and then ground. The dried BSG samples were prepared as described in Section 6.1.3.

7.1.2. Yeast strains, culture media, and growth conditions

The yeast *S. cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and derived mutants used throughout this work are listed in Table 7.1 . These strains were maintained on standard solid yeast extract (1% w/v), peptone (2% w/v), dextrose (2% w/v) and agar (2% w/v) medium (YPD). Yeast cells were grown in 50 mL liquid YPD

medium (without agar) in an Erlenmeyer flask with air-liquid ratio of 5/1, at 30 ^{O}C and orbital agitation at 200 rpm. Growth was monitored by optical density at 600 nm (OD_{600}).

Table 7.1 - *Saccharomyces cerevisiae* strains used in this work

Strain/relevant genotype	Genotype
BY4741	*MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*
Y04964/*apn1*	BY4741 *MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YKL114c*::kanMX4*
Y02806/*rad1*	BY4741 *MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YPL022w*::kanMX4*
Y02806/*yap1*	BY4741 *MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* YML007w::*kanMX4*

7.1.3. Viability assays

A liquid pre-culture of 5 mL YPD medium was prepared with a single yeast colony and grown overnight. The culture was diluted with fresh medium to a density of 1.2 × 10^7 cells/mL and harvested by centrifugation (2 min at 5000 rpm, 4 ºC) after two generations. Cells were subsequently washed twice, each time with the same volume of sterilized deionized H_2O. Pre-treatment with BSG extracts was made by ressuspending the pellet in the same volume of ½ diluted BSG extracts (1:1 (v/v) YPD 2-fold concentrated and BSG extract). Cells with BSG extracts were incubated at 30 ºC, 200 rpm for 90 min. Samples were harvested at 20, 60, and 90 min, serially diluted to 10^{-4} in sterilized deionized H_2O and spread on YPD plates. Plates were incubated at 30 ºC for 48 h and the colonies were counted. Viability was calculated as percentage of colony-forming units (CFU) at each time point in relation to the beginning of the experiment (0 min).

As only for *black* BSG extracts a statistically significant decrease in cells growth rate was observed at 90 min, another assay was carried out under these conditions in order to investigate the cytotoxicity of the extract. Cells were prepared in the same way as for viability measurement, except that they were suspended in phosphate buffered saline (PSB; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), instead of YPD medium. Growth rates were calculated as percentage of CFU, assuming 100% survival for cells of the suspension before any treatment.

7.1.4. Analysis of DNA damage by the yeast comet assay

Oxidative DNA damage in *S. cerevisiae* cells was induced by H_2O_2. H_2O_2 produces hydroxyl radicals ($OH^{\cdot}$) by means of the Fenton reaction in an iron dependent manner, and these radicals cause single strand breaks by attacking the ribose on the DNA backbone [195]. DNA damage was assessed by a recently developed yeast comet assay [189]. Briefly, cell walls were digested with 2 mg/mL zymolyase (20,000 U/g; ImmunO™ – 20T), incubated at 30 ºC for 20 min and collected by centrifugation at 5000 rpm, 4 ºC for 2 min. Spheroplasts were then suspended in 1 mL S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5) and 80 µL of this suspension were distributed by microtubes and collected by centrifugation at 5000 rpm, 4°C for 2 min. Each pellet was suspended in BSG extracts diluted in water and exposed to the oxidant solution (10 mM H_2O_2) for 20 min at 4 ºC. Treated spheroplasts were collected from suspension by centrifugation at 5000 rpm, 4 ºC for 2 min and then embedded in 1.5% (w/v in S buffer) low melting agarose (LMA) at 35 ºC and distributed by glass slides (slide coated with a water solution of 0.5% w/v normal-melting agarose), covered with a coverslip and placed on ice to solidify. Following exposure of the cells to the H_2O_2 treatment, the slides were incubated in the lysing buffer (30 mM NaOH, 1 M NaCl, 0.05% w/v laurylsarcosine, 50 mM EDTA, 10 mM Tris–HCl, pH 10) for 20 min in order to lyse spheroplasts. Samples were incubated in electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris–HCl, pH 10) for 20 min and then submitted to electrophoresis in the same buffer for 10 min at 0.7 V/cm. After electrophoresis, the slides were incubated in neutralization buffer (10 mM Tris–HCl, pH 7.4) for 10 min, followed by consecutive 10 min incubations in 76% and 96% (v/v) ethanol. Then, the slides were dried at room temperature and were visualized immediately or stored at 4 ºC until observation. For visualization in a fluorescence microscope (Leica Microsystems DM fluorescence), the slides were stained with GelRed (10 µg/ mL; Biotium) and representative images were acquired at magnification of 400× in order to obtain at least 20 random comets per sample that were analyzed with the CometScore version 1.5 software for the tail length (expressed in µm). Error bars represent variability between the mean of at least three different slides obtained from biologically independent experiments.

7.1.5. Analysis of antioxidant activity by flow cytometry

Cells were prepared in the same way as for yeast cell viability measurement, except that they were diluted to a density of 1.0×10^6 cells/mL and suspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), instead of

YPD medium. Five hundred microlitres of untreated cells were removed for autofluorescence measurement. Dichlorofluorescein diacetate (H_2DCFDA; Sigma-Aldrich) (50 μM final concentration) was added to the reminder of the cells and cell suspension was further incubated at 30 ºC, 200 rpm for 1 h in the dark, washed twice with the same volume of PBS and distributed in aliquots for the different assay conditions. Cells were treated with diluted BSG extracts, or 5 mM H_2O_2, or both and incubated at 30 ºC, 200 rpm for 20 min. Twenty thousand cells of each sample were analyzed by flow cytometry in an Epics XLTM cytometer (Beckman Coulter) equipped with a 15 mW argon-ion laser emitting at 488 nm. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm bandpass filter. Data were analyzed and histograms were made with the Flowing software version 2.5.0.

7.1.6. Measurement of growth of yeast cultures

Pre-inocula of *S. cerevisiae* strain BY4741 and derived mutants were prepared with a single yeast colony from solid stock culture, suspended in 5 mL of liquid YPD medium and incubated overnight at 30°C and 200 rpm. An appropriate volume of the pre-inoculum was diluted in 10 mL of fresh liquid YPD medium to OD_{600} 0.1 and 3 mL was distributed for each falcon. The culture was then incubated for 45 min at 30 ºC and 200 rpm to ensure that yeast cells were adapted to the new conditions. Cells were then subsequently treated with BSG extracts 20-fold diluted and H_2O_2 (1 mM final concentration). After incubation at 30 ºC, 200 rpm for 1 h, 0.5 mL of the culture was diluted in 0.5 mL of liquid YPD medium and OD_{600} was measured. Cultures growth was followed for 5 h and OD_{600} was measured every hour. Cell growth was expressed as the value of OD_{600} units at each time point.

7.1.7. Statistical analysis

All results are the mean and standard deviation (SD) values of at least three independent experiments. Differences in means were detected using one-way ANOVA and Tukey's test. The software employed for statistical analysis was Graphpad Prism, version 5 for Windows. All asterisks indicate differences considered statistically significant: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, when compared to the respective control.

7.2. Results and Discussion

The evaluation of antioxidant activity and identification of the major phenolic compounds of BSG from six malt types has previously revealed that *pilsen* BSG showed higher TPC and antioxidant activity than *black* BSG, assessed by DPPH, ABTS and deoxyribose assays (Chapter 6). As the main differences were observed between BSG from *pilsen* and *black* malts, in the present work we have investigated the protective effects of these two BSG extracts on genomic DNA upon oxidative shock. We have used *S. cerevisiae* as experimental model to take advantage of the simplicity of manipulation and of the mutant strains. These tools open the possibility for molecular biology approaches in the investigation of mechanisms of action of BSG extracts and protective compounds present in the extracts.

7.2.1. *Saccharomyces cerevisiae* viability is not affected by BSG extracts

To investigate if the BSG extracts were not toxic to yeast cells we assessed the growth rate of yeast cells in the presence of extracts (Fig. 7.1). *Saccharomyces cerevisiae* cells were incubated up to 90 min with BSG extracts diluted 2-fold in YPD medium. Aliquots of the suspension were harvested at different time points, diluted and plated on solid rich medium in order to count colonies after 48 h incubation at 30 ºC. Relative cell viability at each time point was calculated as the percentage of CFU's in relation to the beginning of the experiment (0 min).

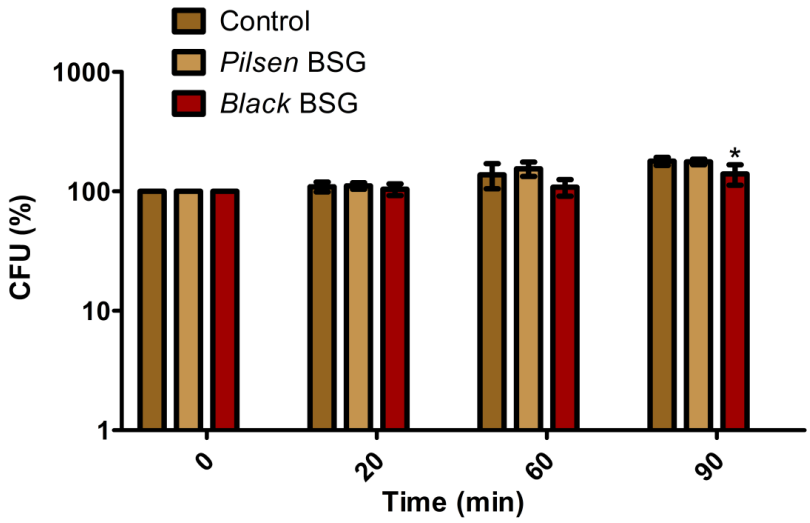

Fig. 7.1 - Viability of yeast cells when incubated with water (control) or with BSG extracts diluted 2-fold in YPD medium. Mean ± SD values are from three independent experiments (* represent $p < 0.05$).

As can be observed in Fig. 7.1, cells without any treatment (control) or incubated with BSG displayed an increase in CFU's during the 90 min incubation. After 60 min incubation yeast cells treated with *black* BSG extracts show less viability than the control and cells treated with *pilsen* BSG, which becomes statistically significant ($p < 0.05$) after 90 min.

To evaluate if the effect of the *black* BSG on the viability was by inhibition of proliferation or by promotion of loss of viability, yeast cells were incubated with *black* BSG extracts in PBS buffer instead of YPD and the viability of cells was assessed after 90 min incubation (Fig. 7.2). Therefore, if a decrease in cell viability is detected it means that BSG extract could cause cell death (cytotoxicity).

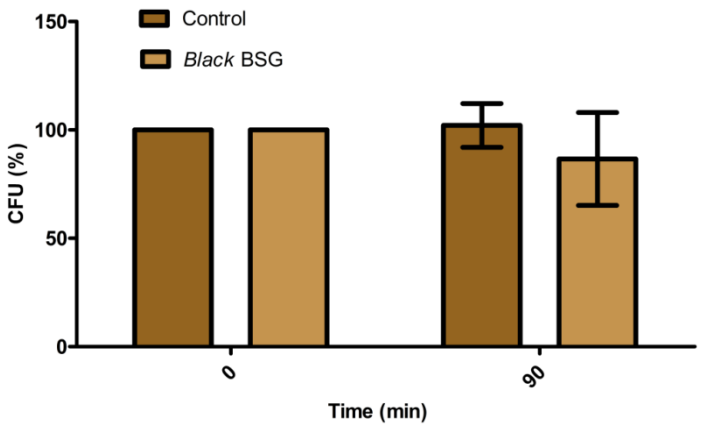

Fig. 7.2 - Viability of yeast cells when incubated with water (control) or with *black* BSG extracts diluted 2-fold in PBS buffer. Mean ± SD values are from three independent experiments.

Comparing the cell viabilities from control with cells treated with *black* BSG extract, the decrease observed was not statistically significant ($p > 0.05$). This decrease may be due to an adaptation of yeast cells to the new conditions.

According to the aforementioned, the use of *black* BSG extract appears to affect cell viability. However, compared to control, the difference of cells treated with *black* BSG was not statistically significant ($p > 0.05$) after 60 min incubation. As in the following assays the incubations were for 20 min, it can be concluded that the extracts did not interfere with cell viability in the assays.

7.2.2. BSG extracts protects yeast cells against DNA damage by H_2O_2

A simple way of quantifying the degree of DNA damage in an individual cell in the comet assay is the determination of tail length. Oxidatively damaged DNA was observed and quantified as electrophoretic migration out of the nucleoid, being the length of migration (comet tails) proportional to the extent of damage [189, 196].

As can be seen in Fig. 7.3, yeast cells treatment with H_2O_2 produced visually different images of comets from cells treated with water or BSG extracts. Comets are also visible in the control, even without treatment with H_2O_2, possibly due to initial DNA damage present or as a consequence of the handling of cells during comet preparation procedure and the presence of replication forks in S phase of the cell cycle. Replication forks are equivalent to single-strand breaks in electrophoretic mobility under alkaline conditions in the comet assay [189].

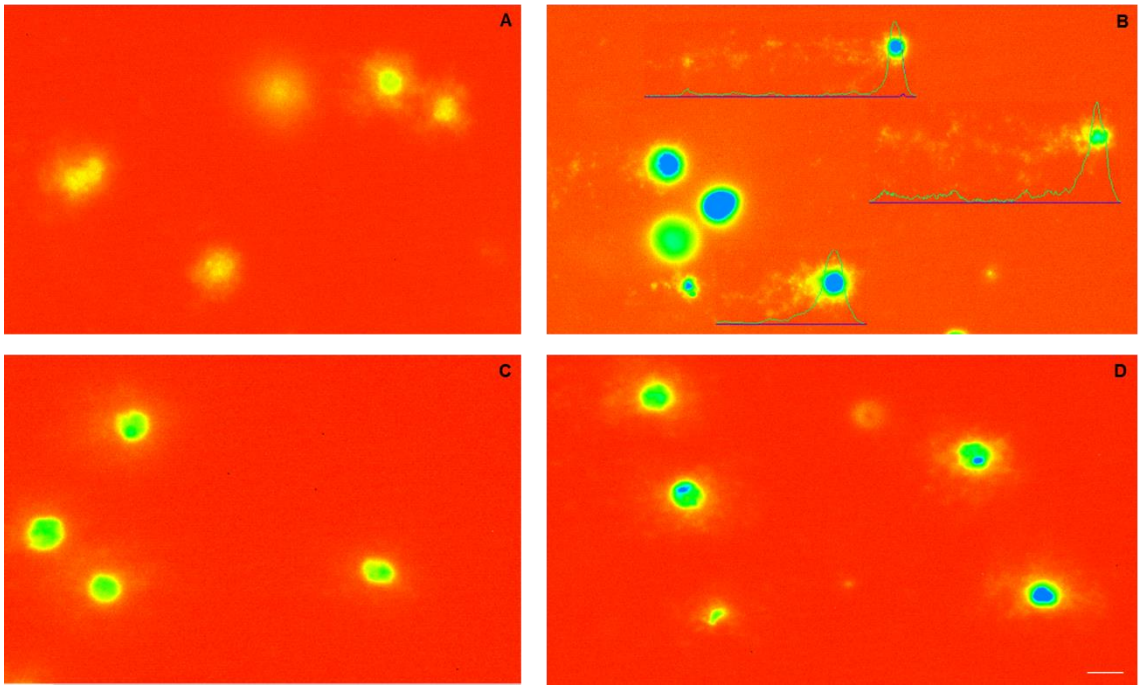

Fig. 7.3 - DNA damage induced by the exposure of *S. cerevisiae* cells to different treatments was assessed by the yeast comet assay and tails were measured for at least 20 comets/sample. The images obtained for untreated cells (A) and cells treated with 10 mM H_2O_2 (B), BSG extract (C) or BSG + 10 mM H_2O_2 (D) were acquired with fluorescence microscopy at ×400 magnification (figure colors are simulated and produced by CometScore software for a better contrast). The images correspond to one representative experiment from three independent experiments. White bar = 10 μm.

Comet tail length obtained for yeast spheroplasts treated with various BSG dilutions in water and simultaneously incubated with 10 mM H_2O_2 for 20 min are represented in Fig. 7.4.

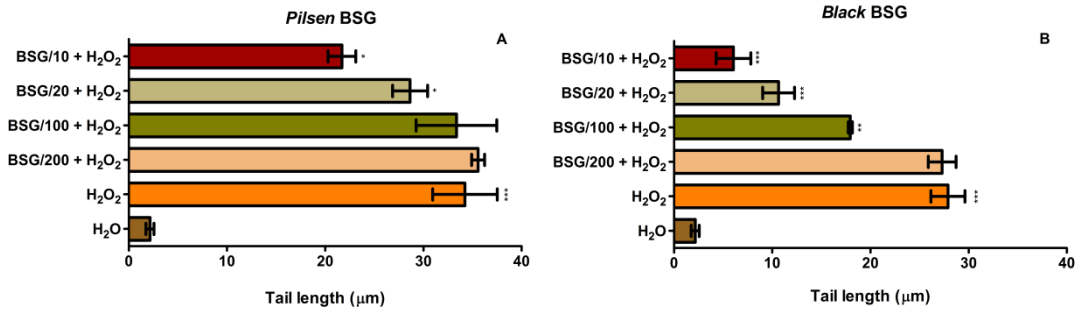

Fig. 7.4 - *Pilsen* (A) and *black* (B) BSG extracts protects DNA of *S. cerevisiae* cells from oxidative damage by H_2O_2. Yeast spheroplasts were incubated with BSG (diluted 10, 20, 100 or 200-fold in water, respectively, BSG/10 + H_2O_2, BSG/20 + H_2O_2, BSG/100 + H_2O_2 and BSG/ 200 + H_2O_2) simultaneously with 10 mM H_2O_2 for 20 min. Positive control (H_2O_2) represents treatment exclusively with 10 mM H_2O_2; and negative control (H_2O) represents treatment exclusively with H_2O. DNA damage was analyzed with the comet assay method (see Section 7.1.4). Mean ± SD values are from three independent experiments (*** represents $p < 0.001$, ** represents $p < 0.01$ and * represents $p < 0.05$).

As can be seen, for both assays deionized water did not cause DNA damage, while the addition of 10 mM H_2O_2 to yeast cells dramatically increased comet tail length (Fig. 7.4). When yeast spheroplasts were incubated with *pilsen* BSG extracts and exposed to H_2O_2 (Fig. 7.4A, BSG/10 – 200 + H_2O_2), a dose dependent decrease in comet tail length was observed, when compared to the positive control (Fig. 7.4A, H_2O_2). Results showed that *pilsen* BSG extract at 10 and 20-fold dilution significantly ($p < 0.05$) reduced the comet tail length in H_2O_2 treated yeast cells, when compared to the value obtained for cells treated only with H_2O_2. For a 10-fold dilution (Fig. 7.4A, BSG/10 + H_2O_2), nearly a two-fold decrease in comet tail was observed. *Pilsen* BSG extracts seem to have some protection effect on DNA even at 100 and 200-fold dilutions, although these differences are not statistically significant ($p > 0.05$). Regarding *black* BSG extract, the oxidative DNA damage was significantly reduced, as shown by a five-fold decrease in comet tail length for a 10-fold dilution (Fig. 7.4B, BSG/10 + H_2O_2). Only for a 200-fold dilution of *black* BSG extract the protection effect on DNA was not statistically different from H_2O_2 ($p > 0.05$).

In our experiments, the results obtained showed that the extracts of BSG from both malt types exert concentration dependent protection against the genotoxic effects of H_2O_2. A slightly less protection in the presence of *pilsen* BSG compared to *black* type was observed (Fig. 7.4), suggesting less antigenotoxic activity of this extract. Some studies [197] have reported that bioactive compounds with antioxidant activity present in both BSG types can contribute to the protection of DNA against the strong reactivity of ROS. Therefore, the difference observed in the prevention of DNA damage for *pilsen* and *black* BSG extracts can be attributed to the composition of these extracts. As

described in Chapter 6, ferulic and *p*-coumaric acids are the phenolic compounds present at highest concentrations in BSG. Previous studies have demonstrated that the standard FA protect against DNA damage caused by H_2O_2, as measured by the comet assay [198]. Recently, McCarthy *et al*. [116] demonstrated that 1 μg/mL of FA produce a 2-fold reduction in the percentage tail DNA in H_2O_2 treated U937 cells measured by the comet assay. In a study conducted by Wang *et al.* [198] feruloyl oligosaccharides, the FA ester of oligosaccharides from wheat bran, protected against H_2O_2 induced DNA damage in normal human peripheral blood lymphocytes, as measured by the comet assay. The authors attributed this protection to the antioxidant capacity of the FA moiety.

The results reported here for yeast comet assay do not directly correlate with the previously reported data in Chapter 6, which found that *pilsen* BSG exhibited the highest antioxidant activity. This difference can partially be explained by the type of the assay used for antioxidant evaluation, which has been shown to interfere with the antioxidant activity of a selected sample [71]. The radical systems used in the assays described in Chapter 6 are based on *in vitro* assays, while the comet assay uses yeast cells where the chemical environment is highly complex. Additionally, the observed difference in the prevention on DNA damage between *pilsen* and *black* BSG extracts can be attributed to the melanoidins compounds generated during kilning by the Maillard reaction, mainly present in *black* malts. These compounds are widely reported to possess reducing properties enhancing the antigenotoxic effect of *black* BSG extracts [182]. The antioxidant properties of melanoidins have been partly ascribed to the metal chelating capacity of these compounds [198, 199]. Melanoidins are brown high molecular weight compounds formed in the last stage of the Maillard reaction of thermally treated foods. The chemical structure of melanoidins has not been completely elucidated yet, although they behave as anionic material and can form stable complexes with metal cations [58, 199]. Transition metals have a major role in the generation of oxygen free radicals in living organisms. Iron exists in two distinct oxidation states – ferrous and ferric ions. The ferric ion (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on the conditions, particularly pH, and oxidized back through Fenton type reactions (see below), with production of hydroxyl radicals, which can lead to DNA damage . Several studies reported that melanoidins might possess the ability to chelate iron and potentially inhibit the metal-dependent processes [6, 200]. Therefore less $^{\bullet}OH$ radicals are produced and oxidatively DNA damage occurs in a less extent.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

In accordance with the aforementioned, this protection from both BSG types can be attributed to the chemical components of the extracts, which would have a scavenging effect on H_2O_2 and/or an induction of oxidative stress response and/or induction of DNA damage repair. The mechanism of the protective action of the bioactive compounds is not clearly understood. Scavenging of ROS by polyphenols is the generally accepted mechanism of their antioxidant activity. However, mechanisms involving metal binding have also been proposed and are gaining popularity [201-203]. Perron *et al.* [202] have confirmed that iron binding is a key mechanism for DNA damage inhibition by polyphenol compounds, such as catechin, quercetin, gallic, protocatechuic and vanillic acids. They reported that the iron oxidation observed upon binding to polyphenol compounds may result in an iron(III) complex that cannot be reduced by cellular reductants to catalytically generate a hydroxyl radical. Thus, in the future the determination how iron coordination controls the antioxidant activity of these extracts is important to better understanding their biological activity.

7.2.3. BSG extracts decreases intracellular oxidation

The antioxidant activity of *pilsen* and *black* BSG extracts was also investigated by flow cytometry with the fluorochrome H_2DCFDA. The diacetate form of dichlorofluorescein is able to diffuse freely through the plasma membrane into cells where intracellular esterases deacetylate H_2DCFDA to dichlorofluorescein (H_2DCF), which accumulates since it does not permeate membranes. Oxidation increases fluorescence of H_2DCF so whole cell fluorescence can be used as a marker for intracellular oxidation.

To investigate if BSG extracts protect cells from oxidative stress, we incubated mid-log growth phase cells with H_2DCFDA subsequently with BSG extracts diluted in PBS or 5 mM H_2O_2 for 20 min and then measured fluorescence in the flow cytometer.

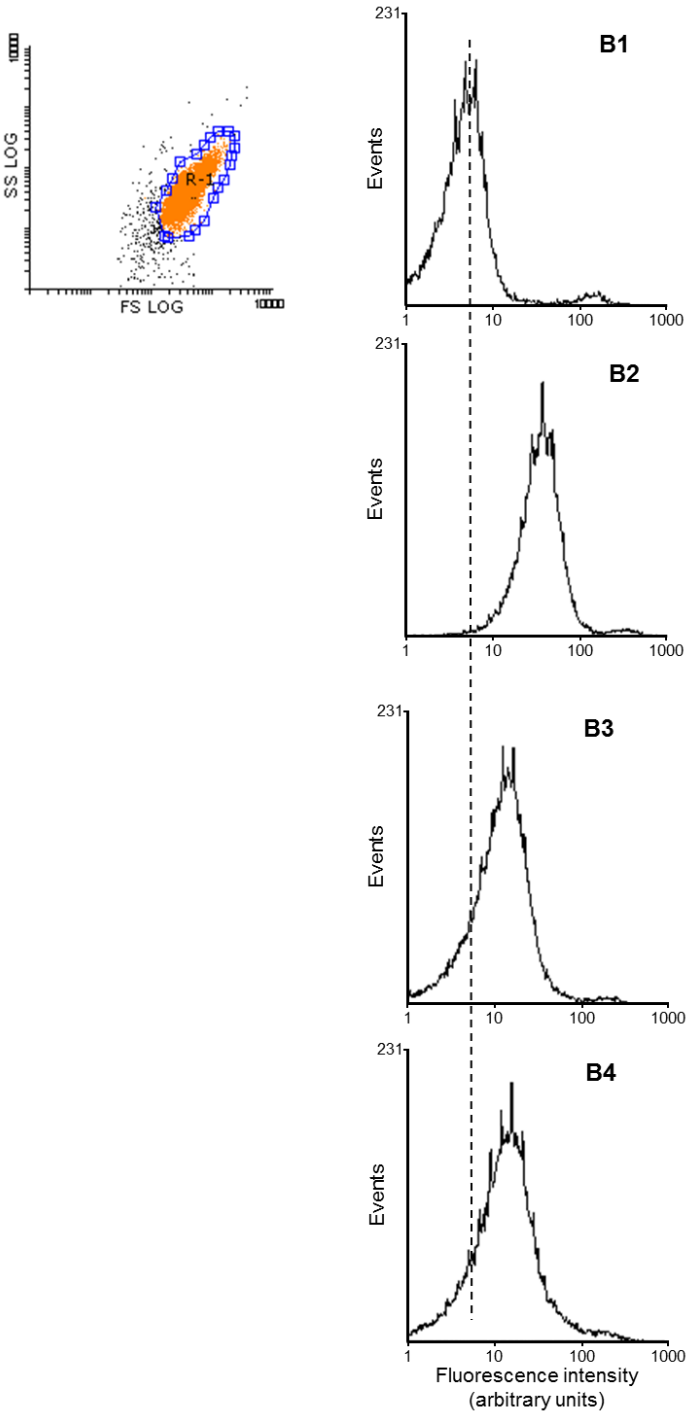

Fig. 7.5 - BSG extracts decrease intracellular oxidation in *S. cerevisiae* cells. Yeast cells were loaded with 50 μM H_2DCFDA for 60 min in the dark. After washing with PBS, cells were analyzed by flow cytometry. (A) - Distribution of the cells according to their size (FS) and roughness (SS), in which the area corresponding to live cells was selected. (B) - Representation of the obtained histogram for fluorescence of the oxidized form of H_2DCF (B-1); or after incubation with 5 mM H_2O_2 for 20 min, washed with PBS and analyzed by flow cytometry (B-2); or incubation with *pilsen* or *black* BSG extract diluted 20-fold and 5 mM H_2O_2 for 20 min, washed with PBS and analyzed by flow cytometry (B-3 and B-4). Data are from one representative experiment from at least three independent experiments.

In order to determine the size of cells two different parameters, size (FS) and roughness (SS), were analyzed. The percentage of cells in regions corresponding to viable and non-viable cells was also evaluated. The results obtained by flow cytometry appear initially in the form depicted in Fig. 7.5A. Each point illustrated in Fig. 7.5A represents a single particle, which is distributed in the graph according to its size (FS) and roughness (SS). Once selected the region corresponding to yeast cells (R1, Fig. 7.5A), it is possible to achieve a histogram similar to that which is shown in Fig. 7.5B. The x-axis corresponds to the fluorescence intensity whereas y-axis is the number of cells. Since the aim of the study was to evaluate the inhibitory effect of BSG extracts on oxidative DNA damage, one would expect a shift of the peaks to the right in the cases where DNA damage was affected by the addition of the different treatments.

As can be seen in Fig. 7.5B, oxidative shock by H_2O_2 induces an increase of intracellular oxidation and, hence, fluorescence of the population of cells shifted right compared to non-treated cells (Fig. 7.5B-1 and B-2). When cells were simultaneously treated with BSG extracts 20-fold diluted in PBS and 5 mM H_2O_2, fluorescence decreased significantly, suggesting that *pilsen* and *black* BSG extracts neutralize or stimulate protection in yeast cells against oxidative shock (Fig. 7.5B-3 and B-4). Antioxidant activity was also present when BSG extracts was further diluted in PBS: 50, 75 and 100-fold. The antioxidant effect was also quantified by determining the fluorescence average in each experimental situation (Fig. 7.6).

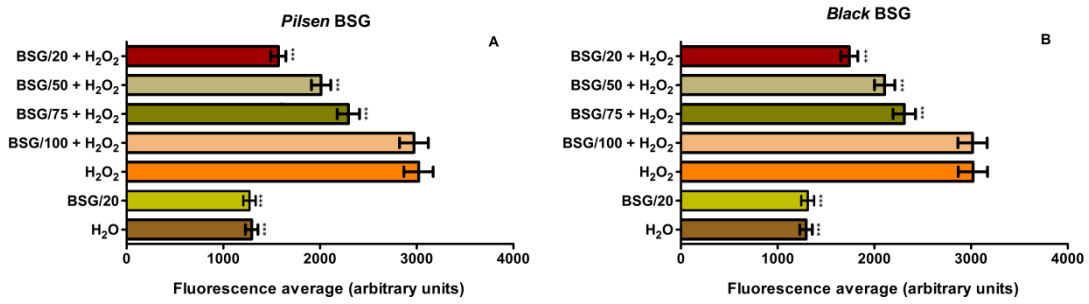

Fig. 7.6 - Treatment of *S.cerevisiae* cells with BSG extracts decreases intracellular oxidation by H_2O_2. Yeast spheroplasts were simultaneously suspended in BSG extracts 20-, 50-, 75- and 100-fold dilutions in PBS and 5 mM H_2O_2 and incubated for 20 min. The negative control (H_2O) reflects the amount of DNA damage in cells without exposure to BSG extracts and H_2O_2. The BSG extract 20-fold diluted reflects the amount of DNA damage in cell only treated with BSG extracts. Oxidative damage was analyzed by flow cytometry (see Materials and Methods). Fluorescence average ± standard deviation (SD) are from three independent experiments (***represents $p < 0.001$).

Results presented in Fig. 7.6 show a dose dependent effect of BSG extracts. A 2-fold increase in fluorescence intensity was observed in yeast cells treated with 5 mM

H_2O_2 for 20 min, as compared to untreated control cells (H_2O). Under the same experimental conditions, the H_2O_2 oxidant activity was reduced to 52% and 58% in yeast cells following the treatment with 20-fold dilution *pilsen* and *black* BSG extracts, respectively. Only for a 100-fold dilution no protective effect was provided ($p > 0.05$). These results strongly suggest that the intracellular ROS in yeast cells were reduced after co-incubation with both *pilsen* and *black* BSG extracts.

Despite no statistically difference was observed in the antioxidant effect of BSG extracts in this study (Fig. 7.5 and Fig. 7.6), these results are in accordance with the previous reported in Chapter 6, with *pilsen* BSG extracts showing a higher antioxidant activity. However, contradictory to that obtained by the yeast comet assay. These results obtained by flow cytometry suggest that the bioactive compounds present in the BSG extracts can scavenge directly endogenous ROS and/or efficiently recycle endogenous scavenger cellular proteins like glutathione, thioredoxin, superoxide dismutase, catalase, and/or induce the pentose phosphate pathway activity in regenerating NADPH [189].

7.2.4. BSG extracts protect cells of mutant strains affected in NER, BER and oxidative stress response

The determination of the mechanism of action of the protective effects previously observed for BSG extracts could be studied by exploring the mutants of *S. cerevisiae* affected in pathways of oxidative stress response and DNA repair. For that we hypothesized that a mutant strain deficient in a target pathway of a given extract would not be protected by the extract when compared with the wild type strain. Yeast cells from mutants affected in the *YAP1* gene involved in the response against oxidative stress, the *APN1* gene from the BER pathway (involved in the repair of, mostly, oxidative DNA damage induced by H_2O_2) and *RAD1* from the NER pathway (involved in the repair of, mostly, damage caused by UV) were incubated for 1 h with 1 mM H_2O_2 and BSG extracts 20-fold diluted. Culture growth was followed for 5 h and OD_{600} was measured every hour to evaluate if BSG extracts were able to protect cells from oxidative stress (Fig. 7.7). To our knowledge, this kind of approach has not been applied to BSG extracts and can provide new information of their mechanism of action in the protection of cells against oxidative stress.

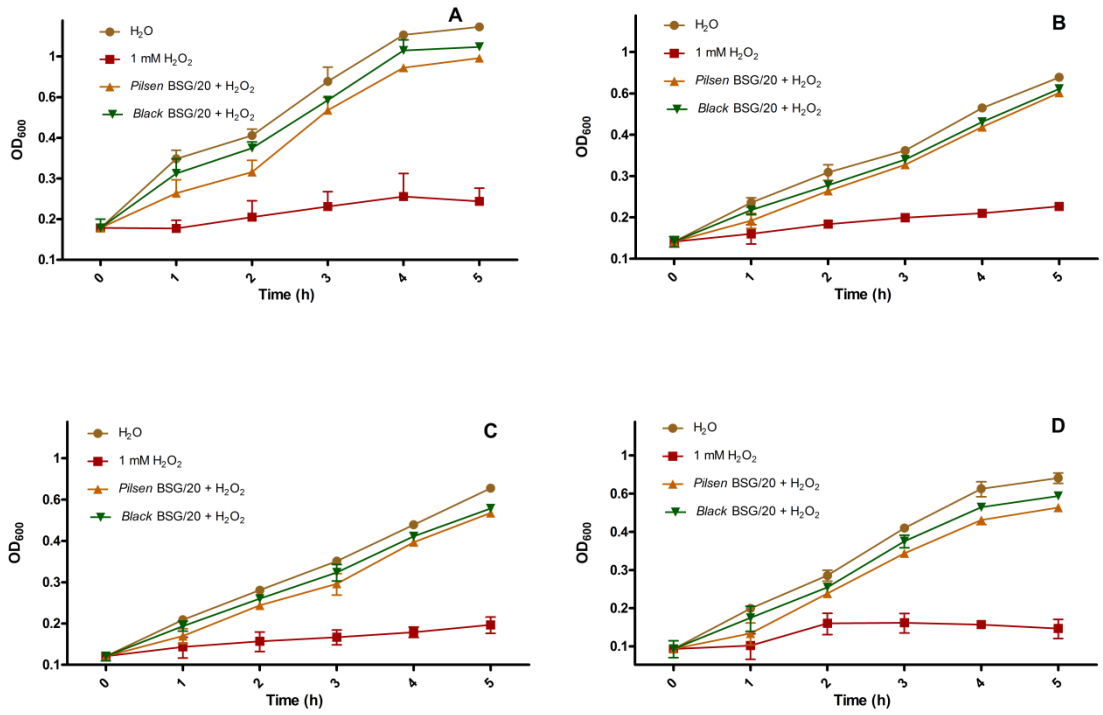

Fig. 7.7 - Treatment of mutant yeast cells with BSG extracts 20-fold diluted improved DNA damage repair mechanisms. Cells from mutant strains derived from strain BY4741 (A) affected in *APN1* (B), *RAD1* (C) or *YAP1* (D) were incubated simultaneously with 1 mM H_2O_2 and *pilsen* and *black* BSG extracts 20-fold diluted for 5 h. The negative control (H_2O) was prepared with cells without exposure to H_2O_2. Cell growth was monitored by OD at 600 nm every hour until 5 h. All results are the mean ± SD from three independent experiments.

As shown in Fig. 7.7, all yeast mutants and the parental strain displayed similar growth to the control when incubated with 1 mM H_2O_2 in the presence of *pilsen* and *black* BSG extracts 20-fold diluted. These results suggest that BSG extracts can protect cells even when NER, BER, or oxidative stress response mechanisms are affected. To our knowledge only one report described an involvement of BSG extracts in the protection against DNA damage, using human lymphotic U937 cells as experimental model [116]. In this work we used yeast cells to study the DNA repair induction promoted by BSG extracts. We have used *S. cerevisiae* as experimental model to take advantage of the simplicity of manipulation and of the availability of mutant strains.

Unlike the antioxidant activity of BSG extracts, the ability of BSG extracts to improve DNA repair mechanisms has never been investigated. Here we demonstrate that yeast cells can be used not only in the study of antioxidant activity of BSG extracts, but also that the use of genetically modified yeast strains can contribute to understand the underlying mechanisms of this activity. The results obtained with the yeast mutants

of BER, NER or oxidative stress response pathways are the first evidence linking BSG activity to specific cellular pathways, potentially providing important insights on its mechanism of action. Our results show that BSG extracts do not need of these cellular pathways to exhibit its protective effect. However, this possibility should not be excluded and it will be interest to test more mutant strains from these pathways and other BSG concentrations.

In conclusion, both extracts of BSG protected against the genotoxic effects of H_2O_2. *Black* BSG extracts exhibited the greatest protection against oxidant-induced DNA damage assessed by yeast comet assay. In addition, *pilsen* and *black* BSG extracts improved DNA repair rate following oxidative shock as measured by cell growth. This suggests that BSG extracts protect genomic DNA against oxidative stress and that the two modes of action are present, direct protection from oxidation and stimulation of DNA repair. According to these studies, BSG may have the potential to be developed as a food additive or dietary supplement with possible antigenotoxic health promoting properties.

III

Concluding Remarks and Perspectives

Concluding Remarks and Perspectives

Increasing efforts are being directed towards the reuse of agro-industrial by-products from both economic and environmental standpoints. The brewing industry generates relatively large amounts of by-products, being the most common spent grains, spent hops and yeast. However, as most of these are agricultural products, they can be readily recycled and reused. Despite all the possible applications that are described, its use is still limited to animal feed or simply in land fill. For this reason, the development of new techniques to use these agro-industrial by-products is of great interest. In the last few years great attention has been paid to the bioactive compounds, in particular to phenolic compounds, due to their ability to promote a number of benefits for human health. Therefore, researches have been intensified in order to find natural resources with antioxidant activity to effectively replace the synthetic antioxidants, which have been related to toxic and carcinogenic effects.

The ultimate purpose of this research work was to obtain extracts from the brewing by-products to be used as a new source of natural antioxidants. In this work it was clearly demonstrated that brewing industry by-products, namely spent grains, are rich sources of phenolic compounds. Hydroxycinnamic acids (caffeic, sinapic, *p*-coumaric and ferulic) were the main compounds contributing to the phenolic composition of that samples. Within the analysed by-products, BSG demonstrated to have the highest antioxidant activity and phenolic content. Furthermore, BSG is the main by-product generated by the brewing industry, and therefore their exploitation as a source of added-value products may be more cost-effective and merits a profounder investigation.

Conventional extraction techniques with organic solvents are the most common method used to recover natural antioxidants from plant materials. However, this type of extraction is time, energy and solvent consuming, generating higher quantities of wastes. In this work a new extraction process of phenolic compounds, and particularly of FA, from BSG based on microwave-assisted extraction was proposed. The developed new extraction methodology enabled us to obtain approximately 5-fold higher FA yield than other extraction methods (0.27 ± 0.02% for alkaline hydrolysis and 1.31 ± 0.04% for MAE). In addition, the MAE approach allows reducing processing time, solvent and energy consumption while generating fewer wastes, indicating that this is an effective alternative to polyphenols recovery from BSG. According to these

results, we suggest that MAE could be used to produce large quantities of BSG phenolic extracts by using available large-scale microwave extraction reactors, which are suitable for 10–100 kg of BSG per batch.

HPLC-DAD-MS analyses reveal that industrial BSG is also composed by several isomeric ferulate dehydrodimers and one dehydrotrimer. These results demonstrated that the BSG phenolic extracts obtained can be fractionated and purified by preparative and semi-preparative chromatography allowing the isolation of bioactive compounds that could be used as natural and inexpensive alternatives to synthetic antioxidants. Moreover, it was shown for the first time that MAE technology could be used in the future to obtain polyphenolic-enriched extracts for application in food, cosmetic and pharmaceutical industries.

As the type and origin of the raw-materials used in the brewing process can affect the content of phenolic compounds extracted, the influence of different malt types (*pilsen*, *melano*, *melano 80*, *carared*, *chocolate* and *black*) in the extracted phenolics was also investigated. The results obtained suggest that the type of malt used as well as the malting process, in particular the kilning regimes and roasting temperatures, may have important impact in terms of the phenolic composition and antioxidant features of BSG. Amongst the BSG studied, extracts from light types (malt KT ≤ 160 ºC) contained higher amounts of total and individual phenolic compounds, in particular *pilsen* BSG (20 ± 1 mgGAE/g dry BSG). This knowledge can surely be valuable for exploring new applications for different types of BSG. It is evident from the results here reported that BSG extracts, especially from *pilsen* malt, can be used as an inexpensive and good natural source of functional substances for human health and the food industry.

The antioxidant properties described for the BSG extracts can be associated with the antigenotoxic activity, determined by the ability of a sample to protect against the oxidative DNA damage in *Saccharomyces cerevisiae* cells, using the yeast comet assay. The results obtained show that the *black* BSG extracts exhibit a 5-fold reduction in the genotoxic effects of H_2O_2, compared to the 2-fold reduction by the *pilsen* BSG extracts. These results suggest that these protective effects from BSG can be attributed to the melanoidins compounds, mainly present in *black* malts, which possess reducing properties and can form iron chelates, reducing the DNA damage. Furthermore, it was observed that BSG extracts can activated the affected DNA repair mechanisms through interaction with the BER or NER pathways. These results indicate

that BSG extracts protects genomic DNA against oxidative stress and suggest that the two modes of action are present, direct protection from oxidation and stimulation of DNA repair mechanisms. Despite the good results obtained in this work, the antiproliferative and genotoxic effects of BSG extracts were performed in yeast cells. Therefore, the study of this extracts on tumor cell lines could be interesting due to the complexity of the mechanisms derived from the compounds present in the extracts.

Although there have been many recent advances to recycle the by-products, the recovery of antioxidants from BSG seems to be a very stimulant field of research. In fact, this BSG extracts, mainly rich in FA, which possess antioxidant properties, may have the potential to be used as food additives or dietary supplements with possible health promoting properties. In this perspective, this application for BSG could reduce treatment costs and even create additional profits to the companies, contributing to the sustainable development.

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