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FORMULATION AND SYNTHESIS OF MATERIALS WITH β-GLUCANS

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ABSTRACT

Nowadays, there is a tendency towards the use of natural products in industrial applications, especially in those related with the environment and human health. These renewable raw materials, usually non-toxic, are present in many structures in nature and therefore are able to mimic well their behavior and act in a more efficient way. Some of the most commonly studied products are polyphenols and polysaccharides, the first ones as active compounds mainly due to their anti-oxidant effect, and the seconds as biopolymers instead of some other processed polymers traditionally used in diverse applications.

In this thesis, the ability of β-glucans to be used in formulation of active compounds and in synthesis of materials was evaluated. β-glucans are polysaccharides composed by D-glucose units linked by glycosidic bonds. They are present in different sources in nature, such as cereals, bacteria or algae. Depending on their origin and bonding, they have very different properties regarding 3D-configuration or biological activity, among others. Unlike other polysaccharides like alginate or starch, the use of β-glucans in these fields is not widely studied. The final applications of the products developed with β-glucans were chosen by analysis of the properties of β-glucans and the advantages that they could provide over other biopolymers on those applications.

In chapters I and II, β-glucans were used as encapsulating materials of active compounds to be used as fungicides in agriculture. Since β-glucans are present in the cell wall of some fungi, a formulation containing β-glucans could improve the penetration of the active compound through the wall, while protecting it from thermal degradation and oxidation, and thus enhance its action against the fungus. Both liquid and solid formulations with β-glucans were developed in this thesis. If the product is kept liquid, the production steps are reduced, because there is no need of a drying process, and it can be applied easier in the plants. However, if the product is further dried, it is better preserved without risk of destabilization and it is easier stored. In all cases, the formulations were performed by supercritical fluids or

high-pressure processes in order to determine the differences in the final product compared to other conventional processes.

In chapters III and IV, β-glucans were used as polymeric matrix which acts as carrier of active compounds for drug delivery and also as scaffold for tissue regeneration. Different studies in literature report the biological activity of β-glucans, which comprises the ability to lower cholesterol or anti-inflammatory effect, but also the modulation of the immune system and the enhanced healing of wounds. Thus β-glucans were chosen for this application in order to add these properties to the produced materials.

Solid formulations with barley β-glucans were studied in **Chapter I**. Resveratrol was the selected active compound. It is a phytoalexin spontaneously produced by some plants as a defense mechanism when they are attacked by a fungal infection. Besides, soy lecithin was also used as encapsulating material, both alone and in combination with barley β-glucans, to assess the influence of the encapsulating material on the final product. First, an oil-in-water emulsion was formed by high-shear emulsification, and upon removal of the organic solvent (ethyl acetate), the suspension was dried by PGSS-drying or spray-drying. Also SAS was performed, but it was not a suitable process to produce dry particles of β-glucan from an organic solution (dimethyl sulfoxide), because great quantity of the organic solvent remained trapped into the β-glucan structure. Well-dried particles of barley β-glucans, lecithin and a mixture thereof containing resveratrol were obtained both by spray-drying and by PGSS-drying. The greatest difference between both drying processes was related to particle size, which was found to be dependent on the drying process rather than on the encapsulating material. Smaller particles were produced by PGSS-drying, although they formed bigger agglomerates (in the range of 10 μm by spray-drying and 100 μm by PGSS-drying). Encapsulation efficiency of resveratrol was high (60-96%) and similar between the drying-processes for each encapsulating material since it depends mostly on the emulsification

process, which was the same in all cases. According to XRD analysis, amorphous resveratrol was obtained in the final dry product due to the interaction of the active compound with the carriers. *Botrytis cinerea* was the fungus chosen for assessing the antifungal activity of the different formulations, because it is one of the fungi containing β-glucans on its cell wall. Previous works reported growth inhibition of *B. cinerea* at 100 ppm of pure resveratrol. This was not observed in our work, but fungal growth was reduced between 50 and 70% with all the formulations developed in this chapter.

Chapter II includes the development of liquid formulations of barley and yeast β-glucans. As in the previous chapter, soy lecithin was also utilized in this one for comparison, and resveratrol as active compound. For the liquid formulation, an oil-in-water emulsion was first developed, and the final suspension was produced by removal of the organic solvent by vacuum evaporation. Three different emulsification techniques were used: high-pressure, high-pressure and temperature and high-shear emulsification. Comparing the emulsification methods, particle size and encapsulation efficiency achieved in the final suspensions were similar for all of them (below 90 nm and between 70-100%, respectively). The encapsulating material affected also the final properties of the product. Nevertheless, the crystallinity of resveratrol depended both on the emulsification procedure and on the encapsulating material. Yeast β-glucans provided higher encapsulation efficiency than barley β-glucans, and also better inhibition of fungal growth of *B. cinerea* (50% for yeast β-glucans and 20% for barley β-glucans). The formulations with lecithin, both alone and in combination with β-glucans, did not reduced fungal growth, which might be a consequence of the presence of crystalline resveratrol in the final encapsulated particles. Therefore, it was concluded that the formulation of resveratrol with β-glucans improved the action against *B. cinerea*, probably through an enhanced absorption of the active compound by the fungus

In **Chapter III**, barley and yeast β-glucans aerogels were produced by supercritical drying with CO_2. First hydrogels were created with both β-glucans, and their rheological behavior was studied. It was determined that the ones produced with yeast β-glucans were more stable, resistant to shear stress and elastic than the ones with barley β-glucans because of their different structure and 3D-configuration in the gel. On the one hand, barley β-glucan is formed by linear chains of polymer that arrange parallel creating a more compact material. On the other hand, yeast β-glucan is a branched polymer which upon gelation creates a structure with more void space due to the crosslinking of the chains. Then, solvent was changed from water to ethanol, and ethanol was further extracted with supercritical CO_2. The noticed differences between both β-glucans in the hydrogels were also observed in the aerogels. Thus, yeast β-glucan aerogels had higher density, were stronger against compression stress and were able to absorb more water. Nevertheless, all the aerogels had similar morphology, with mean pore size between 13-16 nm and BET surface area around 180 m^2/g. Furthermore, supercritical impregnation of acetylsalicylic acid was also performed at the same time as the drying of the alcogels, at different temperature and pressure conditions. Up to 15% impregnation was achieved, with an increasing tendency with CO_2 density. Finally, release of the drug in PBS was analyzed. An initial delay for the first 3h of analysis was noticed, which was indicative of a good impregnation of acetylsalicylic acid into the β-glucan matrix by supercritical impregnation. After that time, 60% of the drug was released for the following 5h, and this value was maintained for 16h more. By analysis of the release profile, it was determined that the release was controlled by relaxation of polymer chains and swelling of the matrix.

The materials produced in chapter III were not porous enough to be used as scaffolds in tissue engineering. Therefore, **Chapter IV** explores the possibility of creating more porous materials with barley and yeast β-glucans by hydrogel foaming with supercritical CO_2. In this process, the hydrogels created with the β-glucans were subjected to high-pressure CO_2, that was dissolved in them in those conditions. Upon depressurization, supersaturation of CO_2 in the hydrogels

promoted the formation of pores inside. After complete water removal by freeze-drying, the structures produced were highly porous, achieving up to 80% porosity, mean pore size of 250 μm and 75% of interconnected pores. These parameters increased with foaming pressure, although at the highest pressure tested (20 MPa) the porosity of the resulting materials was non-homogeneously distributed, having an external crust of polymer while the inner part was mostly hollow. Despite the good porosity achieved, the scaffolds produced with both β-glucans were brittle and had low resistance to compression stress. Scaffolds were loaded also with dexamethasone, which is a compound extensively used because of its anti-inflammatory properties and its ability to induce differentiation of stem cells towards osteogenic linage. The scaffolds provided a controlled release until total dissolution up to 4 days, with higher release rate during the first 8h. Analysis of the mechanism governing the release revealed that it was mainly (above 75%) controlled by diffusion of the drug in the liquid medium, with slight contribution of relaxation of polymer chains.

Thus, in this thesis barley and yeast β-glucans were effectively used as encapsulating agents in antifungal products. Also, they were processed and studied as biomaterials for medical and pharmaceutical applications. In both cases, supercritical fluids were utilized as green technology to achieve the final products.

INTRODUCTION

β-glucans are polysaccharides which are present in several bacteria, fungi, algae and some cereals, including barley and oat. They consist on D-glucose monomers linked by glycosidic bonds. β-glucans are named after the type of bonds they have. They are basically formed by a chain of D-glucose molecules bond in positions (1,3), and usually, they present branches at positions (1,4) or (1,6). Thus, β-glucans present different properties depending on their origin, branching, 3D configuration or molecular weight. For instance, the branching affects the biological activity of the β-glucans, so that the unbranched β-glucans do not present any activity, whereas the branched ones exhibit some activity (Bohn and BeMiller, 1995). Also, β-glucans with very low MW do not show biological activity (Zeković et al., 2005).

Some of the most commonly used β-glucans are: **curdlan**, a linear (1,3)-β-glucan from bacteria; **laminarin**, a side-branched (1,3;1,6)-β-glucan from the brown algae *Laminaria* spp.; another side-branched (1,3;1,6)-β-glucan, **lentinan**, from *Lentinus edodes*, also known as Shiitake mushroom; and **pleuran**, an insoluble (1,3;1,6)-β-glucan isolated from the fungi *Pleurotus ostreatus*. Cereals like **barley, oat and wheat** contain linear (1,3;1,4)-β-glucan. **Fungal and yeast cell walls** (e.g., from *Saccharomyces cerevisiae*, *Candida albicans* or *Aspergillus fumigatus*) are also composed by (1,3;1,4)-β-glucan, but they have as well (1,3;1,6)-β-glucan.

In general, β-glucans are a minor component in the sources where they are present. For instance, β-glucans account for 3-7% in barley, being located in the cell walls of the endosperm (Irakli et al., 2004). Therefore, prior to their use, they must be first extracted and purified in order to achieve a product with higher quantity of β-glucans. The process for the obtention of β-glucans contains 3 main steps: pretreatment, extraction and purification (Zhu et al., 2016). Although optional, the pretreatment is an important step to prepare the feedstock and enhance the yield of the following extraction process. It might comprise from milling to removal of impurities, depending on the physical state and composition of the raw material. The extraction of β-glucans from the natural matrix is commonly performed in hot water. Nevertheless,

depending in the structure of the β-glucans and on the characteristics of the matrix, sometimes it requires other type of procedures, such as alkaline or acidic extraction. Recently, many works explore the use of other techniques to enhance the mass transfer limitations and thus improve the extraction yield, such as ultrasounds (Benito-Román et al., 2013) or microwaves (Du et al., 2014). Finally, the extracted β-glucans have to be purified from other by-products that have been extracted at the same time (mainly some other biomacromolecules such as polysaccharides or proteins) and have to be precipitated. Most of the times, a last drying step is required in order to get a solid product because in this way β-glucans are better preserved than in liquid phase, where they can easily develop bacterial or fungal growth. A detailed revision of extraction methods for cereal and yeast β-glucan can be found in (Benito-Román et al., 2011) and (Varelas et al., 2016), respectively.

APPLICATIONS

Due to their aforementioned origin, β-glucans can be obtained from renewable natural sources, thus avoiding the risk of depletion if they are excessively used in industrial applications. Besides, they possess some other features, as detailed next, that make them good candidates to be utilized directly in the environment or in humans and animals, i.e. in food, agriculture or pharmaceutics. For instance, different studies concluded that they are antioxidant, reduce obesity and cholesterol and activate the immune system (Roupas et al., 2012). The European Food Safety Authority has recognized the ability of oat (OBG) and barley β-glucans (BBG) to lower blood cholesterol (Efsa Panel on Dietetic Products and Allergies, 2010, 2011). β-glucans also can be used as encapsulating material for active compounds, as they trap them into their structure, protecting them from degradation and changing their rate of absorption. Different applications of β-glucans related to these topics are summarized in the following paragraphs.

BIOMEDICAL APPLICATIONS

In the last years, biopolymers are being studied in medical and pharmaceutical applications both as drug delivery vehicles and as scaffolds for tissue regeneration. The aim is to use compounds that are present in the human body, especially polysaccharides and proteins, to achieve certain signaling and trigger natural-occurring reactions in the body (Malafaya et al., 2007).

β-glucans have some well-known biological properties. (1,3;1,6)-β-glucans from fungi and yeast are able to modulate the immune system in order to induce a reaction against a pathogen, rather than attacking it themselves (Bohn and BeMiller, 1995; Zeković et al., 2005). In the same way, β-glucans from mushrooms also induce a cytotoxic effect against cancer-related cells and at the same time promotes the proliferation of normal cells (Roupas et al., 2012). Besides, some works report the ability of several types of β-glucans (from cereals, mushrooms and yeast) to lower blood glucose and cholesterol, although the European Food Safety Authority has also recognize it for BBG and OBG. Thus, by using β-glucans as carriers or scaffolds, the final product may benefit from these properties.

The presence of β-glucans in formulations for oral drug delivery improves the protection of the active compound through gastric fluids and its absorption in the intestine. Ovo- and bovine serum albumin encapsulated on yeast β-glucan (YBG) were prepared by lyophilization, achieving particle size around 3-5 μm (De Smet et al., 2013). The particles were efficiently absorbed by intestinal epithelial cell lines, thus confirming the efficiency of YBG as carrier for oral drug delivery. Besides, the particles were non-toxic in the tested cell lines. Also BBG was not cytotoxic in *in vivo* trials of oral drug delivery in rats (Jonker et al., 2010). Bacterial probiotics cells were encapsulated in BBG together with Tween 80 and polyethylene glycol, with average particles size of 14-16 μm (Shah et al., 2016). Encapsulation efficiencies higher than 75% were achieved, and the encapsulated cells showed higher resistance to simulated gastric acidic fluids. Oral

administration of OBG by rats produced an increase of antioxidant activity in liver and stomach and suggested their use as protective agents for these organs (Suchecka et al., 2016).

Silver nanoparticles were synthesized with mushroom β-glucan, which acted both as reducing and encapsulating agent. The particles showed good antibacterial activity at hemocompatible doses (Sen et al., 2013). Veverka and coworkers reported the complexation of β-glucans with different nutraceuticals (some hydrophilic compounds, such as folic acid, and some hydrophobic, such as curcumin) through hydrophilic (hydrogen bonding) and hydrophobic, non-covalent bonding, depending on the groups of the nutraceuticals (Veverka et al., 2014). This strong interaction prevented degradation of the compounds. BBG with different molecular weight were used as encapsulating material for proteins (Lazaridou et al., 2015). They were successfully prepared by repeated freeze–thaw cycles, and presented a sustained release of the proteins in water. The release rate decreased with increasing molecular weight of β-glucans.

Some studies report the synergistic effect of β-glucans when they are mixed with polyphenols. The mixture of YBG and resveratrol produced bigger modifications in the immune system than the ones observed when using the individual components: increment of phagocytosis, higher synthesis and release of cytokines (proteins related with development and regulation of the immune system and cell signaling) and elevated antibody response (Vetvicka and Vancikova, 2010). Although YBG present anti-tumor activity, the combination of YBG with resveratrol caused a higher reduction of tumor growth and metastases (Vetvicka and Vetvickova, 2012). This reduction was even bigger upon addition of vitamin C to the mixture.

Processing of β-glucans with other polyphenols has also been explored, since polyphenols possess some interesting biological activities to be incorporated in pharmaceutical, cosmetic or food products. Anthocyanins from black currant extract were encapsulated in BBG hydrogels, both in cubes and in beads, and the materials were dried by different methods, namely in a normal and vacuum oven, freeze-drying or infrared drying (Xiong et al., 2006). Formulation of

quercetin with BBG by emulsification-evaporation led to the precipitation of crystals of quercetin. Indicating that BBG was not a good emulsifier for quercetin (Gonçalves et al., 2015). However, when lecithin was also added to the water-phase, the recrystallization of encapsulated quercetin in the final particles was inhibited.

Some patents describe the use of β-(1-3)-glucan nanoparticles in anti-aging cosmetics (Mihranyan et al., 2012; Shin et al., 2001). Also fungal β-glucans were studied as active compound instead of encapsulating agent by ultrasound assisted emulsification due to their anti-oxidant properties for cosmetic and therapeutic applications of skin (Alzorqi et al., 2016). The use of fungal glucans in formulations to enhance skin permeability is reported as well in some patents (Klein, 1999). OBG is reported to improve the penetration of active compounds into the stratum corneum through deeper layers of the skin (Röding, 2006).

The use of YBG in wound healing has been explored. YBG resulted in an effective treatment for burn injuries in *in vivo* tests with rats by oral ingestion (Firat et al., 2013). They also improved the healing of injuries in mice when processed as wound dressing sheet together with chitosan (Kofuji et al., 2010). In this case, the sheets were applied over the injury and produced faster healing than a commercial chitosan wound dressing. Wound repair was dependent on the release of chitosan and β-glucans from the material, which was governed by the biodegradation rate. Thus, after complete application, the dressing would be totally degraded and absorbed by the body, leaving no residues. In membranes of poly(lactic-co-glycolic acid), an enhanced cell adhesion and proliferation was observed when YBG were added (Kim et al., 2012). Due to this, the membranes provided better interaction and integration with the surrounding tissue, which reflected in an improved healing of wounds. β-glucans from barley and fungi were also evaluated for wound healing applications, although they were not as effective as the ones from yeast (Vetvicka and Vetvickova, 2011).

Comin and coworkers investigated the differences of the drying process (sc-drying, freeze-drying or conventional air-drying) on the final properties of BBG gels (Comin et al., 2012a). Supercritical-dried aerogels had lower density than air-dried materials, and were more homogeneous than the materials produced by freeze-drying. They also tested supercritical impregnation of flax oil into the aerogels, as a model nutraceutical compound, and achieved up to 65% encapsulation efficiency (Comin et al., 2012b).

Up to the author's knowledge, β-glucans have never been used alone as polymeric matrix for the fabrication of scaffolds. However, some previous works explore how the addition of β-glucans influences the properties of scaffolds. Przekora and coworkers found out that the introduction of curdlan into a hydroxyapatite scaffold reduced its mechanical properties, but it also increased its elasticity and water uptake capacity, thus enhancing the adaptation of the scaffold into the implant zone (Przekora et al., 2016). Also, the presence of β-glucan in a chitosan-hydroxyapatite composite promoted cell adhesion and growth (Belcarz et al., 2013).

FOOD

OBG was used as fat replacer, and it was reported to increase moisture retention and soften the meat (Álvarez and Barbut, 2013). Barley and oat suspensions containing between 4 and 45% β-glucans were processed by spray-drying and freeze-drying, in an attempt to produce cereal powders that could act as substitutes of traditional milk powder (Chronakis et al., 2004). By freeze-drying, the morphology of the final particles was independent on β-glucan concentration, whereas this parameter did influence the morphology by spray-drying. Also some works studied the influence of adding OBG or BBG to increase the dietary fiber content of doughs and breads in their final properties (Ahmed, 2015; Ronda et al., 2015).

One of the main applications of β-glucans in food is as thickeners or stabilizers, due to their high viscosity in solutions (Lazaridou et al., 2003; Limberger-Bayer et al., 2014; Liu et al., 2016). The addition of OBG and BBG increased the stability of egg-yolk emulsions through an increase of

viscosity of the continuous phase which slowed down the creaming process (Kontogiorgos et al., 2004; Shah et al., 2016). Structure and molecular weight of the β-glucans played a determinant role in the process. BBG contributed to the stabilization of whey protein concentrate emulsions by the increase of viscosity (Burkus and Temelli, 2000). They also observed a decrease in droplet size and reduction of phase separation.

Due to their biological properties, YBG are also used for animal feeding. Some trials about including YBG in animal feeding were conducted and it was observed that they induced the activation of the immune system in some fish, like salmons (Kiron et al., 2016) and trout (Siwicki et al., 2004). Also, chickens fed with YBG showed higher resistance against bacteria (Shao et al., 2016; Tian et al., 2016).

AGRICULTURAL APPLICATIONS

Formulations of new fungicide products obtained from natural, non-toxic and renewable sources are a good alternative to avoid toxic residues in the fruits or in the environment where they are applied. These formulations should aim to reduce the use of products to effectively control the fungus in order to develop agricultural production systems which are sustainable both from an economical and an environmental point of view.

Laminarin and its derivatives and (1,3;1,6)-β-glucans act as elicitors in plant defense, mainly by triggering the production of phytoalexins (Fesel and Zuccaro, 2016). In this sense, laminarin is commercialized as an innovative plant protection product against fungi and bacteria (Goëmar, 2016). The protection is due to the combination of 2 different effects: a strength of cell walls of the plants and the production of phytoalexins and defense proteins that attack the pathogen. It was also reported that laminarin had elicitor effect for protection against pathogens in tobacco plants (Klarzynski et al., 2000). Laminarin was further used to reduce the oxidative stress of biocontrol yeast and thus enhance its action against *Cryptococcus laurentii* on pears (Zeng et al.,

2015). Fungal β-glucan was processed as nanoparticles and applied to control rhizome rot disease caused by *Pythium aphanidermatum* (Anusuya and Sathiyabama, 2015).

THESIS OUTLOOK

As shown in the previous section, owing to their interesting properties, β-glucans can be applied in many different fields. Nevertheless, some aspects which have been barely explored are addressed in this thesis. BBG and YBG (Figure 1) are used in order to assess the differences in the final products derived from those of the type of β-glucan.

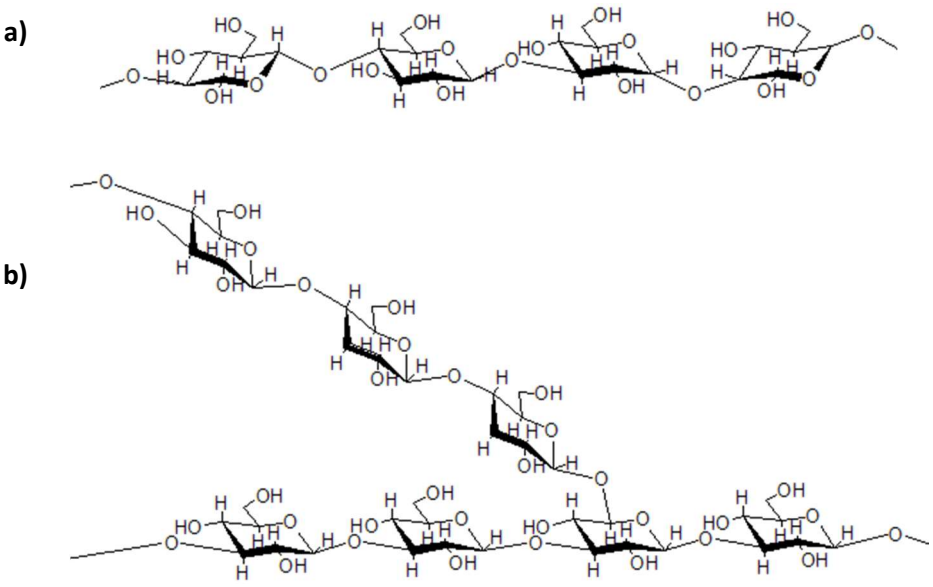

Figure 1. Structure of BBG (a) and YBG (b).

The first half of this thesis studies the use of β-glucans in formulations of antifungal products. As it was mentioned before, (1,3;1,6)-β-glucans are present in the cell wall of yeast and fungi, acting as support for the content of the cell and being responsible of the rigidity of the wall. In an attempt to increase the effectiveness of the product and thus reduce the dose required to achieve good control of the infection, we hypothesize that a formulation containing β-glucans could improve the action of the active compound. The fungus would recognize the antifungal

product as its own compound due to the presence of the β-glucans and thus the active compound would penetrate better through the wall and act more efficiently.

The second half of the thesis contents the processing of β-glucans by several techniques in order to produce materials with different properties focusing on their application as carriers for drug delivery or as scaffold for tissue engineering. These applications require some specific characteristics regarding porous and mechanical properties and release of active compounds, among others. Although the biological activity of β-glucans has been widely demonstrated, they have been scarcely used as drug delivery vehicles and scaffolds, and mostly in combination with other compounds and not as individual components. Hence, their potential for processing and application in this field is studied in this thesis.

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AIMS

The aim of this work is to explore the possibility of using different β-glucans in diverse applications and to assess the influence of the type of β-glucan on the final products. Barley and yeast β-glucans were chosen as raw material due to their different structure, behavior in solution and biological properties. Moreover, high-pressure processes were performed and compared to conventional processes.

With this main goal, this thesis comprises two partial objectives:

- Development of β-glucan formulations for new antifungal products based on natural products. Application to the encapsulation of resveratrol, as phenolic fungicide.
    - Solid formulation by spray-drying, PGSS-drying and supercritical anti-solvent.
    - Aqueous formulation by high-shear, high-pressure and high-pressure and temperature emulsification techniques.
    - Characterization of the aforementioned formulations regarding particle size, resveratrol crystallinity and encapsulation efficiency. Determination of the effect of each processing method and type of β-glucan used on the final product.
    - Evaluation of the antifungal activity of each of the formulations against *B. cinerea*.
- Synthesis of β-glucan-based materials for drug delivery and tissue engineering.
    - Production and rheological characterization of hydrogels.
    - Supercritical drying of hydrogels to produce aerogels.
    - Evaluation of the influence of type of β-glucan and concentration on the morphology, porosity, mechanical properties, degradation and water uptake capability of the aerogels.
    - Loading of the aerogels with a model anti-inflammatory drug (acetylsalicylic acid) by supercritical impregnation in order to study the influence of pressure

and temperature on impregnation yield and the release of the drug from the matrices.

- Hydrogel foaming to achieve highly porous structures and characterization of their porous and mechanical properties. Freeze-drying of the non-foamed hydrogels was also performed for comparative purposes.
- Loading of a model active compound for osteogenic differentiation and anti-inflammatory properties (dexamethasone) in the scaffolds processed by hydrogel foaming to carry out the study of the release of the drug and determine the governing mechanism.

CHAPTER I:

ENCAPSULATION OF RESVERATROL ON LECITHIN AND β-GLUCANS TO ENHANCE ITS ACTION AGAINST *BOTRYTIS CINEREA*

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ABSTRACT

β-glucans and soy lecithin were used as encapsulating materials for resveratrol and tebuconazole, in order to test their antifungal activity against *Botrytis cinerea*. First an oil-in-water emulsion was formed and afterwards the emulsion was dried by spray-drying or by particles from gas saturated solutions-drying (PGSS-drying). β-glucans were precipitated also by supercritical anti-solvent (SAS), but it was not a suitable drying process for this material. Particles were characterized regarding particle size, morphology, crystallinity, encapsulation efficiency and *in vitro* activity against *Botrytis cinerea*. Although the emulsions with β-glucans had bigger droplet size than the ones with lecithin, there was no difference on particle size for each encapsulating material, it was just dependent on the drying process. For all the materials and drying processes, completely amorphous particles were obtained, in spite of the crystalline form of the pure active compounds. Concerning the antifungal activity, the encapsulation of tebuconazole did not improve its action, because it was already very effective. Pristine resveratrol did not reduce fungal growth, but it was inhibited between 50-70% with encapsulated resveratrol, which implies the production of an effective fungicide against *Botrytis cinerea* totally from natural origin substances.

Keywords: resveratrol, β-glucan, lecithin, spray-drying, PGSS-drying, fungicide.

1. INTRODUCTION

Botrytis cinerea is a pathogenic fungus causing gray mold, which affects several fruits and plants all over the world. Once a product is attacked by *Botrytis cinerea*, it cannot be recovered, so it produces great economic losses (Williamson et al., 2007). Also, one of the most important problems when handling with *Botrytis cinerea* is that it infects the plants during the blooming, but it is not noticeable until the ripening (Timperio et al., 2012). Commonly, chemicals are used to fight against this disease, for instance azoles (Stehmann and de Waard, 1996), anilinopyrimidines, phenylpyrroles or hydroxyanilides (Rosslenbroich and Stuebler, 2000). However, resistant strains are developed quickly by the fungus (Elad et al., 1995; Pappas, 1997). Furthermore, as the treatment against *Botrytis cinerea* must be applied in full-grown products, or even post-harvest, there is a tendency towards replacing the use of toxic substances by natural, environmentally friendly products (Ali et al., 2015).

When a plant is attacked by a pathogen, it develops a chemical response which involves the production of some proteins, phytoalexins and other phenolic compounds (Langcake and Pryce, 1976; Montero et al., 2003; Timperio et al., 2012) that show antifungal activity (Mendoza et al., 2013). The main phytoalexin produced is resveratrol, which is found in high quantities on grape skin (Casas et al., 2010; Zheng et al., 2011). Further, it has been found to be active against different fungi (Aldred et al., 2008; Santos et al., 2006), including *Botrytis cinerea* (Adrian et al., 1997). It was observed that, when resveratrol is produced spontaneously to fight against fungi, it is present within the cell wall of the plant to be in contact with the pathogen (Adrian and Jeandet, 2012), or it is even excreted out from the cell in liquid culture (Donnez et al., 2011) to improve the contact between resveratrol and the fungus. Based on this findings, resveratrol has been selected as promising antifungal of natural origin. Nevertheless, when applied externally in the plant to fight *Botrytis cinerea*, resveratrol should be encapsulated in a carrier that reaches easily the cell wall of the fungi and improves its penetration.

Encapsulation of active substances into polymeric matrices provides a controlled release of the compound, since the encapsulating material can act as a carrier for the active compound into the target, as well as protection against thermal or oxidative degradation. Recently, some works focused on the treatment of *Botrytis cinerea* with chitosan (Badawy and Rabea, 2009; Wu et al., 2005; Xu et al., 2007), which is one of the components of the cell wall of the fungus. Furthermore, the encapsulation of essential oil into chitosan improved its antifungal action against *Botrytis cinerea* (Mohammadi et al., 2015). Taking into account that also β-glucans are present on the cell wall of *Botrytis cinerea* (Tenberge, 2007), this polysaccharide is going to be use as carrier in the present work. β-glucans are composed by D-glucose monomers linked by glycosidic bonds. Depending on the bonds they have, they present different structure and branching, which provides them their properties, such as solubility or biological activity (Bae et al., 2013). To the authors' knowledge, there is just one previous study using β-glucans against *Botrytis cinerea* (Aziz et al., 2003). In this work, linear β-1,3-glucans from seaweeds were used as antifungal compound, and they reduced the growth of the fungus by 50% in 4 days with a concentration of 1 g/L. However, β-glucans have never been used as encapsulating material, although some studies have demonstrated that, when they are added to an emulsion, they increase its stability (Burkus and Temelli, 2000; Kontogiorgos et al., 2004).

Encapsulation processes based on supercritical fluids techniques have attracted great interest in the last years, because they allow better control of particle size and morphology and reduce the contamination of the product by working in an inert atmosphere or by an improved removal of the organic solvents (Martín and Cocero, 2008). Among the micronization processes with supercritical fluids, particles from gas saturated solutions-drying (PGSS-drying) and supercritical anti-solvent (SAS) are performed in this work.

PGSS-drying is a supercritical fluid process suitable for the production of particles, in which the suspension is saturated with CO_2 at high pressure and temperature prior to the atomization. The sudden decompression of the fluid in the nozzle promotes the desorption of the CO_2 from the

droplets, breaking them and enhancing the drying process, so it can be performed at lower temperature than with conventional spray-drying (SD), which prevents the product from thermal degradation (Rodríguez-Rojo et al., 2013). Also, all the process is a closed system inerted with CO_2, so it is appropriate for drying substances that are oxidized easily (Martín and Weidner, 2010). Few works used this technology for the production of particles. They were mainly focused on the encapsulation of antioxidants and essential oils (de Paz et al., 2012; Meterc et al., 2008; Varona et al., 2013).

Alternatively, in SAS, the encapsulating material and the active compound are dissolved in an organic solvent, and the liquid is introduced through a nozzle in a high pressure vessel containing CO_2 in supercritical conditions. At these conditions, CO_2 is highly soluble on organic solvents. When the droplets of the liquid stream are put in contact with the supercritical CO_2, the organic solvent is saturated with the CO_2, so the solubility of the solute decreases and this promotes its precipitation (Rodríguez-Rojo et al., 2013).

The aim of this work is to develop the formulation of a product against *Botrytis cinerea* based entirely on natural substances, namely resveratrol, β-glucans and soy lecithin. Soy lecithin has been used as comparison for the β-glucans because it is has been demonstrated that encapsulation essential oils in lecithin can be improved their bactericidal action since it can form liposomes in aqueous media and interact with cells (Varona et al., 2013). Besides, it is biocompatible and non-toxic, and thus it is commonly used in formulation of emulsions in applications related with food processing (de Paz et al., 2012). First, an oil-in-water emulsion of resveratrol on β-glucan, lecithin or a mixture of both substances is produced and afterwards it is dried by conventional SD or by PGSS-drying. Further, tebuconazole, a triazole commonly used in plant protection (Yang et al., 2014), is used to compare the action of resveratrol. Besides, β-glucan particles are also obtained by precipitation by SAS. The particles formed by all these methods are characterized and tested *in vitro* against *Botrytis cinerea*.

2. MATERIALS AND METHODS

2.1. Materials

As encapsulating materials, barley (1-3, 1-4)-β-glucans (75% purity; Glucagel, kindly supplied by DKSH, France) and soybean lecithin (Glama-sot, SOTYA S.A., Madrid, Spain) were used. A molecular weight of 125 kDa was determined by size exclusion chromatography at the conditions indicated on Section 2.3.

Ethyl acetate (99%) and malt extract agar were purchased from Panreac (Spain), and dimethylsulfoxide (DMSO) from Sigma-Aldrich (Spain). Resveratrol with 98% purity was purchased from Pure Bulk (USA). Tebuconazole was kindly supplied by Aragonesas Agro (Spain), both as pure powder (technical grade) and the commercial product Orius 20EW (an oil-in-water emulsion with 20% w/v of tebuconazole).

2.2. Formation of particles

First, an oil-in-water emulsion was formed (IKA Labor Pilot), with the fungicide (tebuconazole or resveratrol) dissolved in ethyl acetate (7.5 g/L) as organic phase, whereas the aqueous phase, saturated with ethyl acetate, contained the different matrixes used (15 g/L): β-glucans, soybean lecithin or a mixture of both of them (50% in weight). Both liquid phases were mixed in a ratio 1:9 at 750 rpm for 5 minutes and then the resulted emulsion was fed into a rotor-stator machine (IKA® LABOR PILOT 2000/4), with 200 mL capacity, and processed at 4200 rpm for 4 minutes. These operating conditions were selected based on some previous works (Varona et al., 2009), where it was found a reduction in droplet size with increasing surfactant concentration, homogenization velocity and time. Afterwards, the organic solvent was removed by vacuum evaporation (Heidolph) at 60º C, 75 rpm and a vacuum of 0.08 MPa, and finally the suspensions were dried either by SD or by PGSS-drying.

By SD (Gea Niro Mobile Minor), the suspension (1 L/h) was introduced into the drying chamber through a rotary atomizer (compressed air at 0.6 MPa). Droplets were formed and water was

removed from them by a stream of hot air (130º C at the inlet and 85º C at the outlet). The dry particles were recovered in a cyclone.

By PGSS-drying the suspension was pumped and put into contact with 10 kg/h of preheated and pressurized CO_2 (Milton Roy membrane pump) at 9.5 MPa and 125º C in a 150 mm static mixer filled with 4 mm glass beads, so that the liquid was saturated with CO_2. Gas to liquid ratio was set at 30 (weight basis). Then this stream was expanded through a nozzle (Spraying Systems, 500 µm) in the drying chamber, which was kept at 65-70º C. The conditions selected for the drying process were based on a previous work (Varona et al., 2011), which contains further details about the experimental device. Figure 1 shows a simplified diagram of the formation of particles by SD and PGSS-drying, with the previous creation of the emulsion and suspension.

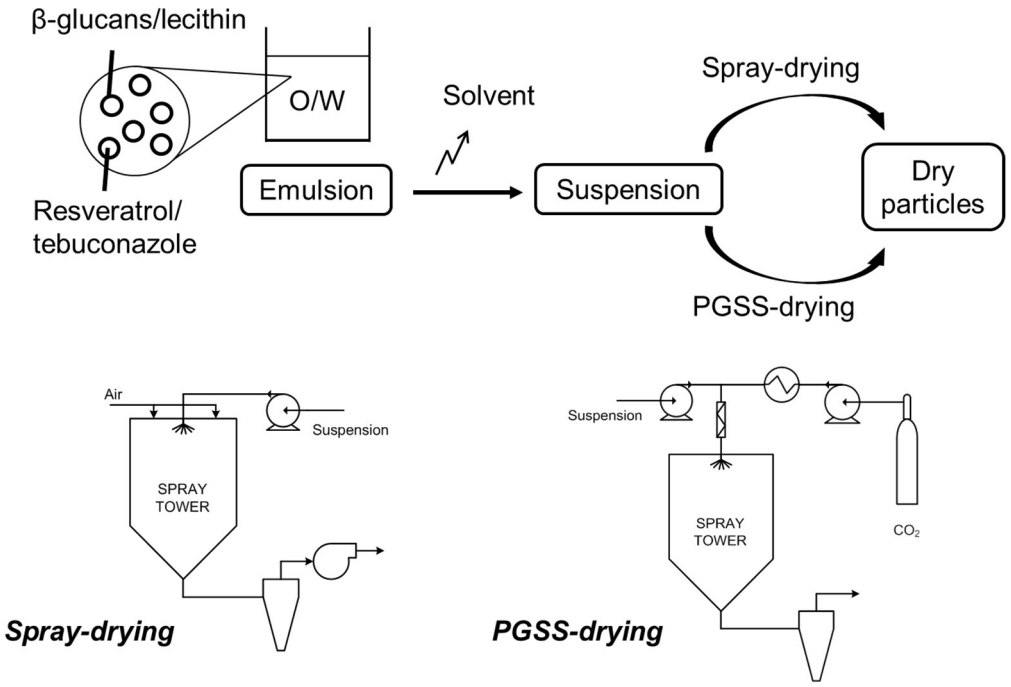

Figure 1. Formation of particles by SD and PGSS-drying.

For the production of β-glucan particles by SAS, DMSO containing β-glucans in a concentration of 2-10 g/L was pumped (HPLC pump, Gilson, model 850) and introduced in a vessel with CO_2. Preliminary tests at 20º C showed limited solubility of β-glucans in ethyl acetate, acetone, ethanol and tetrahydrofuran (lower than 2.6 g/L for every solvent), and higher in DMSO (14.8

g/L), so it was the solvent selected. Prior to introduce the solution, the vessel must be conditioned just with CO_2 until the required pressure and temperature are achieved. Pressure was always set at 10-11 MPa to avoid the formation of a liquid phase in the precipitation vessel. After all the suspension was pumped, CO_2 was still passed through the vessel in order to make sure that there was no remaining DMSO inside. Then, the system was decompressed and the particles formed were recovered from a filter placed at the outlet of the vessel. A scheme of the process is shown in Figure 2.

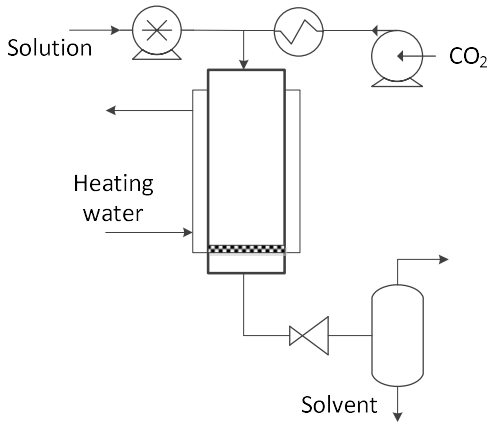

Figure 2. SAS process.

2.3. Molecular weight of β-glucans

Molecular weight of the β-glucans was measured by size exclusion chromatography, with a guard column (Waters Ultrahydrogel Guard Column), a column (Ultrahydrogel 500, Waters Corporation) and a differential refractive index detector (410, Waters Corporation). The column was kept at 35º C and flow rate of the mobile phase (0.1 M $NaNO_3$ and 0.02% NaN_3) was set at 0.4 mL/min.

2.4. Morphology of the particles

The morphology of the particles was analyzed by scanning electron microscopy (SEM, JEOL JSM-820, 20 kV, 23-mm working distance) at vacuum conditions. Prior to the analysis, the samples were covered with gold in an argon atmosphere.

2.5. Particle size distribution

Particle size distribution of the particles and emulsions produced was measured by laser diffraction (Mastersizer 2000, Malvern). In order to perform the measurement, the emulsions were suspended on a solution of water saturated with ethyl acetate, whereas the suspensions were suspended only in water. On the contrary, the dried particles were measured within a stream of air at 0.2 MPa.

2.6. DSC

Differential scanning calorimetry (DSC) profiles of the particles produced by SAS were obtained using a Mettler Toledo 822e calorimeter. Heating rate was 10º C/min in the temperature range of -40 to 280º C. An empty pan was used as reference. The equipment was calibrated with indium.

2.7. X-ray diffraction

X-ray diffraction (XRD) measurements were performed on a Bruker Discover D8 diffractometer to check the crystallinity of the obtained particles, using the Cu Kα radiation (λ = 0.15406 nm). The scattering intensities were measured over an angular range of $5 < 2\theta < 70$ for all the samples, with a step size of 0.02º.

2.8. Encapsulation efficiency

Quantification of tebuconazole and resveratrol in the final particles was done by HPLC, with a guard column (Bio-Sil C18, Bio-Rad), a column (Symmetry C18, Waters) and a UV detector (at 224 nm for tebuconazole and 306 nm for resveratrol). The column was kept at 40º C or 25º C for tebuconazole and resveratrol, respectively, and flow rate of the mobile phase was set at 1 mL/min for tebuconazole (acetonitrile and water) and 0.8 mL/min for resveratrol (acetonitrile and water with formic acid). For each sample, 3 independent measurements were made.

2.9. Botrytis cinerea culture

For the in-vitro culture of *Botrytis cinerea*, the fungus was isolated from vines in our university (Campus La Yutera, Universidad de Valladolid, Palencia, Spain) and it was grown on malt extract

agar by two different procedures. In the first one, the fungus was placed on the surface of the solid agar, and the fungicide was dissolved in water with 4% v/v ethanol (96%, Panreac, Spain) and applied to the fungi. The second method consisted on pouring the agar in the dishes containing the particles, so once the agar solidified, the particles were entrapped on it. In all cases, 5 replicates were grown for every sample and the growth area was calculated by measuring the diameter in two directions after incubation at 22º C for one week. Once measured, the average value and standard deviation were evaluated, and Student's t-test was performed (unpaired samples, unequal variances) in order to check whether there were significant differences between each sample, with a significance p-value of 0.05.

3. RESULTS AND DISCUSION

3.1. Formation of particles

Prior to the drying process, droplet size distribution of the emulsions and suspensions formed was analyzed (Figure 3).

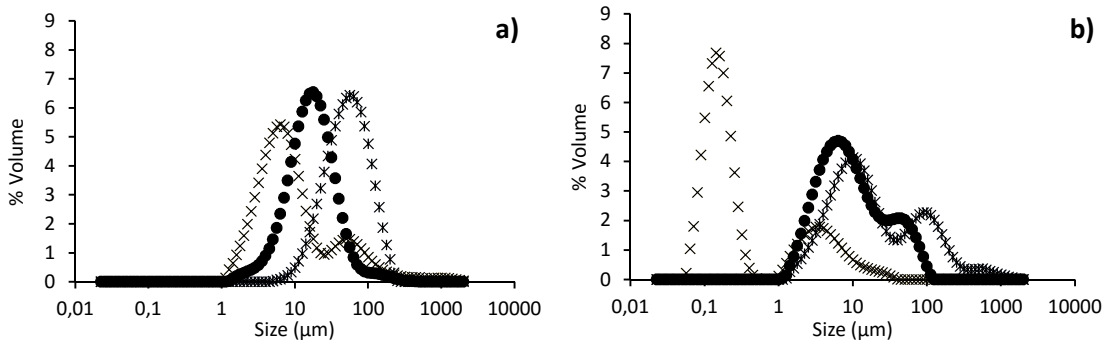

Figure 3. Droplet size distribution of the emulsions (a) and suspensions (b) of resveratrol with different encapsulating material. X: lecithin; ⁑: β-glucan; ●: lecithin and β-glucan.

All of the emulsions (Figure 3 (a)) showed unimodal distribution, although a smaller second peak towards bigger particle size is noticed in the case of the particles of lecithin. Lecithin droplets were the smallest ones (d(0.5) = 7.4 ± 0.9 µm). On the contrary, the emulsions created with β-glucans had the biggest droplets (d(0.5) = 52 ± 8 µm). The emulsions formed with a mixture of both substances had an intermediate value of droplet size between those (d(0.5) = 15 ± 5 µm).

The formation of bigger droplets with β-glucans is explained by the observation of other authors (Burkus and Temelli, 2000; Kontogiorgos et al., 2004) that they form a network structure when used as stabilizers of emulsions. Similarly, after removing the organic solvent, the same trend in particle size is observed for the suspension: smaller sizes for lecithin and bigger ones for β-glucans formulations (Figure***Figure 3*** 3 (b)), although particle size is reduced and particle size distribution is broader in all cases.

Then, dry particles were obtained from the suspensions both by SD and by PGSS-drying. All of the samples had moisture content below 6% (water evaporation estimated by weight difference after 24 hours at 105º C; results not shown here). Figure 4 contains SEM images for β-glucan particles obtained by SD (a) and by PGSS-drying (b).

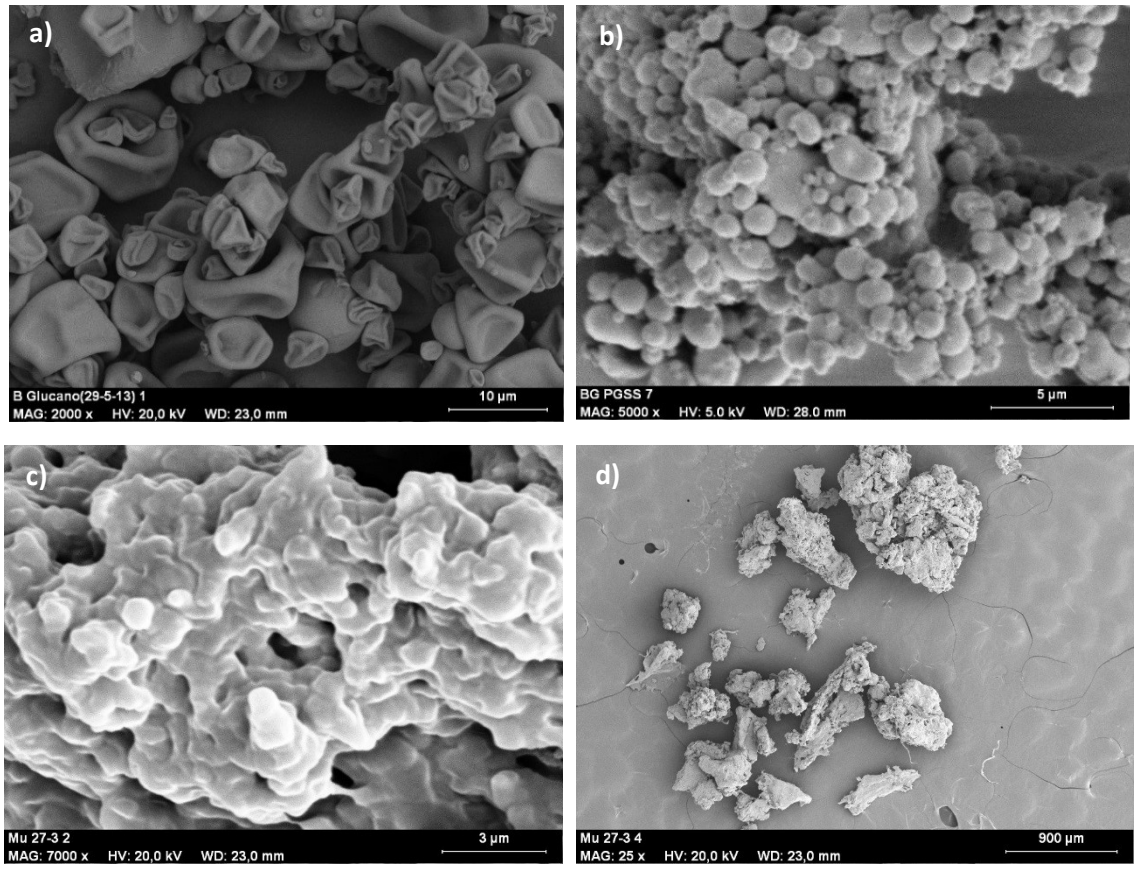

Figure 4. SEM images of β-glucan particles by SD (a), PGSS-drying (b) and by SAS (exp. F: 5 g/L in DMSO, precipitation at 10 MPa, 35^{o} C, 2 mL/min; c and d).

It is observed that the particles obtained by PGSS-drying had more spherical shape, whereas the ones produced by SD are irregular due to a shrinkage effect caused by the evaporation of water

once the crust has started to being formed. This has been also observed by other authors when drying at high temperature (de Barros Fernandes et al., 2014; Porras-Saavedra et al., 2015; Shi and Zhong, 2015), also oat powder suspensions containing between 18-33% (w/w) β-glucans at similar drying conditions (80-82º C in the drying chamber) as the ones used in this work (Chronakis et al., 2004). Neither the particles obtained by SD, nor the ones by PGSS-drying, show cracks in their surface. The morphology results of the PGSS-drying particles are in accordance with the reported ones from previous works (de Paz et al., 2012; Varona et al., 2013). In addition, the particles produced by PGSS-drying are smaller than by SD, but they form agglomerates very easily, producing much larger clusters. This is in accordance with the results reported by other authors (Li et al., 2013), who detected an increase of the agglomeration of particles when drying at lower temperature. The formation of small particles by PGSS-drying is a consequence of the desorption of CO_2 from the liquid when the pressure is suddenly reduced in the nozzle, which breaks the liquid into smaller droplets. Figure 5 shows particle size distributions of the particles formed with the different encapsulating materials by both SD and PGSS-drying.

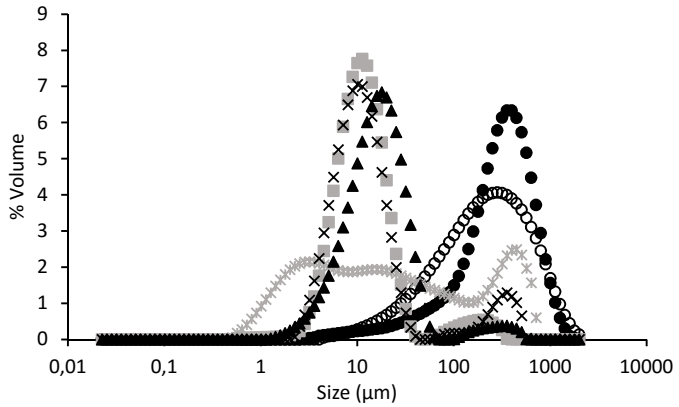

Figure 5. Droplet size distribution of the particles produced by SD and PGSS-drying with different encapsulating material. By PGSS-drying: ○: with lecithin; ●: with lecithin and β-glucans; X: with β-glucans; and by SD ■ :with lecithin; ▲: with lecithin and β-glucans; and X: with β-glucans.

According to these results, despite the different droplet size of the emulsions as a function of the encapsulating material, particle size was just dependent on the drying process: it was in the

range of 10 µm for SD and 150 µm for PGSS-drying. This confirms SEM results that, even though smaller primary particles were created by PGSS-drying, they formed huge agglomerates.

Regarding the formation of particles by SAS, the operating conditions of the different experiments performed are shown In Table 1:

Table 1. SAS experiments.

Exp	β-glucan concentration (g/L_{DMSO})	Dissolution flow (mL/min)	CO_2 flow (kg/h)	T (ºC)
A	2	2	2.5	34-35
B	5	2	2.5	37
C	5	2	2.5	38
D	5	2	2.5	50
E	5	2	2.5	34-35
F	5	2	5	34-35
G	5	4	2.5	34-35
H	10	2	2.5	34-35

Except for experiments B, C and D, which were performed at higher temperature, the rest of them were done at 10 MPa and 35º C in order to have one single phase for the mixture of DMSO and CO_2, according to the data reported by other authors (Andreatta et al., 2007).

By SAS it was not possible to obtain well dried β-glucan particles in the operating conditions tested. At higher temperature, no solid material was obtained. In the experiments at lower temperature, the product recovered from the vessel formed a matrix rather than particles. This is observed with SEM images (figure 4 c and d): there are melted micro-particles (4c) which conform aggregates with particle size around 1 mm (4d).

By DSC it was estimated that after the drying process there was still between 20-30% (w/w) of DMSO in the final product (DSC results not shown), so it was not completely extracted with supercritical CO_2. Several previous studies succeeded on obtaining solid particles by SAS using DMSO with different biopolymers, as dextrans, poly-(hydroxypropylmethacrylamide) (HPMA) (Reverchon et al., 2000), cyclodextrins (De Marco and Reverchon, 2008) or hemicellulosic material (Haimer et al., 2010). However, in all cases, molecular weight of the biopolymers tested was lower than that of the β-glucans used in this work. Thus, with β-glucans, DMSO was entrapped into the chains of polymer, the viscosity of the solution increased and it was not

possible to extract it with CO_2 in the selected operating conditions. This problem when precipitating high viscous solutions of DMSO in supercritical fluids was also observed by other authors (Fernández-Ronco et al., 2014).

3.2. Loading of active compounds in the particles

Results of the loading of tebuconazole and resveratrol for the different encapsulating materials and drying processes are shown on Table 2.

Table 2. Fungicide loading for the different materials and drying processes.

% loading	Tebuconazole		Resveratrol	
	SD	PGSS-drying	SD	PGSS-drying
Lecithin	86.4 ± 1.1	79.1 ± 1.9	71 ± 4	75.3 ± 0.2
Lecithin and β-glucans	82.6 ± 1.6	72 ± 9	65 ± 7	67 ± 2
β-glucans	57 ± 2	49 ± 14	61.3 ± 1.2	94 ± 2

The recovery of the active compound is higher for tebuconazole by SD rather than by PGSS-drying. On the contrary, when using resveratrol PGSS-drying is more effective, because in PGSS-drying lower drying temperature is required and the product is protected from oxidation since it is included in an inert atmosphere of CO_2. Anyway, the differences between both processes are lower than 10%, except in the case of the particles of resveratrol encapsulated on β-glucans (recovery 30% higher by PGSS-drying). These small differences are probably due to the fact that the encapsulation of the active compound into the carrier is done by emulsification prior to the drying process, so there is not influence of the latter on the loading of tebuconazole and resveratrol in the final particles.

When comparing the different encapsulating materials, the loading of active compound is always higher for lecithin than for β-glucans, again with the exception of the case mentioned above. However, the values are high (above 49%) with both materials, probably due to the formation of hydrogen bonds between the active compounds and the encapsulating materials, as pointed out in some previous works for the case of lecithin with antioxidants (Gonçalves, 2015) and polyphenols with β-glucans (Wu et al., 2011).

Furthermore, XRD analysis were performed in order to check whether there were crystals of fungicide remaining on the surface of the particles or they were completely encapsulated. In all cases, the peaks corresponding to tebuconazole or resveratrol were observed in the physical mixtures with the encapsulating material, but they could not be noticed in the particles obtained either by SD or by PGSS-drying. As an example, figure 6 shows the XRD diagram for the particles of resveratrol and lecithin obtained by SD and the physical mixture of both compounds with the same composition.

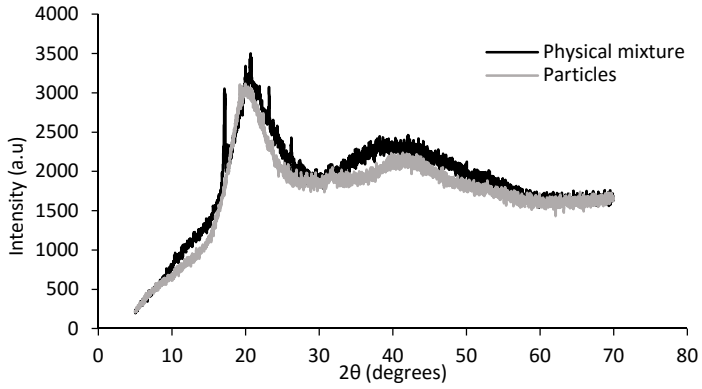

Figure 6. Physical mixture and SD particles of resveratrol and lecithin.

The particles obtained do not show any of the peaks corresponding to crystals of resveratrol. As the structure is totally amorphous, it is assume that resveratrol is correctly included into the lecithin and there is not unbound crystals of resveratrol in the sample. In addition, the encapsulated resveratrol is on amorphous state too, since XRD is a penetrating technique and the corresponding diffraction peaks were not observed. This same behavior is observed for all the particles produced, and it is in accordance with previous results of β-glucan complexes with some nutraceuticals (Veverka et al., 2014).

3.3. Antifungal activity against Botrytis cinerea

According to commercial guidelines of Tebuconazole agricultural products, it is recommended to be applied at a concentration between 100 and 250 mg/L, so the antifungal tests were performed at 100 mg/L of tebuconazole. Figure 7 (a) shows the results of the growth of the fungus when applying pure tebuconazole (Teb), a commercial oil-in-water emulsion (Orius), and

the particles of lecithin (Lec), a mixture of lecithin and β-glucans (LecbG) and β-glucans (bG) produced by SD (SD) or PGSS-drying (PGSS).

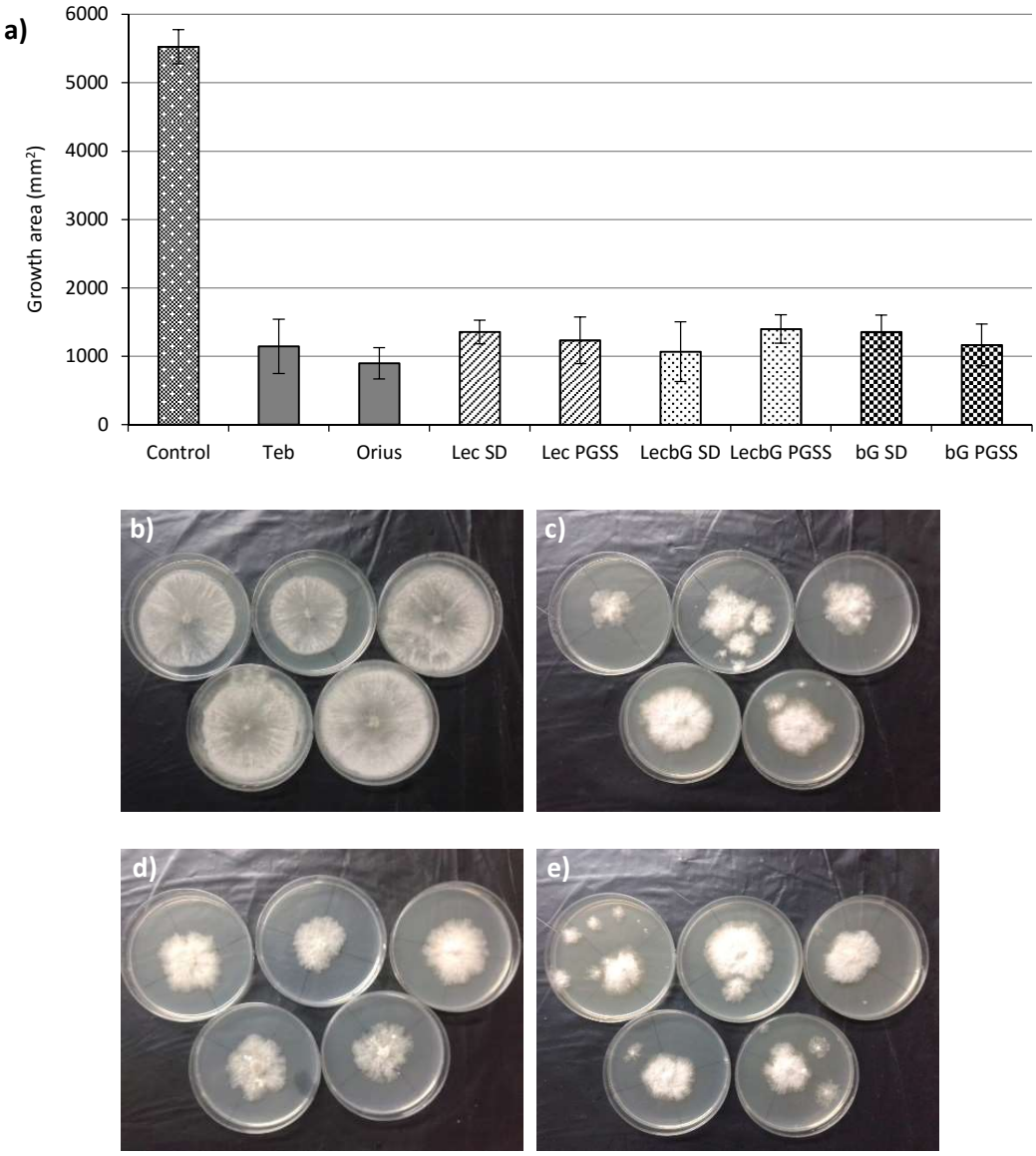

Figure 7. Growth area of Botrytis cinerea for the products of tebuconazole (a) and images of the growth area in the Petri dishes: control (b), commercial tebuconazole (c), tebuconazole and β-glucan particles by SD (d) and by PGSS-drying (e).

All the particles obtained produced a great inhibition of the fungal growth (between 75 and 80% compared to the control sample). However, no significant differences were observed with the commercial tebuconazole products. Therefore, although the encapsulated particles were very effective against *Botrytis cinerea* growth, they did not improve the action of powder and oil-in-

water commercial tebuconazole, because it is already very active against this fungus. Figure 7 shows some images of the Petri dishes of *Botrytis* culture as control (b), with commercial tebuconazole powder (c) and with the particles of tebuconazole encapsulated on β-glucan obtained by SD (d) and by PGSS-drying (e). It is noticed that growth is reduced when applying both pure tebuconazole and the particles, compared to the control dishes, but there are not significant differences among them.

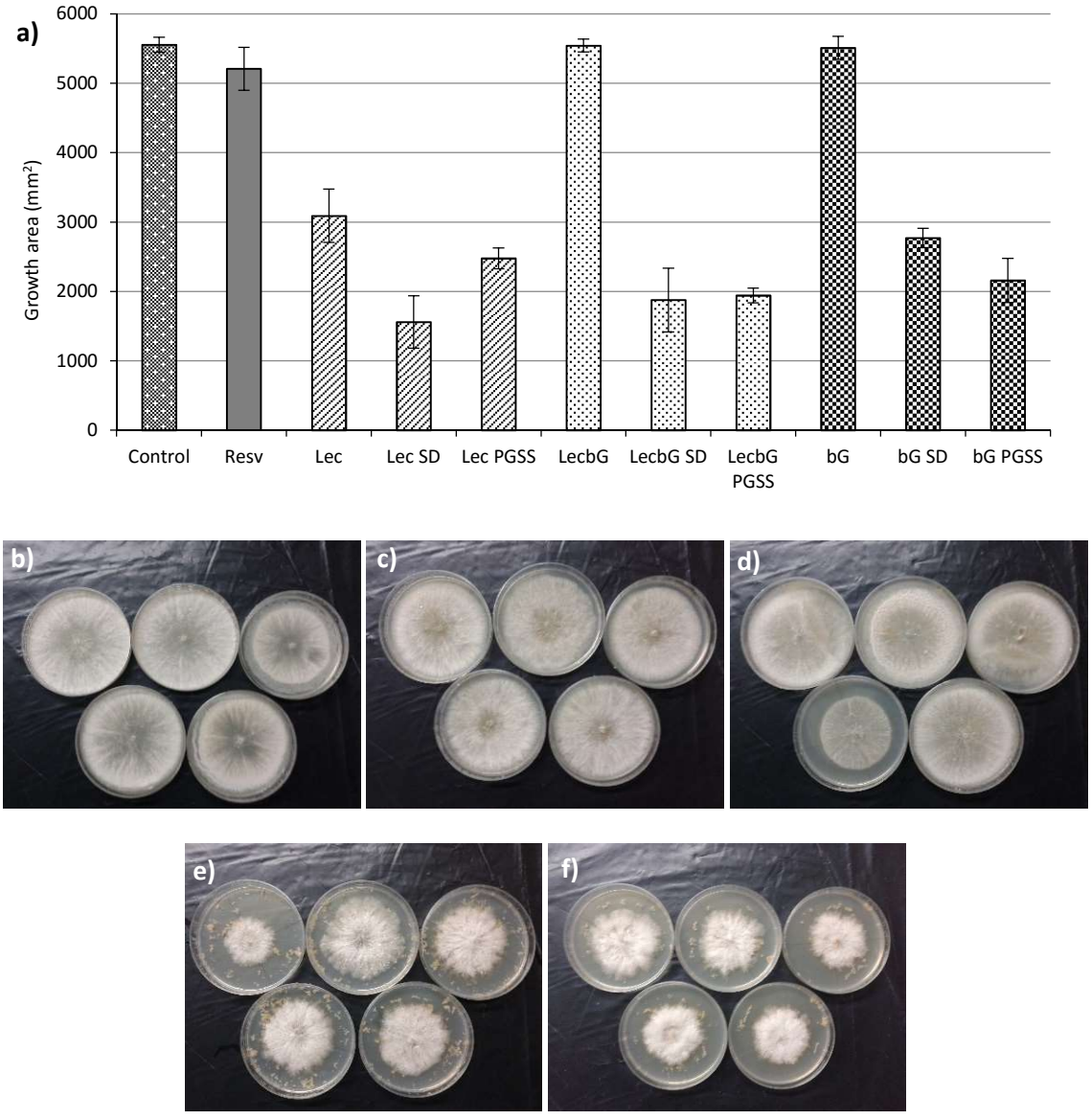

Figure 8. Growth area of Botrytis cinerea for the products of resveratrol (a) and images of the growth area in the Petri dishes: control (b), pure resveratrol (c), β-glucans (d), resveratrol and β-glucan particles by SD (e) and by PGSS-drying (f).

For the activity of resveratrol, it was considered that previous works (Adrian et al., 1997) reported growth inhibition of *Botrytis cinerea* with resveratrol concentration between 60 and 160 mg/L, so 100 mg/L of resveratrol was chosen for the in vitro tests. Figure 8 (a) reveals the results corresponding to the growth of *Botrytis cinerea* in the presence of pure resveratrol (Resv), pure soy lecithin (Lec), pure β-glucans (bG) and a mixture of both substances (LecbG), and the particles created with these encapsulating materials and pure resveratrol by SD (SD) or PGSS-drying (PGSS).

No effect was observed on fungal growth when applying pure resveratrol, although growth inhibition at that concentration range was reported in previous works (Adrian et al., 1997). The main difference is that here it was applied as pure crystals, whereas in that work it was dissolved in an ethanolic solution. However, the growth was reduced between 50 and 70% by all the particles of resveratrol produced in this work. This means that the encapsulation of resveratrol enhanced its antifungal activity. Small differences were noticed between both drying processes for every encapsulating material, although particles of lecithin were more effective by SD, while particles of β-glucans resulted slightly better by PGSS-drying.

Figure 8 contains images of the growth in Petri dishes of some samples. Similar growth is observed between the control sample (b), the one with pure resveratrol powder (c) and the one with pure β-glucan (d). However, the growth area is smaller for the particles of resveratrol encapsulated on β-glucans by SD (e), and even smaller for the ones by PGSS-drying (f).

Pure β-glucans did not affect the growth of the fungus, but lecithin reduced it by 40% (figure 8 (a)). Still, the mixture of both compounds had also no effect on growth inhibition. Taking this into account, the effectiveness of the particles encapsulated on lecithin relays greatly on lecithin. Nevertheless, the action of the particles encapsulated on β-glucans and a mixture of lecithin and β-glucans is related to the synergistic effect of these substances and resveratrol, because neither of them showed effect when applied alone, as well as to the change of resveratrol from crystals

to amorphous state after processing, determined by XRD. Therefore, the shell material improved the absorption of resveratrol, which in this way was active against the *Botrytis cinerea*.

4. CONCLUSIONS

Resveratrol and tebuconazole were formulated as emulsions with lecithin, β-glucans and mixtures thereof. Formulations were successfully dried by SD and PGSS-drying. However, SAS was not a suitable process to obtain β-glucan particles in the conditions tested, because the product had quantities of remaining organic solvent up to 30% (w/w). Final particle size by SD was in the range of 10 μm and in PGSS-drying in the range of 100 μm, showing that the drying processes and not the carrier material determined the final particle size. As first there was an emulsification step, the loading of active compound in the particles was very similar for both drying processes. Furthermore, the interaction of the active compounds with the carriers produced their precipitation in the encapsulated particles in amorphous state instead of their usual crystalline form. This change in crystallinity improved the antifungal activity of resveratrol, which showed no effect against *Botrytis cinerea* as pure crystals, but reduced the fungal growth between 50 and 70% with all resveratrol particles. However, pure and commercial emulsion of tebuconazole inhibited fungal growth by 75-80%, so no further effect could be observed with the particles of encapsulated tebuconazole.

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CHAPTER II:

BARLEY AND YEAST β-GLUCANS AS NEW EMULSIFIER AGENTS FOR THE DEVELOPMENT OF AQUEOUS NATURAL ANTIFUNGAL FORMULATIONS

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ABSTRACT

There is an increasing demand on new antimicrobial and antifungal products from non-toxic and natural origin substances to prevent fruits and vegetables from potential contamination by toxic residues and the formation of resistant strains. In this work, resveratrol was encapsulated on barley and yeast β-glucans and soy lecithin by emulsification-evaporation method. Different emulsification techniques were used: high-shear, high pressure and high pressure and temperature emulsification. The morphology, crystallinity, encapsulation efficiency and *in vitro* antifungal activity against *Botrytis cinerea* of the different formulations were evaluated. Prior to formulations, a study about the solubility of yeast β-glucans in water was performed. No significant differences between each emulsification procedure in particle size (below 90 nm) and in encapsulation efficiency (between 70 and 100%) were observed; only barley β-glucan emulsions showed lower efficiency due to the formation of a gel in which most of the active compound was entrapped. However, a great influence of the emulsification method and the encapsulating material on the crystallinity of the particles was observed. The highest antifungal activity (up to 53% growth inhibition) was obtained by the formulations with yeast β-glucans, indicating an enhanced absorption of encapsulated resveratrol through the cell wall of the fungus at the presence of (1-3, 1-6)-β-glucans.

Keywords: resveratrol, β-glucan, lecithin, encapsulation, high-pressure emulsification, Botrytis cinerea

1. INTRODUCTION

Fungal and bacterial infections are a major concern in agriculture since they produce great losses (Spadaro and Gullino, 2004). On one hand, many fruits and vegetables must be discarded because they cannot be recovered once they are affected, raising an ethical issue due to the increasing global demand of food derived from a bigger population (Bebber and Gurr, 2015). On the other hand, producers must carry out a big investment in order to keep infections under control and to minimize the damages on their crops. Therefore, it is important to develop effective products which reduce the growth of the pathogens and preserve the quality of the food. For many years, several chemical products have been used with this purpose. However, they can leave toxic residues on the plant, which afterwards could affect human health and contaminate the environment (Cruz Cabral et al., 2013). For instance, triazoles are a group of compounds commonly used as antifungal agents which have been reported to be toxic for the environment and have adverse effects in *in vivo* tests in animals (Chen and Ying, 2015; Rieke et al., 2014; Schmidt et al., 2016). Furthermore, fungi and bacteria can develop resistant strains after prolonged application (Panebianco et al., 2015). Thus, in recent years there is a tendency towards the development of alternative antifungal and antibacterial products from non-toxic, natural origin substances (Mari et al., 2003).

In this sense, essential oils have been extensively studied because of their antifungal properties (Soylu et al., 2010; Stević et al., 2014). The main drawback associated with the use of essential oils as biocides is that most of them have strong aromatics compounds, and it could affect the quality and organoleptic properties of the finally product if they are applied in big quantities (Goñi et al., 2009). The biocide activity of essential oils is mainly due to their polyphenolic compounds, which increase permeability of cell membrane and promotes its disruption (Tiwari et al., 2009), produce alkalinization of the medium, stimulate oxidative burst and induce defense genes (Chang et al., 2011). Many works analyze also plant extracts, focusing on their phenolic

content and their activity against different fungi, like *Aspergillus, Penicillium, Botrytis* or *Candida* (Gatto et al., 2011; Martins et al., 2015; Rashed et al., 2014). However, polyphenols are poorly soluble in water and are easily degraded by light, heat and oxygen. In order to overcome these limitations, a proper formulation is required in most of the cases prior to its application, usually with polysaccharides (Parisi et al., 2014). Different techniques are used to process them, such as spray-drying (Liu et al., 2016; Sansone et al., 2011; Sun-Waterhouse et al., 2013), drying with supercritical fluids (Fraile et al., 2014; Sosa et al., 2011), or emulsification (Lu et al., 2016), among others.

Emulsions have been long used for the formulation of active compounds in liquid phase. They consist of two liquids, usually water and an organic solvent, one dispersed as small droplets into the other by means of a surfactant that stabilizes them and contains the active compound. Furthermore, the organic solvent can be removed from the emulsion, promoting the precipitation of the droplets and the creation of a suspension. Different methods are reported in literature to produce suspensions of antifungal products. For instance, *Zataria multiflora* essential oil was loaded into chitosan nanoparticles by ionic gelation (Mohammadi et al., 2015). This method involves the formation of an oil-in-water emulsion of both components and addition dropwise of pentasodium triphosphate, which produced the spontaneous precipitation of the nanoparticles. *Cuminum cyminum* essential oil was encapsulated by sonication on caffeic acid-chitosan nanogels (Zhaveh et al., 2015). A slow release of the oil was achieved, and the antifungal effect against Aspergillus flavus was enhanced compared to the non-encapsulated oil. Freeze-drying was used to form polyphenol-cyclodextrin complexes against *Fusarium oxysporum* and *Botrytis cinerea* (Kfoury et al., 2016). However, low encapsulation efficiency (up to 40%) was observed for the less water-soluble polyphenols.

Lipids (e.g. fatty acids, lecithin) are commonly used as surfactants because they can incorporate both hydrophilic and lipophilic active compounds, are non-toxic and easily absorbed through

biological membranes. Polysaccharides are also used because they enhance the stability of the emulsion through an increase of viscosity that reduces the movement of the droplets within the emulsion (García et al., 2014). β-glucans are polysaccharides composed of D-glucose monomers linked by glycosidic bonds in positions (1-3). They are present in several fungi, bacteria and some kind of plants, including barley, oat and mushrooms, although depending on their origin they have different structure. For instance, cereal β-glucans have (1-4) branching, whereas yeast β-glucans (YBG) have (1-6) branching (Zhu et al., 2016). Also, the variation on their structure provides them different physical and biological properties. For instance, branched (1-3),(1-6)-β-glucans have stronger immune modulatory properties than (1-3),(1-4)-β-glucans from plants (Mikkelsen et al., 2014). Moreover, many works report the ability of β-glucans as elicitors in plants to induce the formation of secondary metabolites and trigger defense responses (Almagro et al., 2016; Aziz et al., 2003).

β-glucans have been used as encapsulating material for anthocyanins by coacervation (Xiong et al., 2006), for proteins through the production of cryogels (Lazaridou et al., 2015) and for resveratrol by spray-drying and PGSS-drying (Salgado et al., 2015). In emulsion, they have been previously used as stabilizers (Burkus and Temelli, 2000; Kontogiorgos et al., 2004; Thammakiti et al., 2004). Barley β-glucans (BBG) were found to reduce surface tension in aqueous solutions, reaching a constant value of 50-55 mN/m at 2 g/L (unpublished work). However, to the authors' knowledge, there is just one work in which they were used as surfactant of emulsions, but it was found out that the active compound (quercetin) precipitated in crystals inside the polymer, so to avoid this lecithin was added as co-surfactant (Gonçalves et al., 2015). In our previous work, BBG were also used as surfactant, although the emulsions were further processed to obtain solid particles (Salgado et al., 2015). In that work, solid particles of resveratrol encapsulated on β-glucans showed antifungal activity against *Botrytis cinerea*. Resveratrol belongs to stilbenes, a group of phenolic compounds. They are included among phytoalexins, secondary metabolites that are synthesized by plants as protective agents against bacterial and fungal attacks (Adrian

and Jeandet, 2012). Resveratrol is present in many plants, although grapes have the highest concentration (Fernández-Mar et al., 2012). Antifungal activity of resveratrol has been previously reported (Jung et al., 2005), also against *B. cinerea* (Adrian et al., 1997). Besides, structural changes in conidia were observed (disrupted plasma membrane, disorganized cytoplasm withdrawn from conidial wall), which could explain the mode of action of resveratrol leading to cell death (Adrian and Jeandet, 2012). However, fungal laccases produce extracellular oxidation of resveratrol and its degradation (Chang et al., 2011; Rivera-Hoyos et al., 2013; Timperio et al., 2012). Therefore, a proper formulation is required in order to encapsulate resveratrol and protect it from laccases until the target.

The aim of this study is to develop a liquid antifungal formulation with the same components, because it would be easier to apply *in situ*. *B. cinerea* is a widely extended fungus that affects several fruits and plants in postharvest stage, causing great losses (Williamson et al., 2007). Different emulsion techniques were used: high-shear, high pressure and high pressure and temperature emulsification. The latter methods were performed because they were reported to provide higher encapsulation efficiencies (de Paz et al., 2013). In that work, β-carotene was encapsulated in modified starch, achieving 8% encapsulation efficiency by high-shear, 30% by ultrasounds and 80% when applying high-pressure. β-glucans are used as encapsulating material because, since they are present in the cell wall of *B. cinerea* (Tenberge, 2007), they may improve the absorption of the encapsulated active compound and thus increase its effectiveness, besides acting as surfactant. Also lecithin was tested, both alone and mixed with the β-glucans, in order to enhance the formation of the emulsion. Furthermore, lecithin can improve the penetration of the active compound into the cell wall of the fungi due to the formation of liposomes, which are very similar to cell membranes (Varona et al., 2013).

2. MATERIALS AND METHODS

2.1. Materials

As encapsulating materials, two different types of β-glucans were used: barley (1-3, 1-4)-β-glucans (75% purity; Glucagel, kindly supplied by DKSH, France) and (1-3, 1-6)-β-glucans from yeast *Saccharomyces cerevisae* (64% purity, determined as indicated in section 2.2.1. β-glucan content; L-Naturae Nutraceutical, kindly supplied by Naturae, Spain). Soybean lecithin (Glama-sot, SOTYA S.A., Spain) was also used as encapsulating material. Ethyl acetate (99%), malt extract agar, acetic acid glacial and sodium acetate were purchased from Panreac (Spain). Resveratrol with 98% purity was purchased from Pure Bulk (USA). Pullulan standards were purchased from Shodex.

2.2. Solubilization and characterization of YBG

BBG were soluble in water, so they could be directly dissolved in water and used in emulsification. However, YBG were insoluble in water, therefore a pretreatment was required in order to solubilize them. For that purpose, a 140 mL stainless steel vessel was used. Beside YBG, sodium acetate (1.41 mg/g of β-glucan) and glacial acetic acid (12.2 µL/g of β-glucan) were charged into the vessel at concentrations reported in (Cox, 2008), with a solid to liquid ratio of 1:10 (w:w). The vessel was heated with an electric external resistance and kept at 135º C and 0.28 MPa for 4.5 hours. Samples of the product were obtained at 3, 3.5, 4 and 4.5 h. Further description of the equipment can be found in (Sánchez, 2015).

2.2.1. β-glucan content

The quantity of β-glucan on the solubilized YBG was analyzed by means of the *"β-Glucan Assay Kit (Yeast & Mushroom)"* from Megazyme (Ireland). Following the described procedure in the kit, the percentage of total glucans and α-glucans is determined spectrophotometrically

(Shimadzu UV-2550), and the percentage of β-glucans is calculated as the difference between the total and the α-glucans.

2.2.2. Molecular weight of the β-glucans

Molecular weight of the β-glucans was measured by size exclusion chromatography, with a guard column (Shodex SB-G), a column (Shodex SB-804 HQ, particle size 10µm, 7.8x300 mm) and a differential refractive index detector (410, Waters Corporation). The column was kept at 35º C and flow rate of the mobile phase (0.1 M $NaNO_3$ and 0.02% NaN_3) was set at 0.4 mL/min. β-glucan standards (123-359 kDa) were used for BBG, whereas the calibration was done with pullulans (5.9-708 kDa) for YBG. All of the standards were purchased from Megazyme (Ireland).

2.3. Preparation of emulsions

2.3.1. High shear emulsification

An oil-in-water emulsion was formed, containing resveratrol dissolved in ethyl acetate (7.5 g/L) as organic phase and the encapsulating material dissolved in the aqueous phase, previously saturated with ethyl acetate. Different concentrations were tested in the aqueous phase (10, 15 and 20 g/L) for each of the encapsulating materials used, BBG, solubilized YBG and lecithin, and mixtures of lecithin and each of the glucans (50% in weight).

Both liquid phases were mixed in a ratio 1:9 (v:v) at 800 rpm for 5 min and then the emulsion was fed into a 200 mL rotor-stator machine (IKA LABOR PILOT 2000/4) and processed for 4 minutes at 4200 rpm, as described in (Salgado et al., 2015).

2.3.2. Precipitation from a pressurized emulsion

High pressure emulsification was also performed with the same aqueous and organic phases aforementioned, at 6 MPa and ambient temperature. The aqueous solution was pressurized with a Dosapro Milton Royal D (18 mL/min, 20 g surfactant/L), whereas a Jasco PU-2080 plus pump was used for feeding the organic phase (2 mL/min, 7.5 g resveratrol/L). After

pressurization, both streams were put in contact in a T-mixer, where the emulsion was formed, and afterwards the product was recovered.

2.3.3. Precipitation from a hot pressurized emulsion

This method of encapsulation was previously used (de Paz et al., 2012) to accelerate the mass transfer kinetics to the time scales of the precipitation process, thus improving the control over the precipitation. Briefly, a solution or suspension of the active compound in the organic phase is put in contact with another stream of pure organic solvent at high temperature and immediately afterwards this flow is mixed with the cold water phase which contains the surfactant. This causes the emulsification of the organic phase and the precipitation of the active compound into the polymer. The emulsion is thus formed by the combined anti-solvent and cooling effect of the water, and the active compound does not undergo thermal degradation during the process because due to the configuration of the equipment, the exposition time to high temperature is lower than 2 seconds. A detailed explanation about the equipment used can be found in (de Paz et al., 2012).

The operation starts with the pressurization of the 3 streams up to 6.0-6.5 MPa and heating up of the organic solvent to the desired temperature (in this case, 85-90ºC). When the operating conditions were reached, the feed was changed from pure water and ethyl acetate to the solutions used in this work: a solution of ethyl acetate with 7.5 g resveratrol/L at 2.75 mL/min, hot ethyl acetate at 4.25 mL/min and a solution of 20 g surfactant/L in water at 20 mL/min. The operating conditions were selected based on a previous work in which quercetin was encapsulated on starch, lecithin and β-glucans (Gonçalves et al., 2015).

2.4. Formation of suspensions

Suspensions were obtained for all the different emulsification methods by removing the organic solvent by vacuum evaporation (Heidolph) at 60º C, 75 rpm and a vacuum of 0.08 MPa.

Afterwards, big agglomerates of polymer and non-encapsulated crystals of resveratrol were removed by centrifugation at 7800 rpm (6461 g) for 10 min.

2.5. Characterization of emulsions and suspensions

2.5.1. Droplet size distribution

Droplet and particle size distribution of the emulsions and suspensions was measured by laser diffraction (Mastersizer 2000, Malvern) by diluting the emulsions on a solution of water saturated with ethyl acetate and the suspensions only in water. Particle size was measured both before and after centrifugation of the suspensions. Results are expressed as % in number. The droplet and particle sizes reported correspond to the value of d(0.5).

2.5.2. CryoTEM

CryoTEM analysis of the aqueous solutions of the different β-glucans and their mixtures with lecithin were performed to check how their chains associated themselves, in a JEOL JEM-FS2200 HRP 200 kV TEM with electron filtering. In brief, 4 µL of sample was deposited on a rack C-Flat 1.2/1.3, which was previously hydrophilized by a plasma cleaner. A blotting is performed on either side of the filter grid during 4 s and then liquid ethane is introduced there in order to freeze the samples, avoiding the formation of crystals (Gatan Cryoplunge 3). Samples are maintained in liquid nitrogen until their transfer to the holder (Gatan Cryotransfer 626).

2.5.3. X-ray diffraction

X-ray diffraction (XRD) measurements were performed on a Bruker Discover D8 diffractometer to check the crystallinity of the particles in the final suspensions, using the Cu Kα radiation (λ = 0.15406 nm). The suspensions were further centrifuged at 20000 rpm for 30 min (Beckman) in order to recover the encapsulated particles. Afterwards, this sediment was dried in a freeze-drier (LyoQuest -55, Telstar) for 48 hours and the final powder was analyzed by XRD. The

scattering intensities were measured over an angular range of $5 < 2\theta < 70$ for all the samples, with a step size of 0.02°.

2.5.4. Encapsulation efficiency

Quantification of encapsulated resveratrol was done by HPLC with a guard column (Bio-Sil C18 HL 90-5, 4.6 x 30 mm, particle size 5 µm, Bio-Rad), a column (Symmetry C18, 4.6 x 150 mm, particle size 5 µm, Waters) and a UV detector (λ = 306 nm). The column was kept at 25º C and flow rate of the mobile phase (acetonitrile and water in ratio 1:3, with 0.2% formic acid) was set at 0.8 mL/min. Encapsulation efficiency was determined as the ratio between the quantity of resveratrol in the centrifuged suspension and the amount of resveratrol in the initial emulsion. Calibration range of resveratrol was between 10 and 40 ppm. Prior to analysis, the centrifuged samples of suspensions were diluted (0.6 mL of sample in 10 mL) and filtered (0.22 µm). The analysis was performed in triplicate, and the data were analyzed by t-Student´s test (unpaired samples, unequal variances) with a significance p-value of 0.05.

2.6. B. cinerea culture

For the *in vitro* culture of *B. cinerea*, the fungus was isolated from vines in our university (Campus La Yutera, Universidad de Valladolid, Palencia, Spain) and it was grown on malt extract agar. After autoclaving, the agar was poured into Petri dishes containing the corresponding sample or control, and when it solidified, the fungus was placed in the center of the surface. The quantity of sample on each plate was determined so as to have a concentration of resveratrol of 100 mg/L, which was reported to be within the range of growth inhibition for *B. cinerea* in previous works (Adrian et al., 1997). Five replicates were grown for every test and the growth area was calculated by measuring the diameter in two perpendicular directions after incubation at 22º C for one week. Once measured, the average value and standard deviation were evaluated, and Student's t-test was performed as mentioned before in order to check whether there were significant differences between each sample.

3. RESULTS AND DISCUSSION

3.1. Solubilization and characterization of (1-3;1-6)-β-glucans

The results of the molecular weight and the quantity of β-glucans in the samples at increasing solubilization time are shown in Figure 1. It can be noticed that both parameters have the same trend: they increase with time, showing a maximum between 3.5 and 4 h, and they decrease again with longer times.

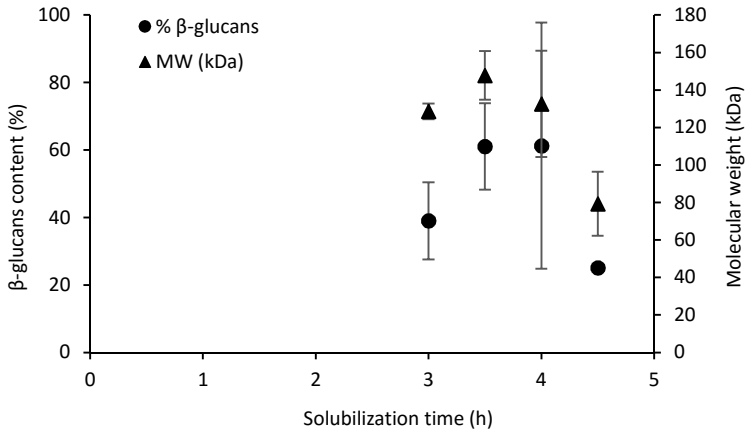

Figure 1. Evolution of b-glucans content and their molecular weight with the time of solubilization.

This behavior is due to the degradation of the product. Initially, the yield of dissolution increases, but after some time in the acidic solution, the β-glucans are broken into smaller compounds, which have lower molecular weight, and the presence of β-glucans is reduced. In order to have a better quality product (higher purity of β-glucans and less degradation) the optimum time for dissolution of the YBG is between 3.5 and 4 h.

3.2. Characterization of the emulsions and suspensions

3.2.1. Droplet and particle size

The influence of the type and concentration of surfactant was evaluated by high-shear emulsification. Unimodal droplet size distributions were obtained in all cases, with narrow peaks

(results not shown here). Regarding average droplet size, there were significant variations depending on the surfactant used and its concentration (Fig. 2).

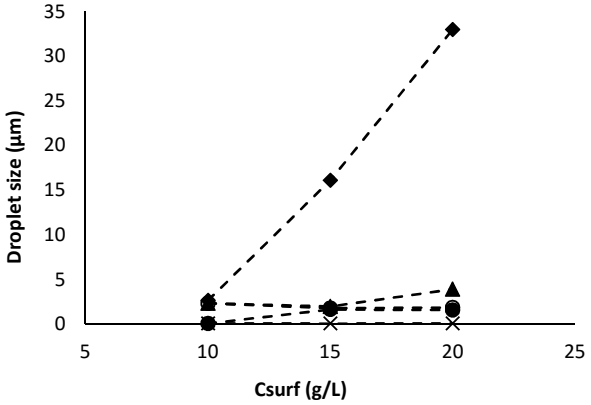

Figure 2. Average droplet size of the emulsions by high-shear emulsification with different encapsulating materials. ● lecithin; ▲ lecithin and barley β-glucan; ◆ barley β-glucan; X lecithin and yeast β-glucan; ○ yeast β-glucan.

The emulsions with lecithin had small droplet size in the concentration range tested, although it increased with concentration from 90 nm to 1.6 µm. Also with YBG small droplets were achieved, between 1.8 and 2.4 µm. However, with BBG, the higher the concentration of glucan, the greater the droplet size, up to 33 µm. Some authors suggest that an increase on the viscosity of the aqueous solution worsens the homogenization process, thus leading to the formation of bigger droplets (Huang et al., 2001). By cryo-TEM it was observed that BBG in solution tend to form a network instead of single droplets (Fig. 3a), which could explain the increase in droplet size. This structure was not observed with YBG (not shown), and therefore smaller droplet sizes were obtained in the emulsions. When lecithin was mixed with BBG, droplet size was greatly reduced in comparison with the emulsions that had only β-glucans. In these cases, the behavior was similar to that of pure lecithin, thus improving the emulsification properties of the BBG. Also, the network of polymer obtained with BBG was not noticed if lecithin was added to BBG (Fig. 3b).

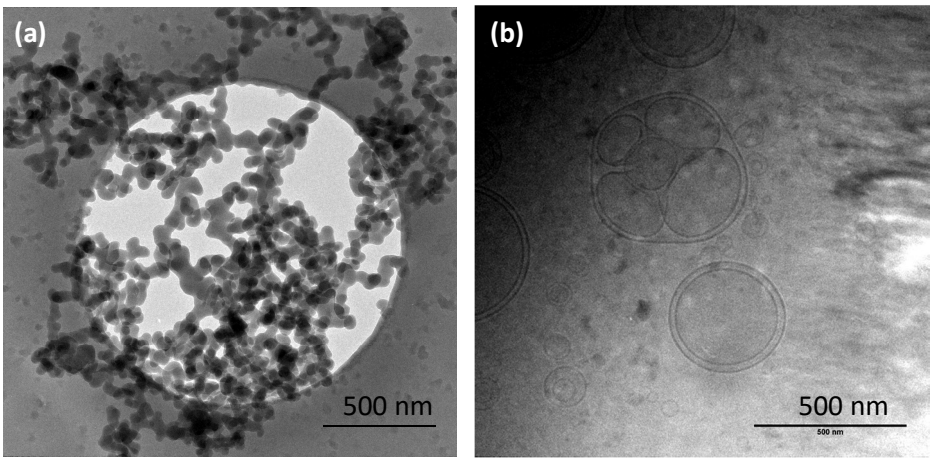

Figure 3. Images of aqueous solutions of BBG (a) and a mix of BBG and lecithin (b).

The same trend was observed for the emulsions produced by high pressure and high-pressure and temperature emulsification: particle size for the experiments with BBG was much bigger than with all the other encapsulating materials, but when they were mixed with lecithin, it was greatly reduced (results not shown).

Table 1. Particle size and encapsulation efficiency for the different emulsification methods (HS: high shear; HP: high pressure; HPT: high pressure and temperature) and encapsulating materials at 20 g/L.

Surfactant	Emulsification method	Particle size (nm)	Encapsulation efficiency (%)
Lec	HS	69	84 ± 11
	HP	88	100 ± 8
	HPT	75	78 ± 9
BBG	HS	63	31 ± 9
	HP	65	54 ± 7
	HPT	63	39 ± 2
Lec-BBG	HS	84	91 ± 11
	HP	62	79 ± 17
	HPT	64	66 ± 9
YBG	HS	65	84 ± 16
	HP	64	74 ± 10
	HPT	64	79 ± 7
Lec-YBG	HS	62	93 ± 14
	HP	61	79 ± 5
	HPT	66	71 ± 5

After centrifugation and removal of big agglomerations of polymer and crystals of non-encapsulated resveratrol, all of the suspensions had narrow particle size distribution, with

average particle size between 60 and 90 nm, regardless of the emulsification process and the encapsulating material (Table 1).

3.2.2. Crystallinity of encapsulated resveratrol

The crystallinity of the particles in the suspensions was analyzed by means of XRD. Different results were obtained depending on the encapsulating material and the emulsification method. Regarding the emulsification methods, a complete amorphous structure was obtained for all the experiments by HPT, whereas with the 2 other methods the main characteristic peaks of pristine resveratrol were noticed with some of the encapsulating materials. As an example, figure 4 shows the XRD pattern for the particles of the suspension of resveratrol in lecithin by high-pressure emulsification (black line) and high pressure and temperature emulsification (gray line), compared to pure resveratrol (dots).

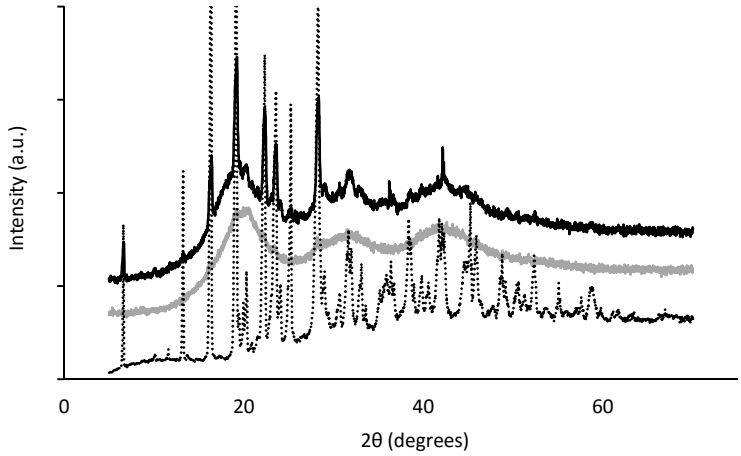

Figure 4. XRD patterns for the suspension of resveratrol and lecithin by high-pressure (black), high-pressure and temperature emulsification (gray) and pure resveratrol (dots).

According to these observations, the application of pressure to the emulsification does not influence the crystallinity of the final particles at ambient temperature. However, by subjecting the organic phase containing the active compound to a heating step followed by a sudden

cooling, the active compound has not time to form crystals and grow. Instead, it precipitates in amorphous state inside the encapsulating material.

Focusing on the different encapsulating materials, in the experiments with BBG, both alone and mixed with lecithin, no peaks corresponding to crystals of resveratrol were noticed, indicating that there was not presence of non-encapsulated crystals of resveratrol and that the encapsulated resveratrol is on amorphous state inside the particles. Among the other encapsulating materials, lecithin showed the highest intensity of crystalline resveratrol peaks, meaning worse encapsulation capacity for resveratrol. However, when it was mixed with any of the β-glucans, the crystallinity of the particles was reduced (Fig. 5).

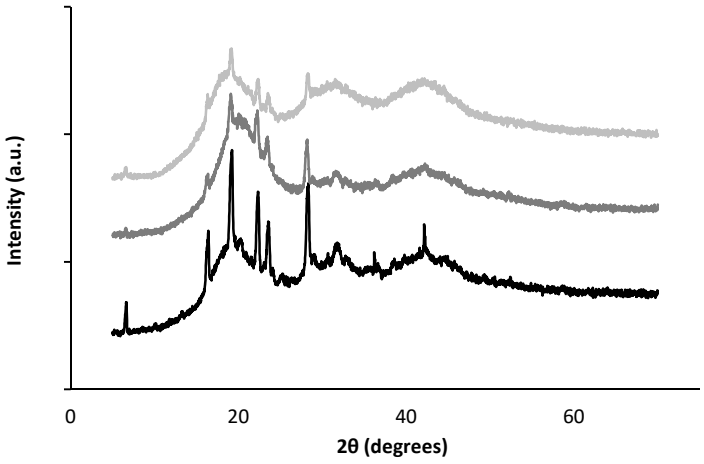

Figure 5. XRD patterns for the suspensions of resveratrol obtained by high-pressure with lecithin (black), yeast b-glucan (light gray) and a mixture of lecithin and yeast b-glucan (dark gray).

Thus, it was found an interaction of β-glucans and resveratrol, which diminishes the formation of crystals during emulsification, although this effect is mitigated when lecithin is also applied. This complexation was previously noticed by (Wu et al., 2008) between calcofluor and oat β-glucan, through the formation of hydrogen bonding between the hydroxyl groups of both compounds and afterwards Van der Waals interactions. Also (Wu et al., 2011) reported the

formation of hydrogen bonding between oat β-glucan and tea polyphenols composed mainly of (-)-epigallocatechin gallate, (-)-gallocatechin gallate and (-)-epicatechin gallate.

3.2.3 Encapsulation efficiency

Results of encapsulation efficiency for the emulsions at 20 g/L of encapsulating material are shown on Table 1. In all cases, the concentration of resveratrol in the suspensions ranges from 550 to 800 ppm, except with BBG, that has between 240 and 440 ppm of resveratrol. Analysis of the centrifuged suspensions after one year revealed that up to 40% of the encapsulated resveratrol was still well preserved in the formulation.

Encapsulation efficiency is much lower with BBG because during centrifugation a gel-like structure was formed and it retained most of the compounds. No significant differences were observed between each emulsification method with any of the encapsulating materials, although in some previous works much higher encapsulation efficiencies were achieved by high pressure and temperature emulsification than by high-shear emulsification in the formulation of β-carotene with modified starch (de Paz et al., 2013). However, in that case the increase was due to the higher solubility of the active compound in the hot organic solvent, whereas in this work the concentration of resveratrol in the organic phase was kept constant in all the experiments.

3.3. B. cinerea culture

For the samples by high-shear emulsification, no significant differences were observed between each product at the lowest concentrations of surfactant (10 and 15 g/L), since none of them had effect on the fungal growth (results not shown). However, some inhibition was achieved with the suspensions at 20 g/L of encapsulating material, also for the samples by high-pressure and high-pressure and temperature emulsification. Figure 6 presents fungal growth area when applying the suspensions of resveratrol formulated with lecithin (Lec), a mixture of lecithin and BBG (LecBBG), BBG, a mixture of lecithin and YBG (LecYBG) and YBG. The results shown here

correspond to the centrifuged suspensions except in the case of BBG. These had much lower encapsulation efficiency, so higher quantity of sample was required to perform the analysis and therefore there was interference in the growth of the fungus due to the dilution of the agar. Therefore, the bars named BBG in Figure 6 correspond to the suspensions without centrifugation.

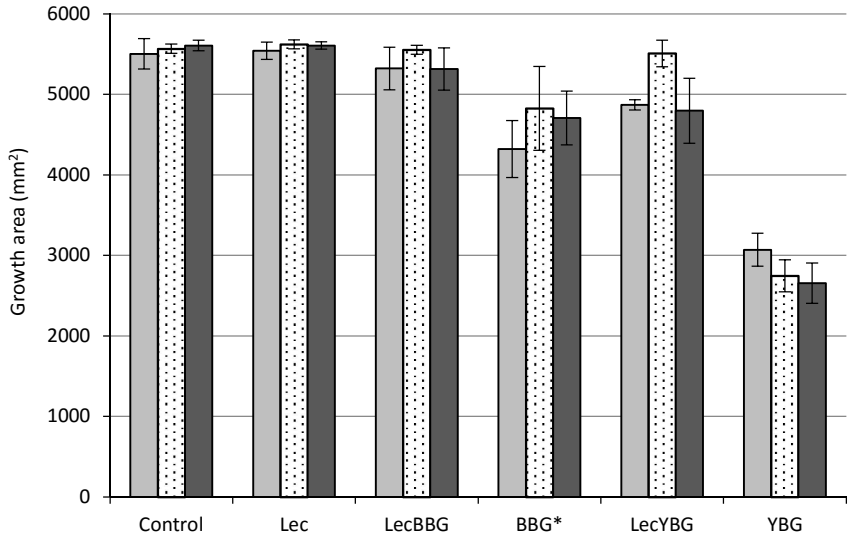

Figure 6. Growth area of B. cinerea for the centrifuged suspensions of resveratrol. Light gray: high-shear emulsification. Dots: high pressure emulsification. Dark gray: high pressure and temperature emulsification. *: suspensions without centrifugation.

The formulation of resveratrol with lecithin and with the mixture of lecithin and BBG had no effect on fungal growth, although some inhibition was achieved with BBG without mixing (up to 23%). The greatest growth reduction corresponded to the suspensions of YBG, ranging the inhibition between 44 and 53%. However, this effect was almost unnoticeable for the mixture of YBG with lecithin: no inhibition was observed by high pressure emulsification, and up to 15% for the other methods. Except in this case, no significant difference was noticed between each emulsification method regardless of the encapsulating material. Pure resveratrol did not produce any inhibition of fungal growth.

According to these results, the presence of β-glucans in the formulation enhances the fungicide action of resveratrol with respect to the formulation with lecithin. Varona et al., 2013 studied the penetration of liposomes formed by lecithin into cell membranes of different bacteria. In that work, liposomes showed ability to cross cell walls when they are constituted by phospholipid layers, as happens in gram-negative bacteria. However, they could not cross into the cell when there were some other barriers, as in the case of the gram-positive bacteria. *B. cinerea* has a two-layer cell wall composed of chitin and β-glucans (Tenberge, 2007). It is possible that liposomes could not penetrate into that barrier and deliver resveratrol inside, and thus the formulations with lecithin had no antifungal effect. On the contrary, resveratrol is better absorbed within the cell wall of the fungus when it is encapsulated in β-glucans, especially YBG, which contains the same β-glucan type as the fungal cell wall (Zhu et al., 2016), so the inhibitory effect on fungal growth is bigger. Nevertheless, the inhibitory effect achieved with the liquid formulations was not comparable to the one obtained with solid particles of the same products reported in a previous work (Salgado et al., 2015). Some studies reported an elicitor effect of β-glucans on the production of resveratrol by some plants, like *Vitis vinifera* (Vuong et al., 2014). Thus, it is possible that when the formulations with β-glucans are applied *in vivo*, the concentration of resveratrol increases, and this could cause a greater fungal growth inhibition.

4. CONCLUSIONS

A liquid formulation of resveratrol was developed and tested against *B. cinerea*, by three different emulsification methods: high-shear, high-pressure and high pressure and temperature emulsification. As encapsulating materials, BBG and YBG were used, both alone and mixed with lecithin. YBG showed good encapsulating properties regarding encapsulation efficiency (74-84%), besides providing 50% inhibition of fungal growth, the best for all the products tested. On the contrary, BBG were not as good as encapsulating material because their chains entangle and

form a net instead of single droplets, thus having low encapsulation efficiency (31-54%). Also they showed lower antifungal activity (around 20% inhibition). The formulation with lecithin also showed high encapsulation efficiency and small particle size, although it did not inhibit fungal growth. When mixing β-glucans with lecithin, a reduction on particle size was observed for BBG. However, the mixing also induces the formation of crystals of resveratrol inside the encapsulated particles in comparison with β-glucans alone. Therefore, it was concluded that the formulation of resveratrol with β-glucans improved the action against *B. cinerea*, probably through an enhanced absorption of the active compound by the fungus. Comparing the emulsification methods developed in this work, there were not big differences between them on encapsulation efficiency and particle size. Nevertheless, by high pressure and temperature emulsification, amorphous resveratrol was obtained inside the particles, whereas it formed crystals with the other methods. Despite this, the effect on the reduction of fungal growth was similar by all the emulsification methods for each material, without significant differences between them. As β-glucans appear as a promising effective encapsulating material to improve the antifungal activity against *B. cinerea*, the formulations could be developed with other polyphenols in future works. It would be also interesting to assess if they are also effective against other fungal species.

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CHAPTER III:

DEVELOPMENT OF BARLEY AND YEAST β-GLUCAN AEROGELS FOR DRUG DELIVERY BY SUPERCRITICAL FLUIDS

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ABSTRACT

Polysaccharide aerogels are a good alternative as carriers for drug delivery, since they allow high loading of the active compounds in matrices that are non-toxic, biocompatible and from a renewable feedstock. In this work, barley and yeast β-glucans aerogels were produced by gelation in aqueous solution, followed by solvent exchange and drying with supercritical CO_2. First, viscoelastic properties and melting profile of the hydrogels were determined. Then, the obtained aerogels were analyzed regarding morphology, mechanical properties and behavior in physiological fluid. Both in the hydrogels and in the aerogels, big differences were observed between barley and yeast β-glucans due to their different chain structure and gelation behavior. Finally, impregnation of acetylsalicylic acid was performed at the same time as the drying of the alcogels with supercritical CO_2. The release profile of the drug in PBS was analyzed in order to determine the mechanism governing the release from the β-glucan matrix.

Keywords: β-glucan, aerogel, supercritical impregnation, supercritical drying, rheology, acetylsalicylic acid

1. INTRODUCTION

Aerogels are solid materials possessing low density, high porosity and high surface area. These properties allow their use in a wide variety of applications, from hydrogen storage (Rueda et al., 2014) to tissue engineering (Quraishi et al., 2015). Among them, aerogels are of particular interest in drug delivery of active compounds, since they offer higher loading capacity due to their surface properties (García-González et al., 2011; Ulker and Erkey, 2014).

Aerogels are formed from an initial gel on aqueous phase which undergoes a drying process. Traditional drying methods, such as air-drying or freeze-drying, produce unwanted changes in the structure of the gel, leading to great shrinkage or even destruction of the network. On the contrary, drying with supercritical fluids avoids network collapse due to the absence of liquid-gas interfaces, so the porous structure is better preserved (García-González et al., 2012). Besides, with supercritical fluids, incorporation of active compounds into the aerogel can be done simultaneously to the drying process, thus reducing processing steps and avoiding the use of organic solvents and high temperatures associated to the preparation of drug-loaded delivery systems (Champeau et al., 2015). The performance of the impregnation in supercritical fluids allows good solubility of the active compounds and diffusion through the matrix, and at the same time the structure of the matrix is well preserved. After the impregnation and upon depressurization, the final product is recovered free of any solvent and no further purification steps are required. Furthermore, supercritical impregnation enhances the penetration of the active compound into the polymeric matrix, providing a homogeneous distribution of the drug in the material (Masmoudi et al., 2011).

To fulfill the requirements of low toxicity, biodegradability and stability for drug delivery applications, polysaccharides are a good option as carriers (Maleki et al., 2016). Many works report the production of aerogels using starch, alginate or chitin (Mehling et al., 2009; Mustapa et al., 2016; Muzzarelli, 2011). However, β-glucans have been barely studied for this purpose.

To the author's knowledge, only Comin and coworkers produced aerogels exclusively with barley β-glucans (Comin et al., 2012a). They observed that supercritical-dried β-glucan aerogels had lower density and more homogeneous structure than the ones air-dried and freeze-dried. They also analyzed the supercritical impregnation of flax oil in the aerogels (Comin et al., 2012b).

β-glucans are polymers formed by D-glucose monomers linked by β-glycosidic bonds. They can be found in cereals, algae, yeast or bacteria, with very different structures and characteristics. β-glucans have some valuable features regarding processing, i.e. they increase the viscosity of solutions and can easily form hydrogels by heating (Lazaridou et al., 2003; Liu et al., 2016b). Several works studied the ability of β-glucans to form gels by hydrogen bonding in junction points (Lazaridou and Biliaderis, 2007; Tosh et al., 2004). However, great differences are observed depending on the origin of the β-glucans, their structure, molecular weight or concentration. For instance, hydrogels are more easily created with low molecular weight β-glucans (from 20 kDa) because they enhance entanglement of polymer chains, whereas high molecular weight β-glucans (above 200 kDa) have less mobility and thus worse gelling capacity (Brummer et al., 2014; Tosh et al., 2004).

β-glucans are being used for medical and pharmaceutical applications due to their interesting biological properties, such as wound healing ability, modulation of the immune system, anti-inflammatory or anti-bacterial properties (Chen and Seviour, 2007; Du et al., 2014). In this sense, the European Food Safety Authority has recognized the ability of oat and barley β-glucans to lower blood cholesterol and thus reduce the risk of heart disease (Efsa Panel on Dietetic Products and Allergies, 2010). Apart from the aforementioned biological properties, some works report the protective effect of different β-glucans in oral drug delivery. On one hand, they protect the stomach against the formation of ulcers derived from intake of some drugs (Ozkan et al., 2010; Suchecka et al., 2016). Further, cereal β-glucans enhance the growth of probiotics in the digestive tract (Lam and Chi-Keung Cheung, 2013). On the other hand, β-glucans also

protect the encapsulated active compounds through the acidic gastric medium to reach undamaged the adsorption sites in the intestine (De Smet et al., 2013; Shah et al., 2016). Also β-glucans from cereals and from fungi are reported to improve the permeability of active compounds through the skin into deeper layers (Klein, 1999; Röding, 2006).

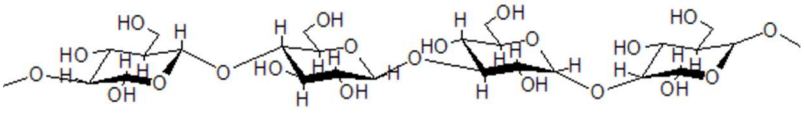
In this work, β-glucan aerogels are produced by supercritical drying in CO_2. Aerogels were characterized and their potential as drug delivery systems was evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Gels were produced from 2 types of β-glucans (Fig. 1): barley (1-3, 1-4)-β-glucans (BBG, 75% purity, 125 kDa determined by size exclusion chromatography as indicated in (Salgado et al., 2015), with β-glucan standards by Megazyme ranging from 40 to 359 kDa); Glucagel, kindly supplied by DKSH, France) and (1-3, 1-6)-β-glucans from yeast *Saccharomyces cerevisae* (YBG, 64% purity, measured with β-Glucan Assay Kit (Yeast & Mushroom), by Megazyme; L-Naturae Nutraceutical, kindly supplied by Naturae, Spain). Acetylsalicylic acid (Sigma, Portugal) was used as model active compound for the impregnation of the aerogels. PBS (pH 7.4) was prepared from tablets (Sigma). Carbon dioxide (99.998 mol%) was supplied by Air Liquide (Portugal). All reagents were used as received.

a)



b)

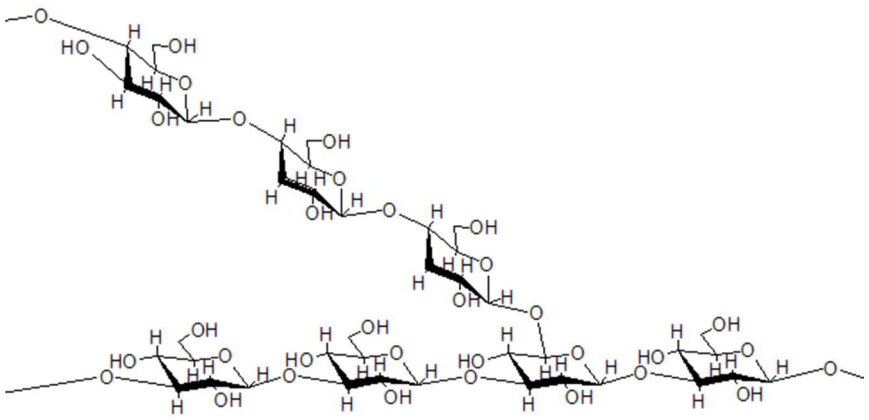


Figure 1. Structure of barley (a) and yeast (b) β-glucans.

2.2. Production of β-glucan aerogels

Fresh solutions of 4 and 5% w/w BBG were produced by mixing the β-glucans with water at 80ºC for 2h with stirring, until complete dissolution. Solutions with concentration lower than that were not able to create a gel. When it was completely dissolved, it was boiled for 5 minutes, and then kept at 75ºC for 1 hour. The hot solution was poured into 96-well plate molds and kept overnight at 4ºC to form the gel. Longer time periods, up to 72h, were required as the polymer concentration decreased. YBG was dispersed in water under stirring for 30 minutes. 5 and 2.5% w/w gels were obtained after heating in a water bath at 90ºC for 1 hour.

Gels samples were taken out from the mold and cylinders with 5 mm diameter and 10 mm height were obtained. Hydrogels were converted into alcogels by subsequently immersing them in 20, 40, 60 and 80% v/v ethanol:water baths for 1.5 h each, and kept in pure ethanol overnight. Then they were dried with supercritical CO_2 in a critical point drier (Autosamdri-815, Tousimis).

2.3. Supercritical impregnation of acetylsalicylic acid

In order to minimize processing steps, impregnation of 4% (w/w) BBG alcogels with acetylsalicylic acid was performed simultaneously to the drying of the alcogels with supercritical CO_2. The alcogels were placed on a high-pressure cylinder immersed on a water bath. At the

inlet, AA was placed on excess and separated from the alcogels with cotton to prevent physical contact with the alcogels. Carbon dioxide was first cooled and pumped (Haskell, MCPV-71) to the desired pressure, and then fed into the high-pressure vessel. A continuous flow of CO_2 was maintained for 1.5 h, which is the time necessary to completely remove ethanol from the structures. Due to the configuration of the drug and the polymer in the vessel, AA was first dissolved on sc-CO_2. When the saturated flow of CO_2 contacted the BBG matrices, they were impregnated with AA. Impregnation yield was determined at different conditions of pressure (8, 12, 16 and 20 MPa) and temperature (35, 40 and 50ºC). Further information about the equipment can be found in the literature (Costa et al., 2010; Duarte et al., 2006b).

2.4. Rheological tests

Viscoelastic properties of β-glucan hydrogels were evaluated on a Kinexus Prot Rheometer (Kinexus Prot, MAL1097376, Malvern) fitted with a parallel plate geometry with 10 mm of diameter (PU8 SR2020 SS). Oscillatory measurements were performed at 1% strain in a range on frequency of 0.01-100 Hz at 25ºC in order to obtain the elastic (G') and loss (G") moduli and the complex viscosity. The thermal stability and the melting behavior of the gels was analyzed in hydrogels cured for 24h, through a temperature ramp with controlled frequency and strain, at 1 Hz, 1% strain and heating rate of 2.5ºC/min. All measurements were performed in triplicate.

2.5. Morphological analysis

The produced aerogels were observed by scanning electron microscopy (SEM) with a high-resolution field emission scanning electron microscope with focus ion beam (Auriga Compact, Zeiss). The aerogels were cut in liquid nitrogen and the sections were placed by mutual conductive adhesive tape on aluminum holders and covered with gold palladium using a sputter coater.

Nitrogen adsorption-desorption isotherms were performed with ASAP 2020 (Micromeritics) to obtain surface area and pore size and volume of the aerogels. Prior to analysis, the samples were degassed at 115ºC for 4h.

Aerogel density was determined with a helium pycnometer (Micromeritics Accupyc II 1340) at 25ºC from 10 replicates (standard deviation lower than 0.5%).

2.6. Mechanical analysis

Behavior of the aerogels under compression stress was analyzed with a universal testing machine (Instron 5540). Compression of the material was carried out at 1 mm/min until the height of the sample was reduced by 70%. The compressive Young modulus was determined as the initial slope in the stress-strain graphs. Aerogels were tested also after rehydrating them in PBS for 2 h, to mimic a physiological environment. In this case, it was also possible to obtain the maximum stress that can be applied until break of the material. The tests were performed in triplicate and the results are presented as the average ± standard deviation.

2.7. Water uptake and degradation test

Aerogel samples were placed in 5 mL PBS, and immersed in a stirred water bath at 37ºC. At different time points (1, 7, 14 and 21 days), samples were taken out (excess water was removed with paper) and weighted.

Water uptake was determined using the following equation:

$$\%\ \text{water uptake} = \frac{w_w - w_i}{w_i} \times 100 \qquad [1]$$

where w_w is the weight of the wet sample and w_i is the weight of the initial sample.

Afterwards, the wet aerogels were changed to ethanol and dried with supercritical CO_2, as indicated in section 2.2, to ensure the complete drying of the matrix. Once the samples were

dried, they were weighted (w_f) to determine the weight loss, which was calculated according to the equation:

$$\%\ \text{weight loss} = \left| \frac{w_f - w_i}{w_i} \right| \times 100 \qquad [2]$$

Water uptake and degradation test was performed in triplicate, and up to 21 days.

2.8. Impregnation yield

The amount of AA impregnated in the aerogels was quantified by UV-Vis at 290 nm using a microplate reader (Synergy HT, Bio-Tek Instruments, USA) in a quartz microplate with 96 wells (Hellma). First, the aerogels were completely dissolved in 5 mL PBS to ensure that all the AA was extracted from them. Then, a sample of the liquid was analyzed by UV-Vis, and the absorbance was adjusted into a calibration curve between 0 and 1 g/L. The influence of BBG on the measured absorption was taken into account in the calculations by measuring the absorption obtained with an aerogel without AA and subtracting this value to the results of the absorbance of the samples with AA.

2.9. In vitro release study

The impregnated aerogels were placed on 5 mL PBS in a bath at 37ºC and 60 rpm. Samples (150 µL) of the liquid medium were taken out at different time points (5, 10, 15 and 30 min, and 1, 2, 3, 4, 5, 6, 7, 8 and 24 h), and replaced by the same quantity of fresh PBS. The amount of AA on the PBS at each time was measured by UV-Vis spectrophotometry as mentioned before. The replacement of the aliquot with fresh PBS was taken into account in the calculations of the cumulative release of AA. All measurements were performed in triplicate.

The kinetics of release of AA was analyzed with the Power Law equation (Eq. 3) (Ritger and Peppas, 1987):

$$M_t / M_\infty = kt^n \qquad [3]$$

Where M_t is the cumulative quantity of AA released at time t, M_∞ is the theoretical amount released at infinite time (maximum AA in the aerogel), k is a constant characteristic of the drug-polymer system and n is the diffusional exponent characteristic of the release mechanism.

3. RESULTS AND DISCUSSION

3.1. Rheological study of β-glucan hydrogels

The variability on origin and chain structure between BBG and YBG leads to different behavior of both β-glucans. For instance, as it was mentioned before, BBG are soluble in water, but YBG are not. This has consequences on the gelling mechanism and chain organization of the polymer in the gels, and reflects on the production of hydrogels with different properties depending on the type of β-glucan used. The differences in the structure of the hydrogels will ultimately have influence also on the structure and properties of the final aerogels.

Figure 2 shows the response of the different β-glucan hydrogels over a frequency range. It can be observed that for all of them the elastic gel network is maintained in a wide range of low frequencies, characterized by higher G' than G" (solid-like behavior), and both with values independent of the frequency. However, at higher frequencies, G' and G" become equal and increase with frequency, revealing the rupture of the gel structure.

Although both β-glucans create the gel structure through junction zones due to hydrogen bonding (Lazaridou et al., 2015; Zheng et al., 2016), these are much stronger in YBG (Liu et al., 2016a), and thus they resist oscillatory stress up to higher frequency (up to 4 Hz for 5% BBG, and up to 10 Hz for YBG). For all samples, at higher frequency, i.e., higher than 10 Hz, rupture of the gels occurred. Up to these values, the gels were able to rearrange their chains around the junction points and resist shear stress, but when the frequency was further increased the junction points were damaged and the gel was broken. This behavior was also confirmed with

the results of complex viscosity (Fig.3a). Complex viscosity decreases with frequency for both β-glucans as a consequence of the rearrangement of the gels up to the values of frequency aforementioned where the structure of the gel was destroyed. Nevertheless, at low frequency, complex viscosity is higher for YBG than for BBG, indicating more interaction between polymer chains in the YBG hydrogel.

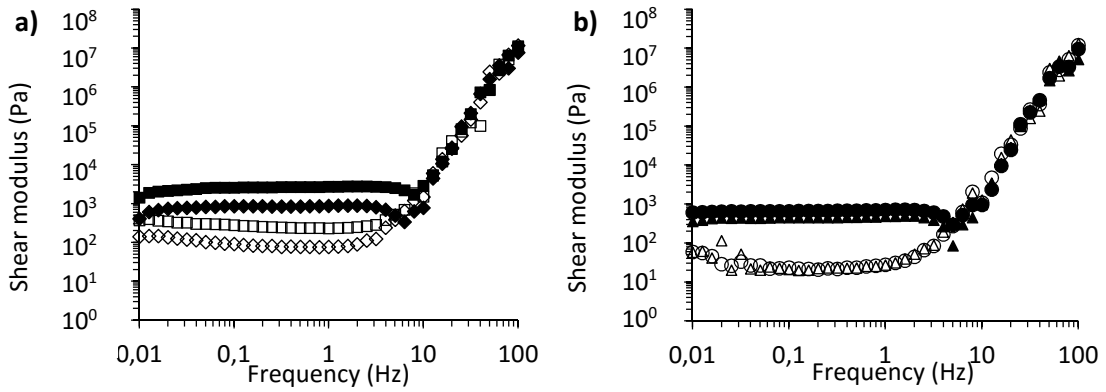


Figure 2. G' (closed symbols) and G" (open symbols) of (a) 2.5% YBG (◆), 5% YBG (■) and (b) 4% BBG (●) and 5% BBG (▲) hydrogels over a range of frequency in oscillatory measurements.

G' increased with the concentration of YBG, indicating more elastic and more stable gel network at higher β-glucan concentration, probably due to more junction points with more quantity of polymer. This difference with concentration was not so noticeable for BBG because the range of β-glucan concentration tested was smaller. Gels of 4% BBG had almost the same moduli as 5% BBG, with the values of G' slightly higher (results not shown). At 5% β-glucan concentration, BBG had much smaller G' than YBG, indicating less elasticity of the gel. Some authors reported that molecular weight of the β-glucan has a great influence on their viscoelastic properties (Brummer et al., 2014). It is also possible that BBG polymer chains are more rigid than those of YBG, because the (1-3, 1-6)-β-glucan structure of the latter induces more voiding space between the chains, which allows better movement and rearrangement of the chains and thus more elasticity of the gel.

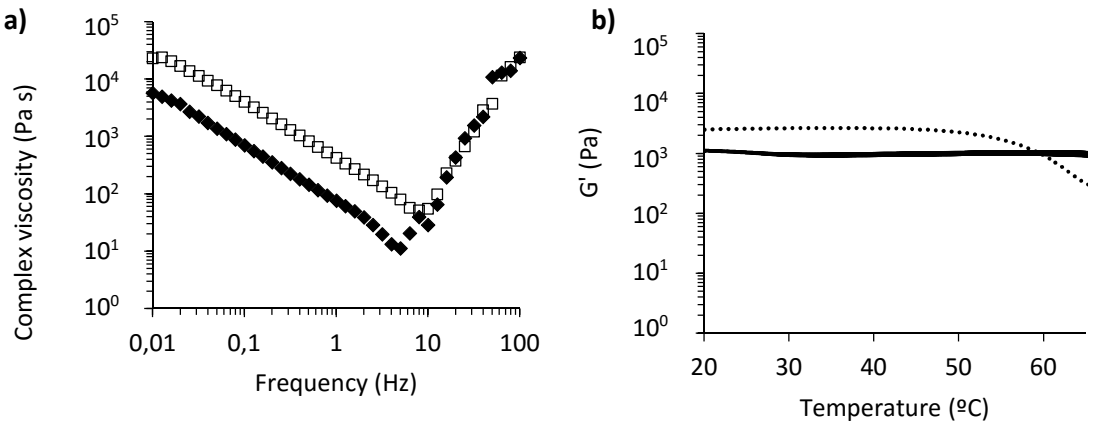


Figure 3. (a) Complex viscosity for 5% (w/w) BBG (closed symbols) and YBG (open symbols). (b) Melting profile of 2.5% YBG (line) and 5% BBG (dots) hydrogels at 0.1% strain, 1 Hz and heating rate 3ºC/min.

The stability of the hydrogels was tested following the mechanical properties as a function of temperature, in a range from 20-70 ºC. Higher temperatures were not tested as water started to evaporate at this point, leading to the solidification of the sample. By heating the hydrogels (Fig.3b) it was observed that G' started to decrease at around 50-55ºC for BBG, reflecting a loosening in the chain entanglements. This point marks the beginning of the melting of the gel. However, G' was kept constant for YBG over the range of temperature tested.

According to this result, the formation of BBG gel is a temperature-reversible process, while the gelling is irreversible for YBG. This is in accordance with the stronger hydrogen bonds in YBG hydrogel aforementioned.

3.2. Morphological characterization of β-glucan aerogels

β-glucan aerogels were successfully dried with supercritical CO_2, preserving the structure without shrinkage during the drying process. However, the gels underwent a noticeable shrinkage during solvent exchange, especially YBG gels, because they were formed from an aqueous suspension instead of a solution. Whereas BBG formed a packed structure of polymer layers, YBG had more free space between the junction points. Thus, when solvent was changed

from water to ethanol, the organization of the network was better maintained with BBG than with YBG.

All of the formed aerogels had a compact network, although the ones with lower concentration presented a more porous structure (Figure 4). Nevertheless, the matrix was thicker with YBG, while BBG aerogels had more spongy-like structure. The same effect of the concentration and type of β-glucan was also observed by the analysis of density (Table 1). When the concentration of polymer increased, the density of the aerogels also increased, especially in the case of YBG, which had a broader concentration range. Similarly, at 5% β-glucan, density was higher for YBG than for BBG, in accordance with the observation of thicker structure by SEM. These results were further confirmed by the mechanical tests. This might be a consequence of the different behavior of both β-glucans during hydrogel formation and solvent exchange from water to ethanol.

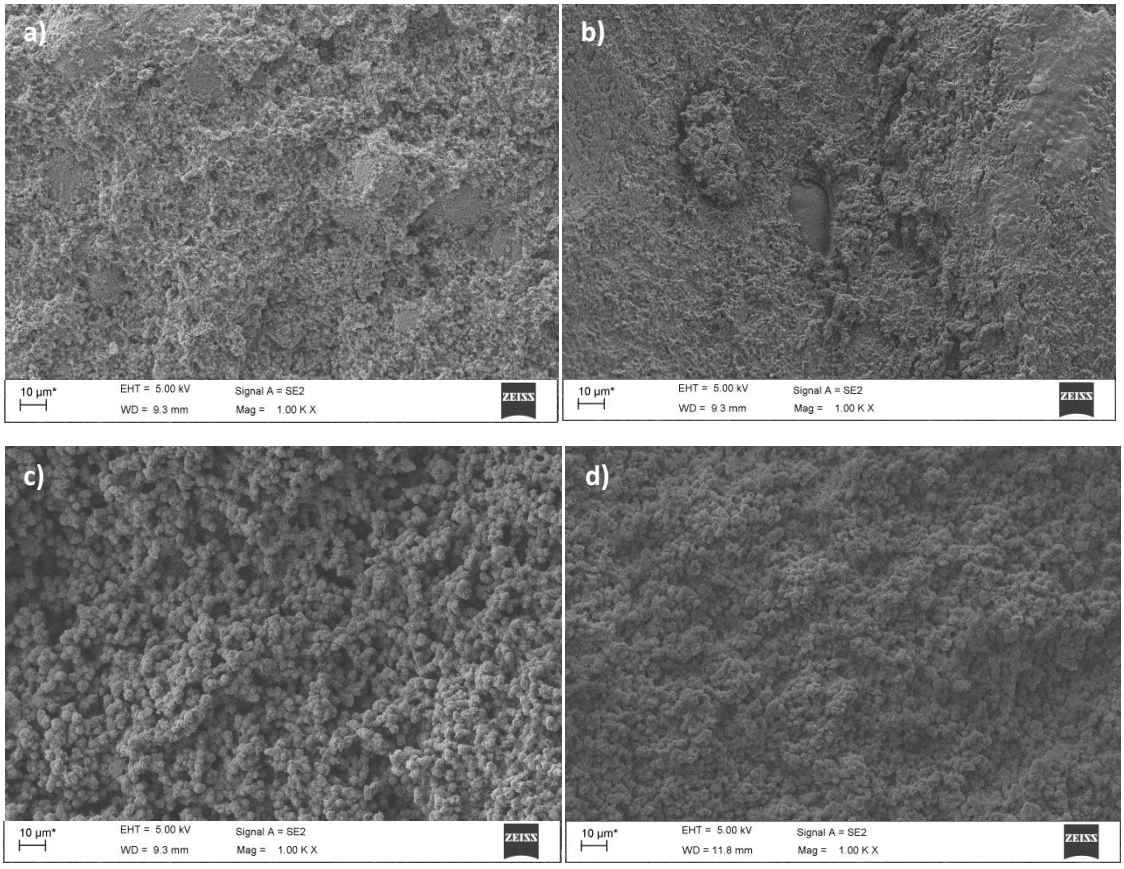


Figure 4. SEM images of 2.5% (a) and 5% (b) YBG aerogels, and 4% (c) and 5% (d) BBG aerogels.

Surface area, pore volume and pore diameter were higher for BBG than for YBG aerogels, although with slight differences between them at the same concentration (Table 1). For each type of material, differences with the concentration could only be noticed for YBG, since the range tested was bigger. The values obtained in this work are in the range of the ones obtained with BBG by (Comin et al., 2012a), except pore size, which was 2.7 nm. The cause of this difference can be associated to the higher pressure of CO_2 that they used to dry the gels, which could reduce the size of the pores and create a more uniform distribution, similarly to the changes in pore size with pressure observed in polymer foaming (Reverchon and Cardea, 2007). Also, depressurization rate is one of the parameters that influences pore size on supercritical drying of aerogels (Martins et al., 2015).

Table 1. Structural properties of the different β-glucan aerogels.

Sample	BET surface area (m^2/g)	Pore volume (cm^3/g)	Pore size (nm)	Density (kg/m^3)
2.5% YBG	173.1	0.563	13.7	34.8
5% YBG	178.2	0.659	15.5	121.1
4% BBG	189.4	0.713	15.8	69.0
5% BBG	184.1	0.705	16.1	79.3

As an example, figure 5 shows the adsorption-desorption isotherm and pore size distribution of the sample of 5% YBG. The shape of the adsorption-desorption isotherms corresponds to a type IV isotherm, according to the IUPAC classification. This type of isotherm is characterized by a hysteresis loop, which is produced due to condensation in the capillaries. The initial part of the isotherm indicates that first there is monolayer adsorption, and, after a plateau, multilayer is formed (Lowell and Shields, 1984). Type IV isotherm is typical of mesoporous materials (Romero et al., 2016), and has also been observed in some other works with polysaccharide aerogels (Comin et al., 2012a; Quignard et al., 2008). All samples had unimodal pore size distribution, although the peak was centered in 8-10 nm in the case of YBG and 18-22 nm for BBG.

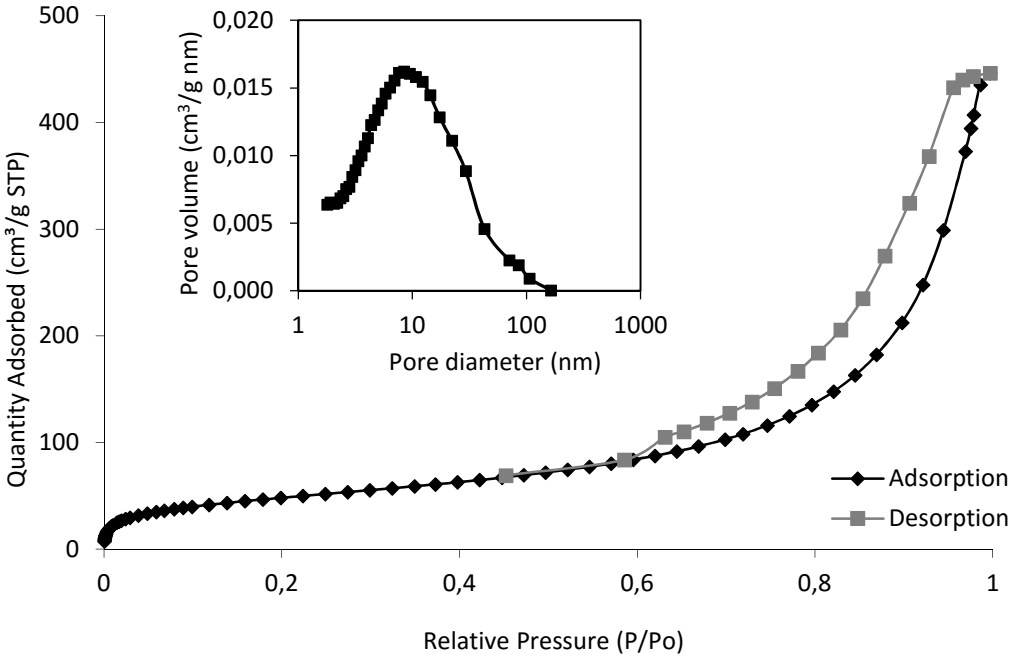


Figure 5. Nitrogen adsorption-desorption isotherm and pore size distribution of 5% YBG aerogel.

3.3. Mechanical properties

The resistance of the aerogels to compression stress has shown to be highly dependent not only on the concentration, as it would be expected, but also on the type of β-glucan. YBG had values of compressive Young modulus almost 1-fold higher than BBG (Table 2). The linear polymer chains of BBG arranged parallel one to each other, and this kind of structure is less resistant. On the contrary, the crosslinked structure of YBG chains allowed the achievement of higher Young modulus, and thus stronger material. With both β-glucans, Young modulus increased with polymer concentration. After rehydration of the aerogels on PBS, Young modulus was greatly reduced in all cases. Although all the materials produced had low stiffness, the values are in the range of those found for other polysaccharides aerogels such as alginate, lignin or starch (Martins et al., 2015; Quraishi et al., 2015), and are higher than others reported for BBG cryogels (Lazaridou et al., 2015).

Table 2. Compressive Young modulus of dry and wet β-glucan aerogels.

	Young modulus (kPa)	
	Dry	Wet
2.5% YBG	286 ± 51	0.38 ± 0.07
5% YBG	448 ± 107	0.36 ± 0.08
4% BBG	58 ± 14	0.21 ± 0.03
5% BBG	69 ± 21	0.27 ± 0.08

The different behavior of the dry and wet aerogels can be noticed by observation of the stress-strain curves. On one hand, dry aerogels had a linear region which corresponds to elastic deformation, and after some point plastic deformation occurred. In these cases, yield strain was between 10-15%. On the other hand, the wet samples had a region of elastic deformation up to higher strain (20-30%), but afterwards they collapsed instead of suffering plastic deformation. Figure 6 shows, as an example, the stress-strain curves of dry and wet samples of 5% YBG. Maximum stress at failure of the wet aerogels was in the range between 5 and 13 kPa. YBG were able to bear higher load than BBG, and also the resistance increased with the concentration of polymer. This is in agreement with the rheological behavior of the hydrogels, which revealed higher resistance to shear stress with YBG rather than BBG.

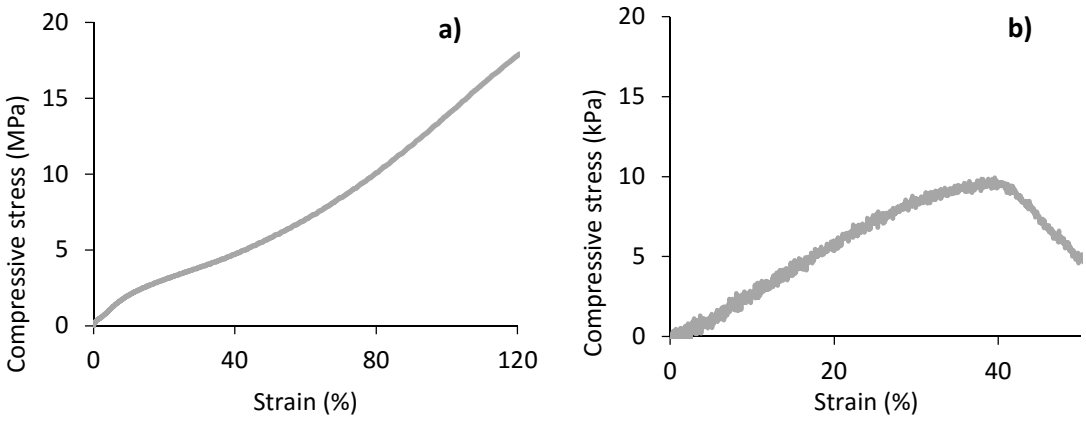


Figure 6. Stress-strain curves for 5% YBG aerogels dry (a) and rehydrated (b), under compression stress.

3.4. Behavior on physiological fluids

Water uptake has a strong influence in the release of active compounds from the matrix: if it absorbs much water, drugs can diffuse more easily to the liquid medium. To the author's knowledge, there are not previous studies about water uptake capability of β-glucan aerogels, although it has been reported for freeze-dried β-glucans. For instance, Lazaridou and coworkers found that swelling and equilibrium water content of BBG lyophilized cryogels decreased with higher β-glucan molecular weight because of a more compact and less porous structure (Lazaridou et al., 2015).

Upon soaking on physiological fluid, it was easily observed that β-glucan aerogels swelled. In all samples, the maximum water uptake was reached after 24h in a physiological solution and it remained around that value without significant differences for the rest of the days (Figure 7). Besides, weight loss was lower than 20% in all cases after 21 days. This high water uptake capacity is related to the hydrophilicity of the β-glucans. Also, some previous works also reported a fast and high water uptake by other polysaccharide aerogels (Quraishi et al., 2015).

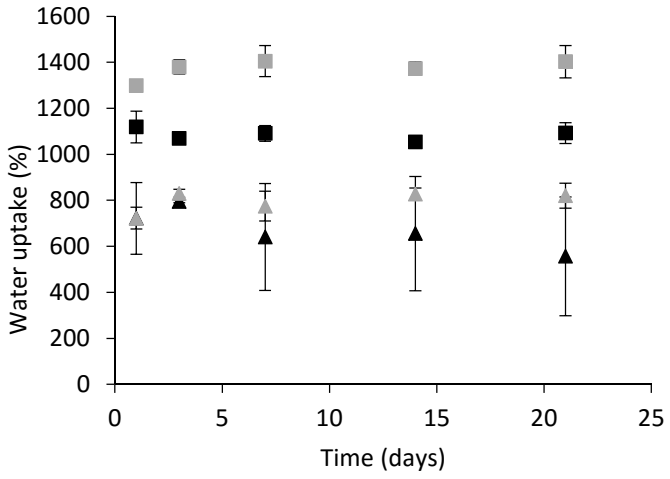


Figure 7. Water uptake of the different β-glucan aerogel samples up to 3 weeks. Squares: YBG. Triangles: BBG. Grey: low β-glucan concentration. Black: high β-glucan concentration.

It was observed that, for each β-glucan, water uptake was higher with lower concentration of polymer. This is in agreement with some previous works reporting slower and smaller swelling of polysaccharide aerogels with higher polymer concentration due to the presence of more chain entanglements (Bilanovic et al., 2016; De Cicco et al., 2016). For 5% w/w β-glucan, water uptake was much higher for YBG.

3.5. Supercritical impregnation of acetylsalicylic acid

Supercritical impregnation of active compounds is composed by 2 main steps. The first one is the dissolution of the active compound in sc-CO_2, which depends on its solubility in CO_2 (and, ultimately, on the density of CO_2). The second step is the penetration of the active compound in the polymeric matrix, and it is influenced by the diffusion of CO_2 into the structure. Both are highly dependent on the properties of supercritical CO_2, and therefore pressure and temperature are key parameters in this process.

The conditions were chosen such that, there was no effect of temperature and pressure studied for the impregnation on the morphological parameters of the aerogels produced. The amount of AA impregnated in the aerogels increased with pressure (Fig. 8). However, the influence of the temperature is not so straightforward. At low pressure (below 12 MPa), impregnation yield is higher at lower temperature. Between 12 and 16 MPa, there is a crossover so that, at higher pressure, impregnation yield increases with temperature. Nevertheless, drug loading in the aerogels was in the range between 8 and 15% (w/w) in all cases. These values are higher than typical loadings of AA by supercritical impregnation reported in other previous works with different matrices (below 4%), even though the impregnation conditions were more severe in those works (Domingo et al., 2002; Duarte et al., 2006a). However, the impregnation yield achieved in our work is comparable to that obtained for the impregnation of ketoprofen in other polysaccharides, namely alginate and starch, with similar processing conditions (Barros et al., 2015; García-González et al., 2012).

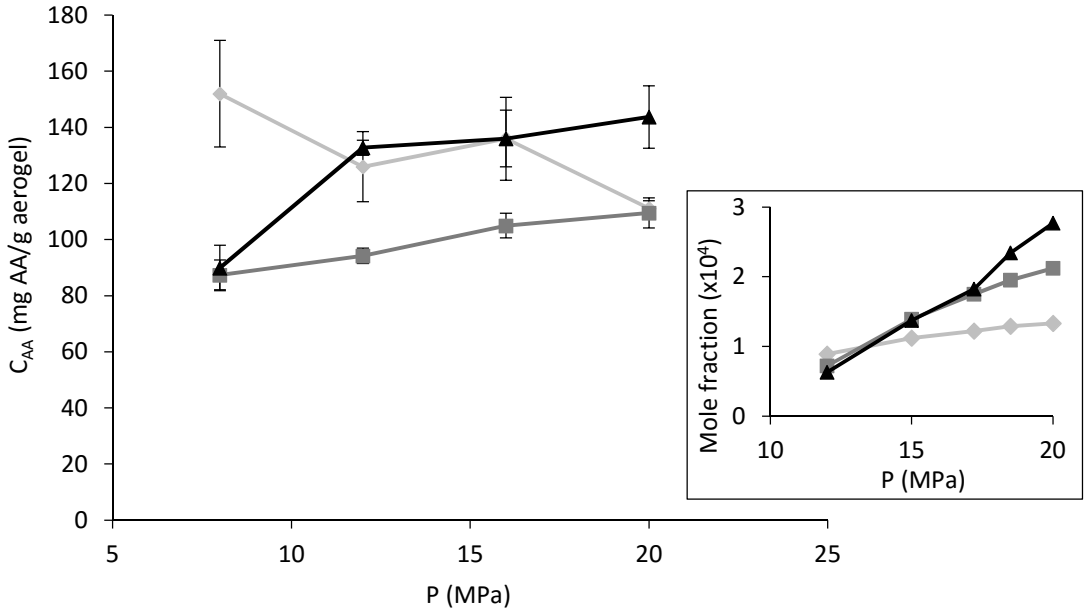


Figure 8. Quantity of AA impregnated per mass of aerogel (4% BBG) at different pressure and temperature. Black: 50ºC; Dark grey: 40ºC; Light grey: 35ºC. Frame: Solubility of AA on CO_2, adapted from (Huang et al., 2004). Black: 55ºC; Dark grey: 45ºC; Light grey: 35ºC.

The same trend with pressure and temperature was observed for the solubility of AA on sc-CO_2, with a crossover of temperature at around 12.5 MPa (Huang et al., 2004). Thus, supercritical impregnation of AA in BBG aerogels is mainly influenced by the dissolution of AA in supercritical CO_2.

When these results are related to CO_2 density (Table 3), it is observed that the quantity of AA impregnated in the aerogel has an increasing tendency with the density of CO_2, because higher density enhances the solubility. Thus, impregnation yield could be increased by choosing a set of conditions of pressure and temperature that results in high density of CO_2. Besides, higher impregnation load of AA could be achieved with longer processing time, although in this work the flow of CO_2 was maintained for 1.5h because this was the time required for a proper drying of the alcogel.

Table 3. Density of CO_2 and impregnation yield of AA in the scaffolds in the different sets of pressure and temperature.

Temperature (ºC)	Pressure (MPa)	Density CO_2 (kg/m^3)	C_{AA} (mg/g aerogel)
35	8	419.1	152.0
	12	767.1	126.0
	16	827.2	135.9
	20	865.7	111.1
40	8	277.9	87.4
	12	717.8	94.2
	16	794.9	105.0
	20	839.8	109.5
50	8	219.2	89.9
	12	584.7	132.7
	16	722.1	136.0
	20	784.3	143.7

3.6. In vitro release of acetylsalicylic acid

The release of the active compounds from the polymeric matrix is dependent on the water uptake capacity of the polymer and the diffusion of the compound out of the matrix (Duarte et al., 2009). As both things are inherent of each material and independent of the impregnation conditions, we chose just one set of parameters to analyze the release of AA, namely 35ºC and 8 MPa, which was the one that render higher impregnation yield.

Release profile of AA on PBS (Figure 9) shows an initial period (up to 3h) of negligible release (lag time). Afterwards, from 3 to 8 h, AA was released from the BBG matrix up to almost 60% of the total amount, and this quantity was maintained for the next 16h. This fast release after the lag time is related to the high water uptake capacity of the aerogel, even at short times (24h). At release time bigger than 48h, crystallization of AA in the medium took place, leading to its precipitation and, thus, lower absorbance values.

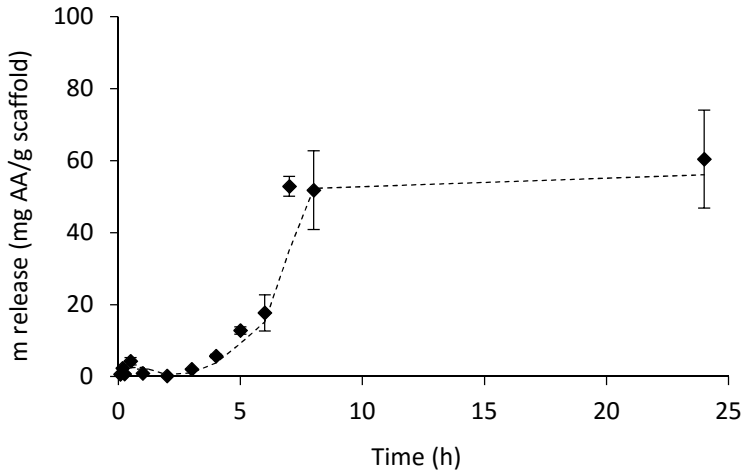


Figure 9. Cumulative release of acetylsalicylic acid per mass of aerogel (4% BBG). Line is added to guide the eye.

The lag time depends on the thickness of the material from the surface to the active compound and not on the impregnated quantity (Kao et al., 1997). Therefore, the initial behavior reveals a good impregnation of AA into the bulk of the β-glucan matrix, instead of being deposited just on the surface (which would be characterized by release of the active compound since the beginning). For the PBS to reach and extract AA to the liquid medium, the matrix must be first well-wetted and swelled. This delayed release can be very interesting in oral delivery, for instance when drug release is supposed to occur after a certain time since administration (Gazzaniga et al., 2008).

In order to analyze the release mechanism in the aerogels, a modification of Eq. 3 introducing the lag time (l) was required (Costa and Sousa Lobo, 2001):

$$M_{t-l}/M_{\infty} = k(t-l)^n \qquad [4]$$

The initial points of release during the lag time were not considered for these calculations, as well as the points corresponding to release higher than 60%. By plotting $Ln(M_{t-l}/M_{\infty})$ versus Ln(t-l), the diffusion exponent *n* obtained was 1.9, with $r^2 = 0.9655$, and k was 0.014 h^{-1}. For a

cylindrical geometry, as it is this case, Eq. 4 can be used in swellable cylindrical matrices (Kosmidis et al., 2003; Park et al., 2011). However, the geometry of the matrix has to be considered in order to analyze the release mechanism governing in the system through the diffusion coefficient *n*. Thus, for cylinders, *n* = 0.45 is indicative of diffusion-limiting release, *n* = 0.89 define pure Case II transport (swelling-controlled release), and values in between represent anomalous release, where both diffusion and swelling influence the release. When *n* is greater than the value of case II, as in this case, the release is said to be super case II transport, with the active compound releasing freely when water penetrates the matrix. This is in accordance with the behavior observed with the release profile: the release started after some initial time in which the aerogel was wetted and relaxation of the chains took place. Once the material was swollen, AA was fast released, without limitations due to diffusion of the active compound through the polymer. Super case II release was also observed for some other polysaccharide matrices, such as carrageenan or alginate, due to relaxation of polymer chains (Baloğlu and Şenyiğit, 2010; Nayak et al., 2016).

4. CONCLUSIONS

Barley and yeast β-glucan aerogels were prepared by supercritical drying of hydrogels, after solvent exchange. Both β-glucans formed different structures due to the differences in their chain configurations: whereas the linear chains of BBG created a more rigid material, YBG arranged in a highly crosslinked configuration, which allowed easier re-arrangement of the chains in the gel network. This difference in the gelation process led to irreversible gelling of YBG, while BBG could be re-dissolved in water. Besides, YBG hydrogels had more stability and elasticity than BBG ones. This also reflected in the characteristics of the aerogels. Although the morphological and structural properties of the aerogels were similar with both β-glucans, YBG had bigger density, were stronger against compression stress, and were able to absorb more

water. Supercritical impregnation of acetylsalicylic acid in BBG aerogels revealed the influence of the process operating conditions on impregnation yield, which increased with the density of CO_2. Release of the drug from the matrix in PBS showed an initial lag time, in which the structure was wetted and relaxation of polymer chains occurred. This delayed release could be an interesting feature for oral drug delivery in cases where a controlled release after a certain time from administration is required, or in order to preserve the active compound through the acid fluids of the stomach to reach undamaged the absorption sites in the intestine. However, a deeper analysis of the dissolution of AA in acidic medium would be required.

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CHAPTER IV:

PREPARATION OF BARLEY AND YEAST β-GLUCAN SCAFFOLDS BY HYDROGEL FOAMING

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ABSTRACT

Porous polymeric materials are being widely studied in tissue engineering, because they can act both as support for cell proliferation and as drug delivery vehicles in the regeneration of new tissues. Hydrogel foaming with supercritical CO_2 is a suitable alternative for the creation of this kind of structures, since it avoids the introduction of organic solvents and the use of high temperature in the processing. In this work, barley and yeast β-glucans were used as renewable raw materials to create hydrogels due to their easily gelation and reported biological properties. The enhancement of porosity was generated by a fast decompression after keeping the hydrogels in contact with supercritical CO_2. Water was further removed by freeze-drying. The effect of the processing conditions and type of β-glucan used in the final properties was assessed regarding morphological and mechanical properties. Although the obtained materials showed good morphological characteristics to be applied in tissue engineering, the mechanical properties did not match those of osteogenic tissue. Finally, the ability of these materials to sustainably deliver dexamethasone was evaluated. The release of dexamethasone from the scaffolds was analyzed and determined to be controlled mainly by diffusion, although there was also some relaxation of the matrix. The scaffolds provided a controlled release of dexamethasone for 4 days, thus being suitable to be used as drug delivery vehicles.

Keywords: β-glucan, supercritical hydrogel foaming, dexamethasone, scaffolds, drug delivery

1. INTRODUCTION

Tissue engineering involves the use of a combination of polymeric scaffolds, cells and bioactive compounds (such as growth factors) in order to mimic the host tissue and provide the necessary cues to induce its regeneration. The aim is to enhance the regeneration of damaged tissue with an implant containing stem or differentiated cells which allow *in situ* growth of new tissue (Duarte et al., 2009b; O'Brien, 2011).

The scaffolds must meet certain requirements to be used in cartilage or bone tissue engineering (Duarte et al., 2013; García-González et al., 2015). They have to be prepared from a biocompatible material that degrades at the same rate as the growth of the tissue, so that it disappears when new tissue is created (Liu et al., 2013). Also they must have similar mechanical characteristics to the tissue they are going to replace, depending on whether it is a soft tissue like cartilage, or a hard and more compact tissue like bone. The mechanical properties of the polymeric scaffolds can be tuned by the addition of ceramic materials (O'Brien, 2011). Not only the incorporation of ceramics like hydroxyapatite or β-tricalcium phosphate improves the mechanical properties but also enhances the biocompatibility and integration of the material in the implant zone (Sharifi et al., 2016). Regarding the structure of the scaffold, macro pores in the order of 150 μm are required. Furthermore, the pores have to be highly interconnected to allow the transport of nutrients and by-products to and from the cells in the matrix, respectively (Okamoto and John, 2013). The scaffolds should not only act as support for cellular growth, but also as drug delivery vehicle for some active compounds. For instance, they could include an anti-inflammatory agent that prevents an undesired inflammatory reaction of the surrounding tissues upon implantation (Duarte et al., 2009b).

β-glucans were chosen in this work as biopolymer for the fabrication of scaffolds. Polysaccharides are widely used with this purpose because they are non-toxic, biocompatible, biodegradable and are obtained from renewable sources in nature. In this context, β-glucans

are a good option, since they are able to create a hydrogel easily by thermal changes. Besides, they offer some interesting biological properties, such as the ability to improve wound healing or modulate the immune system, and their anti-inflammatory and anti-bacterial properties, that increase their potential in medical and pharmaceutical applications. Some previous works explore the use of different β-glucans for tissue engineering. Przekora and coworkers studied the properties of a scaffold composed of bacterial 1,3-β-glucan (curdlan) with chitosan and hydroxyapatite prepared by gelling of the components and air-drying the hydrogels (Przekora et al., 2016). They found out that the addition of β-glucan to hydroxyapatite increased the elasticity and water uptake capacity of the scaffold, suggesting a better adaptation into the implant site, although mechanical strength was decreased (Belcarz et al., 2013). Also curdlan was effectively used for wound dressing in burn injuries (Delatte et al., 2001). Furthermore, regarding cell culture, the presence of β-glucan in the chitosan-hydroxyapatite composite enhanced cell adhesion and growth (Przekora and Ginalska, 2014). Another work also reported an improvement in cell regeneration through a better adhesion and proliferation in membranes of poly(lactic-co-glycolic acid) when they were mixed with 1,3-1,6-β-glucans (Kim et al., 2012).

Several techniques are used to create porous scaffolds, such as salt leaching, phase separation or sintering. However, these procedures imply the introduction of organic solvents or very high temperatures, and further purification steps to completely remove toxic residues (Tai et al., 2007). Foaming of polymers with supercritical CO_2 is an extensively used alternative, since the porosity is produced using CO_2 as blowing agent, and it is easily removed later by decompression. This process is based on the ability of supercritical CO_2 to plasticize and saturate the polymer above its critical point. When pressure is released, supersaturation of the CO_2 occurs, giving rise to bubble nuclei inside the polymer that result in pores upon solidification (Tsivintzelis et al., 2016). Nevertheless, this procedure can only be applied to amorphous or semi-crystalline hydrophobic polymers, but not to hydrophilic polymers that do not experiment any phase transition before thermal degradation (Ji et al., 2011; Tsioptsias et al., 2011). In these

cases, porous structures can be formed with supercritical CO_2 by developing first a hydrogel so that the dense gas is dissolved in the aqueous phase and is also able to penetrate and swell the rubbery structure of the hydrogel (Tsioptsias et al., 2011). Then, when the system is depressurize, pores are created by bubble formation due to supersaturation of CO_2. With this process, highly interconnected structures from natural-origin polymers can be prepared. Different studies reported in the literature show the ability to foam natural-based polymers using this technique, particularly chitosan, elastin and collagen. (Annabi et al., 2009; Ji et al., 2011; Tsioptsias and Panayiotou, 2008).

In this work, porous scaffolds were prepared by hydrogel foaming with 2 different β-glucans: (1-3, 1-4)-β-glucans from barley and (1-3, 1-6)-β-glucans from yeast *Saccharomyces cerevisae.* Both β-glucans differ in their structure and properties: whereas barley β-glucan is a linear, water soluble polymer, yeast β-glucan has a branched conformation and is not soluble in water. Thus, their behavior is different and they have to be processed in a different way in order to create the hydrogel. Also, this will influence the structure and the properties of the final products, which were analyzed regarding morphology and mechanical properties. Besides, dexamethasone was included in the formulations as a model active compound to study the release from the scaffold. It is commonly used in tissue engineering because it promotes the differentiation of stem cells towards osteogenic linage and, at the same time, has anti-inflammatory effect in the implant zone (Shim et al., 2016).

2. MATERIALS AND METHODS

2.1. Materials

Barley β-glucans (75% purity; Glucagel, kindly supplied by DKSH, France) and yeast β-glucans (64% purity; L-Naturae Nutraceutical, kindly supplied by Naturae, Spain) were used as raw

materials to create the hydrogels. Dexamethasone (CAS 50-02-2) was purchased from Sigma. PBS was prepared from tablets (Sigma).

2.2. Production of β-glucan hydrogels

Barley β-glucan hydrogels (4% w/w) were produced by dissolving the β-glucans in water at 80ºC, boiling them for 5 minutes and then keeping them at 75ºC for 1h. After that time, the solution was poured into 96-well plate molds and kept at 4ºC for 3 days until they became gel.

Yeast β-glucan hydrogels (2.5% w/w) were created from a suspension. YBG was first stirred for 30 minutes, in order to correctly hydrate them and achieve a homogeneous dispersion. Then they were heated at 90ºC for 1 h and poured into molds, where they were kept overnight to form the hydrogel.

2.3. Hydrogel foaming with supercritical CO_2

The hydrogels were placed in a high-pressure vessel, preheated at 37ºC. Then the vessel was closed and CO_2 was fed up to the chosen operating pressure, namely 8, 12, 16 or 20 MPa, using a high-pressure pump (Haskell, MCPV-71). The hydrogels were kept in contact with supercritical CO_2 for 1.5h, in order to have enough time to allow the diffusion of CO_2 into the bulk of the hydrogels and saturate them. After that time, a fast decompression was performed to promote the foaming and at the same time freezing of the structures. In this way, the porous structure produced was stabilized and kept overnight at -80ºC. Water was removed by freeze-drying (-80ºC, vacuum lower than 0.5 mbar). As a control, hydrogel samples not subjected to pressurized carbon dioxide were directly frozen and freeze-dried.

2.4. Morphological characterization of the scaffolds

Micro-Computed Tomography (micro-ct) was used to evaluate the porosity and pore size of the 3D structures obtained. The images were acquired on a high-resolution micro-CT SkyScan 1272

scanner (Bruker, Germany) using a voltage of 50 kV and a current of 240 μA. Images were acquired with an exposure time of 160 ms and a pixel size of 15.99 μm. After image acquisition the noise was reduced with nRecon software. CT Analyser software (SkyScan, Belgium) was used to obtain representative data sets of the samples and converting them into 2D images. For each set of conditions, 3 different samples were analyzed. The average values and standard deviation of porosity, mean pore size and interconnectivity were evaluated, and Student's t-test was performed (unpaired samples, unequal variances) in order to check whether there were significant differences between each sample, with a significance p-value of 0.05.

The produced scaffolds were observed by scanning electron microscopy (SEM) with a high-resolution field emission scanning electron microscope with focus ion beam (Auriga Compact, Zeiss). The samples were cut in liquid nitrogen and the sections were placed by mutual conductive adhesive tape on aluminum holders and covered with gold palladium using a sputter coater.

2.5. Mechanical properties

The mechanical behavior of the samples was assessed in compression mode using a universal testing machine (Instron 5540). The scaffolds were compressed at 1 mm · min^{-1} until a maximum deformation of 70% of the initial height. The compressive Young modulus was determined as the initial slope in the stress-strain graphs. The analysis was performed in triplicate, and statistical differences between each were evaluated by Student's t-test (unpaired samples, unequal variances, significance p-value of 0.05).

2.6. In vitro release of dexamethasone

Dexamethasone was loaded in the initial aqueous solution of β-glucans at different concentrations, namely 5 and 10 wt% in respect to the weight of the polymer, and then the

processing of the structures was done as described before (section 2.2 and 2.3). The conditions selected for the foaming were 37ºC and 12 MPa.

The different scaffolds were immersed in 20 mL PBS in a water bath at 37ºC. Aliquots of 150 µL of the liquid medium were withdrawn at different time points (5, 10, 15 and 30 min, 1, 2, 3, 4.5, 6, 8, 24 and 32 h, and daily from 2 to 11 days) and replaced with the same quantity of fresh PBS. The samples were analyzed by UV-Vis spectrophotometry at 245 nm and the absorbance value was adjusted into a calibration curve between 0 and 0.05 g/L of dexamethasone to determine the drug concentration released into the liquid. Three different samples were prepared for each time point. The replacement of liquid with fresh PBS was taken into account in the calculations of the cumulative mass of dexamethasone.

The release kinetics was analyzed with different empirical models in order to determine which one better represented the release behavior from the structures, in particular Korsmeyer-Peppas (Eq. 1), zero order (Eq. 2), first order (Eq. 3), Higuchi (Eq. 4) and Hixson-Crowell's models (Eq. 5) (Costa and Sousa Lobo, 2001).

$$M_t/M_\infty = kt^n \quad [1]$$

$$M_t/M_\infty = kt \quad [2]$$

$$M_t/M_\infty = \exp^{-kt} \quad [3]$$

$$M_t/M_\infty = kt^{1/2} \quad [4]$$

$$(M_\infty)^{1/3} - (M_r)^{1/3} = kt \quad [5]$$

In these equations, M_t is the quantity of drug released at time t, M_r is the quantity of mass retained at time t, M_∞ is the initial quantity of drug and k is the kinetic constant.

3. RESULTS AND DISCUSSION

β-glucan aerogels were prepared in our previous work by supercritical drying of the hydrogels (submitted paper). In that case, the morphological properties were not suitable for tissue engineering approaches, as the mean pore size was too low to allow cell growth and infiltration into the bulk of the matrix, even for the lowest concentrations of polymer tested, which were the ones chosen in this work in an attempt to achieve structures with higher porosity. In this sense, in this work we performed supercritical foaming of the hydrogels in order to improve the morphological parameters of the scaffolds.

3.1. Morphological characterization of β-glucan scaffolds

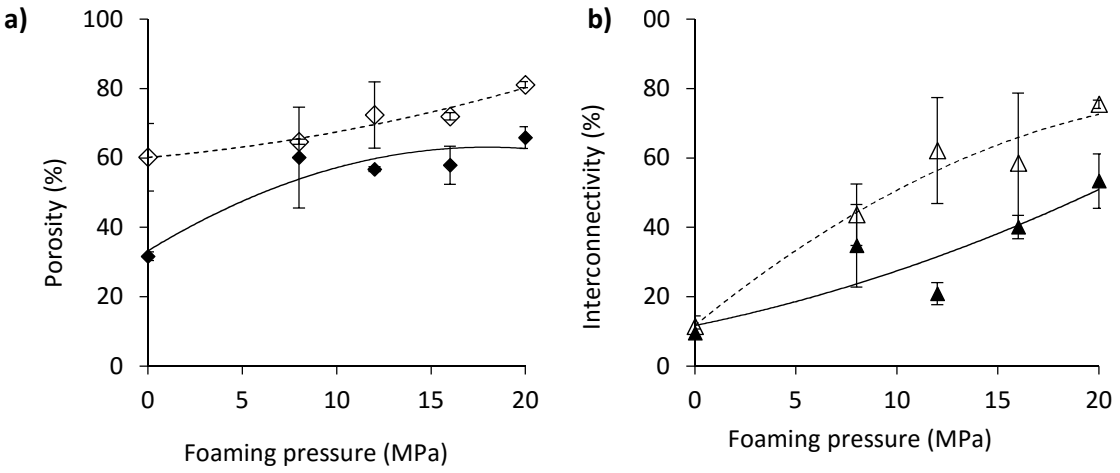
In this work, we successfully obtained β-glucan scaffolds with enhanced porous properties after supercritical foaming of the hydrogels. However, these properties can be further tuned depending on the foaming pressure. An important step in the hydrogel foaming is the stabilization of the structures. The Joule-Thompson effect plays a key role in this process. Upon the fast decompression, the samples were frozen due to the sudden expansion of CO_2 to atmospheric pressure, so that the bigger the pressure drop, the greater the cooling effect and hence stabilization of the materials. Thus, the samples were completely frozen when working at 12 MPa or more, but at 8 MPa it was more difficult to stabilize the structures and this resulted in less homogeneity.

Morphological properties were obtained by micro-ct (Fig. 1). Porosity, pore size and interconnectivity followed an increasing tendency with the foaming pressure for both β-glucans, although some differences were observed between them. For BBG, porosity increased from 30% when the material was just freeze-dried to 55-60% when the structures were foamed with supercritical CO_2, without significant differences with the pressure (Fig. 1a). On the contrary, YBG had higher porosity when it was processed just by freeze-drying (60%), which could be due to the lower concentration of polymer used to create the hydrogel or due to the different gelling

behavior observed between both β-glucans (Chapter III). When the material was foamed, the porosity increased up to 80% with 20 MPa. These values are in the range of those of natural bone, which is between 50 and 90% porosity (Karageorgiou and Kaplan, 2005).

Regarding interconnectivity of the pores, YBG showed a greater increase with the foaming pressure when compared with BBG (Fig. 1b). Although interconnectivity was the same for both β-glucans obtained by freeze-drying (around 10%), after supercritical foaming with CO_2 it was bigger for YBG in all the range of pressure tested, reaching 53% for BBG and 75% for YBG at 20 MPa. Nevertheless, there was a noticeable increase for both polymers when they were processed with supercritical CO_2, compared to conventional freeze-drying.

Despite the differences between BBG and YBG in porosity and interconnectivity, both β-glucans had very similar mean pore size, and the same increasing tendency with foaming pressure, from 50-75 µm by freeze-drying, to 230-290 µm at 20 MPa (Fig. 1c). This increment in pore size makes the scaffolds suitable to be used in bone regeneration regarding pore size, since 100 µm is considered to be the minimum size required for proper bone growth. In addition, pore size up to 300 µm contributes to the formation of capillaries that enhance vascularization of the new tissue (Karageorgiou and Kaplan, 2005). This effect of foaming pressure in pore size was not observed and had not been described in previous works reporting foaming of chitosan and chitin hydrogels (Ji et al., 2011; Tsioptsias and Panayiotou, 2008).



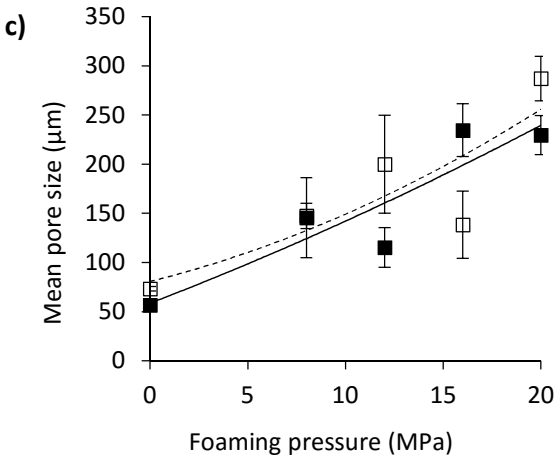


Figure 1. Porosity (a), mean pore size (b) and interconnectivity (c) of the scaffolds at different foaming pressure. Closed symbols: BBG. Open symbols: YBG. Lines are added to guide the eye.

At higher foaming pressure, more quantity of CO_2 saturates the hydrogel (Bamberger et al., 2000). Thus, more nuclei are formed during depressurization, and each one will correspond later to one pore. This partially explains the increase in porosity with the foaming pressure. However, at higher pressures, the amount of CO_2 in the hydrogels is too high and it cannot be totally released during the decompression. Thus dry ice is formed inside the matrix, which will then be sublimated when the freeze-drying takes place (Tsioptsias and Panayiotou, 2008). In this way, it creates channels that link the pores, producing at the same time an increase in the porosity and in interconnectivity. Finally, coalescence of pores and channels generates the increase in pore size.

The increase in porosity and pore size is easily observed on the 2D images of the micro-ct reconstructions of the materials (Fig. 2). By freeze-drying (Fig. 2a and 2d), the samples presented less void spaces, and only the typical channels correspondent to water crystals were noticed. A much porous structure was obtained by foaming with supercritical CO_2 (Fig. 2b and 2e), with the pores well-distributed along the material. However, when the pressure was increased up to 20 MPa (Fig. 2c and 2f), non-homogeneous samples with very large pores were produced, so that

the material was almost hollow on the inside and the polymer being placed mostly on the outer part. According to those results, we can identify pressures of 12 or 16 MPa as the optimum operating conditions in order to establish a balance between the pore size, porosity and interconnectivity required for tissue engineering applications and the homogeneity of the samples produced.

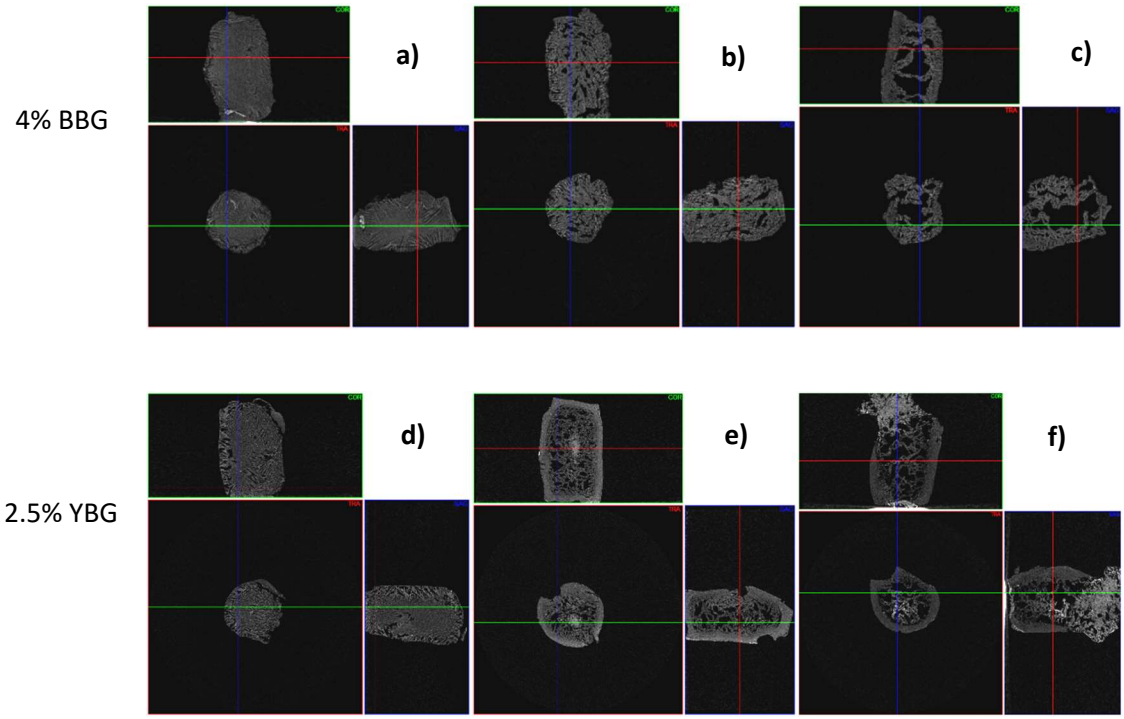


Figure 2. Micro-ct reconstructions of β-glucan scaffolds produced by freeze-drying (a and d) and by foaming with CO_2 at 12 (b and e) and 20 MPa (c and f).

The structure of the scaffolds was also analyzed by SEM (Fig. 3). In the freeze-dried samples not foamed, the pores were smaller, and were oriented following the directions of the crystallization of ice. On the contrary, when the samples were foamed, the pores increased. As it was observed with micro-ct images, the most homogeneous porosity was observed for the samples processed at intermediate pressures (12 and 16 MPa). At 8 and 20 MPa, a combination of small and very large pores was noticed. In the case of 8 MPa, this fact is due to the incomplete freezing of the material upon decompression, which made the structures very unstable and the porosity could

not be well maintained. At 20 MPa, as it was aforementioned, the presence of big pores was a consequence of the joining of the smaller ones because of an excess of CO_2 dissolved in the hydrogel during processing.

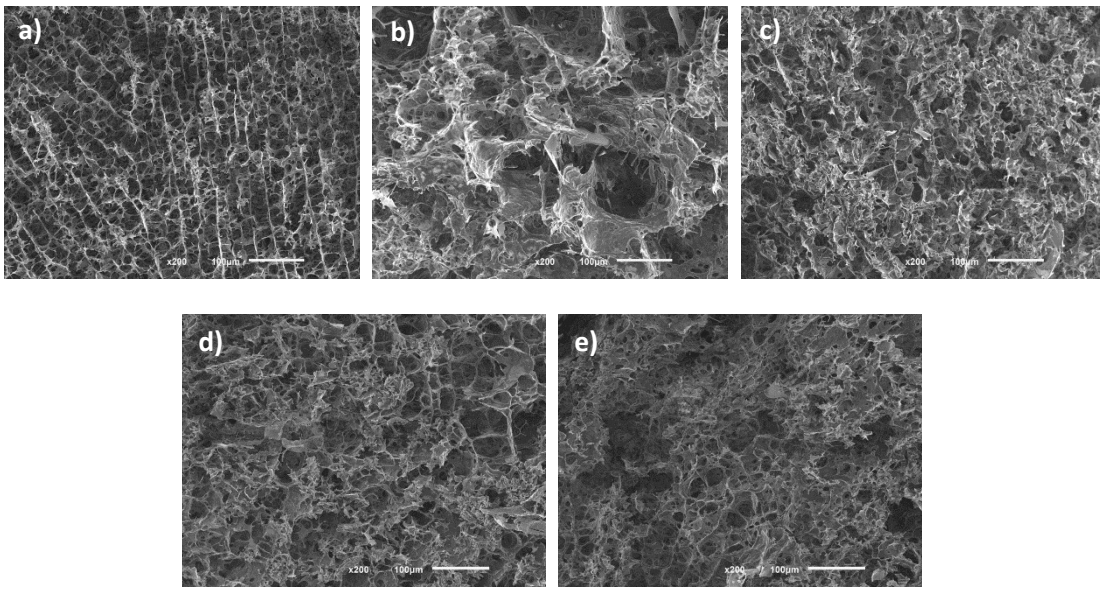


Figure 3. SEM images of 4% BBG scaffolds produced by freeze-drying (a) and by foaming with CO_2 at 8 (b), 12 (c), 16 (d) and 20 MPa (e). Scale bar: 100 µm. Magnification: x200.

3.2. Mechanical properties

The mechanical properties of the scaffolds are highly dependent on the porosity and interconnectivity of the pores. However, the variations observed in the porous properties were not reflected in the mechanical properties. For each β-glucan, there were no significant differences with the foaming pressure, neither in Young modulus, nor in maximum stress until failure. Only the results of Young modulus of BBG at 20 MPa were significantly higher than those at 12 and 16 MPa, even though the materials foamed at this pressure had the greatest porosity and pore size. This increase in Young modulus could be due to the shell effect of the polymer positioned on the outer part of the scaffold, which may have reinforced the sample, although it was almost hollow inside. YBG samples were much more brittle and fragile, probably due to the

lower polymer concentration, and some of them could not even be handled and were therefore not analyzed. Figure 4 shows the results of the compression test.

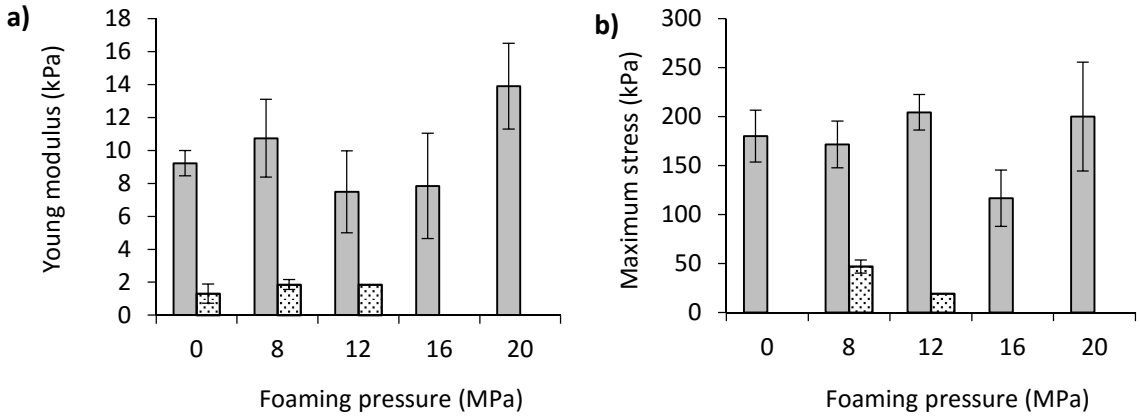


Figure 4. Compressive Young modulus (a) and maximum compression stress until failure (b) of the foamed samples of 4% BBG (grey) and 2.5% YBG (dots).

The values obtained were much lower than those of compact and spongy bone or cartilage (Belcarz et al., 2013; Colombo et al., 2013; Wu et al., 2014). Nevertheless, it has been reported that, although cartilage has values of Young modulus between 0.5-1 MPa, values around 4 kPa enhance the formation of cartilage, whereas Young modulus higher than 40 kPa facilitates osteogenesis (Arora et al., 2015). Higher mechanical properties were obtained for aerogels produced with the same β-glucans (Chapter III). In this case we observe that the enhancement of the morphological properties achieved by foaming dramatically decreased the resistance of the materials to compression stress. However, as mentioned before, porosity, pore size and interconnectivity were suitable for the scaffold to be able to host cells and allow their growth. One of the solutions to overcome this, and in order to balance porous and mechanical properties, is to load a ceramic material to the initial β-glucan solution, so that after processing by hydrogel foaming a similar porous structure would be obtained, but with enhanced mechanical properties (Gantar et al., 2014; Rezwan et al., 2006).

3.3. In vitro release of dexamethasone

The most promising scaffolds for tissue engineering were obtained with 4% BBG prepared at 37ºC and 12 MPa, hence this formulation was the one chosen to proceed with the impregnation of dexamethasone and the study of these matrices as possible drug delivery systems. Two different concentrations of dexamethasone were loaded in the scaffolds, 5 and 10 wt%. We observed that the release profile of dexamethasone into PBS (Fig. 5) was very similar for both concentrations of the active compound tested. This would be expected since it depends mostly on the properties of the polymeric matrix and on the interactions between the active compound and the polymer. First, there was a burst release up to 8h, in which 75 and 49% of dexamethasone was released to the liquid medium for each concentration. After that period, a slower release was obtained until 4 days. At that point, the release reached a constant value and was maintained for the following days, around 100% and 85% for 5 and 10% dexamethasone, respectively. Much faster release of dexamethasone (90% in 2h) was obtained in chitosan scaffolds loaded by supercritical impregnation of the freeze-dried material (Duarte et al., 2009a). In chitosan nanoparticles prepared by mixing dexamethasone in the initial solution and then freeze-drying of the particles, a release profile similar to ours was obtained (Kalam, 2016).

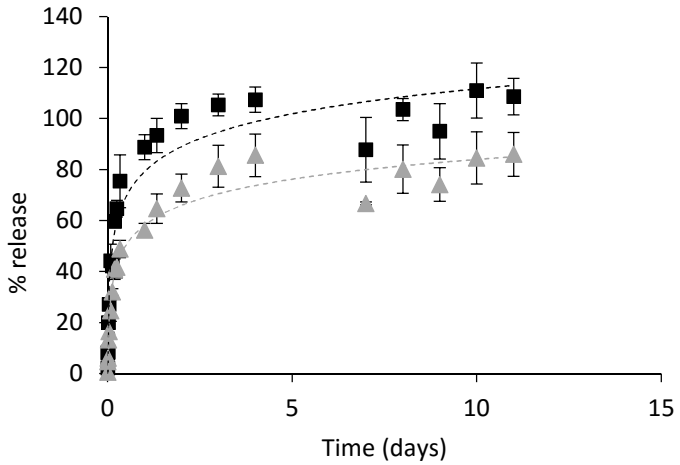


Figure 5. Release profile of dexamethasone from BBG scaffolds. Initial dexamethasone in the scaffolds: 5% (w/w, squares) or 10% (w/w, triangles). Lines are added to guide the eye.

The initial burst release is usually attributed to the active compound present on the surface of the materials. The fast release up to 8h achieved in our work could be a consequence of a combination of the release of the superficial drug and the highly porous matrix, which allowed a rapid penetration of PBS into the scaffold and the extraction of dexamethasone present also in the surface of the more accessible pores towards the liquid medium during the first hours. Afterwards, a sustained release was observed for the following days due to the diffusion of the drug from the polymeric matrix to the liquid medium. In a previous work (submitted paper), swelling-controlled release was reported for β-glucan aerogels that had low porosity, so that the release occurred freely once the sample was completely wetted and swollen.

In order to be able to draw conclusions on the mechanisms governing drug release from the scaffolds, different empirical equations were applied to the experimental data. Results of the fitting of the results of release in PBS (Table 1) revealed that release for 10% dexamethasone could be well described by first-order model, which represents a control by both diffusion and relaxation of the matrix. However, in the case of 5% dexamethasone, the adjustment with Higuchi model was better. Thus, the release was mostly controlled by the diffusion of dexamethasone from the polymer to the liquid. With Korsmeyer-Peppas' model, it is also possible to obtain the diffusion exponent, n, which characterizes the mechanism governing the drug release (Costa and Sousa Lobo, 2001). In both cases the diffusion exponent determined for the BBG scaffolds loaded with dexamethasone fell in the values determined for anomalous transport for a cylindrical matrix, i.e., a value of n between 0.45 and 0.89. This means that the release is governed by both diffusion and swelling of the scaffold.

Table 1. Fitting of the release of dexamethasone to different models.

	r^2					
	Zero-order	First-order	Higuchi	Hixson-Crowell	Korsmeyer-Peppas	n
5%	0.6716	0.9218	0.9718	0.8464	0.9317	0.745
10%	0.7353	0.9187	0.8932	0.8645	0.8330	0.721

In order to complete the information from the Korsmeyer-Peppas model, Peppas and Sahlin proposed another empirical equation to determine the contribution of each release mechanism (diffusion controlled or swelling controlled) to the total drug release (Peppas and Sahlin, 1989):

$$M_t/M_\infty = k_1 t^m + k_2 t^{2m} \quad [6]$$

Where m is the diffusion exponent of the pure Fickian diffusion, which is 0.45 for a cylindrical geometry. In this equation, the first term of the right ($k_1 t^m$) corresponds to the Fickian contribution, and the second ($k_2 t^{2m}$) corresponds to the relaxation contribution. By fitting k_1 and k_2 to the experimental data up to 60% release, it was possible to calculate the relative contribution of Fickian (F) and relaxation (R) mechanisms as follows:

$$F = 1 \Big/ 1 + \frac{k_2}{k_1} t^m \quad [7]$$

$$R/F = \frac{k_2}{k_1} t^m \quad [8]$$

With Eq. 6, k_1 = 0.0328 $min^{-0.45}$ and k_2 = 0.0008 $min^{-0.45}$ for 5% dexamethasone, and k_1 = 0.0277 $min^{-0.45}$ and k_2 = 0.0006 $min^{-0.45}$ for 10% dexamethasone. Introducing these values in Eq. 7 and 8, it was observed that the release was mainly controlled by the diffusion of dexamethasone from the β-glucan matrix to the liquid medium (Fig. 6). At initial time, release was almost totally controlled by Fickian diffusion, although this contribution decreased with time, while relaxation of the matrix gained importance in controlling the release. Nevertheless, the contribution of the relaxation of the polymer was lower than 25%. As it would be expected, the results were similar for both concentrations of dexamethasone, since the matrix was the same.

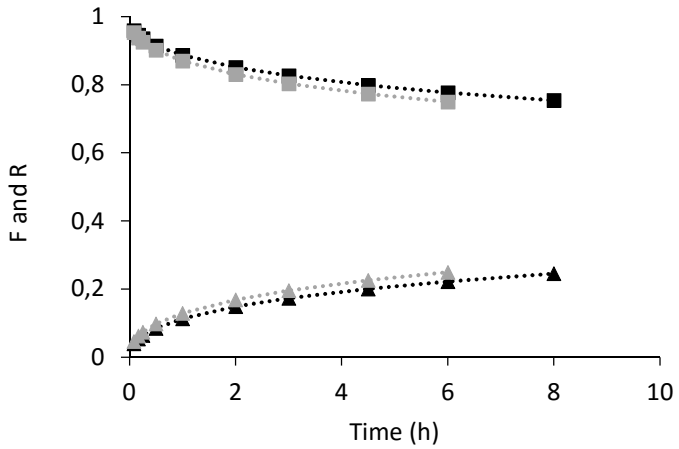


Figure 6. Contribution of diffusion (F, squares) and relaxation of the polymer (R, triangles) to the control of the release of dexamethasone. Grey: 5%, and black: 10% dexamethasone.

These calculations further confirm the observations from the release profiles. According to this, the release rate of dexamethasone was first controlled by the diffusion rate of the drug from the scaffold to the liquid. With time, the scaffold was wetted and relaxation of chains occurred, so that this fact also contributed to the release of dexamethasone from the zones that were initially inaccessible to the liquid. However, the release mechanism governing the process was always the diffusion, possibly due to the high porosity of the scaffolds, which allowed the liquid to easily penetrate into the matrix, be in contact with the drug and extract it to the liquid medium.

4. CONCLUSIONS

Foaming of hydrogels with supercritical CO_2 was applied to the production of scaffolds for tissue engineering. This process is based on the solubility of high-pressure CO_2 in the water present in the hydrogels, thus the porosity is created due to supersaturation of CO_2 upon decompression. Barley and yeast β-glucans were used as biopolymers for the production of the scaffolds.

Influence of the foaming pressure was noticeable on the porous properties of the materials through an increase of porosity, pore size and interconnectivity with the pressure. However no significant changes were observed on their mechanical properties under compression stress. Regarding the type of β-glucan, despite the scaffolds with YBG had greater porosity and interconnectivity, mean pore size was similar for both β-glucans. Due to the differences in porosity, YBG scaffolds had lower resistance to compression. Release of dexamethasone from BBG scaffolds showed a high release (between 50 and 75%) up to 8h, and slower until complete release in 4 days. The release was mainly controlled by diffusion of the active compound, although there was also some contribution due to relaxation of the polymeric matrix.

Although the morphological properties achieved in the scaffolds with both β-glucans were appropriate for support of cells and growth of new tissue, they were brittle and did not reach mechanical properties of bone or cartilage tissue by themselves. The addition of a ceramic material could greatly enhance mechanical properties and, thus, the applicability of the materials in the field of tissue engineering. Nevertheless, the porous properties were greatly enhanced compared to those of β-glucan aerogels. Besides, the scaffolds provided a controlled release of dexamethasone on physiological fluid.

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CONCLUSIONS

This work is a contribution to the development of new β-glucan-based products for their use in agriculture, pharmaceutics and medicine. The main conclusions of this thesis are presented below:

- Development of β-glucan formulations for the encapsulation of resveratrol as new antifungal product. Solid and liquid formulations of resveratrol encapsulated on β-glucans were successfully produced.
    - Particles of resveratrol encapsulated on barley β-glucan (BBG) were obtained by emulsification-evaporation technique and further processing by PGSS-drying and spray-drying. The particles obtained by PGSS-drying were smaller than by spray-drying (10 μm), but they created big agglomerates (100 μm). Encapsulation efficiency was similar between both processes (60-96%), indicating that it is governed mainly by the emulsification technique and not by the drying process. Furthermore, amorphous encapsulated resveratrol was found in all the final particles. On the contrary, SAS was not effective for drying of BBG in the conditions tested.
    - Aqueous formulations of resveratrol with BBG and yeast β-glucan (YBG) were developed by different emulsification techniques (high-shear, high-pressure and high-pressure and temperature). However, the final suspensions had similar characteristics regardless of the emulsification technique (particle size below 90 nm and between 70 and 100% encapsulation efficiency). The main influence derived from the encapsulating material. BBG provided the greatest droplet size in emulsion (30 μm) and lowest encapsulation efficiency (30-50%), although particle size was greatly decreased (below 2 μm) and encapsulation efficiency increased (above 65%) by the addition of lecithin to BBG.
    - Both solid and liquid formulations inhibited fungal growth of *B. cinerea*. Solid particles of resveratrol encapsulated on BBG reduced growth by 70%. On

aqueous formulation, resveratrol encapsulated on YBG provided higher antifungal activity than on BBG (50 and 20% growth reduction, respectively). Thus, the hypothesis of an improved antifungal activity due to a better penetration in the presence of YBG was confirmed.

- Synthesis of β-glucan-based materials for drug delivery and tissue engineering.
    - A study on the rheology of BBG and YBG hydrogels revealed that YBG created more elastic and stable gels, which were able to rearrange and resist higher shear stress without collapsing. Besides, BBG hydrogels could be redissolved in water, but YBG gellation was irreversible. This is a consequence of their different chain structure and 3D-configuration.
    - Upon solvent exchange and supercritical drying, these differences were also noticed on the aerogels. The crosslinking of the branched chains of YBG produced thicker and more resistant structures, whereas the linear chains of BBG created a more spongy material. Thus, at 5% polymer concentration, although the textural properties were similar for both β-glucans (around 180 m^2/g BET surface area, 0.6-0.7 cm^3/g pore volume and pore size 13-16 nm), YBG aerogels had higher density (121 kg/m^3, and 79 kg/m^3 BBG aerogels), were able to withstood higher compression stress (up to 450 kPa, and 70 kPa BBG aerogels) and absorb more water (1100% and 600%, respectively).
    - Supercritical impregnation of acetylsalicylic acid was performed at different pressure (8-20 MPa) and temperature (35-50ºC). Impregnation yield was ranging between 8 and 15%, with an increasing tendency with density of supercritical CO_2. Release of the drug from the aerogels was initially delayed for 3h, as a result of the penetration of the drug into the aerogel during supercritical

impregnation. After that time, the release was controlled by swelling of the matrix once the polymer was completely wetted. Therefore, β-glucan aerogels resulted in a good alternative as drug delivery carriers.

- Hydrogel foaming successfully enhanced the porous properties of the materials in comparison with supercritical- and freeze-drying. The structures produced in this way had up to 80% porosity, mean pore size of 250 μm and 75% of interconnected pores, which are suitable characteristics to be used as scaffolds for tissue engineering, since they are able to host cells and allow the transport of nutrients and wastes needed for their growth and proliferation. The aforementioned porous parameters increased with the foaming pressure, although above 16 MPa the pores were not homogeneously distributed, leading to almost hollow structures. Regarding mechanical properties, the scaffolds were brittle, so they should be blended with some ceramic material that strengths the structures and at the same time promotes their *in situ* integration with the native surrounding tissues.
- Dexamethasone was completely released from BBG scaffolds in a sustained way for 4 days, although release rate was higher during the first 8h. Analysis of the mechanism governing the release revealed that it was mainly controlled by diffusion of the drug to the liquid medium, with minimum limitation due to penetration of liquid into the structure because of its high porosity. The contribution of relaxation of polymer chains in the matrix gained importance with time, but it only accounted for up to 25%.

FUTURE WORK

Further studies could be conducted following the work developed in this thesis. The study about the solid formulations could be completed with the evaluation of the performance of YBG, which was the encapsulating material that provided the best reduction of fungal growth in the aqueous formulations. In order to remove the step of solvent removal from the processing, the formulations could also be developed with essential oils as antifungal active compounds, or with a natural, non-toxic oil as organic phase. Since the formulations with β-glucans successfully inhibited the growth of *B. cinerea*, the test could be repeated with some other fungal species containing β-glucans on the cell wall.

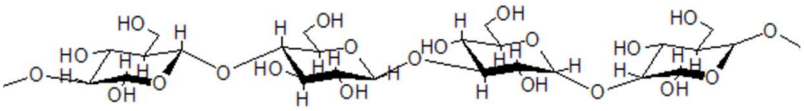
Regarding the production of β-glucan-based aerogels, a deeper analysis of the influence of the solvent exchange step is required, in order to assess the influence in the final properties of the material. It would be also interesting to explore the possibility of producing beads instead of monoliths, since this shape is preferred for drug delivery purposes due to the higher surface area and easy handling (flowability). This could be accomplished by dropping hot aqueous solutions of β-glucans in large volumes of ethanol or in a continuous process in which lower particle size can be achieved (i.e. in a microfluidic device or in a system that mix a stream of ethanol and a stream of aqueous solution of b-glucan, so that the beads are formed by an anti-solvent effect). Besides the beads could be used as well for tissue engineering applications by bonding them together with an elastic polymer such as gellan gum. As the mechanical properties achieved for the β-glucan scaffolds produced in this thesis were quite low for load-bearing applications, the addition of a bioactive glass to the formulation would improve them. Furthermore, the *in situ* applicability of the β-glucan scaffolds could be enhanced by the addition of the glass, because bone-like structures would develop in contact with physiological fluids in the implant site.

RESUMEN

Hoy en día se tiende a potenciar el uso de sustancias naturales en aplicaciones industriales, especialmente en aquellas relacionadas con el medioambiente y el ámbito sanitario. Estas sustancias, que normalmente provienen de fuentes renovables y no presentan toxicidad, se encuentran en distintas estructuras en la naturaleza, de forma que son capaces de imitar su comportamiento e incrementar la efectividad de los productos. Entre dichas sustancias naturales, los polifenoles son de los más estudiados debido a sus destacadas propiedades antioxidantes, mientras que los polisacáridos se estudian como biopolímeros naturales para sustituir en diversas aplicaciones a otros obtenidos mediante síntesis química.

El objetivo de esta tesis es explorar el uso de β-glucanos en formulación y en síntesis de materiales. Los β-glucanos son polisacáridos formados por unidades de D-glucosa unidas mediantes enlaces glicosídicos. Se encuentran en distintas fuentes en la naturaleza, como bacterias, algas o cereales. Dependiendo de su origen y de su estructura y enlaces, pueden tener propiedades muy variadas en cuanto a configuración tridimensional o actividad biológica, entre otras. Por ejemplo, los β-glucanos de cebada (BBG) son polímeros lineales con enlaces en las posiciones 1-3 y 1-4 del anillo de glucosa, mientras que los de levadura (YBG) son ramificados debido a los enlaces en posición 1-3 y 1-6 (Figura 1).

a)



b)

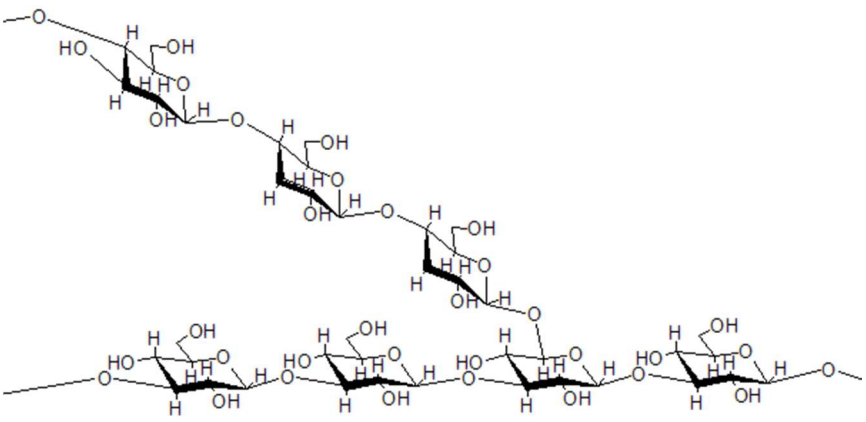


Figura 1. Estructura de BBG (a) y YBG (b).

Al contrario que otros polisacáridos, como alginato o almidón, los β-glucanos no han sido tan ampliamente estudiados. Las aplicaciones finales que se han recogido en esta tesis fueron elegidas atendiendo a las propiedades de los β-glucanos, y a las ventajas que podrían ofrecer frente a otros polisacáridos en dichas aplicaciones.

Así, en los capítulos I y II, los β-glucanos se han usado como materiales encapsulantes de compuestos activos para su uso como fungicidas en agricultura. La encapsulación de los compuestos activos previene su degradación por efecto de la temperatura o por oxidación. Además, como los β-glucanos son uno de los componentes de la pared celular de muchos hongos, se plantea la hipótesis de que su inclusión en la formulación podría mejorar la penetración del compuesto activo a través de la pared celular del hongo, incrementando de esta forma su acción fungicida. Se desarrollaron tanto formulaciones en fase líquida como en fase sólida. Con las formulaciones líquidas se elimina el paso de secado en el procesado, y son más fáciles de aplicar sobre las plantas. Por el contrario, con las formulaciones sólidas, el producto se conserva mejor sin riesgo que se desestabilice. En ambos casos, las formulaciones se realizaron mediante técnicas con fluidos supercríticos o a alta presión para determinar las diferencias en los productos finales comparado con los procesos tradicionales de formulación.

En los capítulos III y IV, se emplearon los β-glucanos como matriz polimérica en aplicaciones biomédicas, por una parte como vehículo para la liberación de compuestos activos, y por otra como estructuras de soporte para regeneración de tejidos. La actividad biológica de los distintos β-glucanos ha sido ampliamente evaluada en trabajos bibliográficos previos, incluyendo tanto su capacidad para reducir el colesterol, como para modular el sistema inmune o su capacidad de acelerar la cura de heridas. Así, los materiales sintetizados con β-glucanos para las aplicaciones anteriormente mencionadas poseerán también estas propiedades.

Objetivos

El objetivo global de esta tesis es estudiar la posibilidad de usar diferentes β-glucanos en distintas aplicaciones y evaluar la influencia que tiene el tipo de β-glucano empleado en las propiedades de los productos finales. Concretamente, se escogieron como materia prima β-glucanos de cebada y de levadura porque tienen muy distinta estructura y propiedades biológicas. Además, se trataron mediante procesos a alta presión para comparar con los productos obtenidos mediante procesos tradicionales.

En este marco, se pueden distinguir 2 objetivos parciales:

- Desarrollo de formulaciones con β-glucanos como nuevos productos antifúngicos basados en compuestos de origen natural. Aplicación a la encapsulación de resveratrol como fungicida fenólico.
    - Formulaciones sólidas mediante secado en spray, secado por PGSS (PGSS-drying) y anti-solvente supercrítico (SAS).
    - Formulaciones acuosas mediante técnicas de emulsificación con un equipo rotor-estator, a alta presión y a alta presión y temperatura.
    - Caracterización de las formulaciones en cuanto a tamaño de partícula, cristalinidad del resveratrol encapsulado y eficiencia de encapsulación. Determinación del efecto que tiene en el producto final cada método de procesado y el tipo de β-glucano.
    - Evaluación de la actividad antifúngica contra *B. cinerea* de cada formulación.
- Síntesis de materiales con β-glucanos para su uso en liberación controlada de compuestos e ingeniería de tejidos.
    - Producción de hidrogeles y caracterización reológica.
    - Producción de aerogeles mediante secado supercrítico.

- Evaluación de la influencia del tipo de β-glucano y su concentración en las propiedades finales de los aerogeles, como morfología, porosidad, propiedades mecánicas, degradación en medio fisiológico y absorción de agua.
- Incorporación de un compuesto anti-inflamatorio modelo (ácido acetil-salicílico) en los aerogeles mediante impregnación supercrítica y estudio de la influencia de la presión y la temperatura en el rendimiento de impregnación. Estudio de la liberación del compuesto de la matriz.
- Producción de estructuras más porosas mediante espumado de los hidrogeles con CO_2 supercrítico y caracterización de sus propiedades porosas y mecánicas. Comparación de dichas propiedades con las de materiales obtenidos mediante liofilización de hidrogeles.
- Incorporación de dexametasona en los scaffolds procesados mediante espumado de hidrogeles, como compuesto modelo con propiedades anti-inflamatorias y de diferenciación osteogénica. Estudio de la liberación del compuesto y del mecanismo que la gobierna.

<u>Capítulo I:</u> Encapsulación de resveratrol en lecitina y β-glucanos para mejorar su acción contra *Botrytis cinerea*.

Este capítulo recoge el estudio de formulaciones en fase sólida con β-glucanos de cebada como material encapsulante y resveratrol como compuesto activo, ya que es una fitoalexina que las plantas producen espontáneamente como mecanismo de defensa cuando son atacadas por agresiones externas, como hongos o radiación UV. Además de estos productos, como comparación se usó también lecitina de soja como material encapsulante, tanto sola como mezclada con β-glucanos, y tebuconazol como producto fungicida modelo. La encapsulación de compuestos activos proporciona una liberación más controlada, al mismo tiempo que los

protege de la degradación. Como hongo de estudio, se eligió *Botritis cinerea*, ya que contiene β-glucanos en su pared celular, de manera que se puede probar la hipótesis de una mejor penetración de los compuestos activos a través de ella por la presencia de β-glucanos en la formulación.

En primer lugar, se produjeron emulsiones aceite-en-agua (ratio 1:9) con un equipo rotor estator a 70 Hz durante 4 min, con el compuesto activo disuelto en la fase orgánica (7.5 g/L en acetato de etilo) y el encapsulante en la fase acuosa (lecitina, BBG y combinaciones de ambos 50% en peso). Después, se eliminó el disolvente orgánico mediante evaporación a vacío, y las suspensiones se secaron por secado en spray (SD) o por PGSS-drying (particles from gas saturated solutions). También se intentó precipitar BBG disueltos en dimetil-sulfóxido (DMSO) mediante SAS (supercritical anti-solvent), pero no se conseguía eliminar bien el disolvente por la alta viscosidad de la disolución, quedando residuos de hasta el 30%, por lo que se determinó que en esas condiciones no era un proceso adecuado para micronizar β-glucanos.

El análisis del tamaño de gota de las emulsiones reveló que se producían gotas de mucho menor tamaño con lecitina (7.4 μm) que con BBG (52 μm), mientras que mezclando ambos compuestos se obtenían valores intermedios (15 μm). Esto es debido a que los BBG en disolución forman redes de polímero en vez de gotas. Esta diferencia de tamaños se observó también en las suspensiones. Sin embargo, una vez que se secaban las partículas por SD o PGSS-drying, no se apreciaban diferencias de tamaño de partículas con los distintos encapsulantes, sino por el proceso de secado. Por SD, se obtenían partículas de en torno a 10 μm de diámetro, mientras que por PGSS-drying se producían partículas más pequeñas, pero que formaban aglomerados de mayor tamaño (100 μm). En la figura 2 se muestran los productos obtenidos con BBG como encapsulante medianto SD, PGSS-drying y SAS.

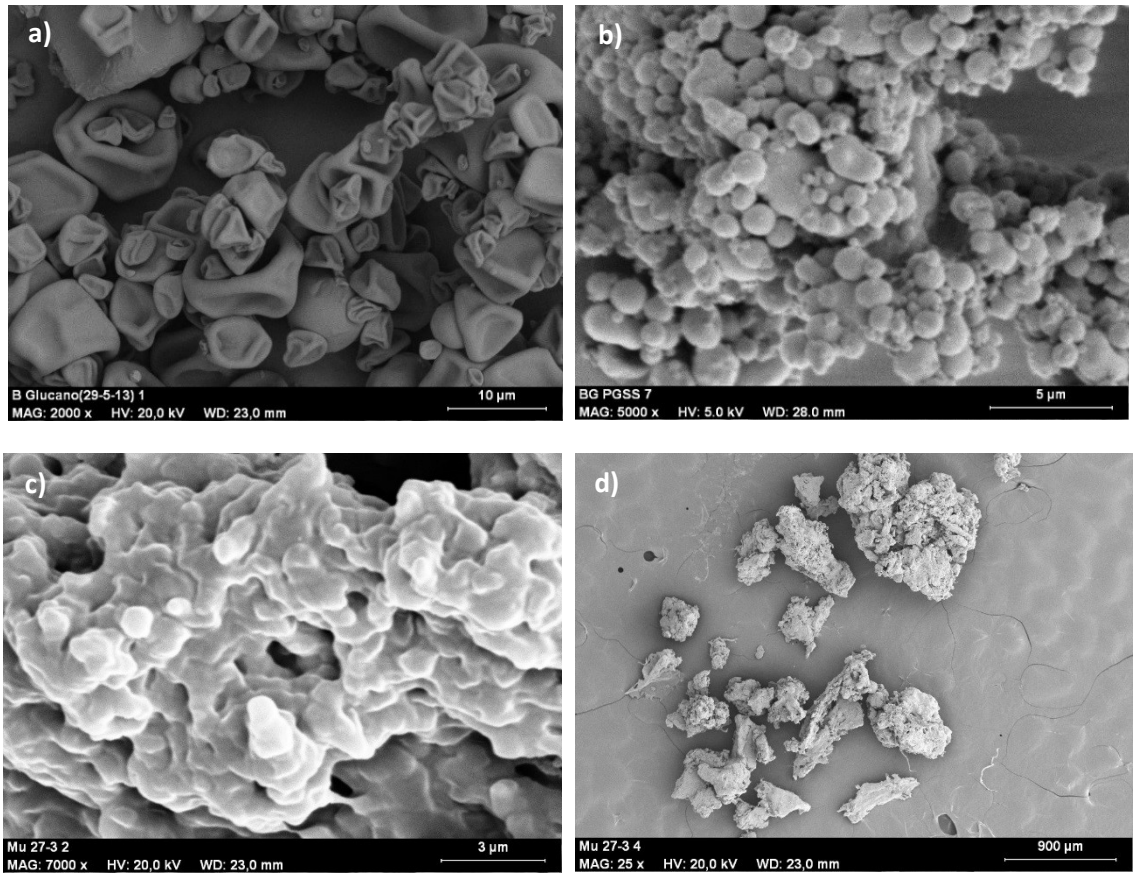


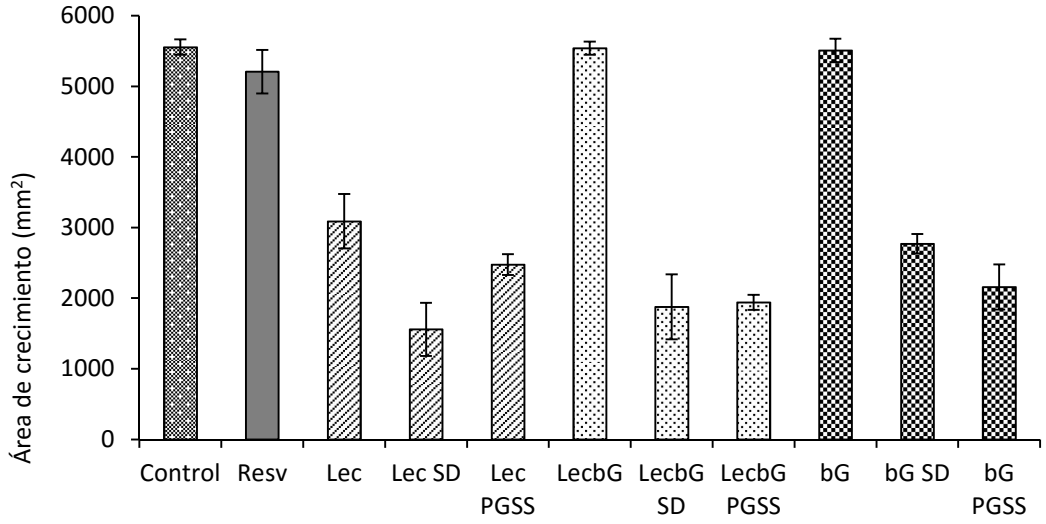
Figura 2. Imágenes de microscopía electrónica de barrido de partículas de BBG producidas por SD (a), PGSS-drying (b) y por SAS (5 g/L en DMSO, precipitación a 10 MPa, 35^{o} C, 2 mL/min; c y d).

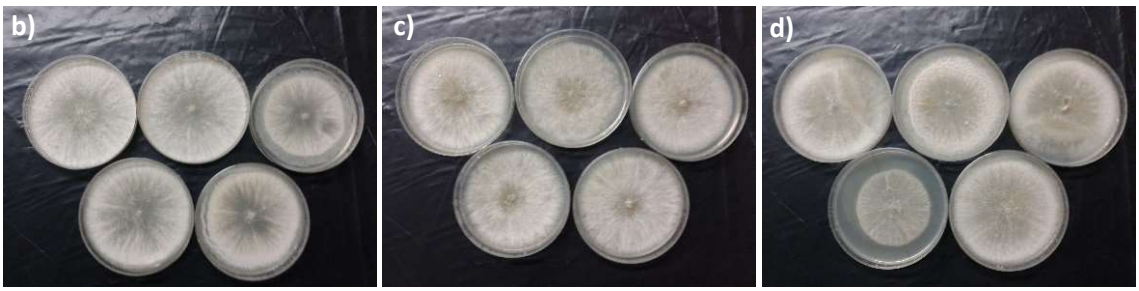
Por otra parte, el porcentaje de encapsulación de resveratrol y tebuconazol en las partículas se mantuvo por encima del 50%. Se apreció que se recuperaba mayor cantidad de tebuconazol por SD que por PGSS-drying, y en cambio el porcentaje de encapsulación de resveratrol era mayor por PGSS-drying que por SD, probablemente por la menor temperatura utilizada en ese caso para el secado y porque el compuesto se preserva mejor de la oxidación por la atmósfera de CO_2. En cualquier caso, las diferencias de carga de compuesto activo entre ambos procesos de secado eran bastante pequeñas, ya que dependen mayoritariamente del proceso de emulsificación, que era igual en todos los casos.

Además, mediante análisis de la cristalinidad de las partículas, se observó que en todos los casos se obtuvieron partículas completamente amorfas, a pesar de que tanto el tebuconazol como el

resveratrol son cristalinos en estado puro. Esto indica que, por una parte, no quedan cristales de los compuestos activos sin encapsular, y por otra, que éstos se encuentran en estado amorfo una vez encapsulados. Esta propiedad es especialmente importante ya que en la mayoría de las aplicaciones los compuestos cristalinos no presentan actividad, sino que se requieren que estén en estado amorfo.

Con respecto a la actividad antifúngica de las distintas formulaciones desarrolladas, las que incluían tebuconazol no mejoraban demasiado la actividad del tebuconazol puro, puesto que ya era muy efectivo y no había mucho margen de mejora (inhibición del 75-80% del crecimiento del hongo en todos los casos). Sin embargo, no se apreció una disminución significativa del crecimiento del hongo con respecto al control al aplicar resveratrol puro. Por el contrario, cuando se encontraba encapsulado, se conseguía una reducción del crecimiento de entre 50 y 70% (Figura 3).





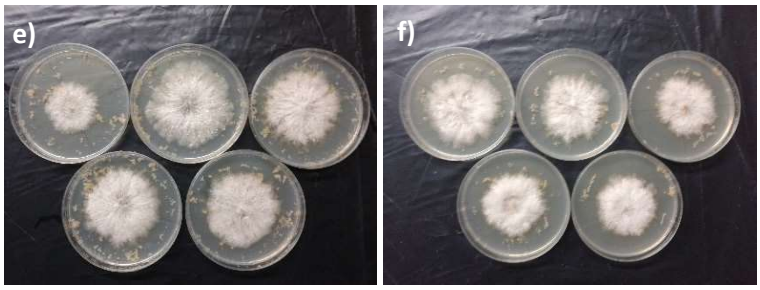


Figura 3. Área de crecimiento de B. cinerea al aplicar los distintos productos con resveratrol (a) e imágenes del area de crecimiento en las places Petri: control (b), resveratrol puro (c), BBG (d), y partículas de resveratrol encapsulado en BBG secadas por SD (e) y por PGSS-drying (f).

Analizando los resultados, se observa que la disolución de lecitina (Lec) ya produce una reducción del crecimiento similar a la de las formulaciones con lecitina, por lo que el efecto se debe principalmente a la presencia de lecitina y no a la formulación. En cambio, tanto los BBG (bG) como la mezcla de éstos con lecitina (LecbG) no tenían ninguna influencia en el crecimiento fúngico en comparación con el control. Por tanto, la reducción de crecimiento que presentaban las formulaciones de resveratrol con estos productos, tanto por SD como por PGSS-drying, se debía a un efecto sinérgico de los distintos compuestos, además de a una mejor absorción del resveratrol por la presencia de BBG. Así, se consiguió la formulación de partículas a partir de productos de origen natural y no-tóxicos con efecto antifúngico contra *B. cinerea*.

<u>Capítulo II:</u> Use de β-glucanos de cebada y levadura como nuevos emulsionantes para el desarrollo de formulaciones acuosas de productos antifúngicos de origen natural.

Este capítulo incluye la formulación de suspensiones acuosas de resveratrol encapsulado en β-glucanos para probar su efecto contra *B. cinerea*. En este caso, además de β-glucanos de cebada, se usaron otros de levadura (YBG). Éstos no eran solubles en agua, por lo que se realizó primero un pretratamiento de hidrólisis ácida a alta temperatura para su solubilización. Para la producción de suspensiones, se creó primero una emulsión aceite-en-agua y se eliminó el disolvente (acetato de etilo) por evaporación a vacío. Las emulsiones contenían resveratrol en

la fase orgánica y β-glucanos como material encapsulante en la fase acuosa. Igual que en el capítulo anterior, se usó también lecitina de soja como encapsulante y mezclado con cada β-glucano. Se realizaron 3 procesos de emulsificación distintos para estudiar las diferencias en el producto final con cada uno: con un equipo rotor-estator, a alta presión y a alta presión y temperatura.

Con respecto a la solubilización de los YBG, se observó que tanto la pureza de los β-glucanos en la solución como su peso molecular aumentaban con el tiempo de solubilización, encontrándose un máximo para ambas variables entre 3.5 y 4h. A tiempos mayores, los YBG se empezaban a degradar en compuestos de menor tamaño, de forma que se reducían tanto la pureza como el peso molecular.

Una vez producidas las emulsiones aceite-en-agua, se estudió la influencia de la concentración de material encapsulante en el tamaño de gota. Pese a que todas las distribuciones eran unimodales, se observaron diferencias en el tamaño medio de gota con el tipo de encapsulante. Las emulsiones con lecitina y YBG tenían tamaño medio por debajo de 2.5 µm en todo el rango de concentraciones estudiado. Sin embargo, con BBG se apreciaba un aumento del tamaño medio de gota con la concentración de surfactante, hasta un máximo de 33 µm, posiblemente por la mayor viscosidad de la solución acuosa. Además, por cryoTEM (Figura 4) se observó que los BBG en disolución acuosa forman redes de polímero en lugar de gotas. Sin embargo, al mezclar los BBG con lecitina, no se observaron estas redes, sino que el comportamiento era más parecido al de lecitina sola. Estas observaciones coincidían con los resultados de tamaño de gota, donde también se apreció en la mezcla de BBG y lecitina tamaño medio en el rango del que se obtenía con lecitina sola.

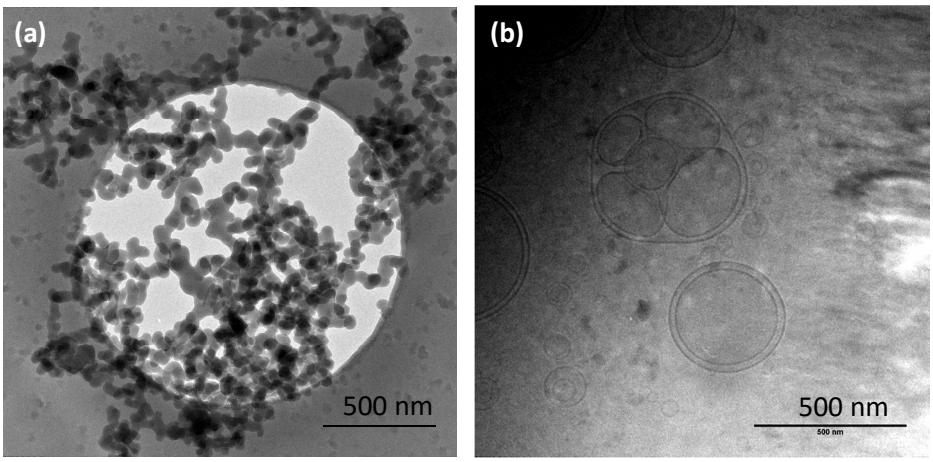


Figura 4. Imágenes de soluciones acuosas de BBG (a) y la mezcla de BBG y lecitina (b).

Con respecto a los distintos métodos de emulsificación, en todos ellos se apreciaba el mismo comportamiento en el tamaño medio de gota: éste era mucho mayor en las emulsiones producidas con BBG, pero disminuía al mezclarlo con lecitina. No obstante, después de retirar el disolvente orgánico por evaporación a vacío y de centrifugar la suspensión resultante para eliminar aglomerados de partículas y cristales grandes sin encapsular, el tamaño medio de partícula estaba entre 60 y 90 nm para todos los materiales encapsulantes y con los tres métodos de emulsificación estudiados.

También la eficiencia de encapsulación de resveratrol era parecida en todos los casos, variando entre 65-100% (550-800 ppm), excepto en el caso de BBG, donde la eficacia era mucho menor debido a la formación de un gel al centrifugar la suspensión que retenía una gran parte de resveratrol. Mediante difracción de rayos X se evaluó la cristalinidad del resveratrol en el producto final. En las emulsiones creadas a alta presión y temperatura, el resveratrol se encontraba en estado amorfo con todos los distintos encapsulantes debido a que no le daba tiempo a recristalizar por el enfriamiento rápido. También precipitaba en estado amorfo en las formulaciones con BBG, para todas las técnicas de encapsulación. En todos los demás casos se obtenían picos correspondientes a cristales de resveratrol en las partículas de las suspensiones finales, aunque se observaba que éstos eran mayores con lecitina sola y disminuían al mezclar

lecitina con cualquiera de los β-glucanos. Así, se apreció una interacción entre los β-glucanos y el resveratrol que disminuye la formación de cristales, posiblemente por la formación de puentes de hidrógeno entre ambos compuestos. Este efecto no era tan fuerte al añadir lecitina.

Por último, se analizó la actividad antifúngica de las distintas formulaciones frente a *B. cinerea* para una concentración de resveratrol en el producto a aplicar de 100 ppm (Figura 5). Las formulaciones que contenían lecitina, tanto sola como con BBG, no producían ninguna reducción significativa del crecimiento del hongo. Al mezclar lecitina con YBG, se conseguía una reducción de hasta el 15% con algunas de las técnicas de emulsificación. Las formulaciones que contenían β-glucanos solos eran las más efectivas, alcanzando una reducción de crecimiento del 23% con BBG y 53% con YBG. No se apreciaron diferencias entre los distintos métodos de emulsificación.

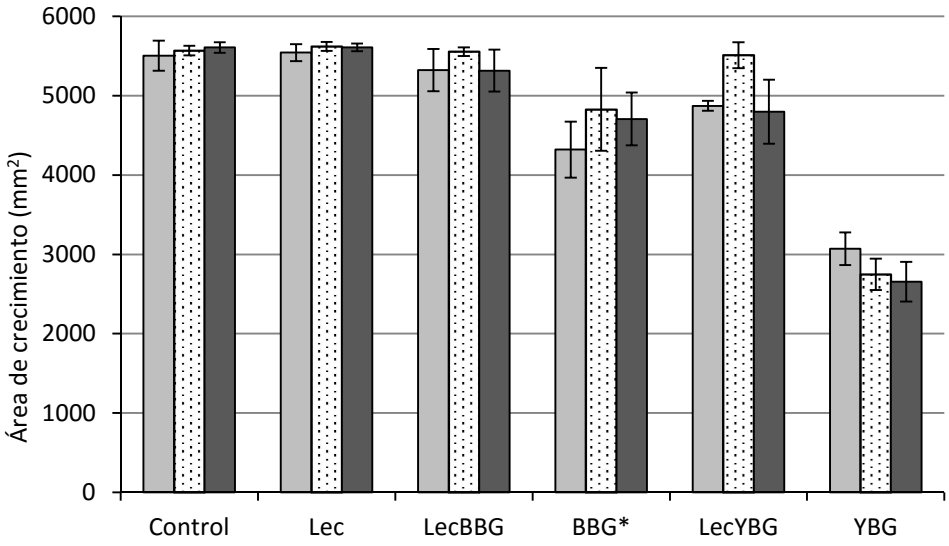


Figura 5. Área de crecimiento de B. cinerea al aplicar las suspensiones de resveratrol centrifugadas. Gris claro: emulsificación mediante rotor-estator. Con puntos: emulsificación a alta presión. Gris oscuro: emulsificación a alta presión y temperature. *: suspensiones sin centrifugar.

De acuerdo a esto, YBG fueron los que mejores propiedades mostraron en formulación, con eficacias de encapsulación de hasta 84%, y además fue la formulación más efectiva contra *B. cinerea*. Los BBG, pese a proporcionar menor eficiencia de encapsulación, también tenían cierta

actividad antifúngica. En cambio, las formulaciones con lecitina, pese a presentar buenas propiedades en cuanto a eficacia de encapsulación y tamaño de gota, no tenían efecto contra el hongo. Además, al añadir lecitina a la formulación con β-glucanos, mejoraban estos parámetros, pero provocaba la precipitación de cristales de resveratrol. De esta forma, se concluyó que la formulación de resveratrol con β-glucanos mejora su acción contra *B. cinerea* posiblemente debido a una mejor penetración del compuesto activo a través de la pared celular del hongo.

<u>Capítulo III:</u> Desarrollo de aerogeles de β-glucanos de cebada y levadura mediante fluidos supercríticos para liberación controlada de compuestos.

En este capítulo se estudia la producción de aerogeles de β-glucanos y su uso como portadores para liberación controlada de compuestos, ya que permiten incorporar una elevada cantidad de compuestos activos debido a su alta superficie específica. Los aerogeles se forman a partir de un hidrogel al que se le elimina la fase líquida. El cambio de la fase acuosa a alcohol y posterior secado con fluidos supercríticos, concretamente CO_2, mantiene la estructura del gel mejor que otros procesos convencionales de secado, como el secado al aire, ya que se eliminan las tensiones interfaciales que pueden provocar cambios en la red de polímero. Además, permite la incorporación de compuestos activos mediante impregnación supercrítica simultáneamente al proceso de secado, de forma que se reducen las etapas de procesado y se evita el uso de disolventes orgánicos.

Los polisacáridos son ampliamente estudiados para aplicaciones de liberación de compuestos debido a su baja toxicidad, biodegradabilidad y estabilidad. Sin embargo, son pocos los estudios de este tipo que emplean β-glucanos. Los β-glucanos presentan ventajas en cuanto al procesado, ya que son capaces de formar un hidrogel sólo por calentamiento sin necesidad de un agente externo como ocurre por ejemplo con el quitosano. Además, tienen propiedades biológicas muy ventajosas.

En primer lugar, se estudió la reología de los hidrogeles, se determinó que los de YBG eran más estables y elásticos que los de BBG por las diferencias entre la estructura de ambos (Figura 6). Los BBG, al estar formados por cadenas lineales de polímero, se disponen en el gel de manera paralela, mientras que los de YBG tienen más ramificaciones y por tanto más entrecruzamiento de cadenas. Esta mayor interacción de cadenas hacía que los geles de YBG fueran más resistentes a los esfuerzos de torsión y que su viscosidad compleja en disolución fuera mayor. Asimismo, se comprobó que el proceso de gelificación era reversible para los BBG, que podían volver a ser disueltos en agua, pero no para los de YBG.

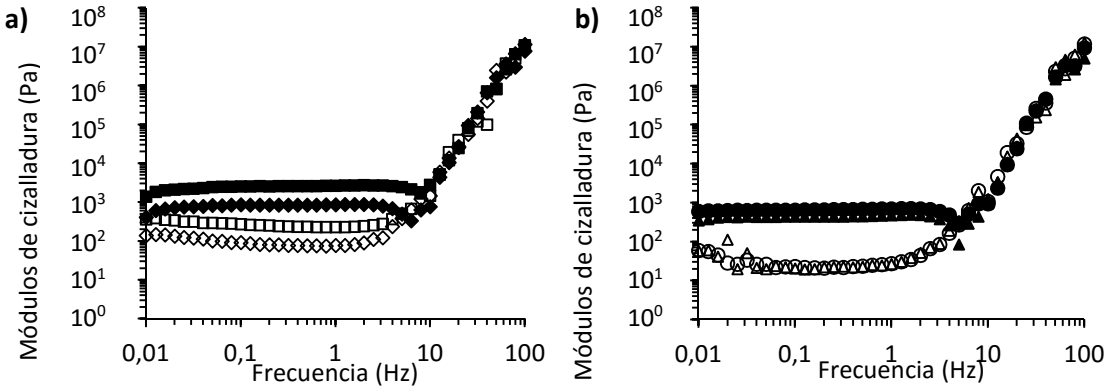


Figura 6.- G' (símbolos rellenos) y G" (símbolos vacíos) de hidrogeles de (a) 2.5% YBG (◆), 5% YBG (■) y (b) 4% BBG (●) y 5% BBG (▲), en análisis oscilatorios en un rango de frecuencia.

Las diferencias con el tipo de β-glucano apreciadas en los hidrogeles se observaron también en los aerogeles. Así, los aerogeles de YBG tenían mayor densidad que los de BBG (121 kg/m^3, and 79 kg/m^3 BBG aerogels), y resistían mayores esfuerzos de compresión (hasta 450 kPa, y los de BBG 70 kPa). También estos parámetros aumentaban con la concentración de β-glucanos. Estos incrementos están asociados a un mayor entrecruzamiento de cadenas en YBG en comparación con BBG, y al aumentar la cantidad de polímero. Pese a estas diferencias, las propiedades texturales eran muy similares en todos los aerogeles, con tamaño medio de poros entre 13-16 nm, volumen de poro de 0.6-0.7 cm^3/g y área BET alrededor de 180 m^2/g, puesto que dependen principalmente de las condiciones de secado.

El proceso de impregnación de ácido acetil-salicílico (AA), como compuesto modelo, con CO_2 supercrítico se llevó a cabo a distintas condiciones de presión y temperatura para estudiar la influencia que tenían ambas magnitudes en el rendimiento de impregnación, que fue de 8-15% w/w en todos los casos (Figura 7a). Se observó que la cantidad de AA impregnada en la matriz aumentaba con la presión. Sin embargo, la tendencia con la temperatura era distinta dependiendo del rango de presión en el que se trabajara, produciéndose un cruce en torno a 12-16 MPa. Por debajo de esos valores, el rendimiento de impregnación era mayor a menor temperatura. Por el contrario, al trabajar a presiones mayores, el rendimiento de impregnación aumentaba con la temperatura. Al relacionar los resultados de rendimiento de impregnación con la densidad del CO_2, se observó que el rendimiento aumentaba con la densidad, ya que se mejora la solubilidad.

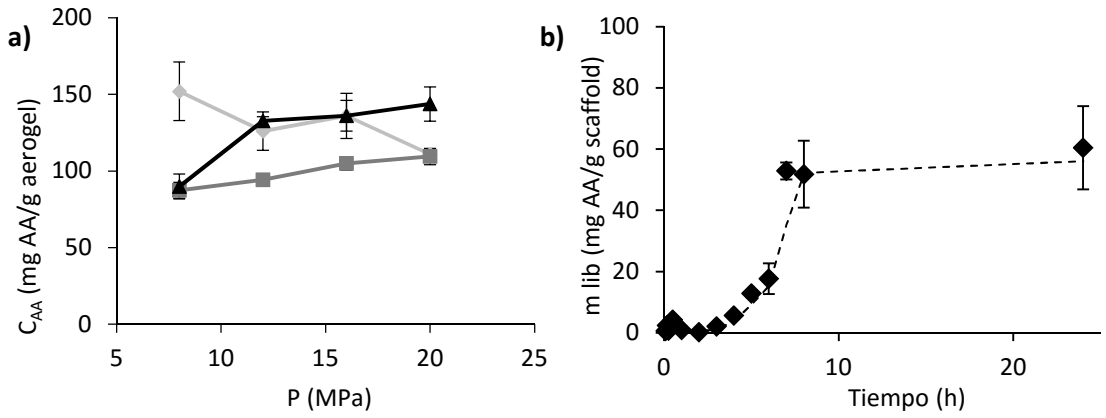


Figura 7.- (a) Cantidad de AA impregnada por masa de aerogel a distintas condiciones de presión y temperatura. Negro: 50ºC; Gris oscuro: 40ºC; Gris claro: 35ºC. (b) Cantidad acumulada de AA liberado por masa de aerogel. Las líneas se añaden para facilitar la lectura.

El análisis de la liberación de AA en PBS (Figura 7b) reveló que durante las primeras 3h no se liberaba nada de AA, un indicativo de que el compuesto había penetrado bien al interior de la matriz en el proceso de impregnación y no se encontraba en la superficie. Pasado ese tiempo, se liberaba el 60% de AA en las siguientes 5h, y este valor se mantenía durante 16h más. Este comportamiento es interesante para algunas aplicaciones de liberación de compuestos en las

que se requiere que el compuesto se libere un cierto tiempo después de su administración y no desde el principio.

Además, a partir del perfil de liberación se estudió el mecanismo de liberación de AA de la matriz de BBG. Se determinó que la liberación del compuesto no estaba controlada por la difusión del mismo desde la matriz hasta el medio líquido, sino por la relajación e hinchamiento de las cadenas de polímero. En estos casos (Super Case II), los compuestos se liberan sin restricciones difusionales una vez que el líquido ha penetrado por completo en la matriz.

<u>Capítulo IV:</u> Preparación de scaffolds a partir de β-glucanos de cebada y levadura por foaming de hidrogeles.

A pesar de que los aerogeles producidos en el capítulo anterior tenías buenas propiedades para su uso como portadores para liberación de compuestos, sus propiedades porosas no eran adecuadas para aplicaciones de regeneración de tejidos. En estos casos, se requieren características muy concretas en cuanto a porosidad o resistencia mecánica. Así, por ejemplo, se necesitan tamaños de poros de al menos 150 µm, para que puedan alojar células en su interior y haya espacio suficiente que permita su crecimiento. Además, tienen que estar muy interconectados para que pueda haber transporte de nutrientes y de desechos entre ellos.

En un intento de aumentar la porosidad de los materiales de β-glucanos, se procesaron mediante espumado supercrítico de hidrogeles. Esta técnica se aplica en polímeros hidrofílicos que no experimentan transición de fase antes de la degradación térmica. En estos casos, se desarrolla primero un hidrogel en el que se crea porosidad al someterlo a CO_2 supercrítico, que se disuelve en la fase acuosa del hidrogel y penetra e hincha la estructura del polímero. Así, al despresurizar el sistema, se crean poros a partir de las burbujas generadas por supersaturación del CO_2. Al final se elimina el agua por liofilización. De esta forma, es posible obtener estructuras de alta porosidad con polímeros de origen natural.

Al realizar el proceso de espumado con CO2 supercrítico en los hidrogeles de β-glucanos, se observó que, en general, la porosidad, el tamaño medio de los poros y la interconectividad de los mismos aumentaban con la presión de espumado (Figura 8). Así, se consiguieron valores de porosidad de hasta 80%, tamaño de poro de 250 µm y un 75% de interconectividad, en el rango de los que se necesitan para regeneración de tejidos.

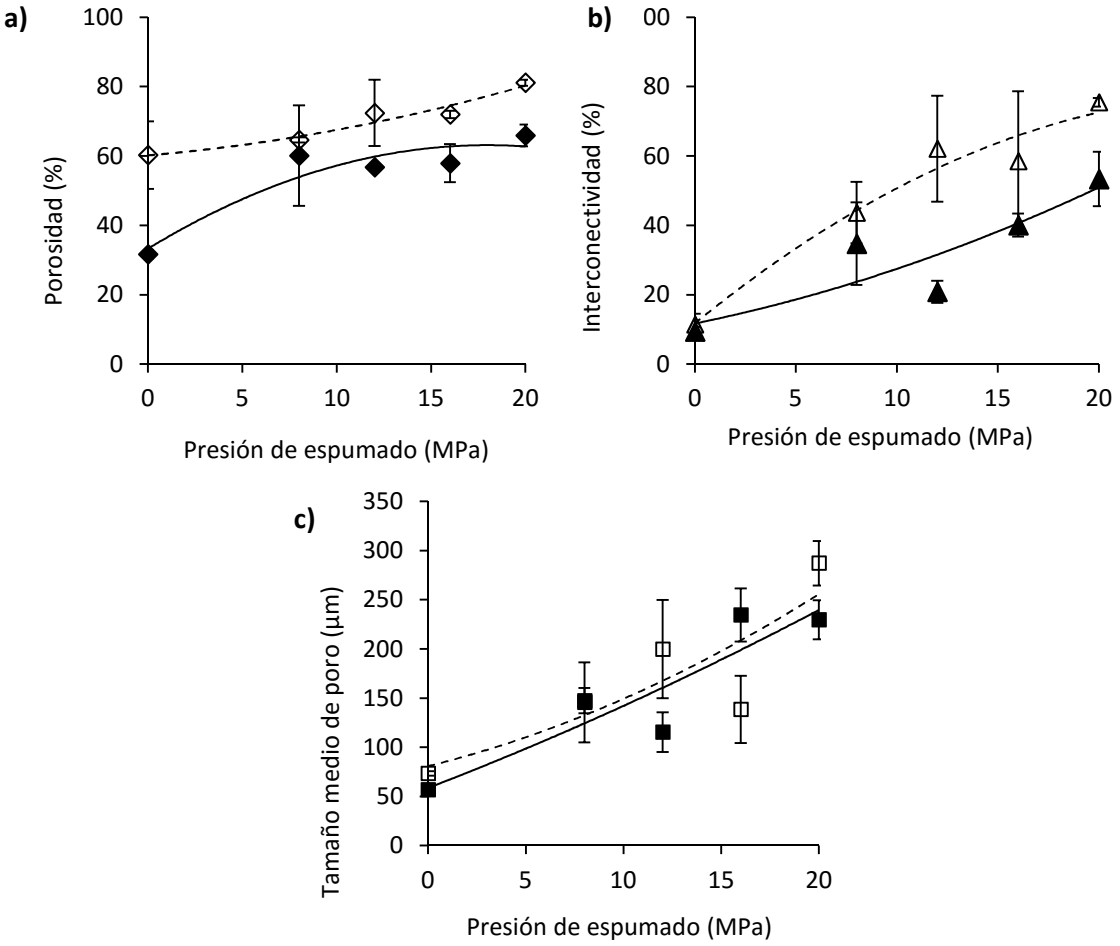


Figura 8: Porosidad (a), interconectividad de poros (b) y tamaño medio de poro (c) de los scaffolds con distintas presiones de espumado. Símbolos rellenos: BBG. Símbolos vacíos: YBG. Las líneas se añaden para facilitar la lectura.

La porosidad aumentaba al someter a los hidrogeles a espumado con respecto a los liofilizados sin espumado, aunque no se detectaban diferencias significativas con la presión de espumado. Por el contrario, la interconectividad de los poros aumentaba con la presión de espumado. Los scaffolds de YBG tenían mayor porosidad e interconectividad que los de BBG, lo cual puede

deberse a que la concentración de polímero era menor, aunque también a una distinta disposición de las cadenas de polímero en los materiales como consecuencia de la diferente estructura de ambos β-glucanos. En cambio, no se apreciaron diferencias en los valores de tamaño medio de poro entre BBG y YBG. En ambos casos aumentaba con la presión de espumado, aunque se observó que si la presión era demasiado alta (20 MPa) se producían estructuras muy heterogéneas, con el interior prácticamente hueco y la mayor parte de polímero posicionada en el exterior formando una costra.

La alta porosidad conseguida mediante espumado repercute negativamente en las propiedades mecánicas de los materiales. Los valores de módulo de Young calculados (en torno a 10 kPa) están muy por debajo de los de los huesos y los cartílagos, aunque sí que son del rango de los que se requieren para potenciar la formación de nuevos huesos y cartílagos. Como las propiedades porosas sí que resultaban adecuadas para emplear estos materiales en regeneración de tejidos, una solución podría ser introducir un material cerámico en la formulación que mejore la resistencia mecánica de los scaffolds.

Las pruebas de liberación de dexametasona (como compuesto modelo con propiedades anti-inflamatorias y de diferenciación de células) de los scaffolds de BBG se realizaron en PBS. Se observó que se producía una liberación controlada de todo el producto durante 4 días, aunque se distinguía una etapa más rápida en las primeras 8h (Figura 9). Se comprobó que el perfil de liberación era similar al cambiar la concentración de dexametasona, ya que depende fundamentalmente de la matriz y de la interacción entre el polímero y el compuesto activo.

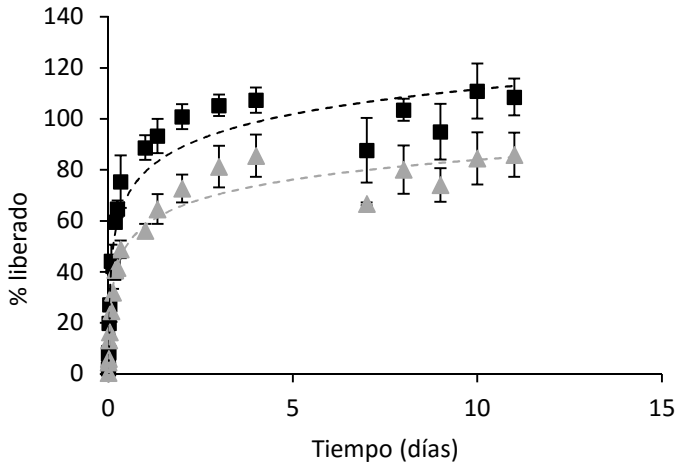


Figura 9: Perfil de liberación de dexametasona de scaffolds de BBG. Dexametasona inicial en los scaffolds: 5% (w/w, cuadrados) o 10% (w/w, triángulos). Las líneas se añaden para facilitar la lectura.

Para analizar el mecanismo que regía la liberación, se ajustaron los datos experimentales a distintos modelos de bibliografía. Con ellos se determinó que la liberación no dependía de un solo factor, sino que dependía tanto de la difusión del compuesto activo hacia el medio líquido como de la relajación de las cadenas poliméricas de la matriz de BBG. En concreto, se calculó que la difusión contribuía en un 95-75%, mientras que la contribución de la relajación del polímero solo alcanzaba el 25%. Además se observó que este último factor aumentaba con el tiempo. Estos resultados en combinación con el perfil de liberación sugieren que la liberación de dexametasona estaba inicialmente controlada por la velocidad de difusión del compuesto desde la matriz hasta el líquido. Con el tiempo, el scaffold se va mojando y las cadenas de polímero se relajan, lo cual contribuye a que se libere dexametasona de zonas que al principio no estaban accesibles al líquido. Sin embargo, en todo momento prevalece en la liberación el control del proceso de difusión, posiblemente porque la alta porosidad de los materiales permite que el líquido penetre la matriz de forma fácil y extraiga el compuesto activo hacia el exterior.

Conclusiones

Este trabajo contribuye al desarrollo de nuevos productos con β-glucanos para aplicaciones agrarias, farmacéuticas y médicas. A continuación se recogen las principales conclusiones que se han obtenido:

- Desarrollo de formulaciones de β-glucanos para la encapsulación de resveratrol como nuevo producto antifúngico, tanto en fase líquida como sólida.
    - Se obtuvieron partículas de resveratrol encapsulado en BBG mediante la técnica de emulsificación-evaporación, y posterior secado mediante PGSS-drying y secado en spray. Las partículas de PGSS-drying eran de menor tamaño que las de secado en spray (10 μm), pero creaban aglomerados más grandes (100 μm). Se calcularon eficiencias de encapsulación similares con ambos procesos de secado (60-96%), de forma que este parámetro está influenciado por la técnica de emulsificación y no por el proceso de secado. Además, se obtuvo resveratrol en estado amorfo en las partículas finales, lo que supone una ventaja en cuanto a la aplicación frente al estado cristalino.
    - Se desarrollaron formulaciones líquidas de resveratrol en BBG y YBG mediante distintas técnicas de emulsificación (equipo rotor-estator, alta presión y alta presión y temperatura). Las suspensiones finales tenían características muy similares independientemente de la técnica de emulsificación que se usó (tamaño de partícula por debajo de 90 nm y entre 70 y 100% de eficiencia de encapsulación). Las mayores diferencias derivaban del material encapsulante. Así, con BBG se conseguía el mayor tamaño de gota en las emulsiones (30 μm) y la menor eficiencia de encapsulación (30-50%), aunque al añadir lecitina a los BBG el tamaño de partícula disminuía (por debajo de 2 μm) y la eficiencia de encapsulación aumentaba (por encima de 65%).

- Tanto las formulaciones sólidas como las líquidas inhibían el crecimiento de *B. cinerea*. Las partículas sólidas de resveratrol encapsulado en BBG reducían el crecimiento en un 70%. En las formulaciones acuosas, el resveratrol tenía mayor actividad antifúngica encapsulado en YBG que en BBG (reducción del crecimiento de 50 y 20%, respectivamente). De esta manera, se confirmó la hipótesis de una mayor actividad antifúngica debido a una mejor penetración del compuesto por la presencia de YBG.

- Síntesis de materiales con β-glucanos para liberación controlada de compuestos e ingeniería de tejidos.
  - El estudio reológico de hidrogeles de BBG y de YBG reveló que los geles creados con YBG eran más estables y más elásticos, de forma que eran capaces de reorganizarse y resistir mayores esfuerzos de torsión antes de romperse. Además, los hidrogeles de BBG podían volver a ser disueltos en agua, mientras que el proceso de gelificación era irreversible en los YBG. Este distinto comportamiento es consecuencia de las diferencias en la estructura de las cadenas y la configuración tridimensional de ambos β-glucanos.
  - Las diferencias observadas entre los hidrogeles de BBG y de YBG también se apreciaban en los aerogeles una vez realizado el cambio de disolvente de agua a alcohol y el secado supercrítico. El mayor entrecruzamiento de las ramificaciones de las cadenas de YBG daba lugar a estructuras más compactas, mientras que los materiales producidos con BBG eran menos densos. Así, para una concentración de β-glucano de 5%, pese a que las propiedades texturales eran similares para ambos β-glucanos (alrededor de 180 m^2/g de área BET, volumen de poro 0.6-0.7 cm^3/g y tamaño de poro entre 13-16 nm), los aerogeles de YBG tenían mayor densidad (121 kg/m^3, y 79 kg/m^3 los de BBG), aguantaban

mayores esfuerzos de compresión (hasta 450 kPa, y los de BBG 70 kPa) y absorbían más cantidad de agua (1100% y 600%, respectivamente).

- La impregnación supercrítica de ácido acetil-salicílico se llevó a cabo en un rango de presión (8-20 MPa) y de temperatura (35-50ºC). Se calculó que se incorporaba entre 8 y 15% de AA sobre la masa total de aerogel, mayor al aumentar la densidad del CO_2 supercrítico. La liberación del compuesto de los aerogeles se inhibía durante las primeras 3h como resultado de la penetración del compuesto dentro del aerogel mediante la impregnación supercrítica. Después de ese tiempo, la liberación estaba controlada por el hinchamiento de la matriz una vez que el polímero se mojaba por completo. Así, los aerogeles de β-glucanos resultaron ser una buena alternativa como portadores para liberación controlada de compuestos.
- Mediante espumado supercrítico de hidrogeles se consiguieron materiales mucho más porosos que los producidos por liofilización y secado supercrítico. Las estructuras resultantes de este proceso tenían hasta un 80% de porosidad, tamaño medio de poro de hasta 250 µm y 75% de interconectividad de poros. Estas propiedades les hacen buenos candidatos como scaffolds para ingeniería de tejidos, ya que con estas propiedades porosas son capaces de alojar células y permiten un buen transporte de nutrientes y desechos que son requeridos y generados, respectivamente, durante el crecimiento y proliferación de las células. Dichas propiedades aumentaban con la presión de espumado, pero si la presión era muy alta (por encima de 16 MPa) la distribución de los poros no era homogénea y se producían estructuras huecas. Con respecto a las propiedades mecánicas, los scaffolds de β-glucanos eran frágiles, por lo que deberían ser mezclados con algún material cerámico que reforzara la estructura y al mismo tiempo mejorara la integración del implante con los tejidos que lo rodean.

- La dexametasona se liberaba totalmente de las matrices de BBG de forma controlada durante 4 días, aunque más rápido en las primeras 8h. Se analizó que dicha liberación estaba controlada principalmente por la difusión del compuesto hasta el medio líquido, siendo mínima la limitación por la penetración del líquido en la matriz debido a su alta porosidad. La contribución de la relajación de las cadenas de polímero a la liberación ganaba importancia a medida que transcurría el tiempo, aunque sólo llegaba a ser del 25% del total.

Posibilidades de trabajo futuro

El trabajo desarrollado en esta tesis podría ser ampliado siguiendo distintas líneas. Por una parte, el estudio de las formulaciones sólidas podría completarse con el uso de YBG, que fue el material encapsulante con el que se consiguió mayor actividad antifúngica en las formulaciones acuosas. Además, se podrían desarrollar las formulaciones con aceites esenciales como compuestos activos, o con un aceite de origen natural que no sea tóxico como fase orgánica, para evitar el paso de eliminación de disolvente del procesado. Como se inhibió el crecimiento de *B. cinerea* con las formulaciones de β-glucanos, las pruebas antifúngicas podrían repetirse con otras especies de hongos que contengan β-glucanos en la pared celular.

En cuanto a la producción de aerogels con β-glucanos, se requeriría un análisis de la influencia de la etapa de cambio de disolvente en las propiedades finales de los materiales. Sería interesante desarrollar esferas de aerogeles en lugar de monolitos, ya que son más ventajosas para liberación de compuestos porque tienen mayor superficie específica y se manejan con más facilidad. Esto se podría hacer de manera sencilla añadiendo gota a gota una disolución caliente de β-glucanos sobre un volumen grande de etanol o en un proceso de producción continua que permita conseguir menor tamaño de partículas (por ejemplo, en un equipo de microfluidos o un sistema de mezclado de las corrientes de etanol y de solución acuosa de β-glucanos, de manera

que las esferas se formarían por efecto anti-solvente). Las esferas podrían emplearse también para regeneración de tejidos si se unen entre sí con un polímero elástico para formar una pieza de mayor tamaño. Para mejorar las propiedades mecánicas de los scaffolds desarrollados en la tesis, se puede añadir un cristal bioactivo en la formulación, que los refuerza y al mismo tiempo facilita su integración con los tejidos nativos, puesto que es capaz de desarrollar estructuras similares a las óseas en contacto con fluidos fisiológicos.

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Marta Salgado Díez (Valladolid, 1987) began her Chemical Engineering studies in 2005 at University of Valladolid. In 2009, she joined the High Pressure Processes Group of the University of Valladolid as student researcher in fluidization experiments. She developed her Master Thesis in the Technical University of Hamburg-Harburg, about spray drying of food suspensions in pilot plant scale. She graduated in 2011 and she was awarded as best student of Chemical Engineering in University of Valladolid.

After finishing her degree, she began her Master's degree on Research in Fluid Thermodynamics Engineering and started working on formulation of active compounds. In 2012, she started her PhD about development of formulations and materials with β-glucans, under the supervision of Soraya Rodríguez-Rojo and María José Cocero. In 2014, she was awarded Jerry King Poster Award, for best poster presentation at 14th European Meeting on Supercritical Fluids in Marseille (France). In the frame of her PhD, she made a stay of research of 4 months at 3B's Research Group, of the University of Minho, under the supervision of Ana Rita C. Duarte and Rui L. Reis.

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PREDOCTORAL STAY

- Stay at 3B's Research Group – Biomaterials, biodegradables and biomimetics, at Universidade do Minho, under supervision of Ana Rita C. Duarte. 01/01/2016-30/04/2016.

PhD GRANTS

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