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Development of natural-based wound dressings impregnated with bioactive compounds and using supercritical carbon dioxide

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ABSTRACT

Film- and foam-like structures of *N*-carboxybutylchitosan (CBC) and of agarose (AGA) were prepared and characterized in order to evaluate their potential application as topical membrane-type wound dressing materials, mostly regarding their sustained release capacities and fluid handling properties. Polymeric biomaterials were loaded with two natural-origin bioactive compounds (quercetin and thymol, which present anti-inflammatory and anaesthetic properties, respectively), separately or as a mixture of these two substances, and using a supercritical solvent impregnation (SSI) method. Impregnation experiments were carried out with supercritical carbon dioxide ($scCO_2$) at 10 and 20 MPa, and at 303 and 323 K. Ethanol (10%, v/v) was employed as a co-solvent whenever quercetin was used. Release kinetic studies were performed for all prepared systems and the obtained results showed that higher amounts of quercetin and/or thymol were loaded when higher pressures and temperatures were employed. Results showed that the separated and the simultaneous SSI loading of these two bioactive substances into CBC and AGA is a feasible and advantageous process and that the relative loaded amounts of these substances can be "tuned" simply by changing the operational pressure-temperature conditions. Quercetin presented more sustained release profiles which can be justified by its higher molecular volume and by its lower water solubility as well as by the specific favourable interactions that can be established between quercetin and CBC. Obtained results showed that the employed SSI process also promoted the size reduction of loaded quercetin particles which can significantly improve the solubility of this compound in aqueous solutions. In addition, prepared systems presented adequate water sorption and water vapor sorption capacities as well as water vapor transmission rates that were in the typical and desired ranges for commercial wound dressings.

1. Introduction

Transdermal systems capable to deliver a bioactive agent into cutaneous/subcutaneous levels are of great interest as a therapeutic or as a cosmetic approach for the effective treatment and prevention of several skin disorders. Topical administration of therapeutic agents offers many advantages over conventional oral drug delivery systems (DDS) and over other more invasive route of administration methods, providing a painless and patient-friendly interface for systemic drug administration (Wagner et al., 2001).

Wound healing is a very special case in which transdermal and topical drug and bioactive species delivery systems can offer several additional advantages. Wound healing refers to the body's replacement of destroyed tissue by living tissue and usually comprises different phases which are not mutually exclusive and that can considerably overlap. Each one of these phases is normally characterized by several specific body response stimuli which are often identified by the generation of some characteristic tissues and/or secretions that may as well require specific treatment agents/conditions (Wiseman et al., 1992). A wound dressing system should present several adequate properties for its intended final application such as flexibility, controlled adherence to the surrounding tissue, gas permeability, durability/biodegradability and the capacity to absorb fluids exuded from the wounded area as well as to, simultaneously, control water loss (Yudanova and Reshetov, 2006; Liu and Huang, 2010). The effective exudates management is one of the principal wound dressing requirements as the accumulation of excess fluid can cause maceration or infection. However, and if the wound is allowed to become too dry, the healing process may be also delayed. This means that, to provide optimum conditions, the dressing should be sufficiently permeable to water vapor in order to prevent the transpired moisture from becoming trapped

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as this could potentially lead to maceration, bacterial proliferation and potential infection (Ovington, 2007).

Depending on the exudation level of the wound, films, foams or combinations of both may be applied (Sibbald et al., 2003) including films with homogeneous structures and composite laminates of two or more materials (Mi et al., 2003). Synthetic polymers like poly(urethanes), poly(ethylenes), poly(ε-caprolactone), poly(lactic acid), poly(glycolic acid), poly(glycolic-lactic acid), poly(acrylonitrile), poly(amino acids), silicone rubbers and natural polymers (such as alginate, chitosan, gelatin and collagen, as well as some of their derivatives), are the most commonly used materials to prepare wound dressing (Hoffman, 2002). Healing of dermal wounds with natural polymers is quite attractive mostly because these polymers (or their degradation products) are usually biocompatible and they present non-irritant and non-toxic properties, thus being their dermis application easy and safe (Sezer et al., 2007). Moreover, natural polymers are normally inexpensive, readily available from renewable sources, potentially biodegradable and capable of a multitude of possible chemical modifications (Prabu et al., 2008). *N*-Carboxybutylchitosan, a modified and more water-soluble chitosan derivative, is already known to present inhibitory and bactericidal/fungal activities (when tested against 298 cultures of various pathogens), to prevent secondary infections and to present limited scar formation (Muzzarelli et al., 1990). This biomaterial also presents relatively good mechanical properties for several potential biomedical applications and can be processed by many different methods, originating materials in different forms and shapes such as particles, thin films and porous monolith structures (Öztürk et al., 2006). This will permit to control the permeation of water vapor and oxygen, the swelling capacity of the membranes to be used as wound dressing materials as well as the rate-controlling release of drugs or bioactive species in a controlled way, simply by varying the thickness and the porosity of the processed layers. Spongeous-type polymeric matrices and scaffolds are advantageous biomaterials for several pharmaceutical and biomedical purposes as well as for tissue engineering applications such as skin or hard-tissue replacement and regeneration (Muzzarelli et al., 1990; Salehi et al., 2008). Agarose is a non-charged neutral polysaccharide that is soluble in hot water and capable to form thermoreversible physical or non-covalent hydrogels when cooled down below its gelation temperature (323–581 K, depending on the molecular weight characteristics of the agarose source) (Wang and Wu, 1997). Because of their non-toxicity, biocompatibility, favourable interactions with living cells and fast degradation into non-toxic metabolites, agarose-based physical hydrogels are widely implemented in many biological and medical fields (Cascone et al., 2001) namely on cell immobilization and cultivation, on surgical lubricants, as wound healing accelerators and as excipients for pharmaceutical tablets (Lindenbaum et al., 1995), drug delivery beads (Wang and Wu, 1997; Hoffman, 2002; Drury and Mooney, 2003) and, more recently, as natural polymer-based scaffolds (Lozinsky et al., 2008; Roman et al., 2008). In this last case, particular attention is being given to the development of porous reinforced sponge-like scaffolds, prepared in different shapes and porosities by directional freezing of preliminarily formed agarose hydrogels, for the *in vitro* culturing of cells (self-assembled into 3D structures) with controlled drug release capacity.

The incorporation of bioactive compounds (namely anti-inflammatories, anti-microbials, anti-septics, supplements, etc.) into wound dressing materials can play an active role in the wound healing process (either directly or indirectly) to prevent and mitigate inflammation, as cleansing agents, to prevent bacterial infection and to accelerate tissue regeneration by stimulating healthy healing responses with the minimum final scar formation (Purna and Babu, 2000; Pranoto et al., 2005; Cabodi et al., 2006; Seydim and Sarikus, 2006; Altiok et al., 2010). Flavonoids are naturally occurring substances that possess already well-known and positive effects on the human health like anti-oxidant, anti-inflammatory, anti-allergic, anti-microbial and anti-cancer activities (Guardia et al., 2001; Faderl and Estrovb, 2003; Alvarez et al., 2004; Zhang et al., 2008). Quercetin is a very common and important dietary flavonoid that is recognized as a intra-arterial pressure reducer and to show anti-inflammatory and anti-oxidant activities. Despite its wide spectrum of potential pharmacological properties, the use of quercetin in the pharmaceutical field is quite limited and this is mainly due to its low aqueous solubility and to its easy degradation in aqueous intestinal fluids (Zhang et al., 2008). Topical and transdermal administration may provide an efficient alternative for quercetin delivery in order to enrich the endogeneous cutaneous protection system, which would represent a successful strategy for diminishing ultraviolet radiation oxidative damages as well as any involved inflammation processes of the skin (Casagrande et al., 2006, 2007). Thymol is a monoterpenic phenol which is usually found in thyme oil and that is known to present strong anti-septic, anti-oxidant, anti-bacterial and anaesthetic properties (Priestley et al., 2003). It is recognized as a safe food additive and it is frequently used in several products as a flavouring and/or anti-microbial agent, showing a broad-spectrum of biological activities against bacteria, yeasts and fungi (Sivropoulou et al., 1996; Nobile et al., 2009). Braga et al. (2006) reported that thymol can have several helpful effects in controlling inflammatory processes present in many infections. Recently, a significant inhibitory effect on gram-positive bacteria and fungi of thymol-loaded water-soluble chitosan nanoparticles was also reported in literature (Hu et al., 2009) which also strengthens the potential applicability of this substance in pharmaceutical and biomedical fields (Pranoto et al., 2005).

The incorporation of drugs or other bioactive species into polymeric matrices is usually performed by mixing these substances during polymeric synthesis and/or processing or by immersing and soaking the previously prepared polymeric materials into a solution containing the bioactive substances to impregnate/load (Gilpin and Pachla, 2003; Li and Jasti, 2006; Braga et al., 2008). Although being relatively simple to implement, these methods can present some drawbacks, as the use of organic solvents (which have to be removed to acceptable limits both for health/safety reasons and for product integrity maintenance), undesired substances reactions and/or degradation, low incorporation yields and heterogeneous dispersion. Supercritical solvent impregnation (SSI), and namely supercritical carbon dioxide ($scCO_2$) impregnation, is being recently proposed as an alternative methodology to overcome most of these problems. Among the main advantages of this technique, it is possible to mention the avoidance of organic solvents, the possibility of working at relatively low operational temperatures and with hydrophobic drugs/substances (which cannot be impregnated by aqueous solution/suspension soaking methods) as well as with most of the polymeric matrices usually addressed for biomedical purposes. Supercritical fluids (SCFs) have physical and transport properties between gases and liquids and possess unique properties that can bring several additional advantages to polymer processing. Besides its greener characteristics, SSI methods also permit to tune additive loading and additive depth penetration by controlling the depressurization step and the impregnation period, or by changing the mobile phase solvent density by the simple pressure and temperature manipulation (Kazarian, 2000; Kikic and Vecchione, 2003; Braga et al., 2008; Natu et al., 2008; Costa et al., 2010a,b). In addition, the supercritical solvent can be completely and easily separated from the substrate and, in most cases, it can be carried properly in order to not alter and/or damage polymeric materials physical, chemical, and mechanical properties and without degrading bioactive additives and polymers. Finally, the high solubility, diffusivity, and the swelling and plasticizing behaviors

of $scCO_2$ in most polymers make it a unique temporary plasticizer that can thus help and accelerate the sorption of bioactive additives into polymers (Zhang et al., 1997; Wang et al., 2002). Therefore, $scCO_2$ offers an advantageous balance when impregnating/loading additives into polymers, by swelling and plasticizing the polymeric matrix and by maintaining a relatively weak solvent power for the additives to be loaded, i.e., it can assure the appropriate plasticization of the polymeric substrate as well as the appropriate solvent power for the additive. This will strongly favour the additive partition into the polymeric phase over the supercritical phase, yielding higher loadings and a more uniform distribution of the additives in the whole polymeric substrate (Shieh et al., 1996; West et al., 1998; Wang et al., 2002).

The main objective of this work was to study the loading and the release capacities of biodegradable and biocompatible natural-based polymeric matrices, namely *N*-carboxybutylchitosan (CBC) and agarose (AGA), that were impregnated/loaded with two natural-origin bioactive compounds, quercetin and thymol, separately or as mixture, by using a supercritical solvent impregnation (SSI) methodology. The long-term goal of this work is to prepare and to develop potential hydrogel-type delivery systems based on natural-origin bioactive species envisaged for wound healing applications. Biopolymer substrate materials were prepared in the shape of films and as foams in order to understand how their different physical–chemical characteristics may influence their final loading, release and barrier properties. The amounts of loaded/released bioactive substances were quantified using UV spectrophotometry and the obtained results were discussed in terms of all the involved $scCO_2$/ethanol/biopolymers/bioactive substances properties and interactions as well as in terms of the employed experimental conditions. In addition, the fluid handling properties of the prepared biopolymeric delivery systems, such as water swelling and water vapor transmission rate, were also investigated.

2. Materials and methods

2.1. Materials

Employed bioactive compounds, quercetin (98%) and thymol (99%), were obtained from Sigma–Aldrich. Chitosan (medium molecular weight), agarose Type III-A, levulinic acid (98%) and sodium borohydrate (99.5%) were also obtained from Sigma–Aldrich. Potassium sulfate (99%) and lithium chloride (99%) were obtained from Fluka. Carbon dioxide (99.998%) was obtained from Praxair, Spain, and ethanol (99.8%) was purchased from Riedel-de-Haen, Germany. MilliQ distilled water was employed to obtain calibration/standard curves and for the kinetics of delivery experiments.

The molecular structures of employed bioactive substances and polymeric biomaterials are represented in Fig. 1. Films of *N*-carboxybutylchitosan (CBC) and foams of CBC and of agarose (AGA) were prepared by the solvent casting method and by freeze-drying, respectively. CBC was synthesized from chitosan, using levulinic acid and sodium borohydrate, and according to the method already described by Silva (2006). The final solution was concentrated using a rotary evaporator (at 100 mbar and at 313 K) and then placed in an ultrasound bath until a complete degasification is achieved. The solution was then poured into Petri dishes that were placed in an oven (at 308 K, until complete evaporation of the solvent) to prepare film samples, or were freeze dried, to prepare foam samples. AGA films were prepared by dissolving agarose in milliQ water (1%, w/v) at 363 K. The obtained gel was then poured into Petri dishes which were freeze dried to prepare AGA foam samples.

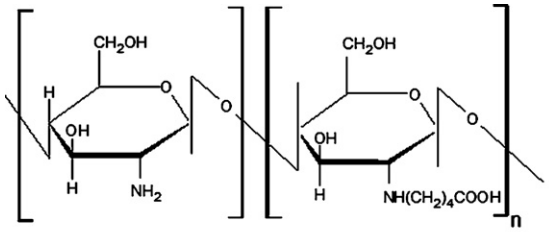
The average thickness of all obtained sample materials was $95 \pm 5\,\mu m$ (for films) and $250 \pm 15\,\mu m$ (for foams). After drying, polymeric samples were cut in rectangular pieces of approximately 10×5 mm and weighing approximately 3 ± 0.5 mg and 5 ± 1 mg, for foams and films, respectively. Nevertheless, all samples were precisely weighed before and after any of the employed processing/characterization procedures. In addition, and between all the processing steps, loaded and non-processed polymeric samples were stored at $-18\,^{\circ}C$, away from light and humidity, and under a nitrogen atmosphere.

2.2. Supercritical solvent impregnation (SSI) loading method

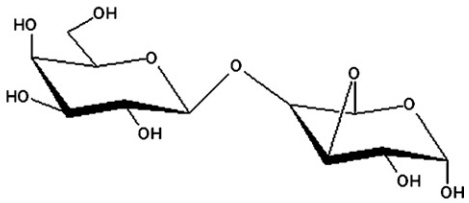
The supercritical impregnation apparatus used in this work was already previously described in the literature (Braga et al., 2008; Natu et al., 2008; Costa et al., 2010a,b). In general terms, it is comprised of a compressed air-operated CO_2 liquid pump, a visual high-pressure stainless steel impregnation cell, a thermostatic controlled water bath and a magnetic stirring plate as an auxiliary tool to dissolve and to homogenize the high pressure mixture (bioactive compounds + $scCO_2$/co-solvent). Ethanol (EtOH), 10% (v/v), was used as the co-solvent for the quercetin system and in order to increase its solubility in $scCO_2$ (Chafer et al., 2004). Two different operational conditions (10 MPa/303 K and 20 MPa/323 K) were tested in order to study their influence on the amounts of loaded bioactive compounds. Previously weighed CBC (or AGA) samples (foams and films) were fitted in stainless steel supports and then placed into the high-pressure cell that already contained known amounts of quercetin, thymol (or of their mixture) and of EtOH (when quercetin is employed). The amounts of employed bioactive compounds were established by taking into account the available cell volume and the compounds solubility in $scCO_2$ (at the tested experimental conditions) and by using amounts that corresponded to 10% of the saturation solubility limit. Employed co-solvent amounts were previously calculated taking again in consideration the available cell volume and the operational pressure and temperature conditions to be employed, in order to achieve the desired co-solvent volume composition (10%, v/v). The system was then closed and pressurized up to the pre-established operational pressure and after bath temperature stabilization. Magnetic stirring (900 rpm) was always employed in order to solubilize and to homogenize the supercritical fluid mixture. All SSI experiments were carried out for 3 h. After this period, $scCO_2$ (or the $scCO_2$/co-solvent mixture) was removed by slow depressurization ($1\,MPa\,min^{-1}$) and in order to not alter or damage the processed polymeric samples.

2.3. In vitro release kinetics

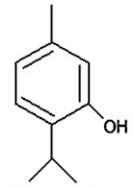
Kinetic studies on quercetin and thymol release (or on the release of their loaded mixture) in milliQ water were performed using a spectrophotometer (Jasco, model 630, Japan), at 366 and 274 nm, for quercetin and thymol, respectively. Rectangular samples of impregnated films or foams with an average weight of 2 mg were immersed in 10 mL milliQ distilled water, at 305 K and under orbital stirring (100 rpm). The loaded polymer weight/volume release solvent ratio was previously optimized in order to avoid saturation of the release solution. At pre-determined time periods, an aliquot (2.5 mL) of the released solution was removed and analyzed being then returned into the release medium. Release experiments were carried out for 9 h. Released quercetin and thymol concentrations were calculated using previously determined calibration curves of known concentrations (in milliQ water and at 305 K). The proportionality between absorbance and concentration was verified in the range from 0 to 100 mg/L and 0 to 4 mg/L for thymol and quercetin, respectively. All release assays were carried out in duplicate.



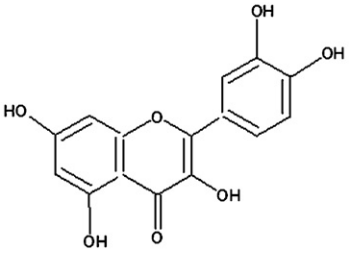
N-Carboxybutylchitosan (CBC)



Agarose (AGA)



Thymol



Quercetin

Fig. 1. Structural formula of employed bioactive substances and polymeric biomaterials.

2.4. Diffusion coefficient calculation procedures

Release kinetic coefficients were estimated for the quercetin/thymol mixture release. Curves were fitted and parameters were calculated using the Korsmeyer–Peppas equation:

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

In Eq. (1), M_t and M_∞ are the absolute cumulative amounts of bioactive substance released at time t and at infinite release time, respectively. This equation was applied for M_t/M_∞ values within the range 10 to 60%. In this equation, n is the release exponent (that provides information on the involved release mechanisms) and k is the so-called kinetic constant (that incorporates structural and geometric characteristics of the release material) (Korsmeyer et al., 1983). The most common release mechanisms can be divided into diffusion-controlled, degradation-controlled or a combination of both (Siepmann and Peppas, 2001; Zuleger and Lippold, 2001). Other equations are generally used to describe the short-time (Eq. (2)) and the long-time (Eq. (3)) approximations for the diffusion-controlled release periods:

$$\frac{M_t}{M_\infty} = 4\left(\frac{tD_1}{l^2\pi}\right)^{1/2} \quad (2)$$

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\exp\left(-\frac{t\pi D_2}{l^2}\right) \quad (3)$$

In these equations, l is the thickness of the sample and D_1 and D_2 are the short-time and the late-time diffusion coefficients, respectively, for each of the considered release periods, and which are assumed to be constant (Higuchi, 1961; Paul and McSpadden, 1976). Eqs. (2) and (3) are just valid for cumulative percentages of delivered substance between 10–60% and 40–100%, respectively.

2.5. Fluid handling properties of prepared polymeric samples

2.5.1. Water sorption ratio

The sorption capacities of CBC and AGA samples were determined gravimetrically after immersing these samples into milliQ water at 305 K. The resulting swollen gels were removed from water after 1, 24 and 48 h and then weighted after removing the surface water. The water sorption (WS) capacity was calculated as the ratio between sample weight at time t and sample initial dry weight. Each assay was triplicated and the corresponding average value was taken as the water swelling capacity for each sample.

2.5.2. Water vapor sorption

Samples were cut into 10 mm × 10 mm squares and dried at 313 K for 48 h until constant weight was achieved. Dried samples were then exposed to a 95% relative humidity (RH) atmosphere, in a desiccator containing a saturated solution of potassium sulfate at 305 K. Samples were weighed periodically (at fixed time periods) and the water vapor sorption capacities (WVS) were calculated using the following equation:

$$\mathrm{WVS} = \left(\frac{W_t - W_0}{W_0}\right) \times 100 \quad (4)$$

In Eq. (4), W_t is the sample weight at time t and W_0 is the sample initial/dried weight. Each experiment was triplicated and the corresponding average value was taken as the water vapor sorption capacity (in percentage) for each sample.

2.5.3. Water vapor transmission rate

Water vapor transmission rates (WVTR) were determined according to a modified ASTM standard (inverted-cup, E96-90, Procedure D) by monitoring the amount/mass of evaporated water through the test-sample membrane and by measuring the weight loss from a water-filled homemade modified Payne Cup. Permeability cells were filled with 5 g of de-ionized and distilled water and the test-sample membrane was fixed onto its opening, with an exposed central circular aperture of 3.14×10^{-4} m^2. Cells were weighed and placed into a desiccator, bottom-filled with a saturated lithium chloride solution at 305 K that promoted an equilibrium relative humidity (RH) of approximately 20%. A digital hygrometer was used to monitor chamber conditions. WVTR daily values were calculated, at pre-determined time periods, by the following equation:

$$\mathrm{WVTR} = \frac{24m}{A\ \Delta t} \quad (5)$$

In Eq. (5), m is the water loss mass (g) along the specified time period, Δt is that time period (h) and A is the effective transfer area (m^2).

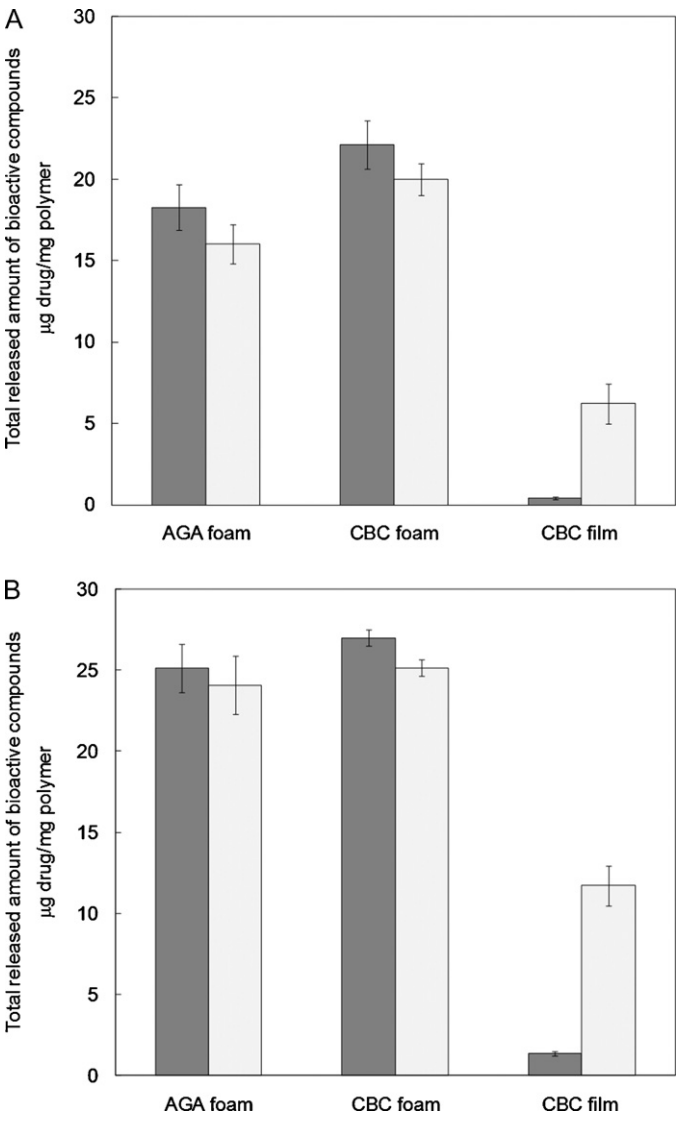


Fig. 2. Quercetin and thymol total/cumulative released amounts from AGA (foams) and from CBC (films and foams) after a 9 h release period. Experiments performed at different SSI experimental conditions: (A) 10 MPa and 303 K; (B) 20 MPa and 313 K. Results for quercetin (■) and for thymol (■).

2.5.4. Microscopic and spectroscopic characterization

Scanning electron microscopy (SEM) (Jeol, model JSM-5310, Japan) micrographs were obtained at 25 kV. Samples were coated with gold (approximately 300 Å) in an argon atmosphere. FTIR-ATR spectroscopy (Jasco, model 4000, UK) was performed at 32 scans and with a 4 cm^{-1} resolution between 500 and 4000 cm^{-1}. Samples were analyzed before and after the impregnation processing experiments.

3. Results and discussion

The effect of employed experimental SSI conditions on quercetin and thymol released amounts from AGA (foams) and from CBC (films and foams), and after the 9 h release period, is shown in Fig. 2A and B (at 10 MPa/303 K and at 20 MPa/313 K, respectively). It is clear that, for all employed polymeric samples, higher pressure and higher temperature led to higher quercetin and thymol total/accumulated released amounts (in 9 h). Despite the fact that we did not quantify the exact total quercetin/thymol loaded amounts by the SSI process, the obtained release profiles (not presented for the separate SSI loading of quercetin and thymol) indicated that almost all quercetin/thymol was released during the above referred 9 h release period. In addition, we also tried to determine these remaining amounts (after the release experiments and after leaching in water for several days) by UV analysis (in concentrated/evaporated solutions) and just negligible residual amounts were detected. Therefore, we can assume that the observed total released amounts do not greatly differ from the corresponding total impregnated/loaded amounts and that the SSI efficiency was somehow affected in the same way by the employed pressure and temperature SSI conditions. At these conditions, the two employed experimental pressure/temperature conditions present similar solvent (or solvent mixture) densities—approximately 17.5 mol/L (NIST Chemistry Webbook). For most systems, solvation and solvent capacity are usually directly related to solvent density, this means that, and for each bioactive compound, the high pressure mobile phase may present quite similar solvent capacities for both experimental conditions. However, at the highest pressure condition (at 20 MPa/313 K), the solvent mixture is in a supercritical state while, at 10 MPa/303 K, it is in a pressurized liquid phase. Thus, the different transport properties (viscosity and diffusivity) which are characteristic of each one of these experimental conditions and corresponding supercritical and liquid states, may justify the observed differences in the release/loading results for each bioactive species. In addition, at higher pressures/temperatures, the $scCO_2$ swelling and plasticization of most polymers is usually favoured and this will also improve the impregnation/loading efficiency (Kazarian, 2000; Kikic and Vecchione, 2003; Fleming and Kazarian, 2005).

It is also clear that, and as expected for the employed impregnation conditions, the higher porosities of foam-like structures permits the loading of higher amounts of quercetin and of thymol, as a consequence of the significant increase in diffusivity and in the available surface area that characterize this type of structures when compared to film-like structures. This is particularly evident for the case of quercetin that, and because of its higher molecular mass and molecular volume, may have more difficulties and founds a higher resistance to penetrate into the CBC film-like structure than into the CBC foam-like structure (at the same operational SSI conditions). In addition, and considering the experimental error (standard deviation), the amounts of loaded thymol and quercetin are very similar for the CBC foams, but not for the CBC film where shape/molecular volume factors may prevail (in this case, by limiting the amount of loaded quercetin). Considering the lower molecular mass/volume of thymol (when compared to quercetin) and its higher solubility in $scCO_2$ it was expected a more efficient impregnation/loading of this bioactive compound into the two employed polymeric materials (and in the shape of foams and films). However, this only happens for CBC dense films. This can occur because the above referred thymol higher solubility and affinity for $scCO_2$ may have also the opposite effect: higher solubilities and affinities in the mobile phase may lead to a low thymol polymer/mobile phase partition coefficient which is promoted by its removal during the depressurization step as the mobile phase is vented. Obviously, and as can be seen, this effect is more pronounced in foam-like structures in which their higher porosities will contribute for the easier removal of this thymol-loaded mobile phase and consequently to lower thymol polymer/mobile phase partition coefficients. Another possible explanation for these results may be the potential favourable interactions that could be established between the employed polymeric biomaterials (CBC and AGA) and quercetin (when compared to the same polymeric interactions with thymol) and may be responsible for an enhanced quercetin polymer/mobile phase partition coefficient despite the diffusional restrictions due to its bulkier nature. These specific interactions as well as their consequences on the release profiles will be discussed later. Therefore, and in conclusion, it is clear that SSI is a quite intricate process and its global efficiency is governed by all the complex physical–chemical properties and

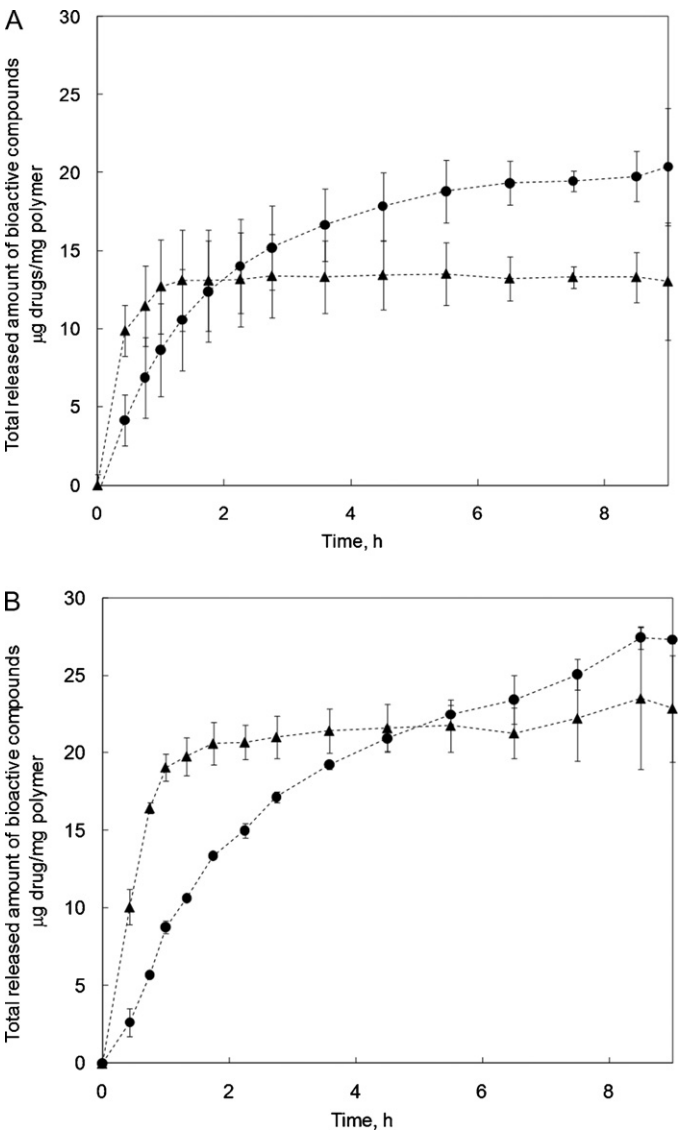


Fig. 3. Quercetin and thymol total/cumulative released amounts from CBC foams for a 9 h release period. Experiments performed with the simultaneous loading of quercetin (●) and thymol (▲), at different SSI experimental conditions: (A) 10 MPa and 303 K; (B) 20 MPa and 313 K. Lines serve only as guides for the eye.

interactions that can be established between all the involved substances in the process: bioactive compounds, biopolymers, $scCO_2$, co-solvents or any other substances which may be present. In addition, the employed operational conditions (pressure, temperature, processing time, co-solvent addition and composition, flow rates, depressurization rate, etc.) can also play important roles in this process (Kikic and Vecchione, 2003; Braga et al., 2008; Natu et al., 2008; Costa et al., 2010a,b).

This work also addressed the characterization of CBC foams regarding their potential ability to be simultaneously SSI-loaded with quercetin and thymol. Fig. 3A and B presents the corresponding released amounts (for a 9 h release period) of these two bioactive compounds from CBC foams (loaded at 10 MPa/303 K and at 20 MPa/313 K, respectively). Although similar release profiles can be observed at the two different operational conditions (for the same bioactive compound) it is clear that the total (accumulated) released amount after 9 h is always higher when CBC foams were loaded at higher pressure/temperature conditions (20 MPa/323 K, Fig. 3B). This was also previously

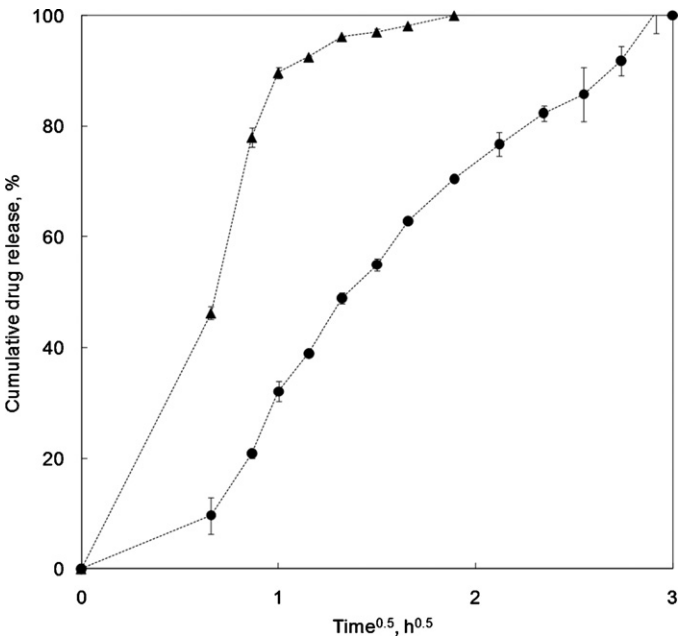


Fig. 4. Higuchi plot for the cumulative percentage of bioactive compounds release. CBC foams loaded simultaneously with quercetin (●) and thymol (▲) at 20 MPa and 323 K.

observed for the separated SSI loading of quercetin or of thymol.

The higher thymol aqueous solubility may justify its observed faster release during the first two monitored hours and for both employed pressure–temperature SSI conditions. On the contrary, a more sustained release profile was observed for quercetin which can be probably due to its lower solubility in water (because of its higher hydrophobic character), to its higher molecular volume (which may difficult its diffusion through the polymeric network and its subsequent release) and also to the some specific favourable interactions between quercetin and CBC (as it was already referred and as it will be discussed later). The characteristic diffusion profiles can also be adequately represented by the corresponding Higuchi plots (cumulative bioactive compounds release, in percentage, as a function of the square root of release time) as shown in Fig. 4 for CBC foams loaded simultaneously with quercetin and thymol at 20 MPa and 323 K. The observed faster thymol release may be an advantageous feature as thymol can work as an enhancer for the intended topical release application, i.e., in order to favour the later quercetin permeation through the skin. The use of skin permeation enhancers in topical drug formulations is a common strategy since it reduces the diffusional penetration barrier by inducing a temporary and reversible increase on its permeability (Olivella et al., 2007; Zhao and Singh, 2000; Chantasart and Kevin, 2010). For example, the inclusion of terpenes in several formulations seems to have a particular and benefic influence in the stratum corneous lipid system by disrupting the hydrogen-bonding network and increasing the hydration levels of the lipid system, probably by forming new aqueous channels (Sunil and Narishetty, 2005). Other example is L-menthol, which was recently identified as an effective skin permeation enhancer for a quercetin-based transdermal therapeutic system that employed a carbopol gel as the polymeric vehicle (Olivella et al., 2007).

A comparison between the total released amounts for each bioactive compound (and thus also assumed as the total loaded amounts of quercetin and thymol, Fig. 3A and B) and the corresponding amounts when the two compounds were SSI-loaded separately (Fig. 2A and B) shows that, and in general terms, the loaded amounts of quercetin are now slightly higher

than the loaded amounts of thymol at both experimental pressure–temperature conditions. In addition, it can also be seen that the highest loading amounts were generally achieved when the two substances were loaded separately and that this decreasing effect was clearly more pronounced for thymol. This can indicate that a "competition" between quercetin and thymol may occur for the mobile phase as well as for the CBC polymer sites. It is known that, and for most situations, as more substance can be dissolved in the mobile phase (either a supercritical phase or a high pressure liquid phase) more efficient will be the SSI process (De Sousa et al., 2006; Duarte et al., 2007; Braga et al., 2008; Costa et al., 2010a). Therefore, the simultaneous solubilization of the two substances will saturate the mobile phase in a faster way and the dissolved amounts of each substance should be smaller than the corresponding dissolved amounts if the substances were dissolved separately. The same type of "competition" between these two compounds can be foreseen for the CBC available sites in which these substances can be deposited. Nevertheless, and based on the obtained results, it seems that quercetin is more specific than thymol for these sites. In conclusion, it is clear that the separated as well as the simultaneous SSI loading of these two bioactive substances into CBC foams is a feasible and advantageous process and that the obtained yields and the relative loaded amounts of these substances can be "tuned" simply by changing the operational pressure-temperature conditions. Despite the fact that we did not perform simultaneous quercetin and thymol SSI-loading into AGA foams, and based on the results for the separated SSI-loading experiments, we may expect that the process should be also feasible and "tuneable" for the impregnation of AGA foams.

By the observation of Figs. 3 and 4, we may assume that the release of quercetin and thymol from CBC foams may be divided into two stages: an initial faster release period (of approximately 2 h) followed by a much slower release period. The individual thymol and quercetin release profiles were fitted to Eqs. (1)–(3) and the correlation results are presented in Table 1. Correlations with Eq. (1) rendered quite similar release exponents ($0.70 < n < 0.87$) for both substances and for the two employed pressure–temperature SSI conditions. This result suggests that quercetin and thymol release occurred by an anomalous non-Fickian diffusion transport mechanism (through diffusion in- and from- the hydrated matrix and by polymer relaxation, i.e., the superposition of Fickian-controlled and of swelling-controlled release) (Ritger and Peppas, 1987; Kuksal et al., 2006). On the other hand, the release rate constant (k) decreased with the pressure and temperature increase (for both substances) despite its value is always much higher for the thymol release results. This is the first numerical evidence that quercetin release is always more sustained than the thymol one and it may indicate a higher affinity between quercetin and CBC than between thymol and CBC. Additionally, the higher aqueous solubility of thymol can also contribute for these obtained values and for its faster release. Similar results and trends were obtained for the quercetin and thymol short-time and long-time approximation diffusion coefficients (Eqs. (2) and (3), D_1 and D_2, respectively) and thus higher diffusion coefficients were always observed for the thymol release, confirming its better ability to diffuse out of the CBC polymeric structure into the bulk aqueous solution.

Considering that the CBC pore-sizes are much larger than thymol molecular size and that thymol does not interact strongly with CBC (Pranoto et al., 2005; Altiok et al., 2010), the most important release rate-controlling factor would be the mass-transfer resistance and thus the hindered diffusion inside the sponge-like porous layer can also be neglected (Peppas and Sahlin, 1989). On the contrary, and since CBC contains the $-NH(CH_2)_4COOH$ groups and still some unmodified $-NH_2$ groups (from chitosan) and $-NHCOCH_3$ groups (from chitin), which are capable of interacting and binding to quercetin hydroxyl and carbonyl groups, the mass transport

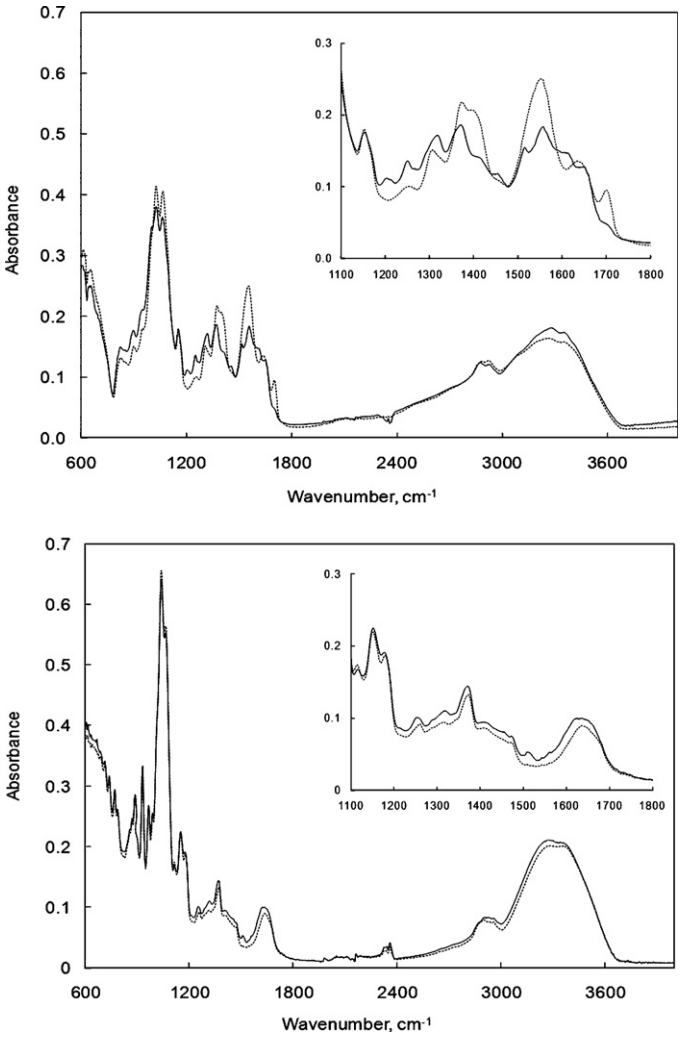


Fig. 5. FTIR-ATR of SSI-quercetin loaded and of non-loaded foams. SSI experiments were performed at 20 MPa/313 K: (A) CBC foams and (B) AGA foams. Full and dashed lines represent the SSI-loaded and the non-loaded polymeric materials, respectively.

resistance will then depend on both chemical/physical and mass-transfer constraints. Previous studies have reported the occurrence of relevant specific interactions (namely hydrogen bonding) and even the ionic complexation in acidic conditions between several phenolic compounds (including quercetin) and chitosan which permitted phenolics isolation from aqueous extraction media as well as the development of potential controlled release systems (Popa et al., 2000; Yamada et al., 2000; Alexandrova et al., 2006; Xia et al., 2007; Zhang et al., 2008; Pasanphan and Chirachanchai, 2008).

The FTIR-ATR spectra of quercetin-loaded and of non-loaded CBC foams (Fig. 5A) show the existence of significant differences in the 1400–1800 cm^{-1} IR range (where the characteristic bands of substituted amino groups are located). These differences include the appearance of some new peaks in CBC-loaded foams (around 1200 cm^{-1}, 1450 cm^{-1}, 1510 cm^{-1} and 1600 cm^{-1},which correspond to the bending and stretching of quercetin aromatic rings), the shift, disappearance or the intensity decrease of some other peaks (around 1700 cm^{-1}, 1400 cm^{-1} and 1310 cm^{-1}, which correspond to the several C=O stretching modes of the carbonyl groups, and around 1550 cm^{-1}, which corresponds to the N–H bending mode of CBC amide II groups). This again suggests the occurrence of the already referred specific interactions between CBC and loaded-quercetin, that led to some structural rearrangements in CBC. An additional evidence for the establishment of specific interactions

Table 1
Correlated release kinetic parameters (n and k) and diffusion coefficients (D_1 and D_2) from Eqs. (1)–(3) and for the quercetin and thymol release from CBC foams loaded at the different employed experimental conditions.

CBC foams	Kinetic parameters			Diffusion coefficients ($cm^2\ h^{-1}$)			
	n	k/h^{-n}	R^2	$D_1 \times 10^4$	R^2	$D_2 \times 10^4$	R^2
10 MPa and 303 K							
Quercetin	0.7871	0.4013	0.9898	0.66	0.9962	0.42	0.9893
Thymol	0.7038	1.1541	0.9979	3.35	0.9557	2.24	0.9975
20 MPa and 313 K							
Quercetin	0.8641	0.2947	0.9692	0.54	0.9866	0.28	0.9799
Thymol	0.8662	1.0045	0.9958	1.83	0.9971	1.78	0.9927

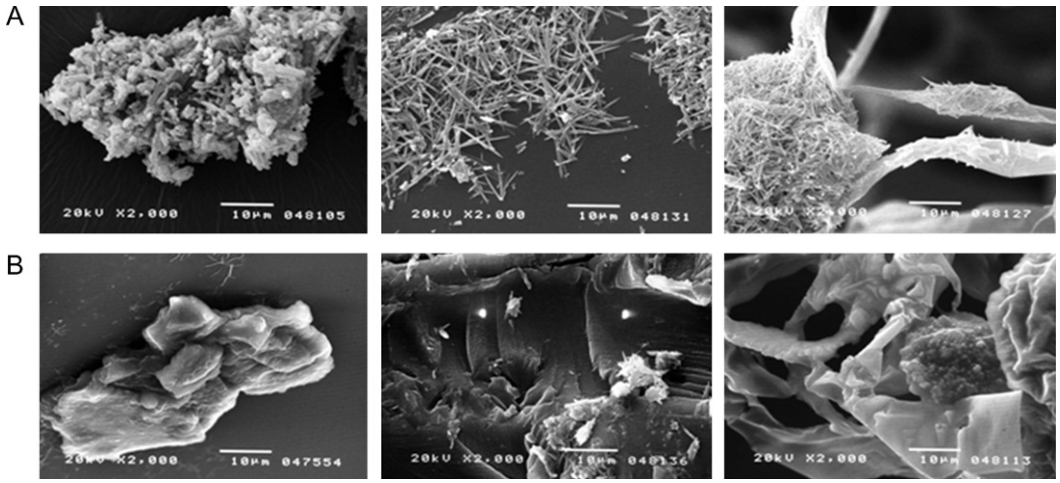


Fig. 6. SEM micrographs for quercetin, thymol and for CBC films and foams. Samples SSI-loaded with quercetin (A) and with thymol (B) (at 20 MPa and 323 K). From left to right: non-processed bioactive compounds, SSI-loaded CBC films and SSI-loaded CBC foams.

between CBC and quercetin comes by opposition and from the FTIR spectra of quercetin-loaded AGA foams which do not present any significant differences in the characteristic AGA IR peaks (as shown in Fig. 5B).

Loaded and non-loaded CBC and AGA matrices were also analyzed using SEM and in order to infer about any possible structural changes that may occur on these polymers as well as on quercetin and thymol morphological properties. It was observed that CBC and AGA morphologies seemed to not be altered by the process (the corresponding SEM micrographs are not presented). However, and comparing Fig. 6A and B (for quercetin and thymol SSI-loaded CBC matrices, respectively), it is interesting to notice that quercetin particle sizes were clearly reduced by the employed SSI process. This is a quite typical occurrence when using supercritical fluids as anti-solvents or as solvents for drugs or any other solid solutes (like in supercritical fluid anti-solvent (SAS) and in rapid expansion of supercritical solutions (RESS) processes), and may have had a great influence on quercetin release from the SSI-loaded matrices. This will happen because the promoted size-reduction will improve the extent and the rate of dissolution of these bioactive substances in aqueous systems, which may lead to a later increased bioavailability and to a subsequent relevant dose reduction in prepared drug delivery systems (Vogt et al., 2008).

It is known that an efficient dressing should produce a moist wound environment in order to enhance wound healing. To guarantee satisfactory conditions at the wound site, the dressing should possess a suitable water vapor transmission rate (WVTR) which, together with the dressing water sorption capacity, will control the extremely important wound fluid balance (Wu et al., 1995). Both properties are directly dependent on the employed dressing material as well as on other environmental conditions such as relative humidity, temperature and local air velocity.

The liquid water sorption capacities of CBC foams/films and of AGA foams were monitored for a period of 48 h after sample immersion in milliQ water at 305 K. As shown in Fig. 7, and after 1 h of immersion, the water sorption ratio was equal to 10, 8 and 4, for CBC and AGA foams and for CBC films, respectively. These water sorption ratio values are in the typical ranges reported in the literature for different polymeric materials and/or membrane processing methods commonly used for these applications (Sripriya

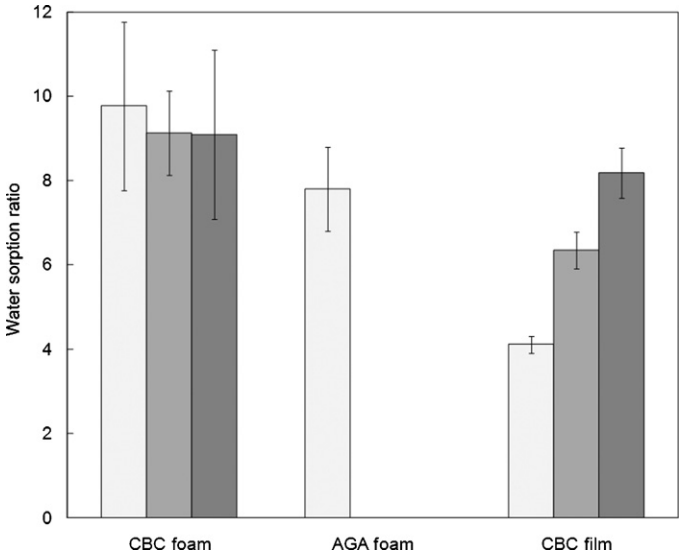


Fig. 7. Water sorption ratio of CBC (foams and films) and of AGA (foams) after 1 h ( ), 24 h ( ) and 48 h ( ) of immersion in milliQ water (at 305 K).

et al., 2004; Sezer et al., 2007; Ruiz-Caro and Veiga-Ochoa, 2009). As expected, the higher porosity of foams led to a faster initial water sorption rate and the sorption equilibrium seemed to be achieved after just 1 h (for CBC foams). Similar results were observed by Park et al. (2006) when studying the influence of foaming/drying methods as well as of cross linking density on the swelling behavior of chitosan (CS) and glycol chitosan (GCS) hydrogels. Nevertheless, the obtained swelling ratio values obtained for CBC are slightly lower than the ones obtained for CS and GCS probably due to the hydrophobicity induced by the alkyl group present on CBC structure (Uragami et al., 1997; Yang et al., 2003). After 24 h of immersion, AGA foams were already significantly dissolved in water being impossible to conclude about its maximum water sorption capacity. On the contrary, CBC films showed a much slower and gradual liquid water sorption behavior which was probably due to the lower diffusivity of water molecules through the smaller pores and denser structure of CBC films. The observed differences between foam- and film-like structures may permit these two distinct morphologies to be applied to different wound types or wound stages, depending on the typical amount/type of exudates produced. Therefore, these two hydrophilic biopolymers can easily swell by absorbing relatively large amounts of water or of aqueous solutions. This feature is essential to allow an efficient release kinetic profile, as it can be responsible for prolonging the residence time of drugs at the absorption site, for sustaining drug release and for improving drug bioavailability for convenient periods of time (Boateng et al., 2008). After the contact of a dry polymeric dressing with a wet wound surface, the wound exudates will penetrate into the polymer matrix causing its hydration and its subsequent swelling, and thus forming a swollen gel over the wound surface that will facilitate the delivery of previously loaded bioactive compounds. In addition, different biomaterials having different (or even slightly different) water sorption properties can be combined in order to develop multilayered structured wound dressing materials having an improved efficiency on the wound healing process. Therefore, the development of asymmetric wound dressing membranes made of these two different biomaterials (CBC and AGA layers) or of different CBC morphologies (foams and films layers) can be a very interesting approach to be addressed in the future development of wound dressings based on these materials (Mi et al., 2002, 2003; Sripriya et al., 2004).

Fig. 8 shows the equilibrium water vapor sorption behavior of CBC (foams and films) and of AGA (foams) from a 95% RH environment. As observed before for the liquid water sorption experiments, CBC and AGA foam-like structures showed a faster initial water vapor sorption rate than CBC films and, once again, CBC films presented a more time sustained water vapor sorption than CBC and AGA foams. However, CBC foams and films presented higher vapor sorption capacities when compared to AGA foams, which achieved their equilibrium vapor sorption capacities after the first two monitoring hours. The water mass loss (or mass of evaporated water) results are shown in Fig. 9. In general terms, the water vapor permeation through the polymeric materials consists of two main steps: adsorption and diffusion. Water vapor is firstly embedded into the polymeric structures (and inside the available void spaces) then it condensates and finally it starts to occur the subsequent diffusion process of the other water vapor molecules. This is the reason why the plots of water mass loss (or mass of evaporated water) against time are always non-linear at the initial periods for hydrogels and for most hydrocolloid dressings. Generally, and after around a 2–3 h period, the water mass loss curves tend to be linear, thus resulting in a constant value for the WVTR. This happens because, and after an initial transient period, the increase in the water vapor diffusivity with hydration is offset by the material swelling and in order to maintain a constant water vapor transmission process (Markin et al., 1998; Mi et al., 2003). Therefore and as can

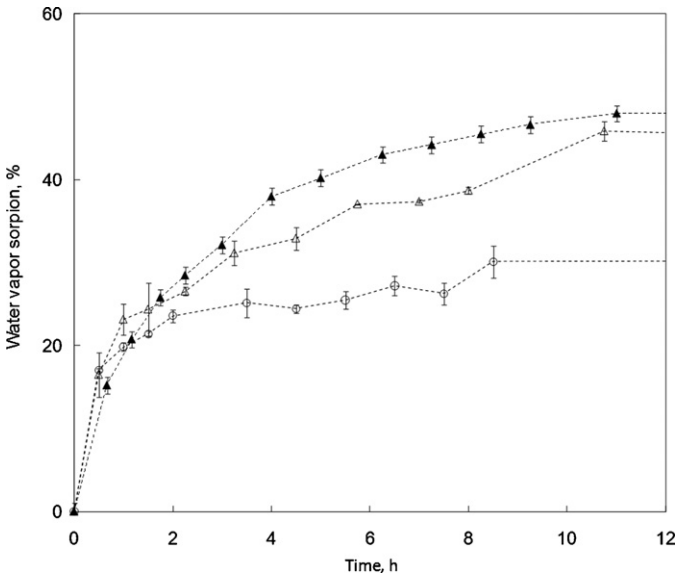


Fig. 8. Equilibrium water vapor sorption for CBC films (▲), CBC foams (△) and AGA foams (○). Experiments were carried out at 305 K and in a 95% relative humidity (RH) environment.

be seen, the obtained results are coherent with this typical water vapor permeation behavior as well as to the water vapor sorption experiments (Fig. 8) in which around 50% of the equilibrium water vapor sorption was attained after ~2.5 h. The much higher porosity of CBC and AGA foam-like structures should be responsible for the initial and enhanced water vapor sorption and adsorption while the lower thickness of CBC film-like structures should promote the water diffusion process, thus resulting in the observed higher WVTR values after the initial polymeric water saturation (Mi et al., 2001, 2002). Daily WVTR results (evaluated at 24 h and under static conditions) were equal to 4352, 4057 and to 3593 g^{-2} day^{-1} for CBC films and for CBC and AGA foams, respectively. An excessive WVTR may lead to wound dehydration and adherence of the dressing to the wound bed, whereas a low WVTR might lead to maceration of healthy surrounding tissue and buildup of a back pressure and pain to the patient. A low WVTR value may also lead

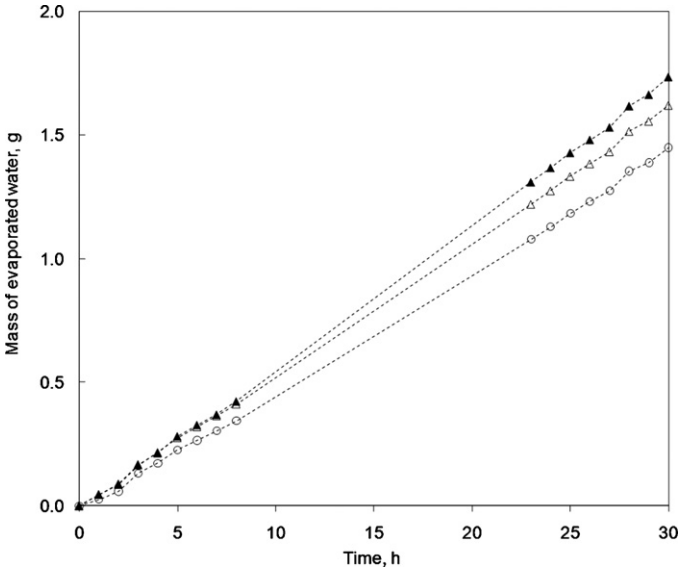


Fig. 9. Mass of evaporated water for CBC films (▲), CBC foams (△) and AGA foams (○). Experiments were carried out at 305 K and in a 20% relative humidity (RH) environment.

to leakage from the edges of the dressing which may result in dehydration and bacterial penetration (Ovington, 2007). It has been recommended that a rate of 2000–2500 g^{-2} d^{-1} would provide adequate level of moisture without risk in wound dehydration (Queen et al., 1987). However, typical ranges usually found for commercial wound dressings made of different biopolymeric materials, vary from 70 up to 9400 g^{-2} d^{-1} (Wu et al., 1995; Hu et al., 2001; Mi et al., 2002; Yudanova and Reshetov, 2006). Such a broad range of WVTR values can be justified by the so many different conditions where a wound dressing may be needed and which includes normal or low-injured skin (typically around 150–200 g^{-2} d^{-1}), first-degree burns (typically around 250–300 g^{-2} d^{-1}) or granulating wounds (around 5000–5200 g^{-2} d^{-1}). In addition, required WVTR can also be quite influenced by other external conditions such as environmental relative humidity and temperature which determine the water vapor partial pressure driving force across the dressing (Lamke et al., 1977; Hu et al., 2001).

4. Conclusions

Biodegradable/biocompatible natural-based *N*-carboxybutylchitosan (CBC) and agarose (AGA) materials were differently processed (as films and/or as foams) and were successfully loaded with two different natural-origin bioactive compounds (quercetin and thymol) by the use of a SSI method. Quercetin and thymol were loaded separately or simultaneously as a mixture.

Results showed that the separated and the simultaneous SSI loading of these two bioactive substances into CBC and AGA is a feasible and advantageous process and the relative loaded amounts of these substances can be "tuned" simply by changing the operational pressure–temperature conditions. Although higher pressures and temperatures allowed the loading higher amounts of both natural substances, the use of mild experimental conditions may be important for more heat sensitive bioactive compounds and/or biopolymers. In addition, by the conjugation of polymeric structure and of process conditions, it is possible to prepare different loaded biomaterials with different release and fluid handling capacities and that may be employed in the healing process of different wounds and/or at different wound stages. The SSI process may also permit to have previously prepared polymeric matrixes/devices that can be subsequently loaded with the desired bioactive compounds, according to the desired application and to patient therapeutic needs, and leaving no harmful solvent residues. Furthermore, obtained results also indicate that these biopolymers may be as well loaded with other quercetin- and thymol-similar bioactive and $scCO_2$-soluble compounds and that, by changing employed biopolymers, bioactive compounds and impregnation operational conditions, several natural-based biomedical and tissue engineering applications can be envisaged.

Quercetin presented more sustained release profiles which can be justified by its higher molecular volume and by its lower water solubility as well as by the specific favourable interactions that can be established between quercetin and CBC. Obtained results showed that the employed SSI process also promoted the size reduction of loaded quercetin particles which can significantly improve the solubility of this compound in aqueous solutions. In addition, prepared systems presented adequate water sorption and water vapor sorption capacities as well as water vapor transmission rates that were in the typical and desired ranges for commercial wound dressings.

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