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## SUPERCRITICAL CO<sub>2</sub> IMPREGNATION OF GELATIN-CHITOSAN FILMS WITH CLOVE ESSENTIAL OIL AND CHARACTERIZATION THEREOF

### Article Highlights

- It is the first study on supercritical impregnation of G/Ch films with clove oil (CO)
- CO in films was affected by CO<sub>2</sub> pressure, impregnation time and G:Ch ratio
- CO loading was favoured at 10-20 MPa, impregnation time of 2-6 h and Gwt% 25-50%
- ScCO<sub>2</sub> treatment had the highest plasticizing effect on G/Ch<sub>50:50</sub>
- Thermal stability of the G/Ch film with 56 mg CO/g<sub>film</sub> to 110 °C was demonstrated

### Abstract

*Supercritical CO<sub>2</sub> impregnation process was used for the first time to fabricate biodegradable gelatin-chitosan (G/Ch) films containing clove oil (CO) for potential use in active food packaging. All the impregnations were carried out at moderately low temperature (40 °C). Aiming to maximize CO loading in the films with acceptable morphological, structural and thermal properties, CO<sub>2</sub> pressure, impregnation time and G:Ch mass ratio were varied. Gelatin fraction in the films of 25-50 wt.%, scCO<sub>2</sub> pressures of 10-20 MPa and impregnation time of 2-6 h impregnation favoured the CO loading. Processing of the film with equal gelatin to chitosan mass ratio (G/Ch<sub>50:50</sub>) at 10 MPa for 2 h yielded sufficiently high loading (56 mg CO/g<sub>film</sub>) without an adverse effect on morphological properties. The G/Ch<sub>50:50</sub> film was therefore chosen for structural and thermal analyses. ATR-FTIR analysis confirmed successful CO incorporation into the G/Ch<sub>50:50</sub> and its interaction with the film. Plasticizing effect of scCO<sub>2</sub> and CO on the film was evidenced by DSC. Incorporation of 56 mg CO/g<sub>film</sub> into the G/Ch<sub>50:50</sub> didn't affect thermal stability of the film. Beside environmental benefits, supercritical impregnation process enables fast fabrication of G/Ch bio-composite films containing CO, thermally stable to 110 °C, which is suitable for most of food packaging applications.*

*Keywords: chitosan, clove oil, composite film, gelatin, impregnation, supercritical CO<sub>2</sub>.*

An increasing global demand for plastics, in particular in the domain of packaging, has raised public concerns about the environmental and economic problems associated with landfill disposal of materials made of non-biodegradable polymers at the end of

their life [1]. The traditional use of polymers derived from fossil-sourced chemicals has therefore been restricted by implementation of new regulations leading to an intensified research on bio-degradable coatings and films obtained from renewable sources such as polysaccharides or proteins, for food preservation from oxidative and microbial spoilage and to the extension of shelf-life characteristics [2].

Chitosan is a polysaccharide composed of repeated acetylated (*N*-acetyl-2-amino-2-*D*-glucopyranose) and deacetylated units (2-amino-2-deoxy-*D*-glucopyranose) linked by β-(1→4) bonds. Com-

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mercial chitosan is obtained by deacetylation of its parent polymer chitin present in green algae, the cell walls or fungi, and in the exoskeleton of crustaceans [3]. Besides being biodegradable, chitosan is known for exceptional biological properties such as antimicrobial and coagulating activities, bioadhesivity, and wound healing ability [4]. Polycationic character allows chitosan interactions with polyanions to produce polyelectrolyte complexes [5]. Chitosan has been used for preparation of functional materials in the form of films, fibres, hydrogels and micro/nanoparticles [6-8]. Presence of OH and NH<sub>2</sub> functional groups in chitosan's structure allows for preparation of various derivatives with tailored properties for specific cosmetic, medical, pharmaceutical, agricultural and food applications [3]. Chitosan is of interest as a potential edible film component for its excellent oxygen barrier and mechanical properties [9,10]. However, the films prepared from chitosan alone are not able to provide an adequate water vapor/oxygen barrier and antimicrobial activity during long term storage due to the hydrophilic nature of chitosan [8]. Gelatin is a protein derived by partial hydrolysis of the fibrous insoluble collagen under acidic or alkaline conditions [11]. Being a biodegradable, relatively low-cost material with well-recognized gelling and viscoelastic properties based on its helix-coil transition, gelatin has found numerous applications in the food industry (edible films, confectionery, low-fat spreads, dairy and meat products), pharmacy and biomedicine (tissue engineering scaffolds, hard and soft capsules, as plasma expanders, in wound care, as injectable drug delivery micro-spheres, and in intravenous infusions) [12-14]. Structural properties of gelatin, and consequently mechanical and barrier properties of its films, depend on amino acids composition and molecular weight distribution [15]. Due to the relatively poor water barrier and mechanical properties, gelatin alone is not suitable as packaging material [16]. On the other side, gelatin films were envisaged for superficial coating to protect food against light and oxygen [14]. Blending of chitosan with other hydrophilic polymers such as gelatin, occasionally followed by alkali cross-linking, has been suggested as an effective strategy for production of biodegradable 'tailor-made' materials for tissue engineering, biomedical, food and packaging applications [14,17-20].

Gelatin-chitosan (G/Ch) blend films were shown to be homogeneous due to their good miscibility and to possess improved mechanical and barrier properties compared to films obtained from either pure gelatin or chitosan [14,21]. These are the result of electrostatic interactions between the ammonium

groups of the chitosan and the carboxylate groups of the gelatin. Interactions between gelatin and chitosan consist in both electrostatic and hydrogen bonding [5]. Hosseini *et al.* [19] reported that the incorporation of gelatin into chitosan films results in more flexible films with lower tensile strength and a higher elongation rate. Their study showed that the increase of G:Ch mass ratio results in a more translucent film due to the reduced light transmission which could expand chitosan-based active film applications as edible coating. Pereda *et al.* [18] developed composite and bilayer edible and biodegradable films based on gelatin and chitosan with improved physico-chemical properties such as water resistance, transparency, and color that had antibacterial activity against *Escherichia coli* and *Listeria monocytogenes*.

Incorporation of plant extracts and essential oils or their components into G/Ch blends can enhance the quality and safety of seafood products primarily for their antimicrobial activity [20,22,23]. Essential oils from aromatic plants and spices represent an attractive natural alternative to chemical additives used in the food industry [24]. Beside antimicrobial or antioxidant activity, the incorporation of plant extracts or essential oils can improve physical and mechanical properties of polysaccharide-protein films [25,26].

Clove (*Syzygium aromaticum*) oil is rich in eugenol (50-90%) [27-29]. The United States Food and Drug Administration (FDA) categorizes clove oil and eugenol as generally recognized as safe (GRAS) for use as a food additive or in a dental cement (U.S. Code of Federal Regulations, 21CFR184.1257) [30]. Eugenol and isoeugenol were proven to have strong antioxidant activity, a protective effect against DNA damage, and antibacterial activity against Gram(+) and Gram(-) bacterial strains at 312.5-625 µg/mL [31-33]. It has been shown that even low weight fraction of the clove oil in the edible polysaccharide films (30 mg oil /g<sub>film</sub>) can result in DPPH inhibition of 72% and antibacterial activity against *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Bacillus cereus* and *Staphylococcus aureus* [34].

Traditional methods for incorporation of bioactive compounds into polymer matrices suffer certain disadvantages, including use of sometimes toxic organic solvents, which have to be removed by heating, undesired drug reactions, drug and polymer photochemical and thermal degradation, low loading and a heterogeneous drug dispersion [35]. Solutions containing volatiles to be incorporated into a polymer by the solvent casting as the most frequently used method can result in a low penetration into the polymer and simultaneous evaporation of the active sub-

stance with the solvent and creation of voids and pits inside the fabricated polymer film [36]. Supercritical carbon dioxide (scCO<sub>2</sub>) is a good solvent for a wide range of natural active substances [37]. At the same time, scCO<sub>2</sub> is also known for its high diffusion ability in organic matter and near zero surface tension as well as for promoting polymer swelling and changing polymer structural properties by a plasticization effect [38-41]. The combination of aforementioned properties makes this fluid a good medium for impregnation of solid matrices with active substances soluble in scCO<sub>2</sub> [42,43]. Supercritical solvent impregnation (SSI), and namely impregnation using scCO<sub>2</sub>, has several advantages over traditional methods for polymer impregnation. SSI process using scCO<sub>2</sub> allows dissolution and incorporation of hydrophobic bioactive substances such as plant essential oils, working at relatively mild conditions (due to the low critical pressure and temperature of CO<sub>2</sub>,  $p_c = 7.39$  MPa and  $T_c = 31.1$  °C) and in an oxygen-free environment. Solvent power and transport properties of supercritical fluid can be tuned by changing operating pressure, temperature and time of contact with the polymer matrix allowing controlled loading and distribution of an impregnating substance [44,45]. Depressurization of the system enables fast and complete phase separation by transition of scCO<sub>2</sub> into gaseous state, resulting in a final product free of solvent traces. The SSI has been used to load poly(lactic acid) and polyethylene and cassava starch films with natural substances with antimicrobial and antioxidant activities present in essential oils (thymol and cinnamaldehyde) [44,46-49] for active food packaging. The SSI was also reported for efficient loading of bark essential oil from *Thujaopsis dolabrata* var. *hondae* and clove oil into poly(lactic acid) and polyethylene based films for food packaging, respectively [49,50].

To the best of our knowledge, there is no data in the available literature on supercritical CO<sub>2</sub> impregnation of G/Ch films with essential oils. For that purpose, this study aimed to investigate the feasibility of SSI process for fabrication of gelatin-chitosan composite films containing clove oil for potential use in food packaging. In this regard, processing pressure, time, and gelatin to chitosan mass ratio (G:Ch) were varied to maximize clove oil loading of the films without an adverse effect on their morphological, structural and thermal properties.

## MATERIALS AND METHODS

### Materials

Chitosan (Ch, low molecular weight, 75-85% deacetylated) was purchased from Sigma-Aldrich

(Germany). Gelatin (G), a yellowish to light brown powder was purchased from Lachner (Czech Republic). Glycerol (purity ≥99%) was purchased from Sigma-Aldrich (Germany). Glacial acetic acid (up to 99.5%, Hemos, Serbia) was used for the solubilisation of chitosan. Commercial clove bud essential oil (*Syzygium aromaticum*) (CO) obtained by hydrodistillation was purchased from Probotanic (Serbia). Commercial CO<sub>2</sub> (purity 99%) was supplied by Meser-Tehnogas (Serbia).

### Film preparation

Composite G/Ch films were obtained by the solvent casting method as follows. Chitosan was dissolved in aqueous acetic acid solution (2 vol.%, pH 3.0) under continuous stirring for 2 h to obtain 1, 2 and 3% solutions, respectively. Gelatin was dissolved in distilled water under continuous stirring for 30 min at 60 °C to obtain 1, 2 and 3 g/100 cm<sup>3</sup> solutions, respectively. Gelatin and chitosan solutions (Table 1) were mixed by magnetic stirrer for 30 min to form homogeneous solutions containing different mass G:Ch ratio of (25:75, 50:50 and 75:25). Subsequently, glycerol (0.3 g/g of dry polymer weight) was added as a plasticizer and solutions were stirred for 30 min. The resulting mixtures were cast into disc-shaped polystyrene moulds with a diameter of 47.18 mm and height of 3.88 mm to be dried in the air at ambient temperature for 24 h and, thereafter, at 70 °C for 18 h. The same amount of glycerol was also used for preparation of pure gelatin and chitosan films (Table 1) which are used for comparative analysis of thermal stability.

Table 1. Preparation of G, Ch and G/Ch blend films

Film	Gelatin solution g/100 cm <sup>3</sup>	Chitosan solution g/ 100 cm <sup>3</sup>	G:Ch, wt. %
G/Ch <sub>25:75</sub>	1	3	25:75
G/Ch <sub>50:50</sub>	2	2	50:50
G/Ch <sub>75:25</sub>	3	1	75:25
G	4	0	100:0
Ch	0	4	0:100

### Chemical composition of clove essential oil

Quantification analysis of clove essential oil was conducted by gas chromatography with a flame ionization detector (GC/FID) on the HP-5890 Series II GC apparatus (Hewlett-Packard, Waldbronn, Germany), equipped with a split-splitless injector and automatic liquid sampler, attached to the HP-5 column (25 m×0.32 mm, 0.52 μm film thickness) and fitted to a ionization detector (FID). Hydrogen (H<sub>2</sub>) was used as a carrier gas with the flow rate of 1

ml/min. Split ratio of 1:30, injector temperature of 250 °C and detector temperature of 300 °C were applied, while the column temperature was linearly programmed from 40 to 260 °C (at a rate of 4 °C/min) and then kept isothermally at 260 °C for 10 min. A 1 µl aliquot of oil was dissolved in the chloroform/methanol mixture (7:3) and injected into the same column. Peak area percentages, obtained by the integration of corresponding chromatograms (FID), were used for quantification of the individual components. The same analytical conditions as above mentioned were employed for the GC/MS analysis. The GC/MS analysis was carried out on the HP G 1800C Series II GCD system (Hewlett-Packard, Palo Alto, CA, USA) equipped with the column HP-5MS (30 m×0.25 mm, 0.25 µm film thickness). Helium was used as a carrier gas. The transfer line was heated at 260 °C. MS spectra were taken at EI ion source of 70 eV; in *m/z* range of 40–450. The sample solutions were dissolved in ethanol and then an amount of 0.2 µl was injected into the column. The components of the oil were identified by comparison of their mass spectra to those from Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for the retention indices were determined by using the calibrated Automated Mass spectral Deconvolution and Identification System Software (Amdis ver. 2.1), compared to those from available literature (Adams) and used as an additional tool to approve the MS findings.

### Supercritical solvent impregnation

Dried G/Ch films (Table 1) were further used for supercritical solvent impregnation (SSI) with clove oil. Batch SSI process was conducted in a 25 ml high pressure view chamber (Eurotechnica GmbH) previously described in detail [51]. Clove oil was placed in a glass container at the bottom of the chamber, and the composite films (0.06 g) were placed in a porous basket above the oil (Figure 1).

After heating the view chamber to 40 °C, CO<sub>2</sub> was introduced and the system was pressurized. The experiments were carried out at pressures of 10, 20 and 30 MPa. The operating pressure and temperature conditions were chosen according to reported liquid-vapour transitions in the clove oil(eugenol)/scCO<sub>2</sub> system within temperature and pressure ranges of 35–45 °C and 10–15 MPa, respectively [52]. Impregnation time was varied from 2 h to 18 h. These times were selected as representative for short, moderately long and long processing of polymeric samples with SSI process according to our previous studies [45,49,51]. The mass ratio of film/clove oil in all the experiments was 1:10. This ratio was chosen with respect to the reported data on CO (eugenol) solubility in scCO<sub>2</sub> at 40–45 °C and 10–30 MPa to saturate supercritical fluid phase with the oil [28,51,52–54]. A slow decompression rate (0.5 MPa/min) was used to depressurize the system to prevent elutriate of the impregnated substance from the polymer matrix. Weight fraction of the clove oil loaded in the G/Ch films (CO%) was calculated using Eq. (1):

$$\text{CO}\% = 100 \frac{m_{\text{CO}}}{m_{\text{F}} + m_{\text{CO}}} \quad (1)$$

where  $m_{\text{F}}$  is the film's mass before impregnation and  $m_{\text{CO}}$  is the mass of loaded clove oil after the SSI process. They are measured using the digital analytical scale with repeatability of ±0.1 mg (Mettler AE100, Mettler Toledo, Columbus, OH, USA).  $m_{\text{CO}}$  presents the differential mass increase of the film before and after impregnation. Each SSI test was run two times and the CO loadings are given as mean values ± average absolute deviations (AAD).

### Attenuated total reflectance Fourier transforms infrared (ATR-FTIR) analysis

Attenuated total reflectance Fourier transforms infrared (ATR-FTIR) spectroscopy was used to study the presence of CO in the films and its influence on

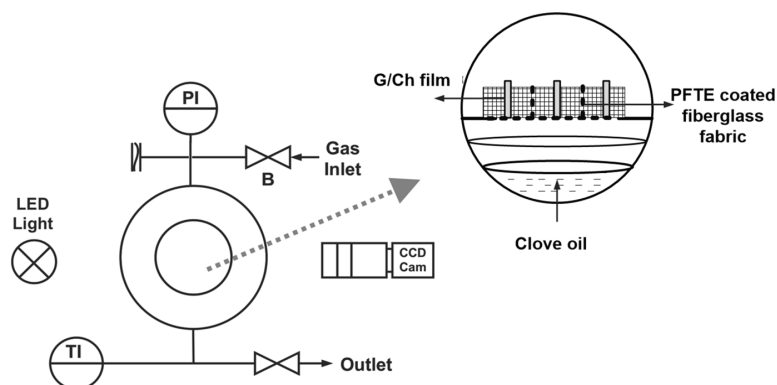


Figure 1. Experimental setup for SSI of the G/Ch films with CO.

the film structure. FTIR spectra of the samples were recorded in absorbance mode using a Nicolet™ iS™ 10 FT-IR spectrometer (Thermo Fisher Scientific) with Smart iTR™ ATR sampling accessories equipped with diamond crystal, within a range of 400–4000 cm<sup>-1</sup>, at a resolution of 4 cm<sup>-1</sup> and in 20 scan mode.

### Scanning electron microscopy (SEM)

Morphological properties of the chosen films were tested in a scanning electron microscope Tescan Vega TS 5130SB. Processing of SEM images was carried out using ImageJ software, developed by National Institute of Health, USA, to determine the change of the film thickness upon impregnation with CO.

### Thermal analysis

Thermal properties of the representative film samples were studied by thermo-gravimetric analysis (TGA) and differential scanning calorimetry analysis (DSC). Thermal stability of the representative film samples were examined by thermal analysis using a SDT Q600 simultaneous TGA-DTA instrument (TA Instruments). The samples were heated in a standard alumina sample pan from room temperature to 700 °C at a heating rate of 10 °C/min under nitrogen atmosphere with a flow rate of 100 cm<sup>3</sup>/min. The influence of scCO<sub>2</sub> and CO on glass transition temperature (*T*<sub>g</sub>) of the G/Ch films was investigated by Differential scanning calorimetry (DSC). DSC measurements were performed using Setaram 151R instrument (software SETSOFT 2000) in the temperature range of 25–300 °C at a heating rate of 5 °C/min under nitrogen atmosphere.

## RESULTS AND DISCUSSION

### The effect of CO<sub>2</sub> pressure, impregnation time and G:Ch mass ratio on the CO loading of the films

Chemical composition of the CO used for SSI of the G/Ch films is given in Table 2. Volatile fraction of the CO used in this study contains mostly eugenol (80.9%), followed by (*E*)-caryophyllene (7.6%), (*Z*)-isoeugenol acetate (7.0%) and isoeugenol (2.2%). Other compounds were present in trace amounts.

Table 2. Composition of the CO; *KI*-Kovats retention index

Compound	<i>KI</i>	Relative content <sup>a</sup> , %
Eugenol	1356	80.9
Isoeugenol	1406	2.2
( <i>E</i> )-Caryophyllene	1417	7.6
( <i>Z</i> )-Isoeugenol acetate	1566	7.0

<sup>a</sup>Corresponds to the % of GC area of each compound

Several factors can affect SSI of the G/Ch films regarding the CO loading efficiency: the partition coefficient of CO between the polymer and the supercritical fluid phase (depending on CO<sub>2</sub> pressure and temperature), the occurrence of specific interactions between scCO<sub>2</sub> and/or CO with the polymer matrix as well as the swelling and plasticizing ability of scCO<sub>2</sub> or the supercritical fluid solution of CO [55–57]. In the initial phase of this research, CO<sub>2</sub> pressure, impregnation time and G:Ch mass ratio were varied to maximize the CO loading at constant temperature (40 °C). The effect of SSI pressure, time and G:Ch mass ratio on the CO loading in the film blends is given in Figure 2. Evidently, gelatin weight fraction (Gwt%) of 25–50 wt.%, low to moderately high scCO<sub>2</sub> pressures (10–20 MPa) and impregnation time of 2–6 h favoured CO loading. At low scCO<sub>2</sub> pressures (10 MPa) the film with the lowest Gwt% (G/Ch<sub>25:75</sub>) and SSI of 6 h yielded the highest loading of 127.8 mg CO/g<sub>film</sub> (Figure 2a). Longer impregnation time at these conditions (10 MPa, 40 °C), except in the case of G/Ch<sub>50:50</sub>, did not favour the CO loading in the films. This could be due to structural changes of the films containing unequal mass fraction of G and Ch (G/Ch<sub>25:75</sub>, G/Ch<sub>75:25</sub>) upon their exposure to scCO<sub>2</sub> and/or CO and their effect on the supercritical fluid uptake. Lower CO loading of the films in the case of short SSI (2 h) at 10 MPa and 40 °C could be due to lower CO solubility in the supercritical phase [54] and lower diffusion coefficients of the supercritical solution compared to higher pressure at constant temperature [58] both leading to decrease of the solute (CO) concentration in the polymer phase. Under isothermal conditions, the increase of pressure promotes CO<sub>2</sub> sorption into the polymer matrix [58–60]. Accordingly, with a pressure increase from 10 to 20 MPa, almost twice as high CO loadings, the G/Ch<sub>25:75</sub> and G/Ch<sub>50:50</sub> films were evidenced after the 2-h SSI (Figure 2b). This is could be due to both the increased solubility of clove oil (eugenol) in scCO<sub>2</sub> with pressure increase at constant temperature [53–55] along with an increased supercritical fluid solution sorption of the G/Ch<sub>25:75</sub> and G/Ch<sub>50:50</sub> films [58]. The slowest SSI rate was observed for G ≥ 50 wt.% (G/Ch<sub>75:25</sub>) at the studied pressure range (10–30 MPa) at 40 °C. It took 6 h of SSI at 20 MPa to achieve the CO loading of G/Ch<sub>75:25</sub> film comparable to CO loading of the films with the G ≤ 50 wt.% for 2-h SSI at the same pressure conditions. At 30 MPa (Figure 2c) it took even longer (18 h) of SSI to achieve CO loading in the G/Ch<sub>75:25</sub> (104 mg CO/g<sub>film</sub>) comparable to 2-h SSI of G/Ch<sub>25:75</sub> and G/Ch<sub>50:50</sub> at 20 MPa (82.6–93.2 mg CO/g<sub>film</sub>, Figure 2b) or 6-SSI of G/Ch<sub>25:75</sub> at 30 MPa (91.7 mg CO/g<sub>film</sub>)

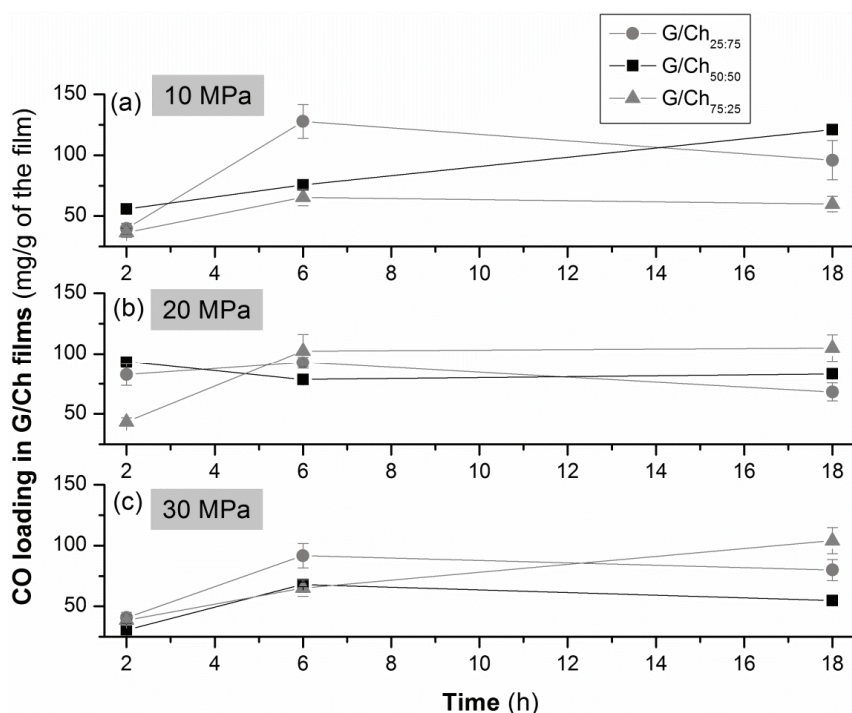


Figure 2. Effect of SSI pressure and time on CO loading in the G/Ch films at 40 °C and: a) 10, b) 20 and c) 30 MPa.

(Figure 2c). Slower impregnation of the film with the highest gelatin proportion (G/Ch<sub>75:25</sub>) could be explained by the formation of a denser matrix and formation of a more stable network due to the attractive interactions between Ch and G in blend films [14]. Accordingly, lowering the available free volume in the matrix could be the reason of lower scCO<sub>2</sub> + CO adsorption. Lower CO loadings evidenced for all the films at 30 MPa and 2–6 h of SSI in comparison to SSI at 20 MPa could be due to two reasons. The first one is an increased solubility of the CO, and consequently its partition coefficient, in the supercritical phase [54]. The other could be decreased scCO<sub>2</sub> diffusivity in the polymer phase due to a higher saturation of the polymer matrix with supercritical fluid [41]. For small CO<sub>2</sub> concentrations in the polymer phase at low pressures, the plasticizing effect of scCO<sub>2</sub> is reflected in the ease of reaching this new equilibrium and therefore in the high values of the diffusion coefficient. On the other side, for high CO<sub>2</sub> concentrations at higher pressures the diffusivity decreases. The hydrostatic pressure may play a role in this decreased diffusivity through reducing the available free volume in the system [42,61].

According to abovementioned, films with gelatin weight fraction (Gwt%) of up to 50 wt.%, pressures of 10 to 20 MPa and impregnation time of 2–6 h at 40 °C were adopted as optimal SSI conditions regarding the CO loading of G/Ch films. Increasing the scCO<sub>2</sub> pressure from low to moderately high pressures (10–20 MPa) can result in an increased mobility of the poly-

mer chains (plasticizing effect) and, consequently, CO<sub>2</sub> sorption. On the other hand, at high pressure free volume, and therefore CO<sub>2</sub> sorption, can be decreased due to an increased hydrostatic pressure [41,42]. Namely, a pressure increase can have two opposite effects on the impregnation process: on the one hand it can enhance clove oil affinity to the supercritical fluid phase, and on the other hand, it increases sorption of CO<sub>2</sub> in the polymer matrix. In addition, interactions between CO and the polymer matrix can contribute to the impregnation yield [55]. For that reason, it was necessary to investigate further structural and thermal properties of the neat and impregnated G/Ch films.

Film G/Ch<sub>50:50</sub> obtained by SSI at the lowest pressure (10 MPa) and for the shortest time (2 h) contains 56 mg CO/g<sub>film</sub> which is expected to be sufficient for antioxidant and antibacterial activity ( $\geq 30$  mg CO/g<sub>film</sub> [34]). Gómez-Estaca *et al.* [20] showed antibacterial activity of the G/Ch films containing only 0.75 ml/g<sub>film</sub>. Therefore, G/Ch<sub>50:50</sub> containing 56 mg CO/g<sub>film</sub> was chosen as a representative sample for morphological, structural and thermal analyses.

#### Characterization G/Ch films

*Morphological analysis.* Analysis of SEM images (Figure 3) indicated that the thickness of G/Ch<sub>50:50</sub> (11.96  $\mu\text{m}$ ) increased to 15.38  $\mu\text{m}$  after being loaded with 56 mg CO/g<sub>film</sub>. Although the thickness increase of  $\sim 3.5$   $\mu\text{m}$  is acceptable due to absence of micron-

sized voids and cracks, it represents a change of 28% of the film thickness. Therefore, higher CO loadings are not suggested in order to preserve film morphology and prevent further swelling and/or foaming of the film upon system depressurization.

**Structural analysis.** ATR-FTIR analysis was performed to evidence presence of CO and its interactions with the polymer matrix. The FTIR spectra of non-treated G/Ch<sub>50:50</sub>, scCO<sub>2</sub>-treated G/Ch<sub>50:50</sub> and G/Ch<sub>50:50</sub> film impregnated with 56 mg CO/g<sub>film</sub> using scCO<sub>2</sub> are shown in Figure 4. The spectra of the blend films showed characteristic peaks of gelatin and chitosan. A broad band 3285 cm<sup>-1</sup> in the spectra of native and scCO<sub>2</sub>-treated G/Ch<sub>50:50</sub> (Figure 4, spectra (a) and (b)) is attributed to the O-H stretching vibration that overlaps the N-H stretching vibration in the same wavelength range [14,62]. Broadening and shifting of the aforementioned peak to the higher wavelength (3351 cm<sup>-1</sup>, Figure 4, spectrum (c)) after loading the G/Ch<sub>50:50</sub> film with CO is assigned to the establishing of hydrogen bonds between clove oil (eugenol) and OH-groups of the G/Ch film. Peaks at 2922 and 2875 cm<sup>-1</sup> evidenced in spectra of all the films arise from symmetric and asymmetric stretch vibration of C-H bonds [62]. An increased intensity of these peaks in the spectra of the impregnated film in comparison to the spectra of the native and scCO<sub>2</sub>-treated film indicates that the addition of clove oil into the film can enhance the hydrophobic groups of the film (CH of CH<sub>2</sub> and CH<sub>3</sub>) [63]. The band at 924 cm<sup>-1</sup> that originates from =CH and =CH<sub>2</sub> out of plane bending characteristic for alkenes has an increased intensity upon G/Ch<sub>50:50</sub> film impregnation which also confirms CO presence in the film.

The characteristic bands at 1639 and 1546 cm<sup>-1</sup> (non-treated and scCO<sub>2</sub>-treated G/Ch<sub>50:50</sub>) correspond to Amide-I, representing C=O stretching vibration coupled with C-N stretch, and Amide-II, bending vib-

ration of N-H groups and stretching vibration from C-N groups, respectively (Figure 4, spectra (a) and (b)) [23]. In the spectra of the G/Ch<sub>50:50</sub> film containing CO the aforementioned bands are shifted to somewhat higher wavenumbers, 1665 and 1559 cm<sup>-1</sup>, respectively (Figure 4, spectrum (c)). The band at 1270 cm<sup>-1</sup> is associated with the stretching of the aromatic C=C groups and the stretching of the phenolic groups present in the eugenol molecules [64]. The peak at 851 cm<sup>-1</sup> associated to ring puckering is also shifted towards a higher wavenumber in the spectra of the impregnated film.

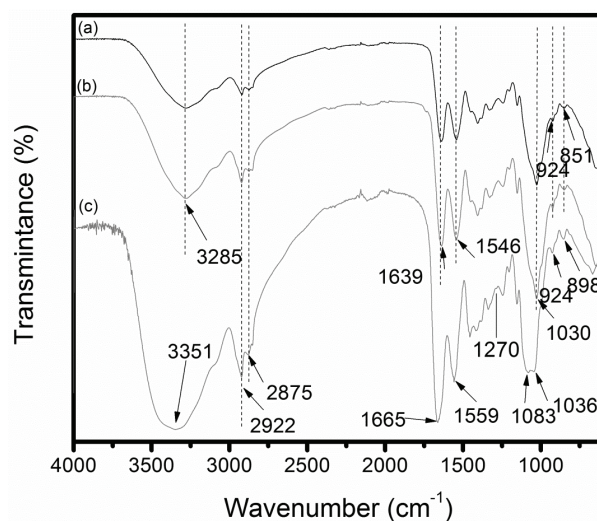


Figure 4. ATR-FTIR analysis: a) non-impregnated G/Ch<sub>50:50</sub>, b) G/Ch<sub>50:50</sub> treated with scCO<sub>2</sub> and c) G/Ch<sub>50:50</sub>+CO.

Beside the aforementioned shifts of the characteristic bands upon the CO incorporation into the G/Ch<sub>50:50</sub>, broadening and an enhanced intensity of the bands at 1083 and 1036 cm<sup>-1</sup> (stretching vibrations of C-O-C bonds in the polysaccharide structure and skeletal stretching vibrations of C-O, respectively) also

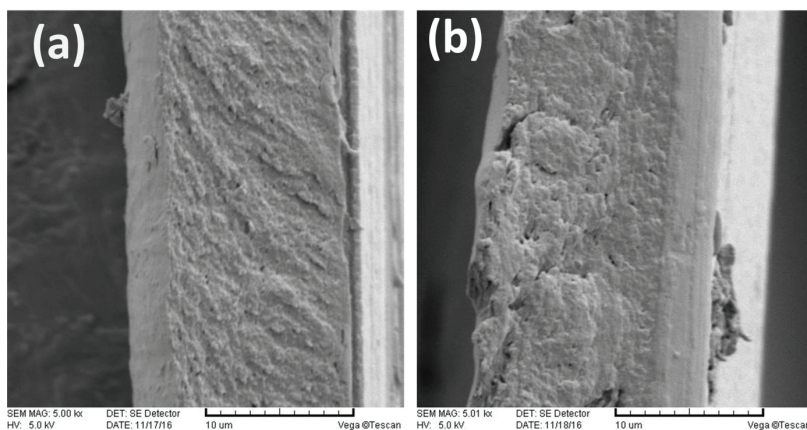


Figure 3. SEM images of G/Ch<sub>50:50</sub> film (a) non-treated and (b) loaded with 5.6 wt. % CO (scale bar = 10 µm).

confirm interaction of the CO (its components) with the polymer matrix.

**Thermal analysis.** Comparative DSC analysis of the non-treated and scCO<sub>2</sub>-treated, as well as the representative G/Ch<sub>50:50</sub> film impregnated with 56 mg CO/g<sub>film</sub>, was carried out to study the effect of the G:Ch mass ratio, scCO<sub>2</sub> treatment and incorporation of CO using scCO<sub>2</sub> on thermal properties of the films. Corresponding DSC curves are given in Figure 5 and corresponding  $T_g$  values in Table 3.

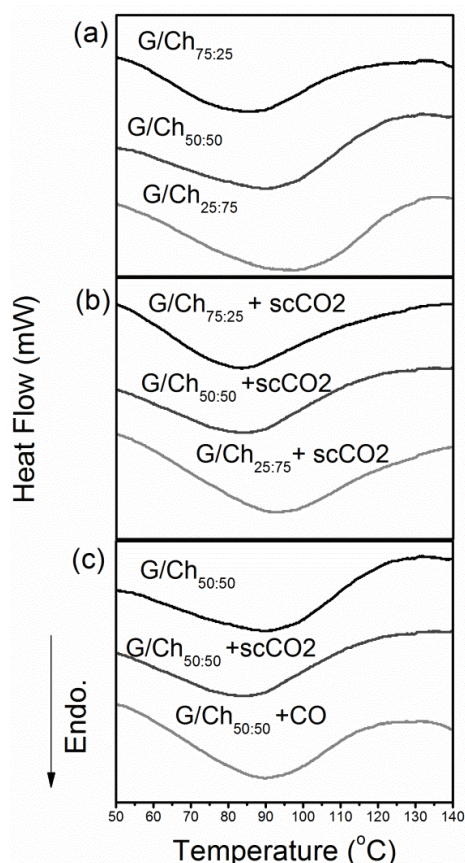


Figure 5. Thermal properties of composite films: a) non-treated, b) treated only with scCO<sub>2</sub> and c) film with mass ratio G/Ch<sub>50:50</sub>.

Table 3. Glass transition ( $T_g$ ) temperature of non-treated, scCO<sub>2</sub> treated and G/Ch films loaded with CO using scCO<sub>2</sub>

Composite film	$T_g$ / °C
G/Ch <sub>75:25</sub>	100.8
G/Ch <sub>50:50</sub>	109.3
G/Ch <sub>25:75</sub>	117.2
G/Ch <sub>75:25</sub> +scCO <sub>2</sub>	94.7
G/Ch <sub>50:50</sub> + scCO <sub>2</sub>	95.2
G/Ch <sub>25:75</sub> + scCO <sub>2</sub>	109.1
G/Ch <sub>50:50</sub> +CO	104.2

Increasing of Gwt% in G/Ch films led to a decrease of  $T_g$ . Exposure of the tested G/Ch films to pure scCO<sub>2</sub> at same processing conditions used to obtain representative G/Ch film with CO (10 MPa, 40 °C, 2 h) resulted in shifting of  $T_g$  to the lower values (6.1–14.1 °C) compared to the non-treated samples. Plasticizing effect of scCO<sub>2</sub> ( $\Delta T_g = T_{g,\text{non-treated}} - T_{g,\text{scCO}_2\text{-treated}}$ ) was the most pronounced in the case of scCO<sub>2</sub> treatment of the film with equal mass ratio of G and Ch, G/Ch<sub>50:50</sub> (Table 3). This is in accordance with the highest CO loading of G/Ch<sub>50:50</sub> achieved for short SSI at low to moderately high pressures (Figure 2a and b). Facilitated plasticization of the G/Ch<sub>50:50</sub> film by scCO<sub>2</sub> enable the highest gas sorption of the film. Accordingly, the SSI at higher operating pressure (30 MPa) and longer impregnation time (18 h) resulted in the lowest CO loading of this film due to the most pronounced effect of the hydrostatic pressure which tends to decrease free volume and sorption of the supercritical solution of CO. Somewhat higher depression of glass transition of G/Ch<sub>25:75</sub> (8.1 °C) than G/Ch<sub>75:25</sub> (6.1 °C) upon scCO<sub>2</sub> treatment (Table 3) can be related to higher CO loading of the G/Ch<sub>25:75</sub> at low pressure 10 MPa in presence of low gas concentration in the polymer matrix (Figure 2a). Being more prone to plasticization by scCO<sub>2</sub> than G/Ch<sub>75:25</sub>, at higher pressures (30 MPa) and longer time (18 h) the hydrostatic effect on the decrease of CO loading of the G/Ch<sub>25:75</sub> is stronger due to the presence of higher gas concentration in the matrix.

Influence of scCO<sub>2</sub> and CO loading on the thermal properties of composite film (G/Ch<sub>50:50</sub>) is presented in Figure 5c. As can be seen,  $T_g$  (95.2 °C) of the scCO<sub>2</sub> treated sample is lower than one for the untreated G/Ch<sub>50:50</sub> film (109.3 °C). On the other hand, loading of CO at 10 MPa and 40 °C for 2 h led to an increase of  $T_g$  value compared to the sample treated with scCO<sub>2</sub> only. Reportedly, the presence of eugenol incorporated in the semi-crystalline LLDPE film by scCO<sub>2</sub> was reported to facilitate mobility of chain segments and interfere in crystal organization, which results in lower shift of the melting point of the impregnated film compared to the sample treated with scCO<sub>2</sub> only [56]. An increase of  $T_g$  of amorphous polymers is related to decrease of free volume of the polymer matrix. Segmental mobility of the polymer matrix consisted of G and Ch could be hindered by addition of CO by an intensified intra- and intermolecular interactions through hydrogen bonding, as well as an increase of the hydrophobic groups of the film as indicated by structural analysis in this study.

In addition to the plasticizing effect of the CO, the thermal stability of the non-impregnated and imp-



regnated G/Ch<sub>50:50</sub> loaded with 56 mg CO/g<sub>film</sub> was also studied and compared to thermal stability of the neat gelatin (G) and chitosan (Ch) films. TGA/DTA thermograms are given in Figure 6. As can be seen in Figure 6, films decompose in two to three stages. Temperatures referring to maximum decomposition rate and corresponding weight losses are given in Table 4. Thermogravimetric measurements under non-oxidizing conditions (nitrogen atmosphere) showed that the films contain water molecules physically adsorbed and/or weakly hydrogen-bonded to chitosan and gelatin (7.9–9.4 wt.%) that are lost in the first stage of film decomposition reaching the maximum rate at 58.8–62.2 °C [65] (Table 4). Vaporization of strongly hydrogen-bonded water and low-molecular-weight compounds (glycerol used for the film

preparation) occur in the second decomposition step at temperatures above 110 °C reaching the maximum at 183.2–184.5 °C (Figure 6, Table 4). For the gelatin film, the second decomposition step with the most pronounced weight loss (69.2%) display maximum at 321.5 °C and can be ascribed to the degradation of gelatin due to decomposition of amino acid fragments [66]. The most pronounced weight loss (48.8 wt.%) of Ch film with a maximum at 280.6 °C is assigned to the thermal degradation of the pyranose ring with the rupture of the glycosidic linkage between the glucosamine and *N*-acetylglucosamine rings on chitosan and the release of volatile products [65].

The presented results are in accordance with literature data obtained from thermal analysis of chitosan under nitrogen atmosphere [65]. Thermal ana-

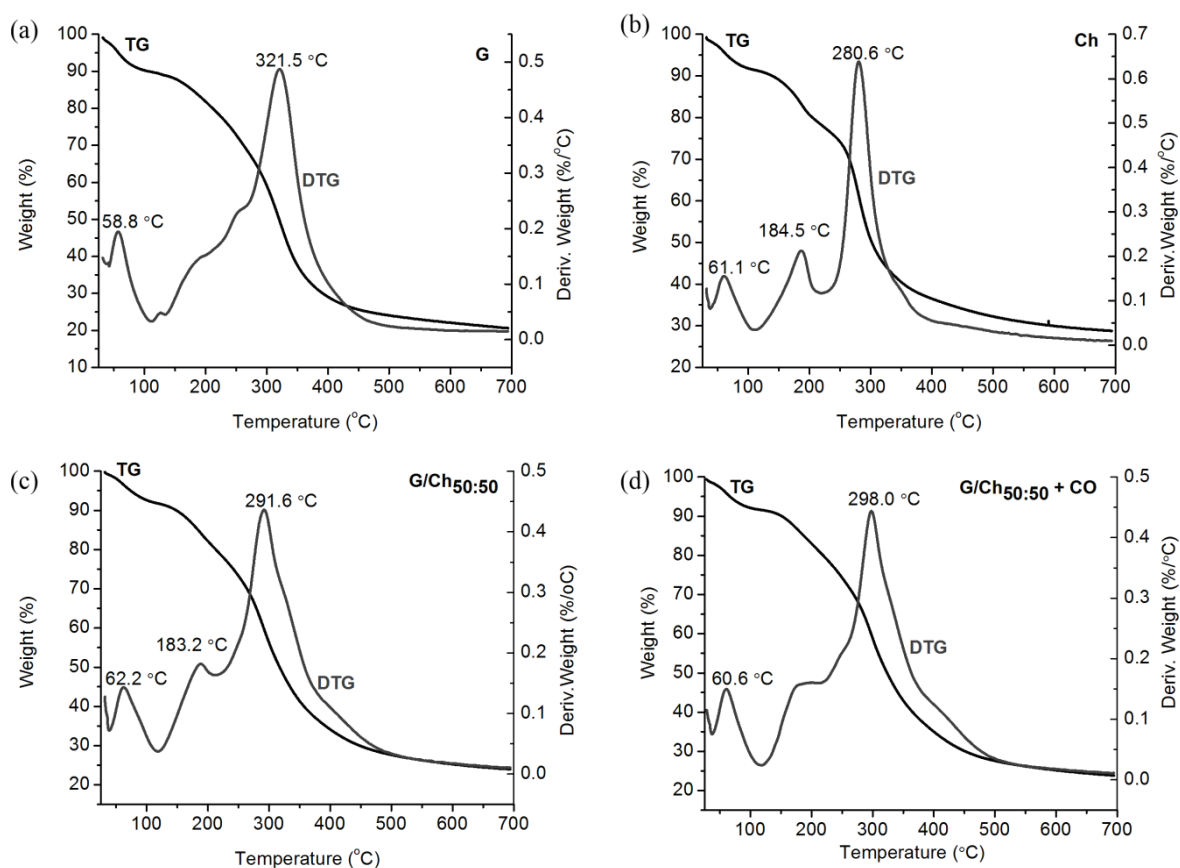


Figure 6. TGA-DTG analysis of non-treated: a) gelatin, b) chitosan, c) G/Ch<sub>50:50</sub> films and d) G/Ch<sub>50:50</sub> film containing 56 mg CO/g<sub>film</sub>.

Table 4. Thermal parameters (characteristic temperatures and weight loss) obtained from thermogravimetric analysis of gelatin (G), chitosan (Ch), blend (G/Ch<sub>50:50</sub>) and G/Ch<sub>50:50</sub> + CO in nitrogen atmosphere

Film	Temperature at maximum decomposition rate (°C) /corresponding weight (wt) loss			Residue, %
	$T_1^{\max}$ °C/wt. %	$T_2^{\max}$ °C/wt. %	$T_3^{\max}$ °C/wt. %	
G	58.8/9.4	-	321.5/69.2	20.6
Ch	61.1/7.9	184.5/13.7	280.6/48.8	28.8
G/Ch <sub>50:50</sub>	62.2/8.2	183.2/11.7	291.6/56.0	24.0
G/Ch <sub>50:50</sub> +CO	60.6/7.8	-	298.0/67.7	23.9

lysis of G/Ch<sub>50:50</sub> films demonstrated that in the observed temperature range (up to 700 °C) weight loss occurs through three steps. Non-treated G/Ch<sub>50:50</sub> shows similar thermal degradation behaviour to Ch film (Figure 6b and c). DTG curve of non-treated G/Ch displays three maxima at 62.2, 183.2 and 291.6 °C with corresponding weight losses of 8.2, 11.7 and 56.0%, respectively. Incorporation of CO into the G/Ch<sub>50:50</sub> seemed to improve the film stability by shifting the degradation temperature to somewhat higher values (298.0 °C) which is in accordance with DSC results. An improvement of thermal stability of G/Ch<sub>50:50</sub> films with CO can be attributed to the interaction between the components of film and clove oil, yielding a more stable network and denser matrix. Accordingly, on the DTG curve of the G/Ch<sub>50:50</sub> neither maximum at 184.5 °C nor at ~200 °C (ascribed to decomposition of clove oil components) is distinctive. Thermal stability of G/Ch<sub>50:50</sub> containing 56 mg CO/g<sub>film</sub> up to 110 °C is suitable for most of food packaging applications. Analysis of thermal stability of the impregnated G/Ch<sub>50:50</sub> film showed clearly that the incorporation of clove oil has no adverse effect and even improves thermal stability of the film.

## CONCLUSION

This study demonstrated for the first time the feasibility of the proposed SSI process for fabrication of bio-composite G/Ch films containing CO. Gelatin weight fraction of 25-50 wt.% in the films, scCO<sub>2</sub> pressures range of 10-20 MPa and impregnation time of 2-6 h favoured the CO loading. Even SSI at low scCO<sub>2</sub> pressure and temperature (10 MPa, 40 °C) enabled relatively high CO loading (56 mg CO/g<sub>film</sub>) of the representative G/Ch<sub>50:50</sub> film for a short impregnation time (2 h) and without adverse effect on the film morphology. Structural analysis confirmed incorporation of CO and its interactions with the G/Ch matrix. ScCO<sub>2</sub> had the most pronounced plasticizing effect on the G/Ch<sub>50:50</sub> film, which enable the highest CO loading (56-93 mg CO/g<sub>film</sub>) of this film at low to moderate pressures and temperature (10-20 MPa, 40 °C) for only 2-h impregnation. Incorporation of 56 mg CO/g<sub>film</sub> in G/Ch<sub>50:50</sub> improved thermal stability of the blend film. The proposed SSI process is advantageous for incorporation of CO into the G/Ch films for being fast, easily controlled by changing process parameters (pressure, temperature, impregnation time) and eco-friendly. Obtained G/Ch films impregnated with CO are thermally stable at 110 °C which is suitable for most food packing applications.

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NAUČNI RAD

## IMPREGNACIJA ŽELATIN-HITOZAN FILMOVA ETARSKIM ULJEM KARANFILIĆA POMOĆU NATKRITIČNOG CO<sub>2</sub> I NJIHOVA KARAKTERIZACIJA

*Procesom impregnacije pomoću natkritičnog ugljenik(IV)-oksida (nkCO<sub>2</sub>) po prvi put su dobijeni biodegradabilni želatin-hitozan (G/Ch) filmovi koji sadrže ulje karanfilića (CO) sa mogućom primenom za aktivno pakovanje hrane. Sve impregnacije su izvedene na umereno niskoj temperaturi (40 °C). U cilju povećanja sadržaja CO u filmovima i postizanja prihvatljivih morfoloških, strukturnih i termičkih osobina istih, varirani su CO<sub>2</sub> pritisak, vreme impregnacije i maseni odnos G:Ch. Udeo želatina u filmovima od 25-50 tež. %, nkCO<sub>2</sub> pritisak od 10-20 MPa i vreme impregnacije od 2-6 h povoljno su uticali na povećanje sadržaja CO u filmu. Natkritičnom impregnacijom filma sa jednakim masenim udelom želatina i hitozana (G/Ch<sub>50:50</sub>) na 10 MPa nakon 2 h postignut je zadovoljavajuće visoki sadržaj ulja u filmu (56 mg CO/g<sub>film</sub>) koji nije imao negativni uticaj na morfološke osobine filma. Iz tog razloga, film G/Ch<sub>50:50</sub> je dalje korišćen za strukturne i termičke analize. ATR-FTIR analiza je potvrdila uspešno inkorporiranje CO u G/Ch<sub>50:50</sub> i interakciju ulja sa filmom. Plastifikujući efekat nkCO<sub>2</sub> i CO na film je potvrđen DSC analizom. Inkorporiranje 56 mg CO/g<sub>film</sub> u G/Ch<sub>50:50</sub> nije uticalo na termičku stabilnost filma. Pored ekoloških benefita, proces natkritične impregnacija omogućava brzu pripremu G/Ch kompozitnih filmova koji sadrže CO i termički su stabilni do 110 °C, što je pogodno za većinu primena pakovanja hrane.*

*Ključne reči: hitozan; ulje karanfilića; kompozitni film; želatin; impregnacija; natkritični CO<sub>2</sub>.*