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Propolis and chitosan as antimicrobial and polyphenols retainer for the development of paper based active packaging materials

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ABSTRACT

The research investigates the potential combination of propolis and chitosan to develop a completely bio-based active food packaging material. Propolis glycolic extract was used as antioxidant and antimicrobial, due to its polyphenols content. Two commercial chitosans with different molecular weights were comparatively used as antimicrobial, wet strength additive substitute and polyphenols retainer. The effects of the addition of carboxymethyl- (CMC) or microfibrillated cellulose (MFC) and two pH values in paper production were investigated for polyphenols retention and paper strength. Chitosan, in replacement of the most commonly used wet strength resin (PAAE), increased polyphenols retention more than 10 times. Paper sheets produced with the highest molecular weight chitosan, at pH 7 and with MFC addition showed the best wet strength (7.4 ± 0.5 Nm/g) and wet resistance (13.3 ± 1.2%). Paper antimicrobial activity was confirmed on thinly sliced raw veal meat, where a decrease of intentionally inoculated *L. innocua* of around 1 log cycle was achieved in 48 h at 4 °C.

1. Introduction

Foodborne microorganisms continue to cause major public health problems worldwide, while a request for shelf life extension is increasing more and more. Therefore, a strong interest is addressed to antimicrobial/antioxidant packaging systems, which can help to reduce food poisoning risks by controlling post-processing contamination on the food surface, as well as oxidation processes and alteration of sensorial attributes (Kujumgiev et al., 1999). Several studies have shown that propolis has the potential to be used in biopolymer-based packaging materials as antimicrobial and antioxidant natural active agent (Juliano, Pala, & Cossu, 2007; Lindström, Wågberg, & Larsson, 2005). Propolis, in fact, is a chemically complex bee product that, in purified form, consists of flavonoids, phenolic acids, their esters, as well as of various organic compounds like terpenes (Burdock, 1998; Kujumgiev et al., 1999). It is a natural source of polyphenols, quite effective and thermostable at moderate temperatures, such as those applied in paper and cellulosic materials manufacturing.

Chitosan, a high molecular mass linear copolymer of N-acetyl-D-glucosamine and D-glucosamine units linked by β (1–4), obtained by the partial hydrolysis of the N-acetyl groups of chitin, is a well-known antimicrobial biopolymer, active against a wide range of microorganisms (yeasts, moulds and bacteria) (Coma, 2013; Dutta, Tripathi, Mehrotra, & Dutta), even if its efficacy can vary considerably. In particular, a degree of polymerization of at least seven monomeric units is required to get significant antibacterial effect, and highly deacetylated chitosan showed higher antimicrobial activity than chitosan with mainly acetylated amino groups (Aider, 2010). Lin, Li, & Chen (2009) demonstrated that the antibacterial effects of chitosan solution against *Staphylococcus aureus* increased as its MW increased between 1×10^4 and 3×10^5 Da, and the optimal pH value was proved to be 6.0 for the highest bactericidal activity. High deacetylation degree, as well, increases either chitosan solubility and charge density, and these two factors are crucial for chitosan adhesion to bacterial cell (Aider, 2010). Low pH values (up to 5.5) also increase chitosan antimicrobial activity because of higher solubility and protonation. On the other side, chitosan is an excellent binder for cellulose fibers thanks to the stereo chemical similarity to cellulose, imparting to paper not only dry strength, but also wet strengthening (Lindström et al., 2005; Nada, El-Sakhawy, Kamel, Eid, & Adel, 2006). This behavior is related to

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chitosan compatibility with cellulose surface; in addition, chitosan does not disrupt conventional hydrogen bonding, and contains functional groups capable of ionic or covalent bonding with the paper fiber surface within the papermaking process (Li, Du, Xu, Zhan, & Kennedy, 2004). Chitosan added in papermaking is almost completely adsorbed onto the surface of cellulose fibers and this adsorption increases with an increasing degree of deacetylation. Charged reactive groups in chitosan can also be exploited to form electrostatic interactions with biologically active molecules such as polyphenols.

Chitosan is also able to interact with some additives used in papermaking: the properties of chitosan and microcrystalline cellulose (MCC) composites have already been investigated (Riedel & Taeger, 1999), as well as wet-strength and wet-stiffness enhancement of chitosan using micro or nanofibrillated cellulose (MFC, NFC) (Dufresne, 2013; Lavoine, Desloges, Dufresne, and Bras, 2012; Nordqvist et al., 2007). Water resistance represents one of the most important characteristics of cellulosic materials to be used in the food-packaging sector. To improve wet strength of paper sheets, a number of resins and polymeric materials have been employed such as urea, phenol- and melamine-formaldehyde resins; nevertheless, concerns remain about the presence of free formaldehyde. Nowadays, paper with permanent wet strength is produced almost entirely using polyamine polyamide-epichlorohydrin (PAAE) resins in a neutral/alkaline pH medium, while glyoxalated polyacrylamide (GPAM) resins are mainly used to provide temporary wet strength in particular to short life-time tissue-like materials (Crisp & Riehle, 2009). However, as the main materials for paper production are bio-based, it should be of great interest to choose additives of renewable resources.

The combination of propolis and chitosan, therefore, can impart antimicrobial as well as antioxidant capacity to paper and cellulosic packaging materials, improving, at the same time, some fundamental features of such food packaging materials (Coma, 2013; Fernández Pan, Maté, Gardrat, & Coma, 2015; Iturriaga, Olabarrieta, Castellan, Gardrat, & Coma, 2014; Mascheroni et al., 2014).

The aim of the present study was to evaluate the potentialities of chitosan samples with different molecular weights as wet strength resin substitute and propolis retainer in a cellulose-based material. The effect of the type of chitosan, pH conditions during paper production, and addition of carboxymethylcellulose (CMC) or microfibrillated cellulose (MFC) was assessed on polyphenols retention capacity as well as on dry and permanent paper wet strength, leading to the selection of the best performing combination. Antimicrobial performance of the selected paper was finally tested both *in vitro* and in a real food system (carpaccio, a thin-cut veal meat), to assess the actual efficacy of the developed active cellulose-based material.

2. Materials and methods

2.1. Materials and supplies

Commercial bleached hardwood and softwood Kraft pulps were used in papermaking. Sodium carboxymethylcellulose (CMC) was purchased from Mikro-Technik (Bürgstadt, Germany); microfibrillated cellulose (MFC) suspension (2% w/w) was prepared from a commercial bleached hardwood Kraft pulp (Cellardenne, France) after enzymatic hydrolysis by endoglucanase and intensive beating up to a freeness value of 80° Shopper Riegler (SR), followed by treatment in a lab scale high pressure homogenizer model ARIETE NS 3006 (Gea Niro Soavi, Parma, Italy). Two types of crab shell chitosan (CS) were used: CS1 purchased from Sigma Aldrich, CS2 kindly supplied by Faravelli (Milan, Italy). Propolis was used as glycolic extract (Specchiasol, Bussolengo). PAAE resin Maresin PP125 was kindly supplied by Mare SpA (Milan, Italy).

2.2. Chitosan characterization

CS molecular weight (MW) was determined by size exclusion chromatography (SEC), with a Dawn-Mals detector Wyatt-Heleos-II coupled with a refractometric detector (Optilab rEX WYATT) with 4 columns in series including the guard column: TSKgel PWxl Guard column 12 µm, TSKgel G6000PWxl 13 µm, TSKgel G4000Wxl 10 µm, TSKgel G3000PWxl 7 µm, flow 0.6 mL/min. For MW between 10,000 and 100,000, chitosans were dissolved in a buffer solution of AcOH (0.3 M) and AcONa (0.2 M) with a concentration of 2 mg/mL. For higher MW, the concentration of the sample was reduced to 1 mg/mL with the same buffer solution. Samples were filtered on cellulose (0.45 µm) before analysis.

CS deacetylation degree was determined by using elemental analysis and infra-red spectroscopy. CS elemental composition was determined using a Thermo Finnigan Flash EA 2000. All determinations were triplicated. Only carbon and nitrogen percentages are presented in this paper. CS deacetylation degree was calculated from the carbon and nitrogen mass percentage with the following formula established for pure CS containing only D-glucosamine and N-acetyl glucosamine units (Dos Santos, Caroni, Pereira, da Silva, & Fonseca, 2009):

$$\text{Degree of deacetylation (DA)} = 100 \times (4 - 0.583093\, W_{C/N})$$

$W_{C/N}$ is the mass ratio between carbon and nitrogen present in the sample.

CS infrared spectra were registered on a Thermo Nicolet Avatar 370 FTIR coupled with a Nicolet Centaurus IR microscope and Omnic software (Thermo-Nicolet, Courtaboeuf, France) between 400 and 4000 cm^{-1} using 64 scans at a resolution of 8 cm^{-1} using the KBr disc technique with CS in powder form. The acetylation degree was measured according to the methods involving the absorbance of the amide I band (around 1655 cm^{-1}) and the absorbance of the hydroxyl band (around 3450 cm^{-1}) as an internal standard (Baxter, Dillon, Taylor, & Roberts, 1992; Domszy & Roberts, 1985). The acetylation degrees were evaluated applying the two following formulae comparatively:

$$[(A1655/A3450) \times 100]/1.33 \text{ (Domszy \& Roberts, 1985)}$$

$$(A1655/A3450) \times 115 \text{ (Baxter et al., 1992)}$$

The baselines, to determine the absorbance of each band, were taken as described by the same authors for each formula.

2.3. Microorganisms and culture conditions

Staphylococcus aureus ATCC 29213 (American Type Culture Collection, USA), *Listeria innocua* DSM20649 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany), *Escherichia coli* CECT434 (Spanish Type Culture Collection, Spain) and *Pseudomonas putida* ATCC12633 were chosen as model strains to test antimicrobial activity. Strains were maintained at −80 °C on TSB (Tryptic Soy Broth, Scharlau Chemie – Barcelona) added with glycerol (1 v/v) and propagated twice in TSB at 30 °C for 24–72 h before use.

2.4. Paper production

A pulp slurry (5 g/L) was prepared by mixing the commercial bleached hardwood (64%) and softwood (36%) pulps previously beaten to 30°SR in a Valley beater according to ISO 5264-1:1979. CMC solution (7.5 g/L) was prepared by adding the powder into distilled water under magnetic stirring at 80 °C for 20 min. MFC suspension (2% w/w) was diluted with distilled water to 7.5 g/L. Chitosan solutions (0.5 g/L) were prepared by adding the powder into 2% (v/v) and 0.4% (v/v) acetic acid solutions under magnetic stirring in order to obtain a pulp slurry of pH 5 and pH 7 respectively, according to the 2^3 full factorial experimental design.