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Manufacture of active multilayer films made of functionalized pectin coated by polyhydroxyalkanoates: a fully renewable approach to active food packaging

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Abstract:	Biodegradable active packaging, i.e. materials able to promote food preservation while avoiding plastic waste accumulation are expecting to play a key role for the manufacture of new generation materials. Bioactive films composed of citrus pectin (CP) added with extracts from spent coffee grounds (SCGs) were herein developed. To address the limitations of pectin-based materials, i.e. hydrophilicity and poor water barrier properties, an active multilayer film was prepared by coating the functionalized pectin by a two-steps dipping into polyhydroxyalkanoates (PHA) solutions characterized by different monomeric compositions. The PHA coating affected the stiffness of the film and its opacity, while improving its hydrophobicity and water vapor permeability. The dipping procedure did not compromise the stability of the bioactive compounds, rather it allowed to preserve their antioxidant and antimicrobial activities for longer times in comparison with uncoated functionalized film. Further, the multilayer material functionalized with extracts was able to delay the carotenoid degradation in mashed carrots likely because of the increased film opacity conferred by both phenols (caffeoylquinic acid isomers including chlorogenic acid) found in SCGs and PHAs layer suggesting that the new material may be used to extend the food shelf-life and potentially to enhance product health credentials.

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II
Dipartimento di Scienze Chimiche
Prof. Cinzia Pezzella

Napoli, 14th June 2023

Dear Prof. Xiao Hu,

following your mail dated 8th May, on behalf of all authors, I am submitting the revised version of the manuscript "**Manufacture of active multilayer films made of functionalized pectin coated by polyhydroxyalkanoates: a fully renewable approach to active food packaging"** (**POLYMER-23-550**)
We have answered the points raised by the reviewers, addressed the main criticisms, and modified the manuscript accordingly (see Point-to-point response to reviewers' comments). All the changes in the revised manuscript have been highlighted.

Following reviewers' advice, the manuscript has been widely improved, including new data about the LC-MS characterization (§2.3 p. 6; §3.1 p. 14-15) of the SCG extract and the assessment of the light-shielding properties of the developed multilayer films (§2.14 p.11-12; §3.4.3 p.23-25). For this reason, two new authors that contributed to the experimental upgrading of the paper have been added to the manuscript (Lucia Panzella and Elisabetta Borselleca).

The point-by-point answers to the comments raised by the reviewers are reported below, with the corresponding changes in the text marked in yellow (for Reviewer 1), and cyano (for both Reviewers).

Consequently, we do believe that thanks to the reviewers' inputs and suggestions, this revised version has been upgraded and may deserve publication in **"Polymer"**.

Cinzia Pezzella, PhD

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Point-to-point response to reviewers' comments

Reviewers' comments:

Reviewer #1: The development of biodegradable active packaging is a promising solution to reduce plastic waste accumulation while promoting food preservation. The use of citrus pectin and extracts from spent coffee grounds to develop bioactive films is an interesting approach. The use of a multilayer film with a coating of PHA solutions with different monomeric compositions is a clever way to improve the properties of the functionalized pectin films. Overall, this study shows a promising approach to the development of biodegradable active packaging that could have a significant impact on reducing plastic waste accumulation while improving food preservation.

The article is written in a very verbose manner, but does not effectively explain the innovation of the research. Extracting active substances from coffee grounds, improving the water-repellent property of membrane materials through hydrophobicity coating, and using multi-layer materials to reduce food decay by providing higher water and gas transfer resistance than single-layer materials, none of the above is the innovation of this work.

If only the research content mentioned above is emphasized, it can only be said that this paper has carried out a relatively complete research work, but it cannot well reflect the characteristics and significance of this research.

For example, the innovation of this article could be to solve the challenges faced by multi-layer materials at the current stage, improve the possibility of delamination, and enhance the interfacial bonding strength. Therefore, the research background and material selection can be centered around these challenges, and be written in a concise and attractive way to the reader.

Reply to reviewer 1:

We thank the reviewer for the helpful and constructive comments aimed at improving the impact of our research. Following his/her advice, the paper was widely revised to make it more fluent and focused on its significance in the field of challenges faced by multi-layer materials.

To this aim, the introduction section was reorganized and modified (p. 3-5) and additional discussion has been added in the abstract (p.1) and conclusion ones (p. 33).

The changes addressing the points raised by reviewer 1 are highlighted in yellow in the revised manuscript.

Further points raised by reviewer 1:

1、Will the phenolic substances extracted from coffee grounds affect the performance of multi-layer materials besides providing antioxidant activity?

> The results provided in the manuscript indicate that the presence of phenolic substances within the pectin layer not only affects the antioxidant properties of the resulted material, but also results into a thicker film more resistant and less flexible with respect to the neat PHA/pectin multilayer (p.29, Figure 7). In the same manner the phenolic compounds further influence the material hydrophobicity by lowering the moisture content (p.29, Figure 7), water vapor permeability and uptake (p.30, Figure 8).

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Beside the mechanical, hydrophobicity and active properties conferred to the material, the phenol additives also contribute to improve the interfacial adhesion between the pectin and PHA layer by means of multiple bonding mechanisms, such as weak interactions, chemical adhesion and mechanical interlocking (Lee, 2021; ref [63] p.29). Following the reviewer's comments, this aspect has been better discussed in the manuscript (p. 29) also mentioning the possible future approaches to address the observed material drawbacks (p.29). Furthermore, the relevance of the obtained results and its future perspective have been highlighted in the conclusion section (p.33).

Minor points raised by the reviewer:

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Although colored film materials may limit their widespread application in the field of food packaging films, they can be used as light-shielding food film materials. If the protective effect of a typical food that needs to be stored away from light is also given, the conclusion of the research will be more convincing.

Following this comment, we set up an experiment of accelerated photo-degradation of carotenoids in carrots, to test the efficacy of the developed multilayer in protecting them. These new data are reported in § 3.4.3. Results indicate that the colored multilayer has a potential as light-shielding film for food.

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water barrier ability of the films by coating polyhydroxyalkanoates. The content of this study is interesting.

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The manuscript has been carefully checked for typos and grammar mistakes, furthermore the headings of Materials and Methods section have been reorganized taking into account the introduction of new paragraphs (the changed headings are highlighted in cyano).

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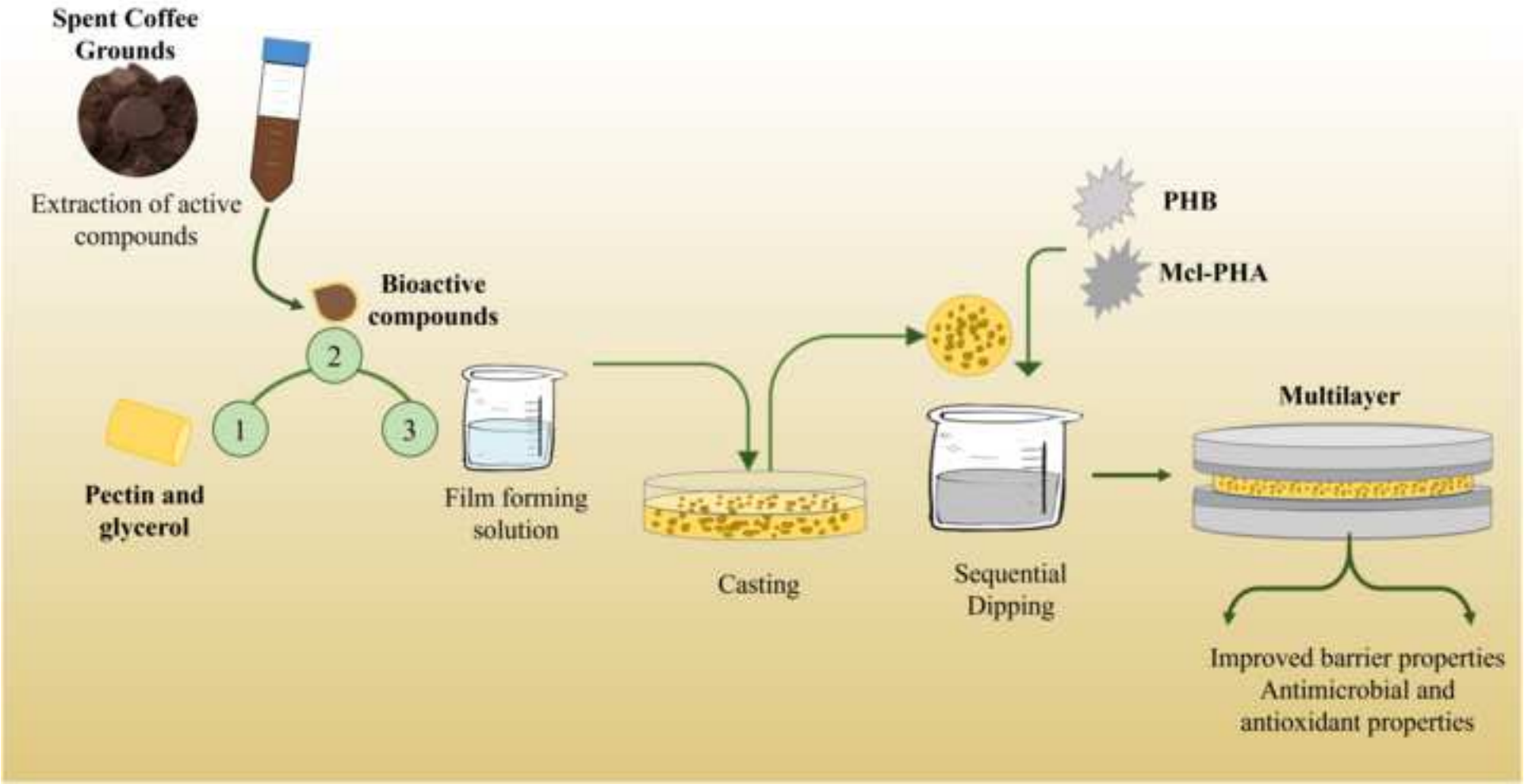
Graphical Abstract



Manufacture of active multilayer films made of functionalized pectin coated by polyhydroxyalkanoates: a fully renewable approach to active food packaging

Seyedeh Fatemeh Mirpoor[a*], Iolanda Corrado[a*], Rocco Di Girolamo[a], Giovanni Dal Poggetto[b], Lucia Panzella[a], Elisabetta Borselleca[a], Cinzia Pezzella[a**], C. Valeria L. Giosafatto[a,$]

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[b]Institute for Polymers, Composites and Biomaterials, National Council of Research, Via Campi Flegrei 34, 80078 Pozzuoli, Italy

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[*]Equally contributing authors

****Correspondence: Cinzia Pezzella, cpezzella@unina.it**

Abstract

Biodegradable active packaging, *i.e.* materials able to promote food preservation while avoiding plastic waste accumulation are expecting to play a key role for the manufacture of new generation materials.

Bioactive films composed of citrus pectin (CP) added with extracts from spent coffee grounds (SCGs) were herein developed. To address the limitations of pectin-based materials, *i.e.* hydrophilicity and poor water barrier properties, an active multilayer film was prepared by coating the functionalized pectin by a two-steps dipping into polyhydroxyalkanoates (PHA) solutions characterized by different monomeric compositions. The PHA coating affected the stiffness of the film and its opacity, while improving its hydrophobicity and water vapor permeability. The dipping procedure did not compromise the stability of the bioactive compounds, rather it allowed to preserve their antioxidant

and antimicrobial activities for longer times in comparison with uncoated functionalized film. Further, the multilayer material functionalized with extracts was able to delay the carotenoid degradation in mashed carrots likely because of the increased film opacity conferred by both phenols (caffeoylquinic acid isomers including chlorogenic acid) found in SCGs and PHAs layer suggesting that the new material may be used to extend the food shelf-life and potentially to enhance product health credentials.

Keywords: Pectin films, polyhydroxyalkanoates, multilayer material

1. Introduction

Nowadays the concept of sustainability and the substitution of non-biodegradable plastics using biodegradable packaging systems has attracted an enormous interest. The need for developing innovative packaging solutions addressing both functional and circularity criteria is particularly urgent in the food sector, where the concept of sustainability must coincide with the unaltered product shelf-life and quality in order to meet consumer acceptance. Food industry currently relies on the use of multilayer materials, as they can provide higher resistance to water and gas transfer than single layered materials, thus reducing food spoilage.[1,2] The multilayer technology is one of the most efficient approaches to combine the benefits of two or more dissimilar materials into improved one. Depending on the type of material, multilayers can be obtained by different methods, i.e. extrusion or solvent casting followed by lamination as well as film extrusion, where each layer is usually glued by adhesives or tie-layers.[3] However, due to their heterogeneity, the recycling of conventional multilayer structures is especially challenging with current standard technologies, that are not able to identify, sort and separate the diverse layers that are not prone to easy delamination [4]. While bioplastics can be used to replace the fossil-based counterpart in the main packaging material or as coating for different substrates, the combination of multiple and biobased and biodegradable polymers into a fully-biobased/biodegradable multilayer material would represent a step forward towards the circularity concept, providing lower carbon footprint and sustainable End of Life options [5].

Among biopolymers, pectin, ~~the main components of the plant cell wall, contributing to tissue rigidity and integrity, is anionic, hydrosoluble, and high-molecular-weight heteropolysaccharide that~~ is receiving much attention for the development of active and edible food packaging materials. However, this polysaccharide possesses limited physicochemical and mechanical performances [6,7] so that, several investigations have been conducted to improve pectin-based filming and coating properties [8]. To enhance the technological features of the film and their surface adhesion, pectin

has been blended with food-grade plasticisers (e.g., glycerol, polyethylene glycol, and sucrose) [9,10] and polymers (e.g., polyvinyl alcohol and cellulose derivatives). [11,12] Sharaby et al. have manufactured novel pectin-based films reinforced with crystalline nanocellulose and activated with zinc oxide nanoparticles demonstrating that the blending of the film matrix with the additives was able to improve UV-blocking, thermal, and antibacterial properties against well-known foodborne pathogens.[7] As well, pectin has been combined with hydrophobic compounds such as lipids to enhance its moisture resistance and water vapour transmission.[13] The improvement of barrier properties of hydrophilic materials (protein or polysaccharide based) by means of adding layers of more hydrophobic polymers (biobased or not) has been addressed by several reports.[14,15]

In this scenario, Polyhydroxyalkanoates (PHA), a family of microbial polyesters produced from renewable sources and completely biodegradable, have emerged as interesting biobased materials to be exploited in multilayer materials taking advantage of their hydrophobicity and good water permeability properties.[16,17] Furthermore, PHA are a family of multipurpose polymers, whose properties range from the very brittle and stiff Polyhydroxybutyrate (PHB) to the more elastomeric medium-chain length (mcl)-PHA. This versatility is associated to their different monomeric composition, that in turn depends on the microbial process they derive from. Recently, multilayers systems based on electrospun coatings of a PHB copolymer, the Polyhydroxybutyrate-co-valerate (PHBV), have been shown to significantly improve the barrier performance of several biopolymers and of fiber-based packaging.[18–20] This electrospun interlayer acts as a tie layer after mild annealing post-processing step.[3] Further, an mcl-PHA based coating added with apple extract has been applied by casting to cellulose nanopapers resulting into an improved hydrophobicity and antioxidant activity.[21]

The current work explores the use of dip-coating as an easy approach to obtain a fully-biobased multilayer material, wherein the PHA layers are applied on both sides of Citrus pectin (CP) films to

improve their water resistance and barrier performance. In particular, the properties of PHB and mcl-PHA were exploited, respectively, to provide a mechanical resistant crystalline layer and to promote the adhesion with the pectin film. The developed multilayers were characterized according to different physicochemical and functional features, including. mechanical, thermal, hydrophobicity, as well as optical properties. Furthermore due to the increase concern for food safety and security for consumers the produced films were functionalized with extracts obtained from spent coffee grounds (SCGs), as potential by-product source of active molecules.[22,23].

The effect of the SCGs extracts on multilayer biological activity, *e.g.* antioxidant and antimicrobial properties, was also considered to further exploit the new multilayer materials as biobased and functional preservatives for some kinds of fresh foods such as vegetables. ~~e.g. antioxidant and antimicrobial properties, was also considered in order to potentially exploit the new multilayer materials as natural bioresources and functional preservatives for some kinds of foods.~~

2. Materials and methods

2.1 Materials

Spent Coffee Grounds (SCGs) were obtained as a domestic waste deriving from the ordinary coffee pods. To prevent microbial spoilage during storage, SCGs were oven-dried at 40°C for 16 h and placed in dark glass containers at room temperature until use. High degree of methoxylation (>50%) pectin from *Citrus* peel was supplied by Silvateam (Rende, Italy). Reagents was purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise specified.

2.2 Preparation of spent coffee grounds extract (SCGE)

The SCGs oil fraction was extracted by using n-hexane in a Soxhlet apparatus at 120°C [24]. Phenols extraction was carried out on defatted SCGs by conventional maceration (25 g L^{-1}) at 60°C using water-methanol (50%) as solvent or distilled water. After 90 min the hydroalcoholic extract was recovered by centrifugation followed by filtration (0.2 µm). Before characterization the methanol was evaporated under nitrogen flux up to 24 h.

2.3 HPLC and LC-MS analysis

HPLC analysis was performed on an Agilent instrument equipped with a UV-Vis detector set at 254 nm. A Phenomenex Sphereclone ODS column (250 × 4.60 mm, 5 µm) was used at a flow rate of 1.0 mL min^{-1}; the gradient elution was as follows: 0.1% formic acid (solvent A)/methanol (solvent B); 5% B, 0-10 min; from 5 to 80% B, 10-47.5 min.

LC-MS analysis was performed in both positive and negative ionization mode on an Agilent LC-MS ESI-TOF instrument; the following conditions were adopted: capillary voltage 3500 V; drying gas (nitrogen) 5 L min^{-1}, 325°C; fragmentor voltage 175 V; nebulizer pressure 35 psig. Analysis were performed on an Agilent Eclipse Plus ODS column (150 × 4.6 mm, 5 µm) at a flow rate of 0.4 mL min^{-1}, with the same eluant as above.

2.4 Total phenolic content

The total phenolic content (TPC) was determined by Folin-Ciocalteu assay. Briefly, 0.1 mL of sample were added to 0.4 mL of sodium carbonate Na_2CO_3 (75 gL^{-1}) to alkalize the solution, and to 0.5 mL of a 10-fold diluted Folin-Ciocalteu reagent, while the blank was 0.5 mL of sodium carbonate solution and 0.5 mL of Folin-Ciocalteu reagent.[25] Samples were delicately shaken and incubated at room temperature for 30 minutes. Then, each sample was measured at λ=765 nm using a spectrophotometer (Jasco V-530 UV/VIS spectrophotometer). Gallic acid (GA) was taken as reference standard. Measures were obtained in triplicates; the TPC content is expressed as equivalent mg of GA per g of SCGs (mg GAE/g SCGs).

2.5 Production of Polyhydroxyalkanoates

Poly(3-hydroxybutyrate) (PHB) was produced by microbial fermentation of *Cupriavidus necator* DSM 428 in a 5L BioFlo/CelliGen®115 (Eppendorf New Brunswick). The strain was grown on rich medium (TSB, Tryptic Soy Broth) for 24h at 30°C and for additional 24h in minimal medium MM_{Cn} containing 20 gL^{-1} fructose.[26] The inoculum was then transferred into the bioreactor at an OD600 of 0.1, the agitation rate was set to 220 rpm and the DO concentration was maintained at 30% of air saturation. The cells were harvested by centrifugation (6000 rpm, 20 minutes) after 72 hours of fermentation and lyophilized. Polymer extraction was performed according to Turco and co-authors.[27]

Medium chain length PHA (mcl-PHA) was produced by microbial fermentation of *Pseudomonas resinovorans* NRL B-2649 in a 5L bioreactor. Briefly, the inoculum was grown overnight in LB medium and then transferred to the bioreactor at an OD600 of 0.1 in Minimal Medium E supplemented with 0.6% v/v oleic acid as C-source. [28] The agitation rate was set to 200 rpm, and the DO concentration at 30% of air saturation. The cells were harvested after 48 hours and polymer extracted as already described.[27]

Gel permeation chromatography (GPC) was performed with a GPC Max Viscotek equipped with a Malvern TDA 305 triple detector array composed by refractive index (RI), right angle laser light scattering (RALS), low angle laser light scattering (LALS), and intrinsic viscosity (IV) detectors. Samples were dissolved (≈5 $mgml^{-1}$) and eluted in $CHCl_3$ (Romil) at a flux of 0.8 mL min^{-1}, with injection volume of 100 µL and analyzed through a column set composed by a precolumn and two columns, Phenogel Phenomenex, with exclusion limits 106 and 103 Da. Temperature of columns and detectors was set at 35°C. All samples were run in duplicate and evaluated with triple point calibration (polystyrene standard Mn = 101.252 kDa and Mw = 104.959 kDa).

2.6 Preparation and characterization of pectin-based film forming solutions

Pectin stock solution (25 $mgmL^{-1}$) was prepared by dissolving pectin in distilled water under constant stirring at room temperature, overnight. The pH of the solution was then brought to 3 by adding 1N HCl. Pectin (500 mg) film forming solutions (FFSs) were prepared by adding different concentration of glycerol (GLY) (0, 20, 33, 50%, *w/w* of pectin), as plasticizer. Further FFSs were also prepared, after finding the best concentration of plasticizer (50%), by addition of different concentration of SGCs phenolic extract (2, 4, 8 % w/w of pectin). The effect of both GLY and SGCs on Z-average and zeta potential of each FFS, diluted with an acidic water solution (pH 3) to reach a concentration of 1 mg pectin/mL, was determined by using a zeta-sizer (Nano-ZSP, Malvern, Worcestershire, UK).

2.7 Preparation and characterization of pectin-based films

2.7.1 Pectin-based film preparation

The FFSs were cast into polyester Petri dishes (8 cm diameter) and left to dry at 25°C and 45% RH for 24 h in a climatic chamber. Then, the dried films were conditioned in a glass chamber containing saturated magnesium nitrate solution at 25°C and 45% RH for further 24 h. Finally, the pectin films, herein labelled as CP films were peeled and their features were analysed. Different concentrations (2,

4, 8 %) of the SCGE phenolic content were added to the CP films to get p2CP, p4CP, p8CP films, respectively.

2.7.2 Coating of CP film with polyhydroxyalkanoates

Dip coating technique was applied for the production of multilayer films based on neat CP or the extracts added one, p8CP. The pectin films were immersed for 2 minutes in a solution of medium chain length polyhydroxyalkanoates (mcl-PHA) in chloroform 20 ($mgmL^{-1}$), dried for 5 minutes at room temperature and then, dipped for additional 2 minutes in a solution of Polyhydroxybutyrate (PHB) in chloroform 20 $mgmL^{-1}$. Multilayer films (PHA/CP/PHA and PHA/p8CP/PHA) were then characterized as described below.

2.8 Fourier transform infrared (FTIR) spectroscopy

The structural properties of neat CP, p8CP films and the multilayer PHA/p8CP/PHA were investigated by means of transform infrared spectroscopy (FTIR) analysis performed in ATR (Attenuated Total Reflection) modality. The spectra were acquired using FTIR Nicolet 5700 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Infrared spectra analysis and spectroscopic manipulation (*i.e.*, baseline adjustment and normalization) were performed using the OMINC 9 software in the range of 4000–500 cm^{-1} with a spectral resolution of 2 cm^{-1}.

2.9 Scanning Electron Microscopy analysis

The cross-sections of neat CP and phenol containing p8CP films and the corresponding multilayers PHA/CP/PHA and PHA/p8CP/PHA were obtained by fracturing the films in liquid nitrogen and subsequently images were obtained by scanning electron microscopy (SEM) using an FEI NovaNanoSEM 450 microscope with FEG source. The images were acquired with a voltage equal to 5kV by means of an ETD detector.

2.10 Differential scanning calorimetry (DSC)

The calorimetric thermograms of coated and uncoated films were obtained by scanning with a differential scanning calorimeter Mettler DSC-822 in flowing N2 atmosphere and heating rate of 10°C/min.

2.11 Mechanical properties and thickness

The mechanical properties of the films including tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were determined by using Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). Five specimens (8 cm × 1 cm) for each sample were then tested (4 cm gage length, 1 kN load and 5 mm/min speed) according to the ASTM method of D882.[29] The average film thickness was measured using an electronic digital micrometer (IP65 Alpa Metrology Co., Pontoglio, Italy, sensitivity 0.001 mm). The films were conditioned in a glass chamber containing saturated magnesium nitrate solution at 25 C and 45% RH for 24 h before being tested.

2.12 Water susceptibility

Film moisture content was evaluated according to the method described by Zahedi et al.[30] The film specimens (2 ×2 cm^2) were placed in the aluminium plates and dried at 105 °C in an oven for 24 h. The weight of the film samples before (W_i) and after (W_d) drying was recorded. Moisture content was calculated by dividing the percentage of the film weight loss due to oven drying to the initial weigh of the film.

Water uptake curve was determined according to the method described by Manrich et al., with some modifications.[31] The weight of the dried films at 105°C in an oven for 24 h was recorded and then they were placed in a desiccator equilibrated at 50% RH with a saturated $Mg(NO_3)_2$ solution. Afterwards, the films were weighted at different intervals over 5 days and water uptake (*WU*) was calculated according to the following equation:

$$WU(\%) = \frac{Ws - Wd}{Wd} \times 100 \qquad (1)$$

Where W_S is the weight of the swollen sample at each time point and W_d is the weight of the dry samples.

Contact angle between water and films was determined as previously reported.[25] Five droplets (10 μL) of distilled water were deposited on both sides of each film and the picture was captured at the moment that the drop was in contact with the film surface. The mean value of contact angle was acquired with ImageJ software.

The water vapor permeability of the films was investigated at 90% RH, 38°C and 6 kPa by using a Total Perm apparatus (ExtraSolution s.r.l., Pisa, Italy).[14] Sample preparation was carried out by placing the previously conditioned films (50% RH for 24 hour) in the aluminium mask with 2 cm^2 area.

2.13 Color and opacity

The colour parameters of neat CP and phenol containing p8CP films and of the corresponding multilayers PHA/CP/PHA and PHA/p8CP/PHA were measured according to Mirpoor et al., using a Mightex® HRS series compact CCD spectrometer HRS-VIS-025 (Mightex, Toronto, ON).[25] All measurements were made at 5 random positions of each film.

The opacity values of the films were recorded by measuring the absorbance of the films, at a wavelength of 600 nm, divided by the film thickness (mm), using a UV/visible Spectrophotometer (SmartSpec 3000 Bio-Rad, Segrate, Milan, Italy). Five film rectangular strips (1 cm × 4 cm) of each film were placed on the inner side of the plastic cuvette to measure their absorbance; air was considered as a blank reference.

2.14 Evaluation of light-shielding properties

An accelerated photodegradation experiment on carotenoids in carrot samples was set up. Carrots were hot-air dried (45 °C, 16 h), shredded and then exposed for three days to light emitted from a led panel (Mondo Leo, Natural light 4000k Cr, 80 PF>0.9). Samples of about 3 g of carrots were covered

with different materials: PHA/CP/PHA; PHA/p8CP/PHA; pristine pectin-based films. Uncovered sample and a sample covered by aluminium foil were used as control.

For carotenoid extraction, 1 g of each carrot sample was taken under stirring in 10 mL of *n*-hexane in the dark for 10 min. The supernatant was then analyzed by UV-Vis spectroscopy on a Jasco V730 instrument after 1:1 v/v dilution in methanol.

2.15 In vitro antioxidant and free radical scavenging activity

The antioxidant activity of both the SGCE and functionalized films was determined by quantifying DDPH* radical scavenging in terms of percentage inhibition of a pre-formed free radical. Different concentrations (from 1.7 µgmL^{-1} to 3.4 µgmL^{-1}) of the SCG extract were tested. 0.005% DPPH (2,2′-diphenyl-1-picrylhydrazyl) radical was prepared in methanol (reaching an absorbance of 0.7 at 517 nm) as previously described by Mirpoor et al.[25] The evaluation of scavenging activity of film containing phenols from SCGs was performed by using 5 mg of film into 2.5 mL of DPPH diluted solution. After 30 min in the dark, the absorbance was measured in a 96 wells plate at 517 nm using Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). All determination were performed in triplicate. The scavenging activity was calculated using the equation:

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_S}{A_0} \times 100 \qquad (2)$$

where A_0 is the absorbance of the control and A_s is the absorbance of the tested sample.

The free radical scavenging activity of phenolic extract was expressed using EC_{50} value that corresponds to the concentration of the sample that could scavenge 50% of DPPH free radical. The free radical scavenging activity was compared with that of ascorbic acid used as a reference.

2.16 Antimicrobial activity

Antimicrobial activity of both SCGE and films loaded with SCGE was evaluated against *Micrococcus luteus* strain. The latter is an opportunistic pathogen, colonizing human and animal skin, as well as

found in soil and water. It has been used for many years as a model system for bacterial cell wall studies and has been more recently found to contaminate aquatic products and also cause severe infections in immunosuppressive patients.[32–34]

A series of dilutions of SCGE were prepared in distilled water and transferred into 24 well culture. The bacterium was grown on Nutrient broth medium for 16 h at 37°C and used to inoculate the plate at 10^6 CFU (final volume 2 mL).[35] After 24h at 30°C and 200 rpm 10 µl spots were transferred on NB agar plate and incubated for additional 24 h at 30°C.

The antimicrobial activity of functionalized films (p8CP, PHA/p8CP/PHA) was carried out using the disk diffusion method as previously described.[36] Briefly, 10 mL of soft nutrient agar (0.8% w/v, Bioxon) was mixed with 200 μL each of 50% (v/v) Tween 20 and Tween 80 solutions. Bacterial population was adjusted to 10^7–10^8 CFUmL^{-1}; the suspension (0.1 mL) was added to the soft agar and then poured into plates containing solidified agar. The films were cut into 1x1 cm small pieces, gently placed on top of the soft agar layer and incubated first for 2 h at 4°C and then at 30°C for 48 h. The neat pectin and PHA film were used as controls.

2.17 Statistical analysis

In order to determine the significant difference between treatments, SPSS19 (Version 19, SPSS Inc., Chicago, IL, USA) software was used for all statistical analyses. One-way analysis of variance (ANOVA) and Duncan's multiple range tests (p <0.05) were used to determine the significant difference among the samples.

3. Results and discussion

3.1 Extraction and characterization of phenols from SCGs

Hydroalcoholic extract from defatted SCGs, SCGE, was obtained by Soxhlet extraction using aqueous methanol (50:50) as solvent, due to its recognized efficiency for phenolic compounds extraction from plant materials.[37] The total phenolic content (TPC) of extracts was 22.6 mg GAE/g SCG. To exclude any interference with further antimicrobial activity assessment, methanol was removed from the extract resulting in the recovering of up to 76% of the phenolic content (17.3 mg GAE/g SCG). When the extraction was carried out using only water as solvent, an extraction yield of 3 mg GAE/g SCG was obtained, thus confirming the efficiency of the hydroalcoholic extraction.

The phenolic content of SCGE is closely associated to the extraction method, as well as to the coffee variety. According to literature, extracts with high TPC can be obtained from SCGs rich in *Arabica* variety. The SCGs used in this work comes from pods containing blends of *Arabica* and *Robusta* high content type.[38,39] As regards the solvent, it is widely reported in literature that choosing the appropriate solvent with polarity near the polarity of solute is crucial for extraction efficiency. As a fact, the extraction efficiency was increased by about 7-fold when water was replaced by aqueous methanol. This result indicates the improvement of solubility of a wider range of phenolic components, maybe due to a higher affinity of these compounds for hydroalcoholic solvent than for pure water as well as to a solute-matrix interactions perturbation that occurs in presence of solvent. Finally, the TPC of hydroalcoholic extract is consistent with those reported in literature, ranging from 17.1 to 35.5 mg GAE/g.[40]

HPLC analysis (Fig. 1) revealed the presence of several compounds in SCGE, eluted between 20 and 30 min. The main species eluted at ca. 27 min was identified as caffeine, whereas those eluted at ca. 20, 24, and 26 min were identified as caffeoylquinic acid isomers, including chlorogenic acid (CGA), by LC-MS analysis (Supplementary S1) and/or comparison of the chromatographic properties with

those of authentic standards. Compounds eluted at ca. 29 min were instead tentatively identified as feruloylquinic acid isomers based on their MS spectrum ($[M+H]^+$ at *m/z* 369). Quantitative HPLC analysis indicated a content of caffeine and caffeoylquinic acid isomers of about 25% and 8% w/w, respectively.

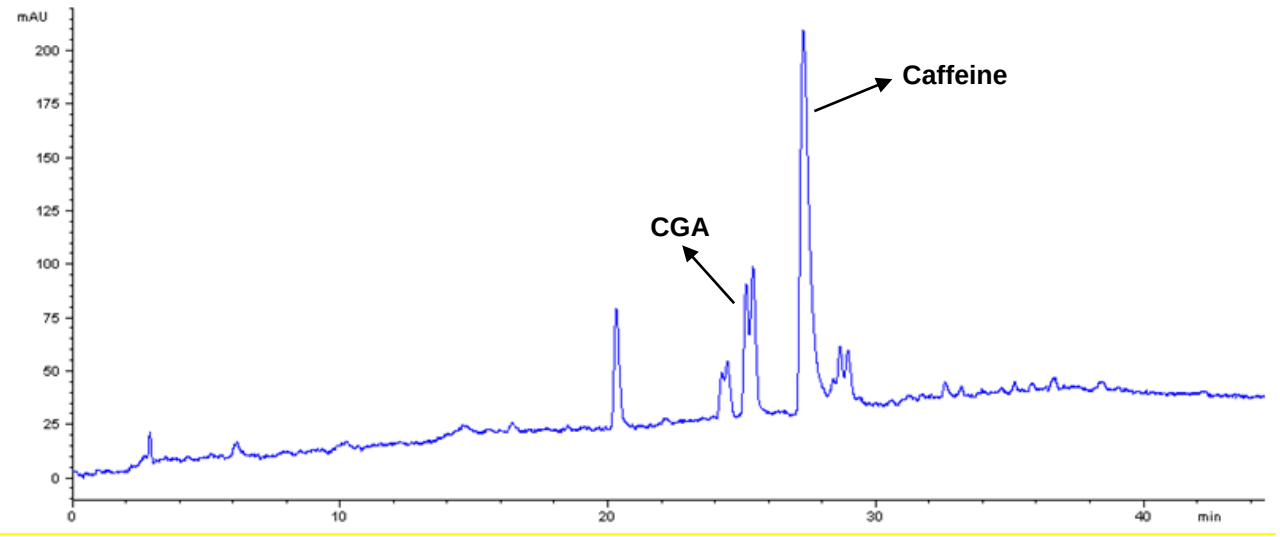


Fig.1. HPLC profile of SCGE.

Antioxidant properties (EC_{50}) and antimicrobial activity (MIC, MIB) of the SCGE deriving from hydroalcoholic extraction were evaluated after removing the alcoholic fraction. Antioxidant activity was evaluated using DPPH radical scavenging activity expressing the phenols concentration (mg GAE equivalent) needed to scavenge 50% of DPPH free radical (EC_{50}). The extract displays an EC_{50} value of 27 μgmL^{-1}, comparable to that of ascorbic acid used as standard (EC_{50} 30 μgmL^{-1}). The scavenging activity against DPPH is generally associated to the presence of caffeic and chlorogenic acids.[41] The EC_{50} of the extract is comparable to values reported by Caballero‐Galvan and co-authors, ranging from 10 to 32 μgmL^{-1} depending on extraction method used.[41]

The antimicrobial activity of the SCGE was evaluated against *M. luteus*, a Gram-positive bacterium chosen as test microorganism, resulting into a MIC of 180 µg GAEmL^{-1}, and a MIB of 240 µg GAEmL^{-1}. This value is slightly lower than MIC value reported for other SCG extracts on different Gram-positive bacteria ranging from 200 to 400 µg GAEmL^{-1}.[42]

3.2 Characterization of CP films

~~*3.2.1 Zeta potential and particle size of film forming solutions (FFSs)*~~

Pectin-based FFSs made with different concentrations of GLY were analysed to assess their stability and average particle size. The solutions are stable at all the concentrations of plasticizer (Supplementary, S2). Furthermore, the particle size and their polydispersity index (PDI) did not change significantly by varying the GLY content~~. These data are in line with previous results about the effect of plasticizer on the particle size and stability of FFSs made of hemp proteins~~, as reported for FFS made of plasticized hemp proteins. [14] ~~(*Cannabis sativa*).~~

~~*3.2.2 Thickness and mechanical properties*~~

CP films containing increasing concentration of GLY (0%, 20%, 33%, 50% *w/w* of pectin) were characterized for their thickness and mechanical properties (tensile strength (TS), elongation at break (EB), and Young's modulus (YM). The film thickness was not affected by the variation in the GLY concentration (Supplementary, S3). On the other hand, tensile strength (TS) and Young's modulus (YM) decreased whereas EB increased as function of concentration of GLY, as a result of enhancement of the free volume and the polymer mobility in the film matrix.[43] ~~The same result was reported for chitosan/starch blended films by Sun et al. who have shown that the films become more extensible in the presence of plasticizer.~~ Considering the results, the film containing 50% GLY with the highest EB, was selected for further investigations.

3.3 Characterization of CP films functionalized with different concentrations of SCGE

~~*3.3.1 Zeta potential and particle size of FFSs*~~

Different concentrations of SCGE (0, 2, 4, 8 % *w/w* of pectin) were added to the FFSs containing 500 mg of CP and 50% of GLY. ~~It can be seen from Table 1, that t~~The solutions are stable for all the different concentrations of extract and there is not a significant difference of different FFSs. However, the particle size and PDI of the solution decreased significantly as a function of the SCGE concentration, due to the interaction between the phenolic compounds and the polysaccharide molecules, thus, preventing the formation of pectin colloidal aggregates (Table 1).[44]

Table 1. Mean particle size and zeta potential of film forming solutions (FFSs) obtained by using 500 mg of pectin, 50% of GLY (w/w of pectin) and different concentrations of SCGE phenolic content (% *w/w* of pectin). (Lowercase letters indicate the result of Duncan's test; means sharing the same letter belongs to the same group, so the differences between those means are not significant).

SCGE phenolic content (% w/w of pectin)	Mean particle size (nm)	Zeta potential (mV)	PDI (%)
0	698.73 ± 9.68 [a]	-32.22 ± 0.87 [a]	0.28 ± 0.02 [a]
2	517.73 ± 5.88 [c]	-32.11 ± 0.34 [a]	0.24 ± 0.02 [b]
4	480.76 ± 4.36 [b]	-31.23 ± 1.02 [a]	0.21 ± 0.01 [c]
8	381.23 ± 5.31 [d]	-32.63 ± 0.37 [a]	0.18 ± 0.01 [d]

~~*3.3.2 Thickness and mechanical properties*~~

Mechanical properties of CP films added with different concentrations of SCGE (p2CP, p4CP, p8CP) were studied and compared with those of neat CP film (Table 2). The thickness of the pectin film increased significantly by the addition of the SCG extracts. The addition of SCGE to the films matrix slightly increased the TS value of the film that might be attributed to the increment of interfacial interaction among pectin polymers and SCGE phenols promoted by the formation of hydrogen bonds.[45] Similar results were reported by Al-Maqtari et al. who have incorporated *Pulicaria*

jaubertii extract in chitosan/gelatin films and Lin et al. who added *Morinda citrifolia* extract to the pectin films blended or not with chitosan.[45,46] On the other hand, a significant increase in the YM was observed in pectin film containing 2% of phenolic extract (p2CP), whilst the addition of 4% SCGE reverted the YM to the same level as the neat pectin film, finally reaching the lowest value in the presence of 8% of the SCG extract. This increased flexibility, also reflected in the increases EB values at the highest SCGE concentrations, could be related to the possible plasticizing effect of the phenolic extracts, that over a concentration threshold reduce the intramolecular forces between pectin molecules increasing their overall mobility. A similar behaviour was observed when alizarin was added to chitosan film matrix.[47]

Table 2. Thickness and mechanical properties of CP, with different concentration of SCGE phenolic extract. (Lowercase letters indicate the result of Duncan's test; means sharing the same letter belongs to the same group, hence the differences between those means are not significant).

Sample	Tensile Strenght (MPa)	Elongation at break (%)	Young's modulus (MPa)	Thickness (μm)
CP	4.7 ± 0.6 ^{a}	5.5 ± 0.8 ^{b}	490.7 ± 64.8 ^{d}	52.5 ± 4.8 ^{a}
p2CP	7.8 ± 0.5 ^{b}	5.7 ± 0.9 ^{b}	621.1 ± 36.0 ^{c}	64.6 ± 6.2 ^{a}
p4CP	8.7 ± 0.3 ^{bc}	7.5 ± 1.1 ^{c}	435.8 ± 53.4 ^{b}	73.0 ± 5.3 ^{a}
p8CP	9.1 ± 0.2 ^{cd}	16.1 ± 1.5 ^{d}	306.0 ± 32.4 ^{a}	81.0 ± 5.5 ^{a}

~~3.4~~ ~~Antioxidant activity~~

CP films with different concentrations of SCGE were tested for their DPPH radical scavenging activity over time (Fig. 2). The antioxidant activity increased with the concentration of the SCGE. Noteworthy, at the highest SCGE amount, it remained constant after 5 days storage, whilst sharply decreased in the films containing 2 and 4% SCGE. ~~These results are in agreement with those reported~~

~~by Liu et al. who have investigated the effect of protocatechuic acid, a potent antioxidant agent from fruits and vegetables, on physical, mechanical, structural and antioxidant features of chitosan films. The authors demonstrated that the DPPH radical scavenging activity released from chitosan films increased as the active molecules concentrations increased in the matrix remaining stable for at least 24 h.~~ Considering these results, the p8CP was chosen for further coating step.

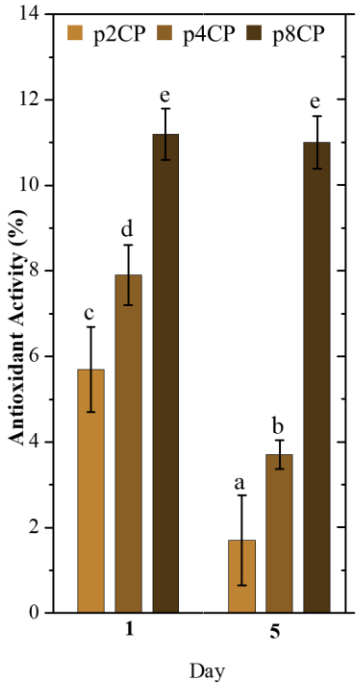


Fig. 2. Antioxidant activity of CP films with different concentrations of SCGE phenolic content. (Lowercase letters indicate the result of Duncan's test; means sharing the same letter belongs to the same group, hence the differences between those means are not significant).

3.5 Preparation of pectin-based multilayer films by means of polyhydroxyalkanoates coating

The most known limitation for the exploitation of pectin-based films in the food sector is their poor water/humidity resistance, being easily soluble in water.[7,48] A reinforcement by development of multilayer films by dip-coating technique was evaluated due to the intrinsic waterproofing

characteristics of PHA. Two different classes of polymers among PHA family were used: a homopolymer, stiff and brittle in nature (PHB), and a medium chain length PHA, endowed with elastomeric and adhesive properties.[16,49] The GPC analysis highlights that PHB (Supplementary, S4) exhibits a main single peak with a Mn of 108 kDa and Mw 252 kDa. Regarding mcl-PHA, chromatogram (Supplementary, S5) shows a bimodal distribution with a Mn of 5 kDa and Mw 549 kDa. It can be speculated that this broad polydispersity with this low value of Mn possess a quantity of low molecular weight material with strong chain mobility increasing some adhesive properties.[50] Preliminary experiments were carried out to define the conditions allowing the production of homogeneous and adhered coating on CP film. From a macroscopic point of view, coating performed by dipping the CP film within a 20 $mgmL^{-1}$ PHB solution was not effective since, after drying at room temperature, the separation of the layers was observed. Delamination also occurred when the CP film was dipped into a 20 $mgmL^{-1}$ mcl-PHA/PHB (10:90) blend in chloroform. Then, a two-step dipping procedure was tested. Firstly, CP films were dipped in a solution of mcl-PHA (20 $mgmL^{-1}$) for 2 minutes. This resulted in a macroscopically homogeneous sticky coverage, difficult to handle (Supplementary, S6). Next, the film was dipped in 20 $mgmL^{-1}$ PHB solution and let dry under the same conditions, resulting in an opaquer and easy to handle multilayer PHA/CP/PHA structure.

It can be hypothesized that the amorphous (Supplementary, S7) and sticky nature (glass transition temperature (T_g) = -48°C) of the mcl-PHA is crucial in promoting the physical adhesion between the layers. As a fact, the mcl-PHA could fill the voids within the pectin matrix and potentially also at the interphase with the outer PHB layer, favouring the interaction and welding between them. A similar role was proposed for lignocellulose nanofibers added in films made of cellulose nanofibers when coated by hot-pressing with electrospun PHBV biopolymer mats.[51] Furthermore, Urbina et al. discussed on the role of a mcl-PHA from *Pseudomonas putida* in filling the gaps and interstices of bacterial cellulose films when cast on such nanopaper.[21]

3.4.1 Scanning electron microscopy analysis

The images of all the films manufactured and their structural morphology are depicted in Fig. 3.

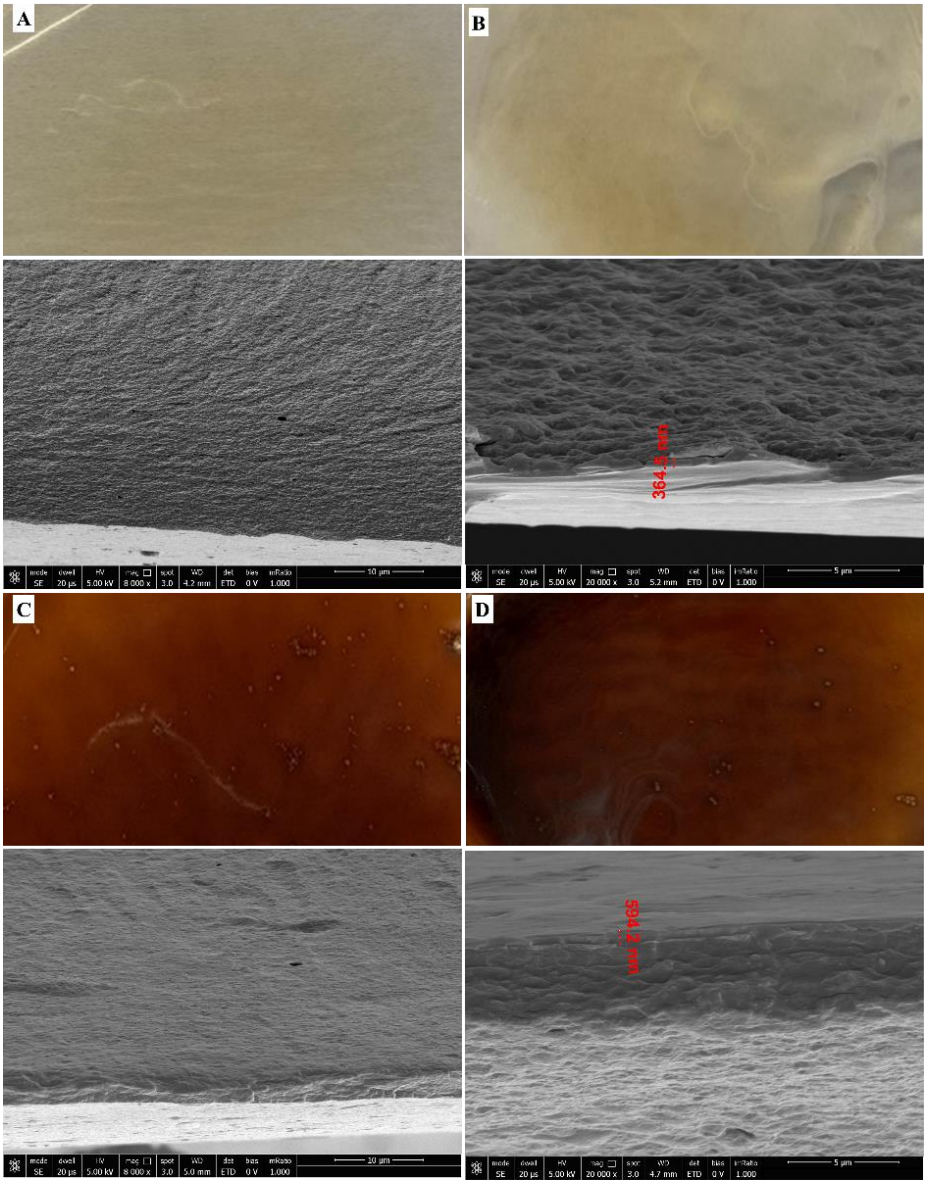


Fig. 3. Images of films and their SEM cross section: neat (A) and coated (B) CP films; neat (C) and coated p8CP films (D). The thickness of PHA layer is indicated in red.

The films containing the SCGE (Fig. 3 C and D) appeared smoother, homogeneous and more compact in SEM cross section analysis, compared to those of neat CP films (Fig. 3 A and B), regardless of the coating indicating a good dispersion of the active molecules as also found by Ribeiro et al., who

prepared films by means of mango by-products, namely mango pectin functionalized with mango peels polyphenol-reach extracts and they demonstrated that the functionalized films had a very dense and continuous structure.[52] In contrast Bermúdez-Oria et al. produced films with pectin and fish gelatin that appeared less homogeneous in their structure in the presence of the olive phenols hydroxytyrosol and 3,4-dihydroxyphenylglycol.[53] The presence of the PHA layer is detectable on both CP and p8CP dipped films (Fig. 3 B and D). The average thickness of the layers measured on both sides and for both films is ~ 0.5 µm. Although some samples can undergo delamination during the preparation for SEM analyses, they show good interlayer adhesion, even after the cryofracturing.[51]

3.4.2 Color and opacity

The color of the films was measured, and values reported in Table 3. A significant change in the colour of the films was observed after both the addition of SCGE and PHA coating. The films containing SGC extract had lower L* and b* (yellowish/bluish) values and higher a* (reddish/greenish) values comparing to the neat pectin films (either coated or not) due to the addition of the brown SCGEs.

Table 3. Color parameters and opacity of CP films added or not with SCGE and corresponding multilayers.

Sample	L	a	b	ΔE	Opacity (mm^{-1})
CP	3.34 ± 0.34^{c}	0.16 ± 0.05^{a}	3.11 ± 0.25^{c}	96.61 ± 0.34^{a}	1.06 ± 0.29^{a}
p8CP	0.97 ± 0.06^{b}	0.8 ± 0.02^{c}	0.33 ± 0.04^{b}	99.04 ± 0.61^{c}	4.11 ± 0.73^{b}
PHA/CP/PHA	8.76 ± 0.41^{d}	0.58 ± 0.03^{b}	6.19 ± 0.23^{d}	91.22 ± 0.41^{b}	12.98 ± 0.82^{c}
PHA/p8CP/PHA	0.55 ± 0.04^{a}	0.98 ± 0.08^{d}	0.14 ± 0.03^{a}	99.47 ± 0.04^{d}	18.17 ± 1.08^{d}

The differences in colour, proved by ΔE values that are greater than 3, are significant and they can be even seen with the naked eye (Fig. 3).[54] ~~This effect could be advantageous should these materials~~

~~be used for packaging some food products because it might increase the consumer attraction and in the same way protect the foods from the negative effect of the light.~~

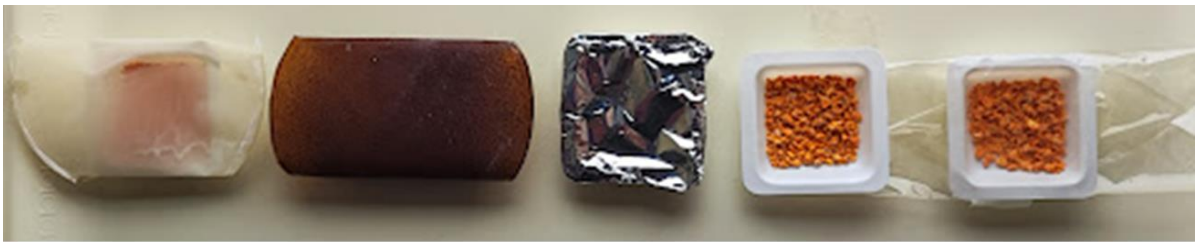
Opacity of materials is a critical property in various applications, particularly if the films will be used as food coatings to improve the product appearance.[55] The film opacity was affected also with both addition of SCGE to the film matrix and coating with PHA. As expected, the coated films were opaquer compared to the uncoated ones and the films containing SCGE extract were less transparent compared to the neat CP films. The effect of polyphenols on film opacity increase has been widely reported. In fact, similar results were observed in chitosan-phenolic composite films incorporated with apple polyphenols, green tea extract, quercetin or tea polyphenols.[56–59] Similarly, Melendeze-Rodriguez observed a yellowing in electrospun films of PHBV containing eugenol encapsulated in silica nanoparticles, ascribing such effect to the intrinsic yellow color of eugenol.[60] On the other hand, the observed increase in opacity is in contrast with what obtained by Urbina et al. for a mcl-PHA based coating on cellulose nanopapers.[21] This could be a consequence of the two-step dipping procedure, including, besides mcl-PHA also the PHB, the latter stiffer and more crystalline, thus probably responsible of the opaquer appearance of the PHA/CP/PHA materials.

3.4.3 Evaluation of light-shielding properties

Although the lack of transparency may limit the widespread application in the field of food packaging, colored films could actually be used as photoprotective materials for light-sensitive food products. Therefore, an accelerated photodegradation experiment of dried carrots was set up to test the protective properties of the developed multilayers against carotenoids photodegradation. When the samples were covered with the multilayer PHA/p8CP/PHA (Fig.4. sample b), the highest retention of carotenoid content was measured after 3 days of light exposure. The value was comparable with the one obtained for carrots covered with aluminium foil (Fig.4. sample c). The PHA/CP/PHA multilayer (Fig.4. sample a) also provided protection from carotenoid photodegradation, although

with a lesser extent, probably due to its opacity. On the other hand, a substantial reduction of carotenoid content was observed in samples covered with pristine pectin-based films (Fig.4. sample e), almost overlapping with the value obtained from uncovered carrot sample (Fig.4. sample d). This evidence represents a further added value of the designed functionalized multilayer, pointing out its potential in packaging application of foods requiring to be stored away from light.

A



a b c d e

B

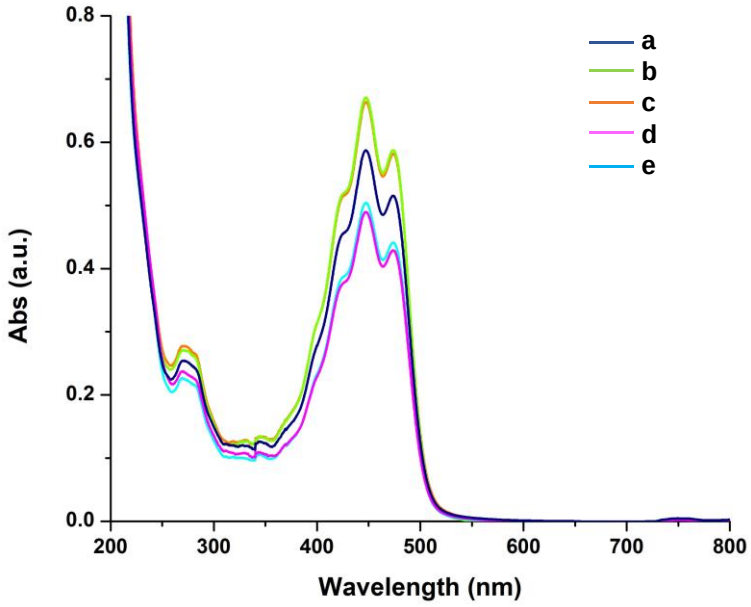


Fig.4. Samples of shredded dried carrots tested in the accelerated photodegradation experiments. The samples were covered with the following materials: (a) PHA/CP/PHA multilayer; (b) PHA/p8CP/PHA; (c) Aluminium foil; (e) Pristine pectin-based films. (d) represents the uncovered control sample (A). UV-vis spectra of *n*-hexane extracts from the different carrot samples as in A (B).

3.4.4 FTIR spectroscopy

The FTIR spectra obtained for CP, p8CP, and the multilayer PHA/p8CP/PHA are reported in Fig. 5. A broad peak ranging from 3600 to 3000 cm^{-1} was detected in all the samples and could be attributed

to the stretching vibration of -OH because of hydrogen bonding interactions in the galacturonic acid of pectin. The characteristic signals of pectin can be detected at 1730 cm^{-1} in both CP and p8CP films that appear as a major band and that correspond to stretching vibration of C=O bonds, while the broader band ranging from 1700 to 1600 cm^{-1} can be attributed to stretching of free ones. Bands at 1143,1095 1012 and 952 cm^{-1} suggest pectin polysaccharides rich in uronic acids.[61] In the presence of SCGE (p8CP) a clear pick at 1645 cm^{-1} is detectable, that could be related to C=C stretching vibration of aromatic rings of polyphenols.

Overall, the addition of SCGE did not clearly affect the FITR spectra of pectin, in accordance with results observed after the incorporation of noni fruit extract in pectin/chitosan composites.[45]

The analysis of multilayer displays bands that could be related mainly to PHA. In particular, the intense band at 1716 cm^{-1}, typical of ester carbonyl groups (C=O) of PHA is clearly detectable in both PHA/CP/PHA and PHA/p8CP/PHA films.[27] Moreover, the stretching vibration of C=C bound due to the aromatic ring deformation can be found when SCGE is present. Despite that, the FTIR analysis did not reveal a chemical interaction among components as suggested by no significant changes in the chemical shifts.

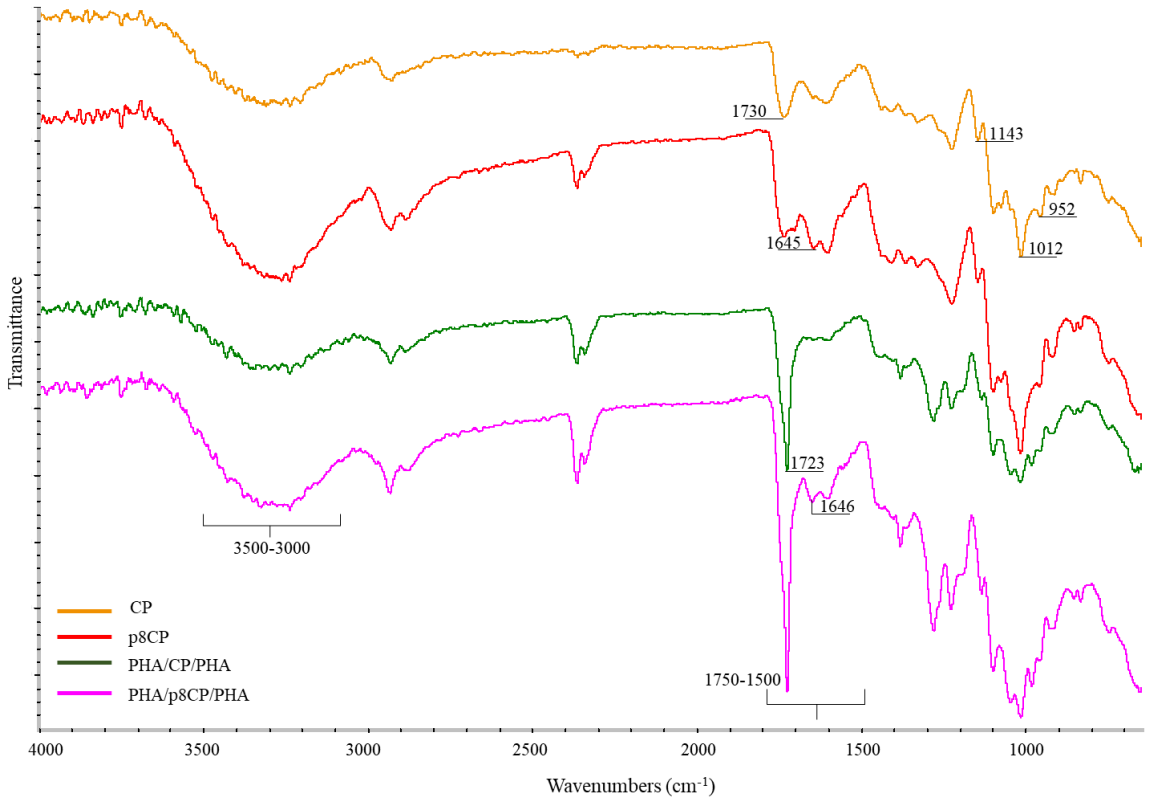


Fig. 5. FTIR spectra of films CPfilms, CP films functionalized with 8% SCGE (p8CP), and the functionalized multilayer (PHA/p8CP/PHA).

3.4.5 Differential scanning calorimetry (DSC)

DSC first heating thermograms of coated and uncoated pectin-based films are reported in Fig. 6 (A). All the samples present a broad endothermic peak (80-95°C) associated with the glass transition temperature (T_g) relaxation phenomena and with the elimination of bound water in the pectin sample. A glass transition onset, related to the pectin phase, has been evaluated at ~ 35°C for all the samples regardless the presence of phenolic extracts and/or PHA. It is wort nothing that the DSC traces of the coated films with PHA show a second endothermic peak with low intensity, at ~ 170°C (indicated with an asterisk in Fig. 6 A) due to the melting of PHA crystals formed during the coating procedure. Indeed, DSC scans performed on the as prepared PHA sample (Supplementary, S7) show melting endotherms at ~ 170 °C.

In order to estimate the degradation temperature of the pectin component, DSC traces of uncoated films have been collected up to 360 °C (Fig. 6 B) showing that degradation phenomenon, highlighted by the changing of the baseline slope, appear at temperatures higher than 170-180 °C.

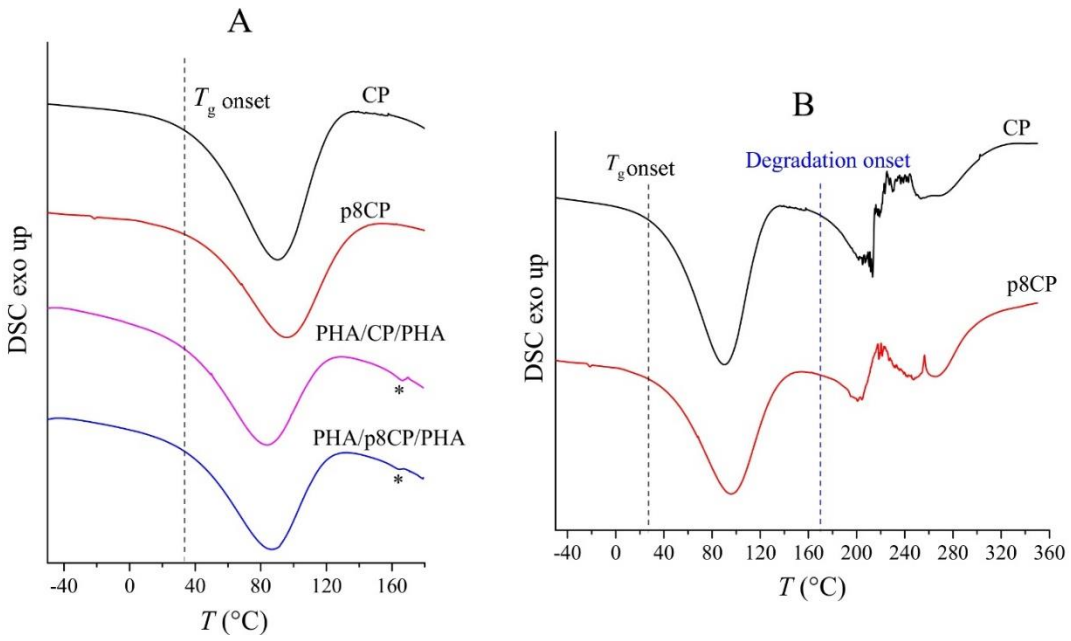


Fig. 6. DSC first heating thermograms of coated and uncoated pectin based films collected up to 180°C (A) and up to 360 °C for uncoated films (B).

3.4.5 Mechanical properties

The mechanical properties of CP and p8CP films and the corresponding multilayers PHA/CP/PHA and PHA/p8CP/PHA are summarized in Fig. 7. Coating the films with PHA caused a significant increase in terms of mechanical strength together with a reduction in their flexibility compared to the uncoated films. This effect is even more marked in functionalized films, where a sharp increase in TS and a drastic drop in EB were observed.~~, probably due to the modification of the pectin film matrix caused by SCGE addition.~~

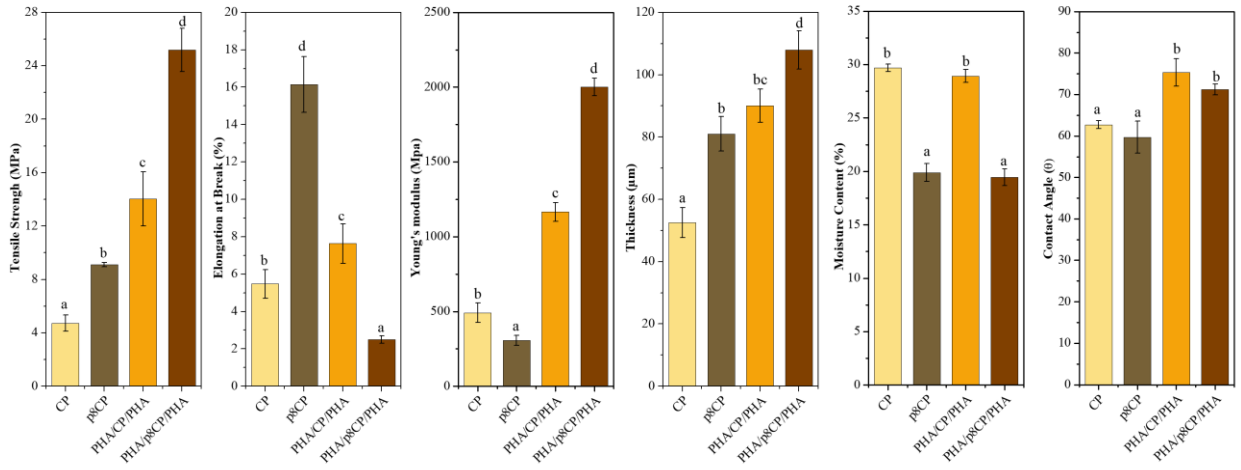


Fig. 7. Mechanical properties, moisture content and contact angle of CP and p8CP films and the corresponding multilayers PHA/CP/PHA and PHA/p8CP/PHA.

Whilst a good interfacial adhesion between pectin and PHA can be responsible of the observed increase in TS and YM, the properties of the PHA layer seems to prevail on the overall mechanical behaviour of the material, resulting into a reduced flexibility.[62]

The increased strengthening observed in functionalized films could be the result of a cooperative effect of the weak interactions established between pectin and PHA at the interface, together with the ones promoted by the presence of the SCGE components. The latter, when exposed on the pectin surface at the interface may contribute to reinforcing the interaction between the pectin and the PHA layer, being the interfacial adhesion an outcome of multiple bonding mechanisms including weak interactions, chemical adhesion and mechanical interlocking. [63].

On the other hand, while the presence of the SCGE strengthens the material interface, an impairment in material flexibility is also observed. In a future perspective, replacing the PHB layer with a scl-mcl PHA copolymer such as PHBV, or its plasticization with compatible additives will be explored as feasible strategies to reduce material stiffness.

3.4.6 Water susceptibility

The moisture content of the pectin films was only affected by the addition of the SCGE extracts and not by PHA coating (Fig. 7). Regardless the PHA layers, we can assume that the linkages between pectin and phenols allow to entrap less water inside the film matrix, resulting in a lower moisture content, compared to the samples without phenols. The same results were reported by Riaz et al. who incorporates apple peel polyphenols in chitosan film matrix.[64] Water contact angle was measured to evaluate the water hydrophobicity of pectin-based films (Fig. 7). While the presence of the phenolic extract did not affect the contact angle value, the hydrophobic nature of PHA significatively contributes to increase the water contact angle of the multilayer film. As far as WU, tracked for 5 days (Fig. 8), the presence of extracts and even more of the polyester layer reduced the water adsorption because of the synergic effect of reducing the number of free hydroxylic groups in the pectin matrix together with the hydrophobic nature of PHA.[65]

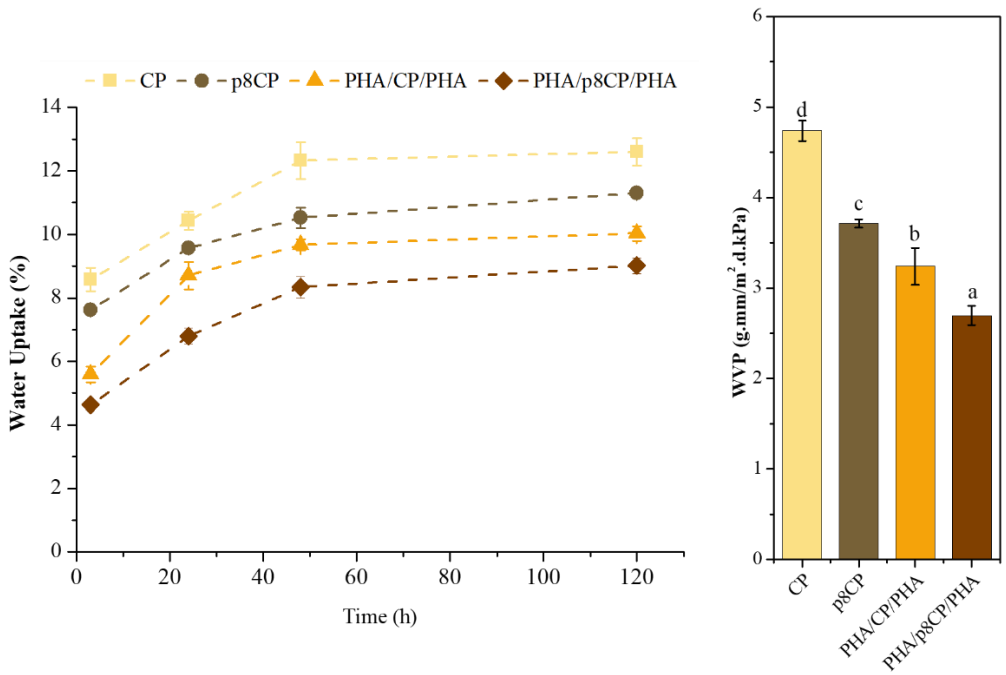


Fig. 8. Water uptake (WU) (%) of the CP, p8CP, PHA/CP/PHA and PHA/p8CP/PHA films over 120 hours and Water Vapor Permeability (WVP).

The WU of neat pectin films is around 13% following 5 days under 50% humidity as reported also by Manrich et al. and it decreased up to 9% for the PHA/p8CP/PHA. [31] In the same way SCG extracts and PHA lamination both led to a significant reduction of WVP as shown in Fig. 8. The same trend of reduction was observed by Sharaby et al. who found out an improvement of water barrier properties of CP films when the latter were grafted with crystalline nanocellulose suggesting that our technology led to results comparable with those obtained with different approaches.[7] Nevertheless, the WVP data provides evidence for the applicability and performance of the produced active film so that they may compete with the biobased Mater-Bi, a material frequently used and commercially available made of corn starch.[14] These results are quite interesting for applying these films in the food sector to protect different kinds of foods.

3.4.7 Bioactivity of pectin-based films

Antioxidant activity of p8CP films and the corresponding multilayer PHA/p8CP/PHA were evaluated (Fig. 9).

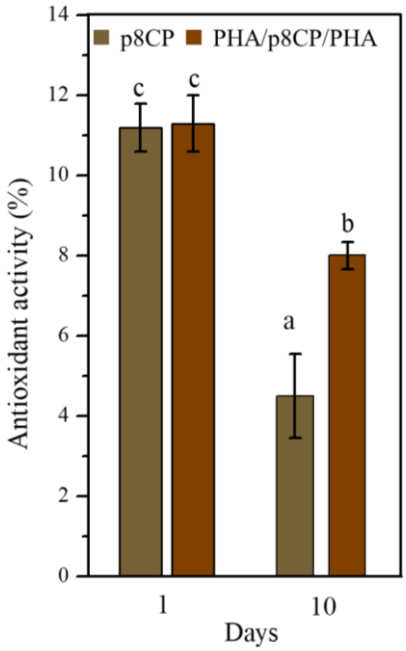


Fig. 9. Antioxidant activity of p8CP film and PHA/p8CP/PHA multilayer after 1 and 10 days.

The results obtained one day after coating did not reveal a significant difference between the coated and uncoated films in terms of antioxidant activity. On the other hand, after 10 days, although a reduction in such activity was observed for both films, the multilayer system retained antioxidant activity to a certain extent, while antioxidant activity of the uncoated film was halved (Fig. 9). Thus, the PHA coating does not interfere with the film antioxidant activity, but even preserves it at least for a short period, because it probably keeps the film from the loss of volatile phenolic compounds. This result was also confirmed by antimicrobial activity of films assayed after 10 days from the preparation by disk diffusion method. Both films loaded with phenolic extract showed the inhibition halo, with a wider diameter in the case of multilayer respect to the neat one (Fig. 10). No inhibition halo was detected in the case of neat pectin and PHA films used as controls (data not shown). Although the assessment of the antimicrobial activity of the designed multilayer will deserve further deepening, this achievement represents a proof of concept of the effectiveness of the dip coating procedure to both do not compromise the stability of the active compounds as well as to preserve the bioactivity of the material for longer time.

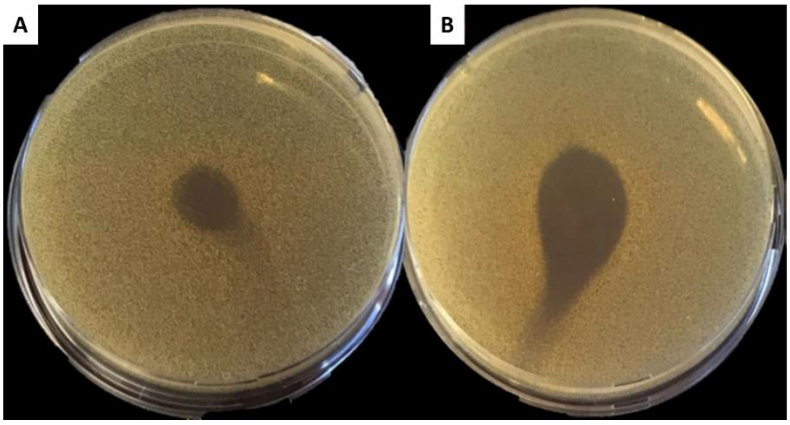


Fig. 10. Disk diffusion method in agar: antimicrobial activity against *Micrococcus. luteus* of p8CP film and PHA/p8CP/PHA multilayer after 10 days from the preparation.

3.6 Conclusions

Coating CP films by a two-steps dipping into PHA solutions provided an efficient technology to conventional ones, *i.e.* electrospinning or lamination extrusion, to achieve double-side coated pectin films with improved technological properties. The easiness and rapidity of the procedure, carried out at room temperature, also allowed to preserve the integrity of the active additives used for film functionalization. In fact, the multilayer films successfully functionalized with SCGE (containing mainly caffeoylquinic acid isomers including chlorogenic acid) were able to keep the antioxidant and antimicrobial property over time while maintaining their mechanical resistance. In addition, the polyester layers conferred to the manufactured films improved water sensitivity features, whereas the SCGE were able to extend fresh carrots shelf-life by delaying carotenoid photodegradation. The obtained results provide important insights about the manufacture of novel bioplastics produced from different bio-wastes that may be utilized as an added value source in the frame of circular bioeconomy. Further work is currently underway to optimize the process to better control the coating thickness and to improve the interlayer adhesion by chemical functionalization of the involved biopolymers. In addition, exploiting the heterogeneity of properties offered by different PHA-based polymers, further improvement in material properties can be achieved by modulating the PHA composition or by the addition of compatible additives. In conclusion, the study opens the possibility to extend the PHA-based coating to other combination of biobased materials/active extracts, contributing to widen the range of biobased/biodegradable multi-layered materials usable in diverse application fields. Ultimately, being PHA polymers degradable by the action of microbial PHA depolymerase enzyme, their exploitation as adhesive layers may address the challenges faced by multilayer material recycling, promoting the possibility of using enzyme-based delamination for the stepwise recovery of the individual components from multilayer materials [4].

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Author contributions

Seyedeh Fatemeh Mirpoor: Investigation, Formal analysis, validation; writing original draft; **Iolanda Corrado**: Investigation, Formal analysis, validation; writing original draft; **Rocco Di Girolamo**: Investigation; **Giovanni Dal Poggetto**: Investigation; **Lucia Panzella**: Investigation; **Elisabetta Borselleca**: Investigation; **Cinzia Pezzella**: Conceptualization; Writing - Review &

Editing; Visualization, Supervision; **C. Valeria L. Giosafatto**: Visualization, Writing-Review & Editing, Supervision.

Abbreviations

CP, citrus pectin (CP); SCGs, spent coffee grounds (SCGs); PHA, polyhydroxyalkanoates; PHB, Poly(3-hydroxybutyrate); mcl-PHA, Medium chain length PHA; PHBV, Polyhydroxybutyrate-co-valerate; SCGE, spent coffee grounds extract; TPC, total phenolic content; GAE, Gallic acid equivalent; GPC, Gel permeation chromatography; RI, refractive index; IV, intrinsic viscosity; FFs, film forming solutions; GLY, glycerol; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; FTIR, Fourier transform infrared; ATR, Attenuated Total Reflection; SEM, scanning electron microscopy; DSC, Differential scanning calorimetry; TS, tensile strength; EB, elongation at break; YM, Young's modulus; WU, water uptake; PDI, polydispersity index; T_g, glass transition temperature; WVP, Water Vapor Permeability.

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Figure 1

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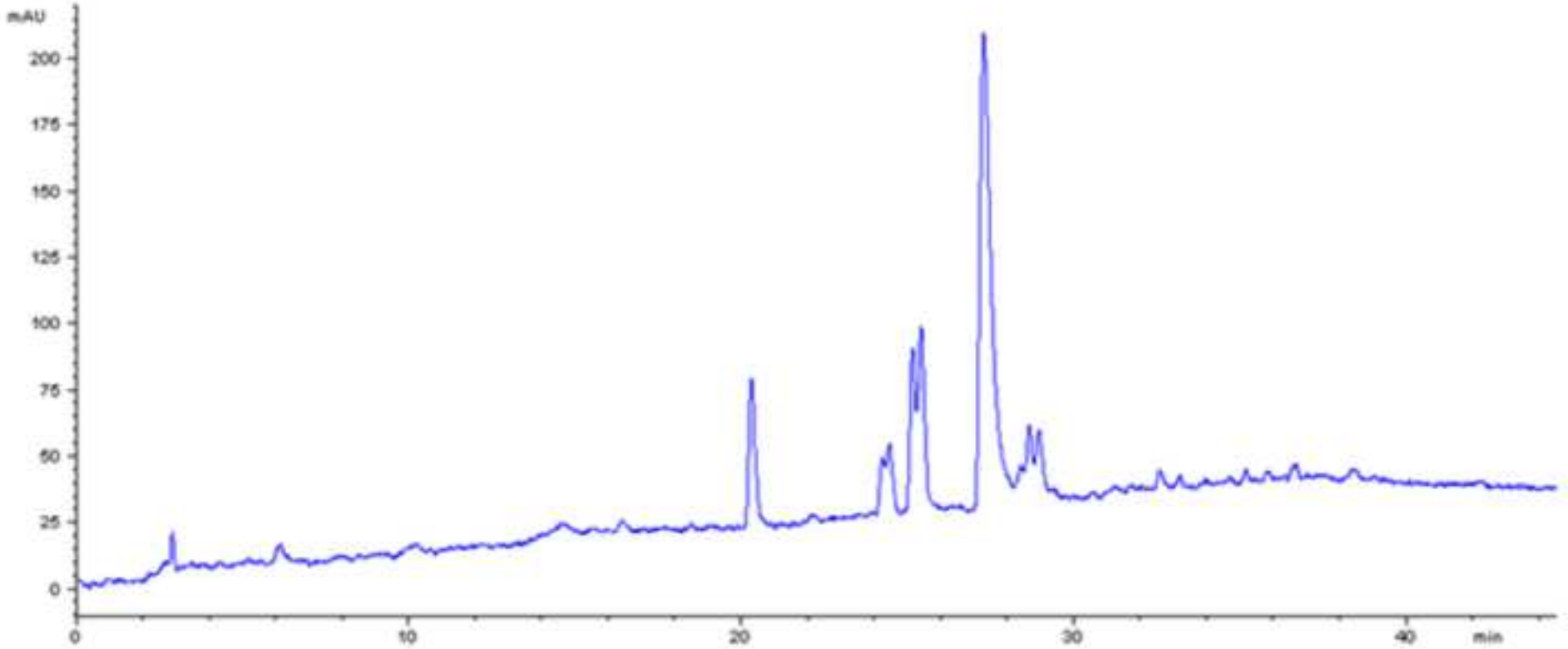

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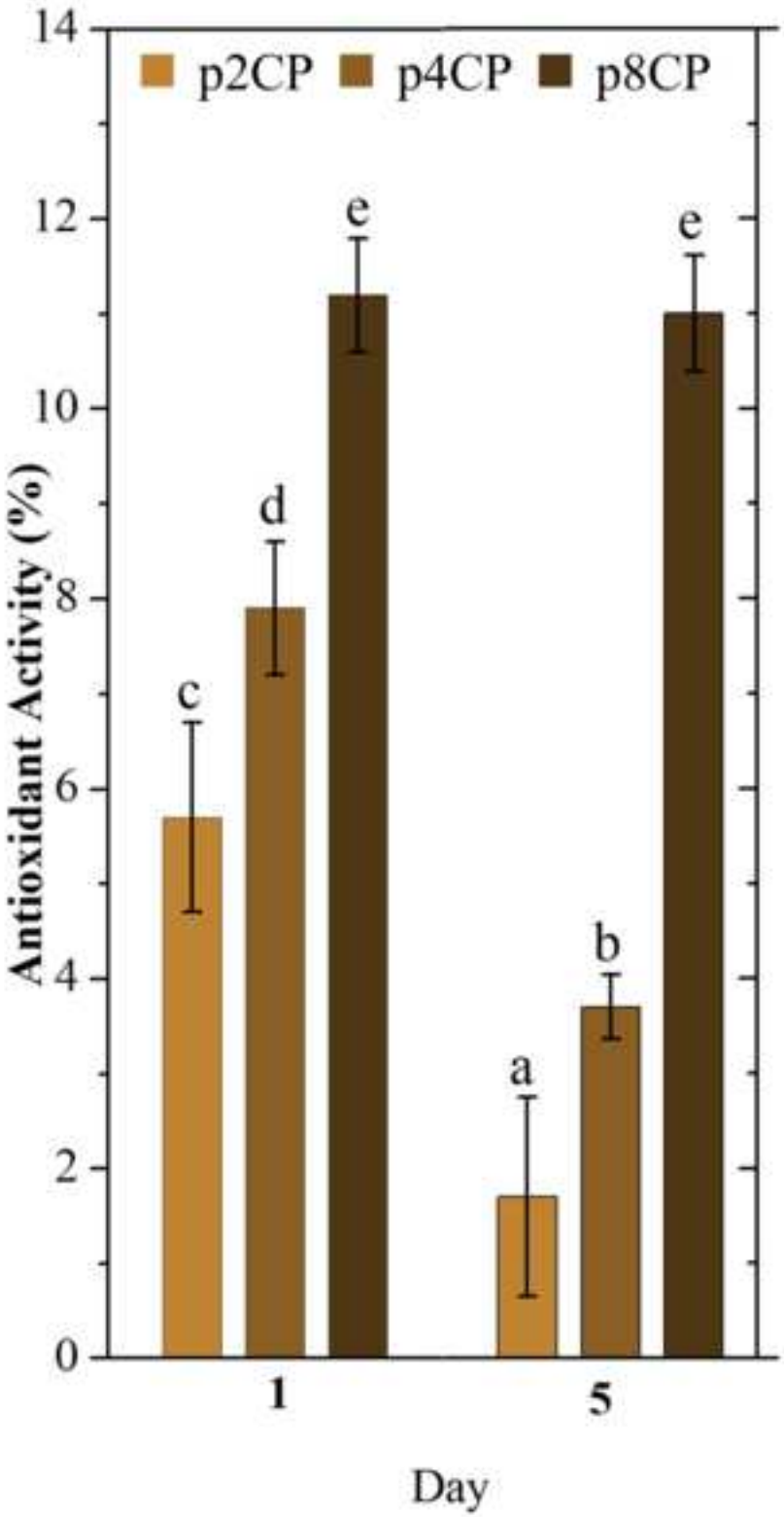

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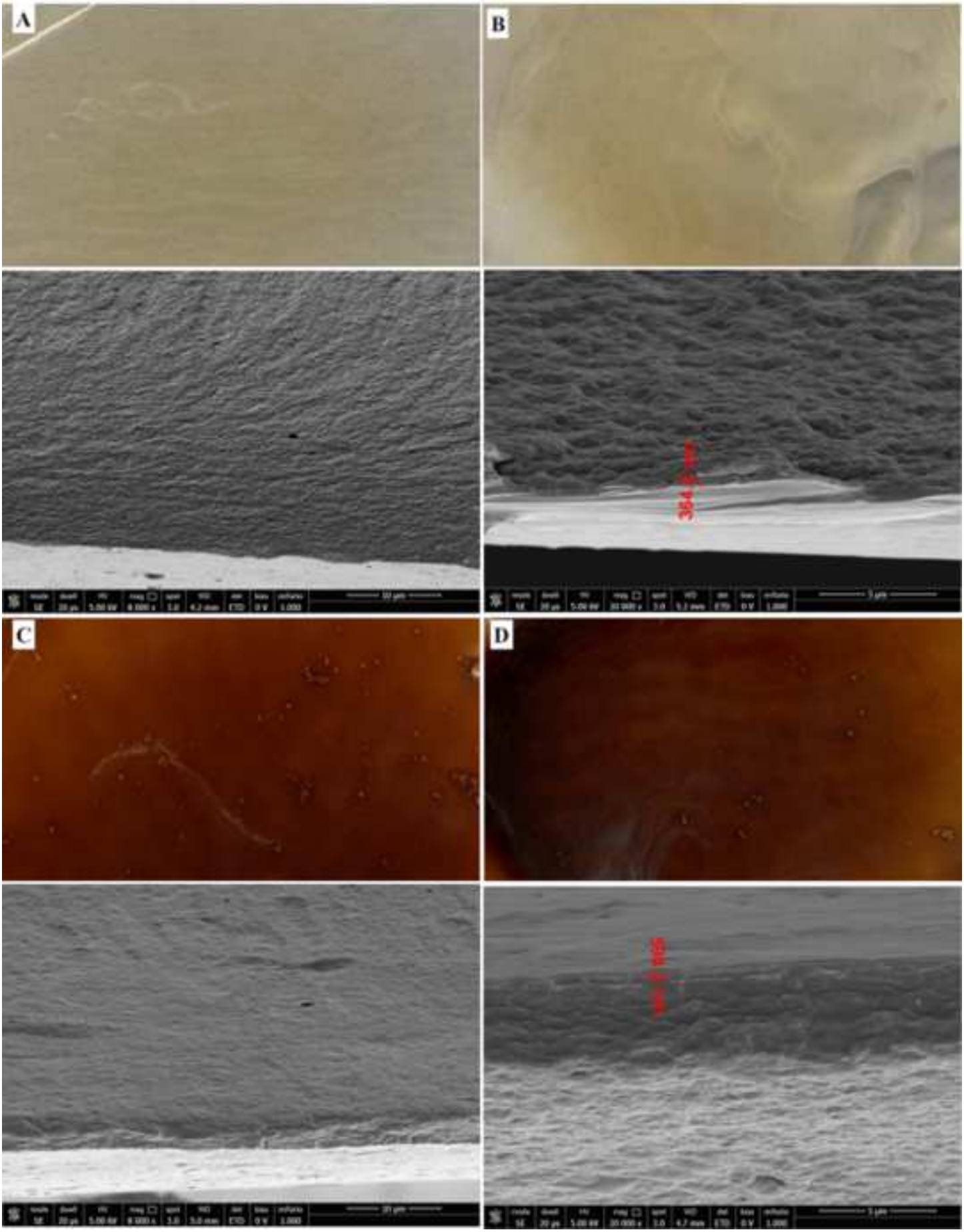
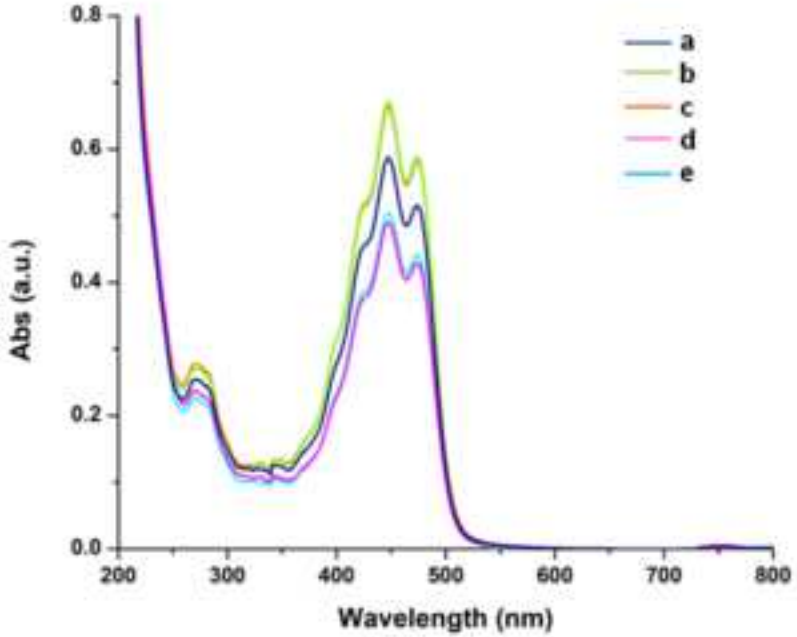


Figure 4

A

a b c d e

B



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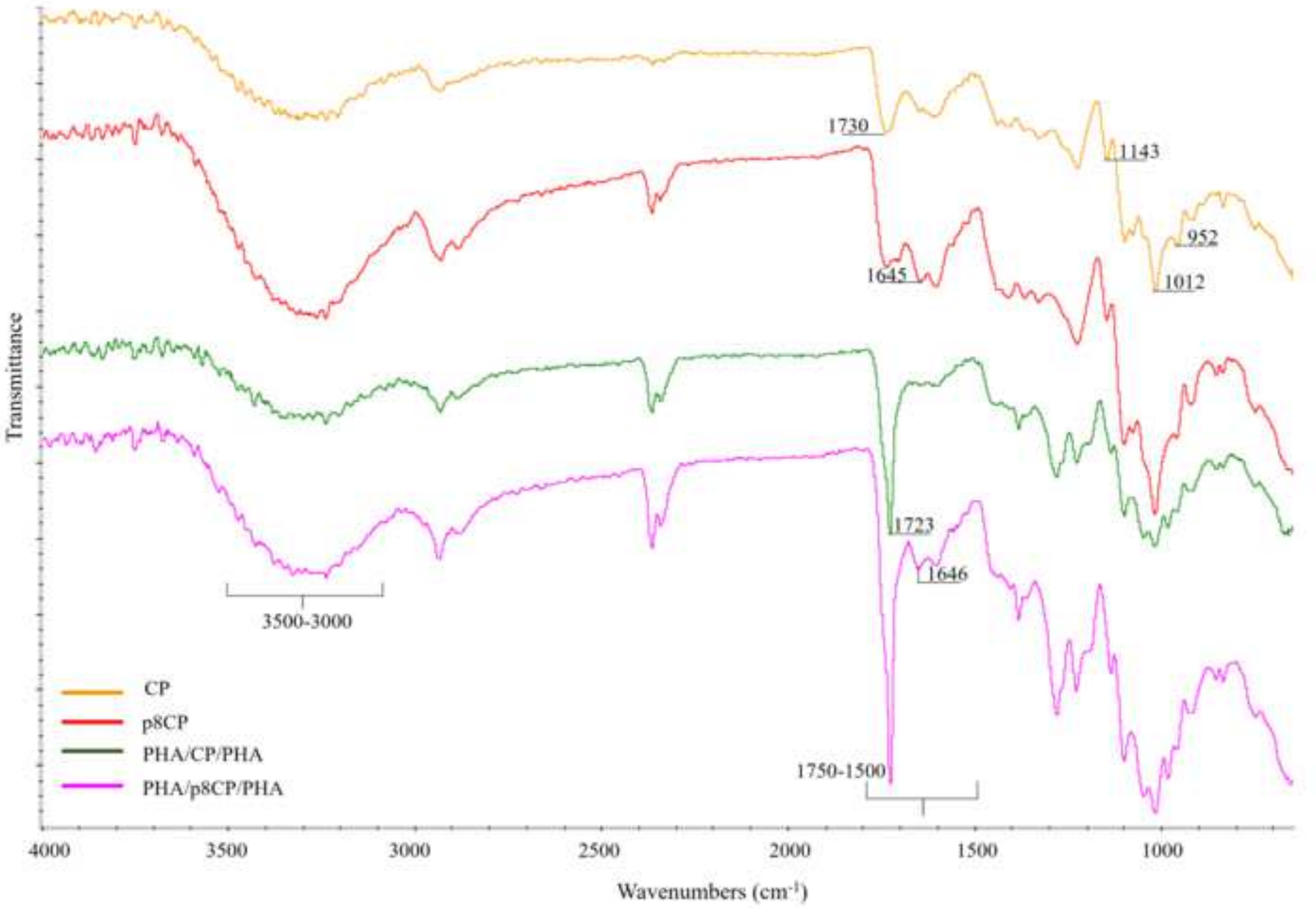

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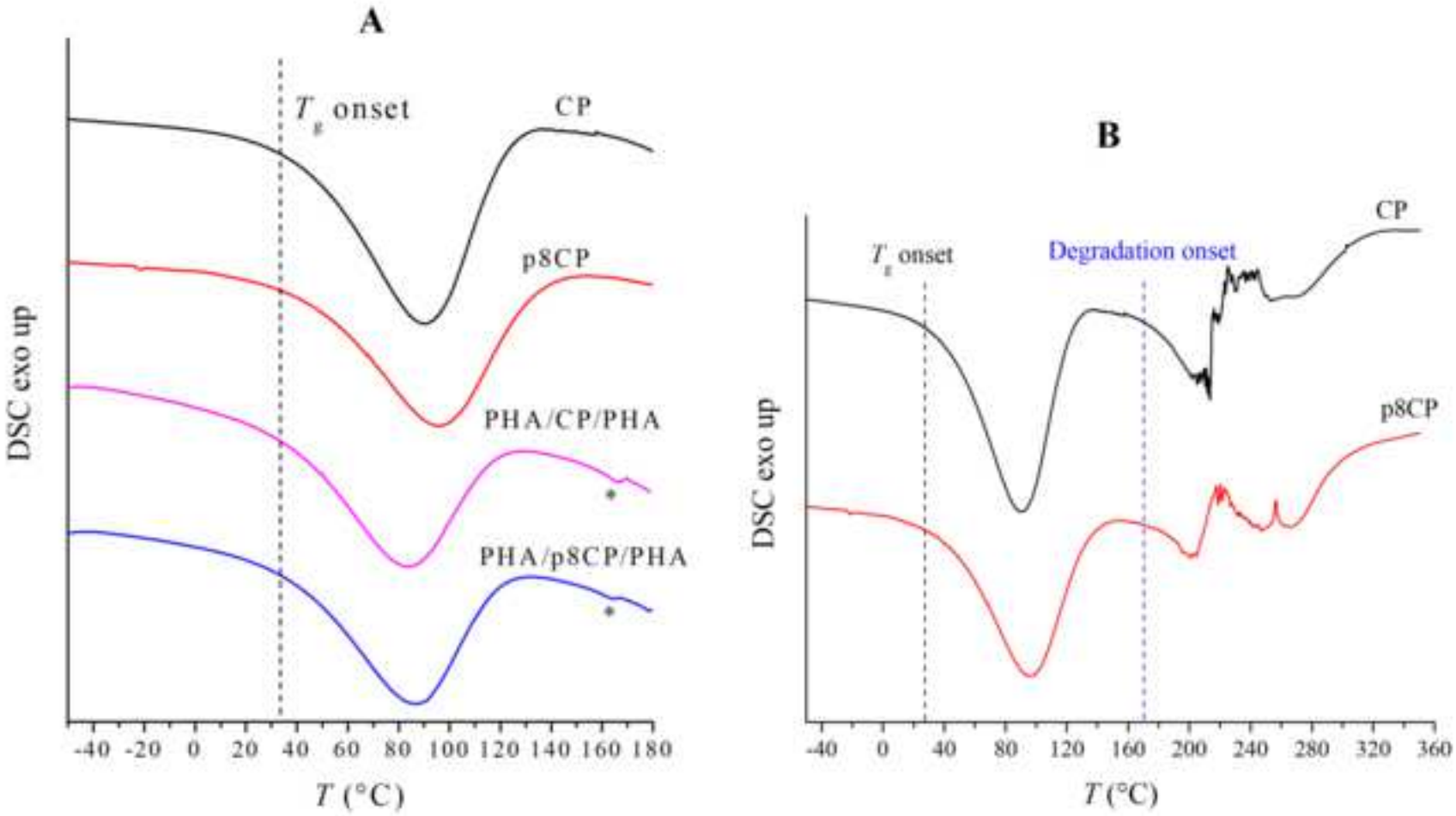

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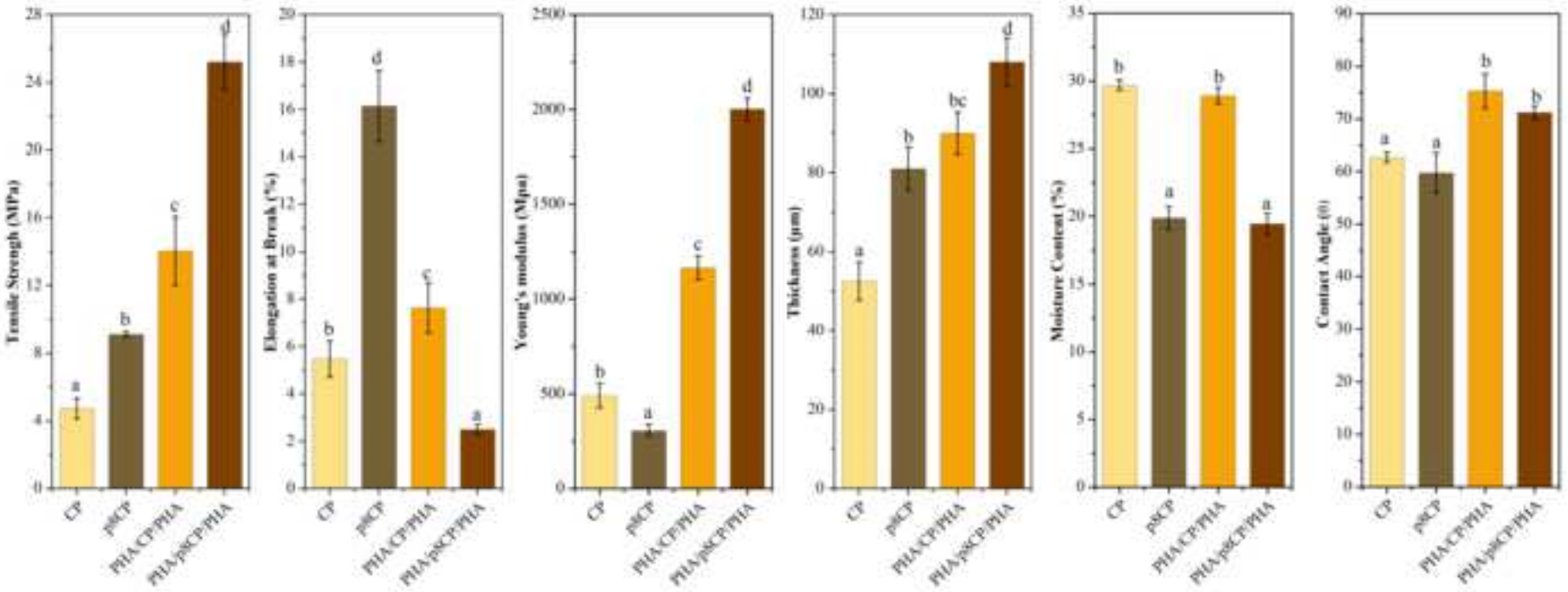

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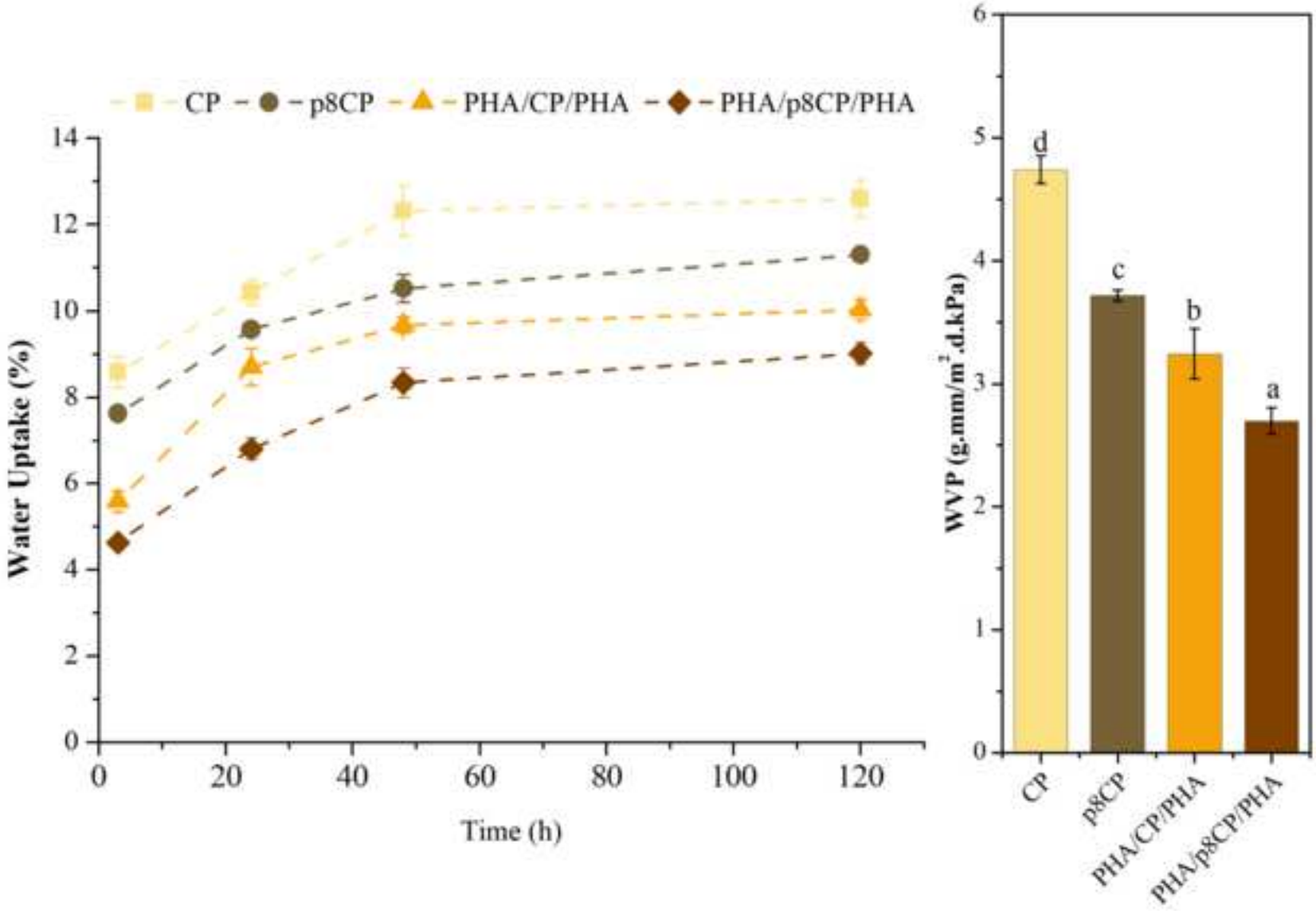

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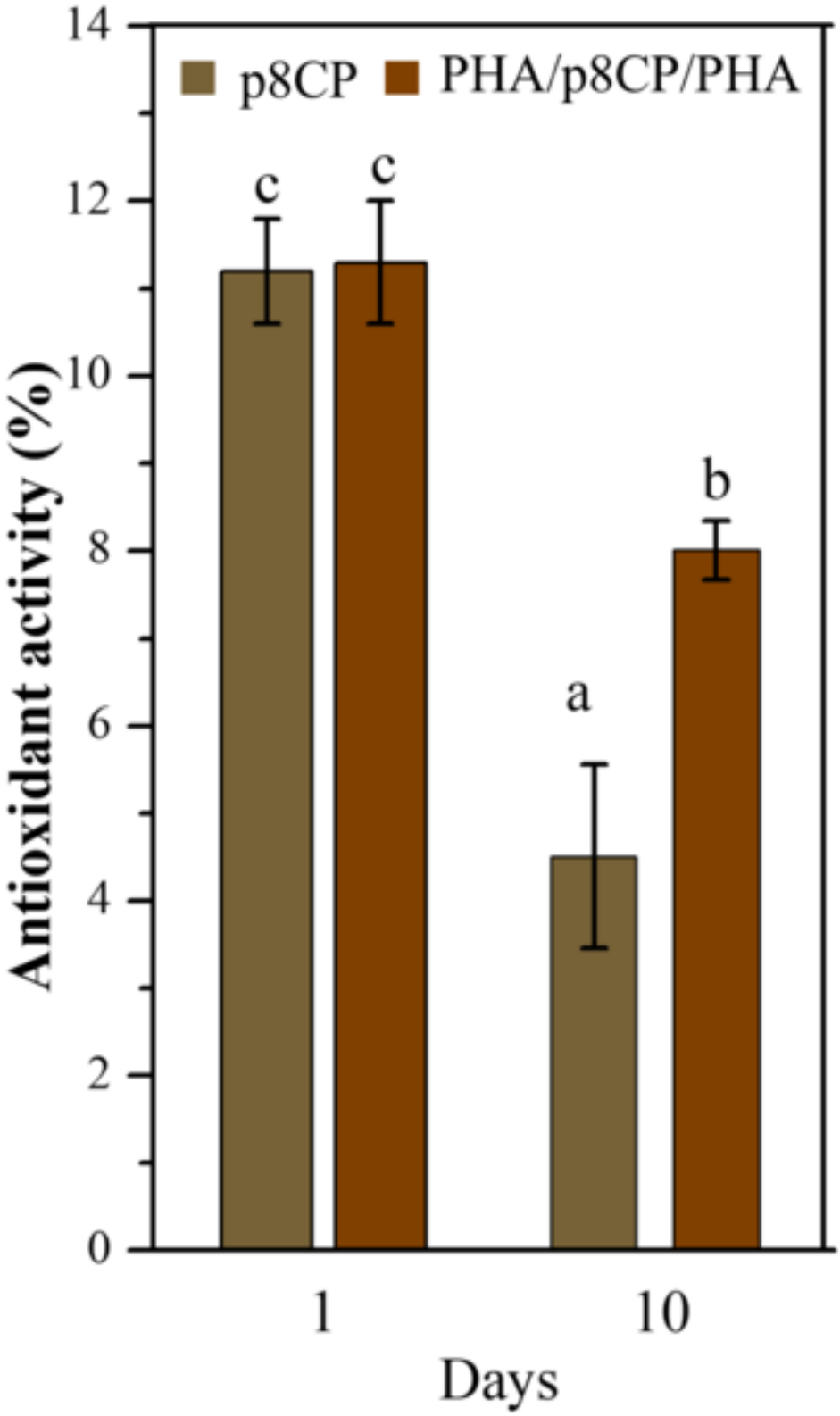

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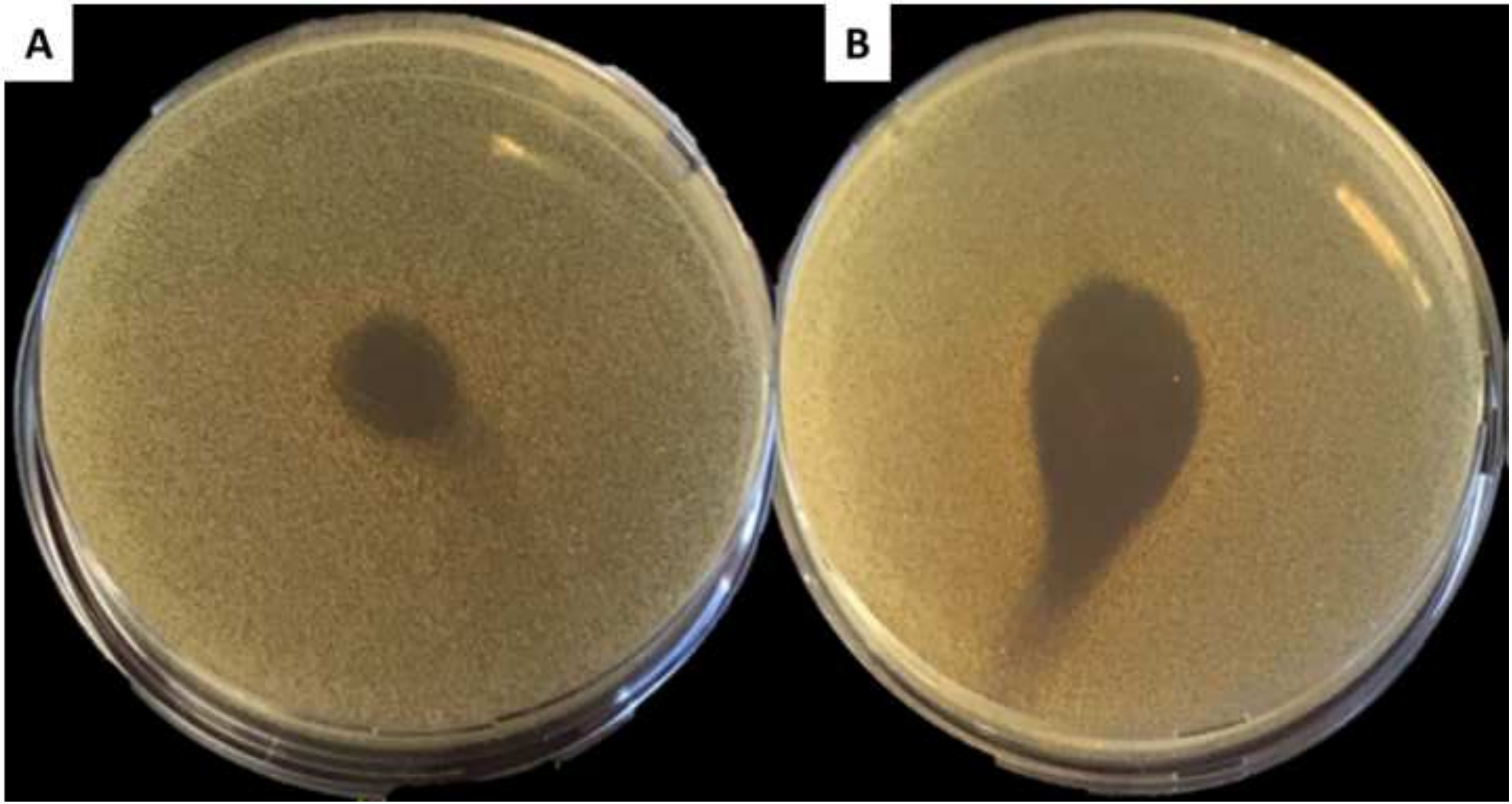

Highlights

- Pectin films were functionalized with phenolic extracts from Spent Coffee Grounds
- ~~Antioxidant properties of the functionalized pectin films were validated~~
- The films were ~~then~~ coated by dipping into polyhydroxyalkanoates (PHA) solutions
- The functionalized multilayer displayed improved water sensitivity features
- Light-shielding properties of the functionalized multilayer were demonstrated
- The PHA layer allowed to keep the antioxidant and antimicrobial activity over time

Credit Author Statement

Seyedeh Fatemeh Mirpoor: Investigation, Formal analysis, validation; writing original draft; **Iolanda Corrado**: Investigation, Formal analysis, validation; writing original draft; **Rocco Di Girolamo**: Investigation; **Giovanni Dal Poggetto**: Investigation; **Lucia Panzella**: Investigation; **Elisabetta Borselleca**: Investigation; **Cinzia Pezzella**: Conceptualization; Writing - Review & Editing; Visualization, Supervision; **C. Valeria L. Giosafatto**: Visualization, Writing-Review & Editing, Supervision.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: