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New Active Packaging Films Made from Gelatin Modified with Fungal Melanin

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ABSTRACT

Fungal melanin was used to prepare gelatin-based composite films. The films of various melanin concentrations (0.1%, 0.5% and 1% w/w) were prepared using a solution casting method. The mechanical, antioxidant, antimicrobial, water vapor, oxygen and UV-Vis barrier properties, as well as any available surface polyphenolics were studied. FT-IR and Raman spectroscopy studies were carried out to analyse the chemical composition of the resulting films. The hydrophobicity, solubility, colour response, optical properties and opacity were also determined. The results of this study showed that the modification of gelatin with fungal melanin had no influence on mechanical and water vapor barrier properties, oxygen barrier properties were improved. The UV-Vis barrier properties of modified films were significantly improved. Modified gelatin films showed good antioxidant activity but were inactive against microorganisms. The modification with melanin caused changes in colour values, decreasing lightness and increasing the redness and yellowness of the films. Based on the results of this study, fungal melanin has good potential to be utilised as a value-added modifier that can improve the properties of gelatin films.

Keywords: gelatin, melanin, packaging, antioxidant, barrier properties, mechanical properties, *Agaricus bisporus*

1. INTRODUCTION

Natural polymers, biopolymers, and synthetic polymers based on annually renewable resources are the basis of a 21st-century portfolio of sustainable, eco-efficient plastics. These biosourced materials are hoped to gradually replace the currently existing family of petroleum-based polymers as they become less competitive in regards to cost performance. Biodegradable polymers from renewable resources have attracted a great deal of attention in research. They are defined as polymers that undergo microbially-induced chain scission leading to mineralization [1].

The development of biodegradable packaging materials is an effective alternative to synthetic packaging material from petrochemical products, which are non-biodegradable and have a negative impact on the environment. Moreover, biodegradable materials are eco-friendly, non-toxic and have been shown to have many desirable physico-chemical characteristics over their synthetic counterparts [2-4]. In the last few decades, there has been a marked increase in the use of natural polymer-based film materials and coatings in packaging for the food industry, which protect food from external contamination, impeding its deterioration by extending shelf life and maintaining quality and safety [4]. In addition to consumer requirements and in order to substitute petroleum-based plastic packaging, a wide variety of biopolymers that come from agro-food industrial wastes and renewable low cost natural resources have emerged [5]. Packaging is widely used for the protection of food quality, thereby ensuring hygiene and extending the shelf life of perishable items, especially those susceptible to oxidative and microbiological deterioration [6]. In this context, the formulation of films and coatings for food packaging applications must include at least one component capable of forming a cohesive three-dimensional matrix [4]. Biopolymers directly extracted from biomass mainly used in edible films for food packaging are proteins, polysaccharides, lipids and their combinations. The physical and chemical properties of the biopolymer used determine the final properties of the developed films [2-4,6,7]. The protein-based films from various sources have excellent oxygen, carbon dioxide and volatile compound barrier properties, than compared with synthetic films under low relative humidity conditions [2,4,5].

Gelatin is a natural water soluble protein characterized by the absence of an appreciable odour and the random configuration of the polypeptide chains in aqueous solution. It is obtained from the denaturation and partial hydrolysis of collagen, a fibrous protein found mainly in certain parts of vertebrate and invertebrate animals as bones, skins, connective tissues and tendons. Its structure consists of rigid bar-like molecules that are arranged in fibres inter-connected by covalent bonds [6,8-10]. It has a triple-helix structure mainly stabilized by the formation of inter-chain hydrogen bonds between carbonyl and amines [4,9-12]. With respect to collagen, it does not express antigenicity in physiological conditions, it is completely resorbable *in vivo*, and its physicochemical properties can be suitably modulated. Furthermore, it is much cheaper and easier to obtain in concentrate solutions [13]. The abundance, availability and low cost of gelatin promote its use in a wide variety of applications [12]. It is unique among hydrocolloids as its melting point is so close to body temperature [8]. At a temperature of about 40°C, gelatin aqueous solutions are in a sol state and change into gels when they are cooled at room temperature, provided that their concentration is high enough [13,14]. The sol-gel transformation is due to a conformational

disorder–order transition of the gelatin chains which form thermo-reversible networks by associating helices in junction zones stabilized by hydrogen bonds [13].

Gelatin is accepted as a “Generally Recognized as Safe” (GRAS) substance by the U.S. Food and Drug Administration (FDA) [11]. Gelatin is widely used in the manufacture of edible films due to its excellent film forming ability, low gelling and melting point and biodegradability [4,8]. Gelatin films exhibit good oxygen barrier properties at low or intermediate relative humidity (RH) and satisfactory mechanical properties, making them suitable for use as coating or food packaging materials [4,11,12]. Depending on the processing method, gelatin can be classified into two types: (1) type A: with an isoelectric point at pH ~8–9, obtained from acid treated collagen; and (2) type B: with an isoelectric point at pH ~4–5, derived from an alkali treated precursor which converts asparagine and glutamine residues into their respective acids, resulting in higher viscosity. Gelatin derived from pig skin is normally referred as type A, and it is derived from beef skin or pig or cattle hides, as well as bones, and is referred to as type B [4].

The food, pharmaceutical, and photographic industries are the main users of gelatin, which has several other technical applications. Its most frequent uses in the biomedical field include hard and soft capsules, microspheres, sealants for vascular prostheses, wound dressing and adsorbent pads for surgical use, as well as three-dimensional tissue regeneration [4,14]. In particular, gelatin is used to provide gelling, stabilization, texturization and emulsification for bakery, beverages, confectionary and dairy products for the food industry. However, the limited thermal stability and mechanical properties of gelatin, especially during processing, limit its potential application [4].

A number of recent studies have dealt with extending the functional properties of biodegradable films by adding different compounds with antioxidant or antimicrobial activities in order to yield a biodegradable active packaging material. In order to reduce the use of synthetic chemical additives in the food industry, the use of natural food additives without any negative effects on human health has increased in recent years [4,15]. Those additives may not only influence the mechanical, barrier, optical properties and thermal stability of the blends but also enhance the microbial stability of the packaged foods by using active packaging developed by the incorporation of antimicrobial compounds into the polymer matrix. Contact between the active materials and food, which has the ability to change the food composition or the atmosphere around it, represents an active packaging system that inhibits the growth of microorganisms present on the surface of food products [1].

Several properties of gelatin films, such as mechanical, permeability, light absorption, transparency, antimicrobial activity and antioxidant ability, are not only influenced by the addition of active substances, but also by physical methods [4,6,12,16-18]. A wide spectrum of additives (nano or microsized) have been used for the preparation of gelatin composites including: hydroxyapatite [19], polysaccharides [20,21], rice flour [22], vegetable carbon black [23], carbon nanotubes [24], metal nanoparticles [25,26], fatty acid sucrose esters [27] and genipin [13].

Additionally, gelatin films can be used as vehicles for the release of antioxidant compounds such as: curcumin and its derivatives [12], essential oils [2,5,8,28-36], esculetin [37], butylated hydroxytoluene and α -tocopherol [38], lignin [9,39], liquid smoke [40], carvacrol [41], tannin [11,42] vanillin [43], riboflavin [44], gallic acid [45], aloe vera gel [46,47], tea polyphenols and green tea extracts loaded into chitosan nanoparticles [15,47-50], grapefruit seed extract [51] and tomato pulp [52].

Currently, naturally occurring bioactive compounds are preferred by both consumers and companies due to concerns over the potential risks of synthetic compounds.

In this context, fungi and plants are a valuable source of active compounds, such as antioxidants and antimicrobials, used for pharmaceutical, medical and food applications [4,12,53]. Melanins have been isolated and characterized from a variety of phylogenetic sources, such as animal [54], plant [55], bacteria [56] and fungi [57-59]. Melanins are commonly represented as black and brown pigments, high molecular weight heterogeneous polymers derived from the oxidation of monophenols and the subsequent polymerization of intermediate *o*-diphenols and their resulting quinones [60,61]. Melanins are types of pigments, possessing broad biological properties including antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory [54-61]. Potentially, these melanin attributes could also be imparted to polymers, and in the case of biopolymers, potentially enhancing performance as well as sustainability credentials, explore its other nonconventional applications such as cross-linking during polymerization, antioxidant and antimicrobial activity, radioprotective ability and improving the biological properties of the polymer. The use of melanins remains relatively unexplored with few examples of these compounds blended with packaging polymers or use them to modify coatings [1,62-66], and no studies on gelatin/melanin composites are available in the scientific literature.

The aim of this study was to investigate the influence of fungal melanin on the properties of modified gelatin films. To evaluate the potential functionality in food packaging applications, the mechanical, barrier, antioxidant and antimicrobial properties were all evaluated. Spectroscopic studies were performed to elucidate melanin addition in the chemical composition of modified blends. Additionally, the goal of the study was also to evaluate the influence of fungal melanin on the colour, solubility, opacity and optical properties of the films.

2. MATERIAL AND METHODS

2. 1. The Isolation of Melanin from *A. bisporus* Waste

Agricultural waste from the production of *A. bisporus* (ABW—Agaricus Bisporus Waste) in the form of fruiting bodies stipes was obtained from a local producer in Wolsztyn (Wielkopolskie voivodeship, Poland). 500 g of ABW was first homogenized (Heidolph Brinkmann Homogenizer Silent Crusher, Germany) in 500 mL of distilled water and incubated (24 h, 37 °C) to allow enzyme tyrosinase action (hydroxylation of monophenols to *o*-diphenols). After incubation, the homogenate mixture was adjusted to pH = 10 by 1 M NaOH, and incubated (24 h, 65 °C) to allow a spontaneous oxidative polymerization of the resulting *o*-diphenols and quinones to form melanin. Afterward, the mixture was filtered, centrifuged (6000 rpm, 10 min), and an alkaline ABW raw melanin mixture was used to purify the melanin. An alkaline ABW raw melanin mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a resulting pellet was collected. The pellet was then hydrolyzed in 6 M HCl (90 °C, 2 h), centrifuged (6000 rpm, 10 min) and washed in distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids

and other residues. Finally, the purified melanin was dried, ground to a fine powder in a mortar and stored at $-20\text{ }^{\circ}\text{C}$ until testing.

2. 2. The Preparation of Modified Gelatin Films

Gelatin films were prepared by the use of a solution casting method. Commercial bovine gelatin was purchased from Rousselot SAS (Saint-Michel, France). Firstly, ammonia water was added to the distilled water and the pH was adjusted to 10 (to enable the solubilization of melanin). Next, fungal melanin was added to alkaline solutions to obtain a 0.1% (sample "1"), 0.5% (sample "2") and 1% (sample "3") (on gelatin dry basis), and the solutions were placed in a stirrer (120 rpm), at $50\text{ }^{\circ}\text{C}$ for 24 h. The solution devoid of melanin addition served as a control film forming solution (sample "0"). When melanin was dissolved, the solutions were filtered through Whatman filter paper under vacuum. 8 g of gelatin were added to 100 mL of filtered alkaline solutions and kept for 2 h at $60\text{ }^{\circ}\text{C}$ under continuous stirring (120 rpm) to obtain a homogenous solution. Then, 10 % w/w (based on gelatin dry mass) of glycerol was added (this was used as a plasticizer). The film forming solutions were stirred for 15 min and finally poured into polypropylene Petri dishes (90 mm diameter), and kept at $25\text{ }^{\circ}\text{C}$, 50% relative humidity (RH) in controlled chamber during 48 h to evaporate water and ammonia, and form the films. Before all experiments all films were conditioned in a chamber 48 h, at $25\text{ }^{\circ}\text{C}$ and 50% RH.

2. 3. Water solubility of the films

Prewighted (W_0) dried films ($2\text{ cm} \times 2\text{ cm}$) were immersed in 15 mL of distilled water with the addition of 0.01% of sodium azide (as antimicrobial agent) at $30\text{ }^{\circ}\text{C}$ under constant agitation (50 rpm) for 24 h. The undissolved matter was separated by centrifugation at 1000 rpm for 5 min and the pellet was dried at $105\text{ }^{\circ}\text{C}$ to determine the weight (W_1) of the insoluble contents. All tests were carried out in triplicate and water solubility was calculated using the following Equation:

$$\text{Water solubility (\%)} = [(W_0 - W_1) / W_0] \times 100\% \quad (1)$$

2. 4. The Mechanical Properties of the Films

Mechanical measurements were tested by the use of Zwick/Roell 2,5 Z equipment (Zwick/Roell, Germany) and they included tensile strength (the gap between tensile clamps was 25 mm and tensile speed was 100 mm/min), elongation at break, and burst strength (transducer diameter 0.75 mm, speed 50 mm/min).

2. 5. The Water Vapor Transmission Rate and Oxygen Transmission Rate of the Films

The Water Vapor Transmission Rate (WVTR) was measured by means of a gravimetric method that is based on the sorption of humidity by calcium chloride and a comparison of sample weight gain. Initially, the amount of dry CaCl_2 inside the container was 9 g. The area of film was 8.86 cm^2 . Measurement was carried out for a period of 4 days, each day the containers were weighed to determine the amount of absorbed water vapor through the films.

The results were expressed as average values from each day of measurement and each container. Analyses were carried out at ten independent containers for each type of films, calculated as a standard unit $\text{g}/(\text{m}^2 \times \text{day})$ and presented as a mean \pm standard deviation.

The Oxygen Transmission Rate (OTR) was measured by means of Ox-Tran 2/10 instrument (Mocon, USA) equipped with a coulometric sensor. The method is based on a standard ASTM D3985 – appropriate for films and laminates.

2. 6. The Contact Angle (CA)

The surface properties of modified and pure gelatin films were measured through a contact angle analyzer. The following measurement was carried out by means of a laboratory goniometer (Haas μL). Film samples were cut into 3 cm x 9 cm and fitted on a sample stage and leveled horizontally. A drop of distilled water was placed on the surface of the film using a microsyringe. Analyses were carried out at three independent times and presented as mean \pm standard deviation.

2. 7. Spectral Analysis

2. 7. 1. UV-Vis spectroscopy

The UV-Vis spectra of the films samples were measured by the use of a UV-Vis Thermo Scientific Evolution 220 spectrophotometer at 200–1100 nm.

2. 7. 2. FT-IR Spectroscopy

Fourier transform infrared (FT-IR) spectra of the unmodified and modified film samples were measured using a FT-IR spectroscopy (Perkin Elmer Spectrophotometer, Spectrum 100, USA), operated at a resolution of 4 cm^{-1} , over 64 scans. Film samples were cut into square shapes (2 cm \times 2 cm) and placed directly at the ray-exposing stage. The spectra were recorded at a wavelength of 650–4000 cm^{-1} . The spectra were normalized, baseline corrected and analyzed using SPECTRUM software.

2. 7. 3. Raman spectroscopy

Pure and modified films were analyzed using a Raman station (RamanStation 400F, Perkin Elemer, USA) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size. Film samples were cut into square shapes (2 cm \times 2 cm) and placed directly at the ray-exposing stage. The spectra were recorded at a wavelength of 250–3300 cm^{-1} . The spectra were normalized, baseline corrected and analyzed using SPECTRUM software (10, PerkinElmer, USA).

2. 8. Color response analysis

The color changes of the films were measured by using a colorimeter (CR-5, Konica Minolta, Japan). The results were expressed as L (lightness), a (red to green), and b (yellow to blue) parameters to evaluate color changes in the modified films. All of the measurements were determined at three random points on both sides of each film, and the experiments were performed five times and presented as a mean \pm standard deviation.

To determine other color properties of the films, ΔE (color difference), YI (yellowness index), WI (whiteness index), hue angle (h^*ab) and chroma (C^*ab) values were calculated using following Equations (where pure gelatin film served as a standard) [1,34]:

$$\Delta E = [(L_{\text{standard}} - L_{\text{sample}})^2 + (a_{\text{standard}} - a_{\text{sample}})^2 + (b_{\text{standard}} - b_{\text{sample}})^2]^{0.5} \quad (2)$$

$$YI = 142.86b \times L^{-1} \quad (3)$$

$$WI = 100 - [(100 - L)^2 + a^2 + b^2]^{0.5} \quad (4)$$

$$h^*ab = \arctg \frac{b_{\text{sample}}}{a_{\text{sample}}} \quad (5)$$

$$C^*ab = [(a_{\text{sample}})^2 + (b_{\text{sample}})^2]^{0.5} \quad (6)$$

2. 9. Opacity measurements

The opacity of modified gelatin/melanin and pure gelatin films was carried out in Opacimeter EE Model 12 (Diffusion Systems LTD, UK). The opacimeter was initially calibrated using standard white plate (value 100 ± 1 , Diffusion Systems LTD) and measurements were performed on each film six times, and presented as mean \pm standard deviation.

2. 10. The Antioxidant Activity of Gelatin/Melanin Blends

2. 10. 1. Determination of Available Phenolic Groups on the Films Surface

The method for the determination of available phenolic groups (APG) on the non modified and modified gelatin/melanin films was carried out according to Łopusiewicz *et al.* [1]. 100 mg of gelatin/melanin or pure gelatin films was taken in a volumetric flask. Sequentially, 1 mL of 10% Folin-Ciocalteu reagent and 4 mL of 2% sodium carbonate solution were added to the flask. Finally, the volume was made up to 25 mL with distilled water and mixed well. The reaction mixture was kept at room temperature for 48 h and the resultant absorbance was determined at 760 nm. A control absorbance was also measured where the aforesaid reaction mixture, devoid of any film, was kept under the same reaction conditions. To determine the available phenolic groups on the modified films surface, a calibration curve was prepared using gallic acid standard solutions and the results were expressed as μ moles of gallic acid equivalents (GAE) per gram of dry film. All experiments were performed in triplicate and presented as mean \pm standard deviation.

2. 10. 2. A Determination of the Free Radical Scavenging Activity of the Films

The free radical scavenging property determination of melanin incorporated gelatin films was carried out using ABTS according to Łopusiewicz *et al.* [1]. Radical 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid ($ABTS^+$) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was then incubated for 16 h in the dark, at room temperature and subsequently diluted with water to an absorbance of maximum 1.00 at 734 nm. To determine

the antioxidant capacity of the films, 1 g of the film was put into 25 mL of ABTS⁺ solution and incubated up to 24 h at room temperature. Control sets without the film were also kept under identical conditions. After incubation period, the film samples were removed from the ABTS⁺ solution. Absorbance for both sets was taken and antioxidant activity (AA%) was calculated using the Equation:

$$AA\% = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100 \quad (7)$$

2. 11. The Antimicrobial Activity of Films

The test microorganisms used in this study were obtained from the American Type Culture Collection (ATCC). The strains used in this study were *Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC2783, *Pseudomonas putida* ATCC31753. To verify the antimicrobial properties of films Mueller-Hinton agar and broth (Merck, Germany) were used. The media were prepared according to the Merck protocol (medium was weighted according to the manufacturer's instructions, suspended in 1000 mL of distilled water, and autoclaved at 121 °C for 15 min). Antibacterial activity of films was evaluated by the use of two methods. Firstly, 50 mL of Mueller-Hinton broth was inoculated by a single bacterial strain and incubated at 37 °C for 24 h and after incubation time 200 µL of bacterial suspension was added to agar surface by a glass spreader. The film samples were cut into square shapes (2 cm × 2 cm), and put directly on the bacteria. The plates were incubated at 30 °C for 24 h. The positive antimicrobial activity was considered as growth inhibition zones around the films. Second test was performed to evaluate the influence of the films of bacterial growth. The film samples (1 g) were incubated with single bacterial strain suspensions (1.0×10^6 CFU/mL) in sterile physiological saline (0.9% NaCl) for 24 h at 30°. After incubation period the bacterial cells concentration was evaluated on solid Mueller-Hinton agar.

2. 12. Statistical Analyses

All determinations were carried out in triplicate as a minimum. Statistical significance was determined using an analysis of variance (ANOVA) followed by Duncans's test. The values were considered as significantly different when $p < 0.05$. All analyses were performed with Statistica version 10 (StatSoft Polska, Kraków, Poland).

3. RESULTS

3. 1. Mechanical Properties

In order to evaluate the potential influence of melanin on the mechanical features of non-modified and modified gelatin-based films, three measurements were carried out: tensile strength, elongation at break and burst strength. The results of tensile strength, elongation at break and burst strength of the films are shown in Table 1. No significant differences ($p > 0.05$) in the results of tensile strength were observed for the samples, the reference sample and those containing 0.1%; 0.5% and 1% of melanin, respectively. This confirms any lack of impact of melanin on the tensile strength. Regarding the brittle/elastic behaviour of the samples, the lowest (0.1%) addition of melanin to the gelatin matrix increased elongation at break value – from 19.31% (reference sample) to 35.65%. The other samples (0.5% and 1%) exhibited

lower values: 23.8% and 24.14%, respectively. Nevertheless, after considering statistical analysis, no significant differences between all of the results were detected, and this was confirmed by the Duncan test ($p>0.05$). As shown in Table 1, no significant influence of melanin was indicated in the results of maximum burst strength ($p>0.05$).

Table 1. Tensile strength (TS), elongation at break (EB), burst strength (BS), Water Vapor Transmission Rate (WVTR), Oxygen Transmission Rate (OTR) and solubility (%) of pure gelatin and gelatin/melanin modified films.

Sample	TS (MPa)	EB (%)	BS (MPa)	WVTR ($\frac{g}{m^2 \times day}$)	OTR ($\frac{cm^3}{m^2 \times day}$)	Solubility (%)
0	111.92 ±16.01	19.31 ±7.89	20.95 ±3.96	36.22 ±8.20	5.44 ±0.41	72.16 ±3.24
1	111.00 ±4.35	35.65 ±9.25	16.76 ±3.60	28.14 ±3.95	4.88 ±0.25	66.25 ±2.15
2	107.17 ±7.10	23.80 ±8.33	18.22 ±1.79	30.47 ±5.41	4.09 ±0.11	43.56 ±4.08
3	118.00 ±12.40	24.14 ±7.61	18.35 ±1.68	30.71 ±7.53	3.85 ±0.16	31.05 ±2.75

3. 2. Solubility of the films

Table 1 shows films solubility. In the present study, the control film showed the highest solubility (72.16±3.24%) among all of the films and the addition of 0.1%; 0.5% and 1% of melanin decreased the solubility of the films significantly ($p<0.05$) to 66.25±2.15; 43.56±4.08 and 31.05±2.75%, respectively.

3.3 . Surface Properties—Contact Angle

Three repetition tests were performed for each sample of the gelatin-based films. The average values of the contact angle obtained for distilled water were as follows: 53.3°; 69.9°; 71.5°; 72.9° for samples “0”, “1”, “2”, and “3”, respectively. The Duncan’s test was applied to demonstrate that these differences of averaged values were statistically significant ($p < 0.05$).

3. 4. Barrier Properties—WVTR and OTR

The water vapor transmission rate of all four samples was measured by means of a gravimetric method, which is based on the sorption of humidity by calcium chloride and a weight gain comparison of the samples.

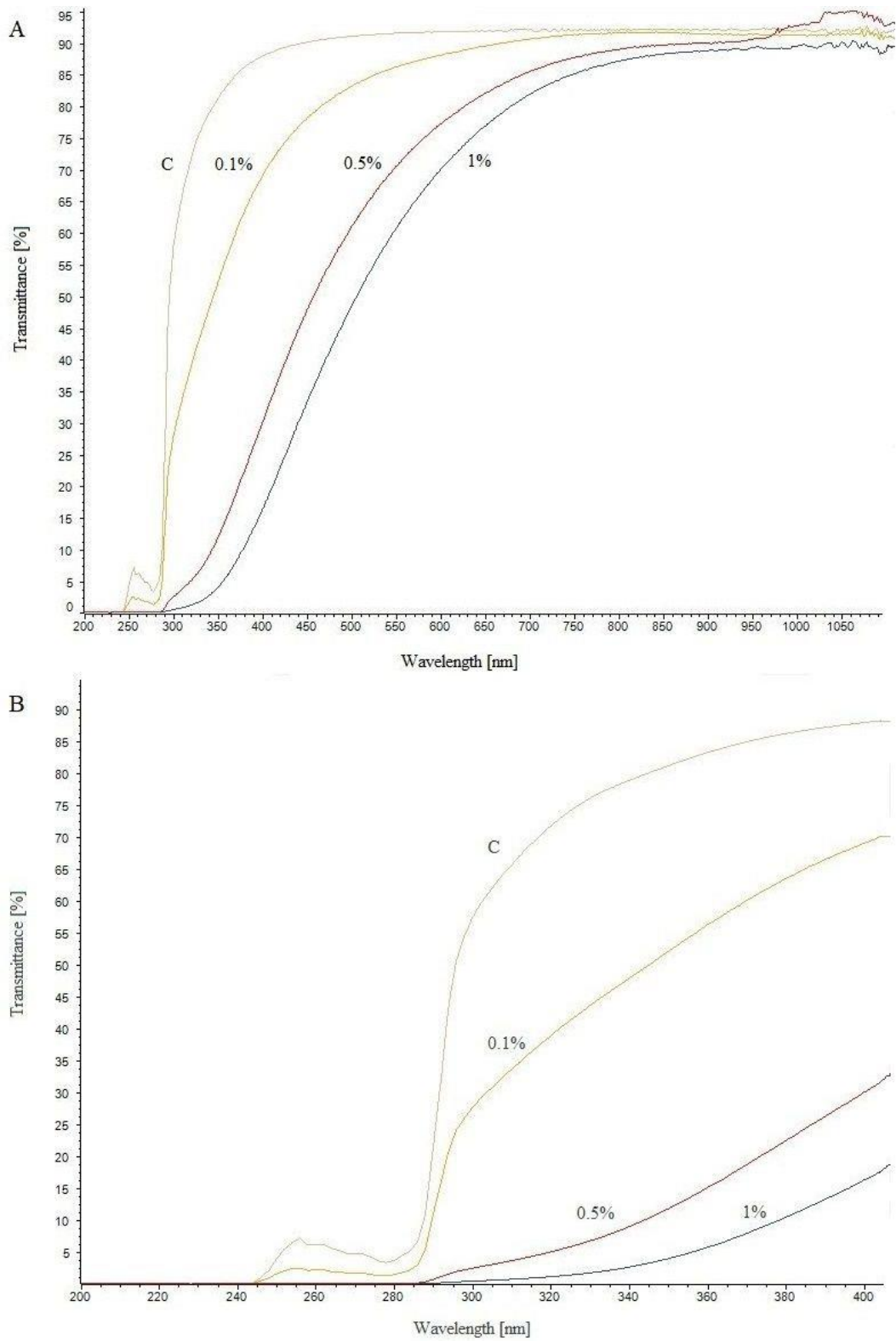


Figure 1. The UV-Vis spectra of pure gelatin and gelatin/melanin films (A)200-1100 nm (B) 200-400 nm

As reported in the Table 1 sample “0” exhibited the highest values of WVTR, 36.22 ± 8.20 g/(m²×day), whereas the values obtained for the modified samples (with increasing melanin content) were 28.14 ± 3.95 ; 30.47 ± 5.41 and 30.71 ± 7.53 g/(m²×day) respectively. However, after considering the statistical analysis, the differences between all the samples were not statistically significant ($p > 0.05$). As shown in Table 1 the oxygen transmission rate values of the films were influenced by the addition of melanin in comparison to the control sample. Sample “0” (devoid of melanin) exhibited the highest value of OTR (5.44 ± 0.41 (cm³/(m²×day))), whereas the values obtained for the modified samples were growing with increasing melanin content and were 4.88 ± 0.25 ; 4.09 ± 0.11 and 3.85 ± 0.16 cm³/(m²×day), respectively. The Duncan’s test was applied to demonstrate that these differences of averaged values were statistically significant ($p < 0.05$).

3. 5. The Spectral Analysis of Modified Films

3. 5. 1. The UV-Vis spectra

UV-Vis spectra of pure gelatin and gelatin/melanin films in selected wavelengths from 200 to 1100 nm in UV and visible ranges are shown in Figure 1a and 1b. The addition of melanin caused noticeable improvement of the light barrier properties. Decreases in light transmission of films modified with melanin were observed at all wavelengths, compared with control film. A noticeable peak at 280 nm in control sample is resulted from aromatic amino acids. The results indicated that melanin was able to impede the light transmission through the films.

3. 5. 2. The FT-IR spectra

FT-IR spectra of pure gelatin and gelatin/melanin films are shown in Figure 2. The addition of melanin caused noticeable changes in intensity of Amide I band at 1633.38 cm⁻¹, Amide II band at 1538.39 cm⁻¹, and Amide III band at 1237.36 cm⁻¹ than compared with pure gelatin film. In addition, absorption bands at the wavenumbers of 1033.28 cm⁻¹ were found. Moreover, amide-A band, arising from the stretching vibration of NH group appeared at wave numbers of 3294.26 cm⁻¹. The amide-B bands were observed at wavenumber 3074.88 cm⁻¹. The asymmetric and symmetric CH₂ vibrations at 2937.05 cm⁻¹ and 2877.50 cm⁻¹, respectively are noticeable.

3. 5. 3. The Raman spectra

The Raman spectra of pure gelatin and gelatin/melanin films are shown in Figure 3. The addition of melanin caused noticeable signal increasing at wavenumbers ranges $2400-2200$ cm⁻¹, $2200-1800$ cm⁻¹, $178-1380$ cm⁻¹, $1380-900$ cm⁻¹, $870-250$ cm⁻¹

3. 6. The Visual Appearance and Color

The visual appearance of pure gelatin and gelatin/melanin modified films is shown in Figure 4. As can be seen in Figure 4 the polymer matrix was homogenous. The color, chroma, hue angle, ΔE , YI and WI values are presented in Table 2. The growing addition of melanin influenced the color values in comparison to pure gelatin film, causing a reduction in the lightness (L) and an increase in the redness (a), yellowness (b), chroma (C^*ab) and hue angle (h^*ab) values. Color differences were statistically significant ($p < 0.05$). ΔE values ranged

from 4.67 (sample “1”) to 32.39 (sample “3”). The yellowness (YI) increased with increasing melanin amount, in contrast, the whitening index (WI) decreased when the melanin content was increasing.

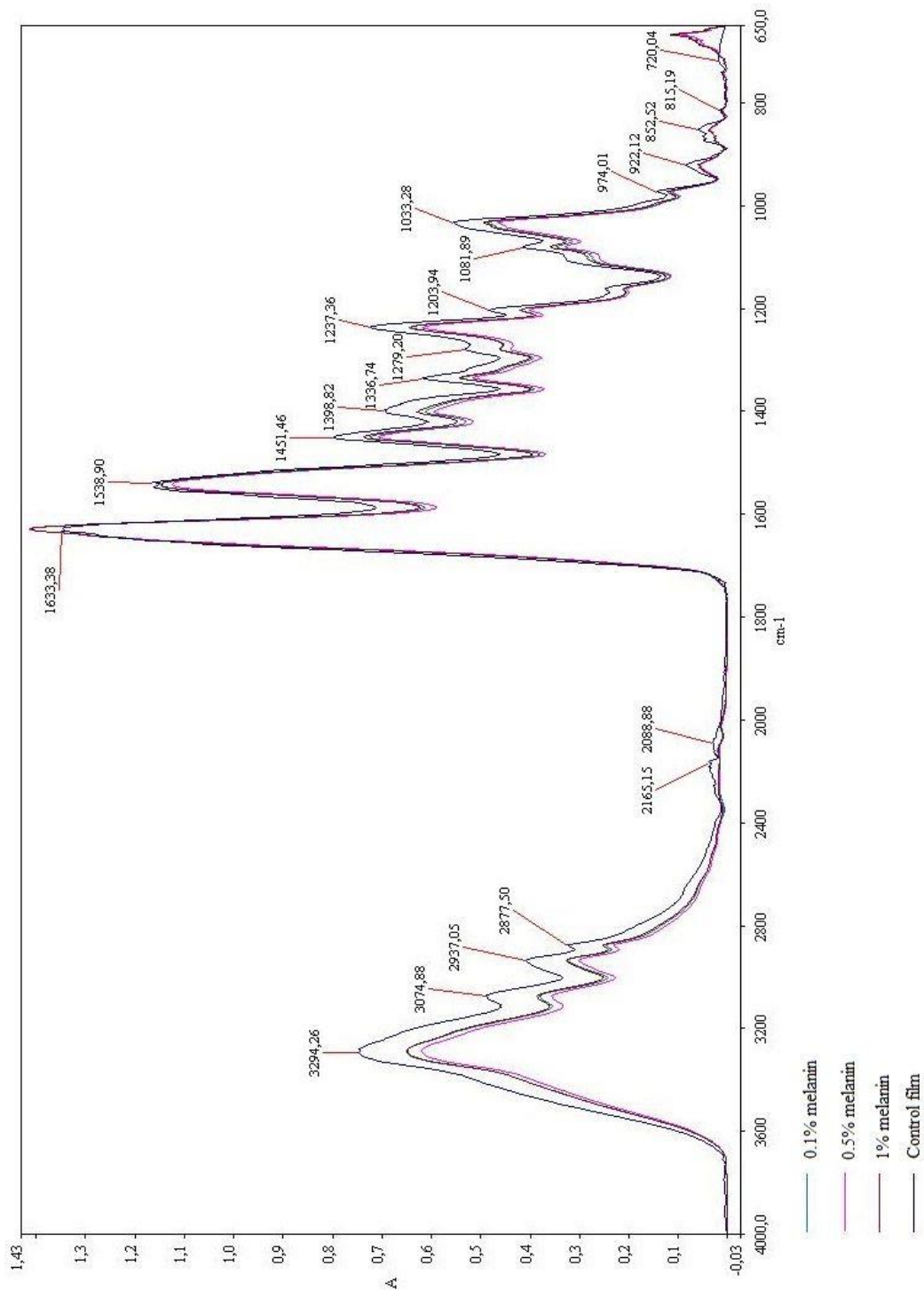


Figure 2. The FT-IR spectra of pure gelatin and gelatin/melanin films

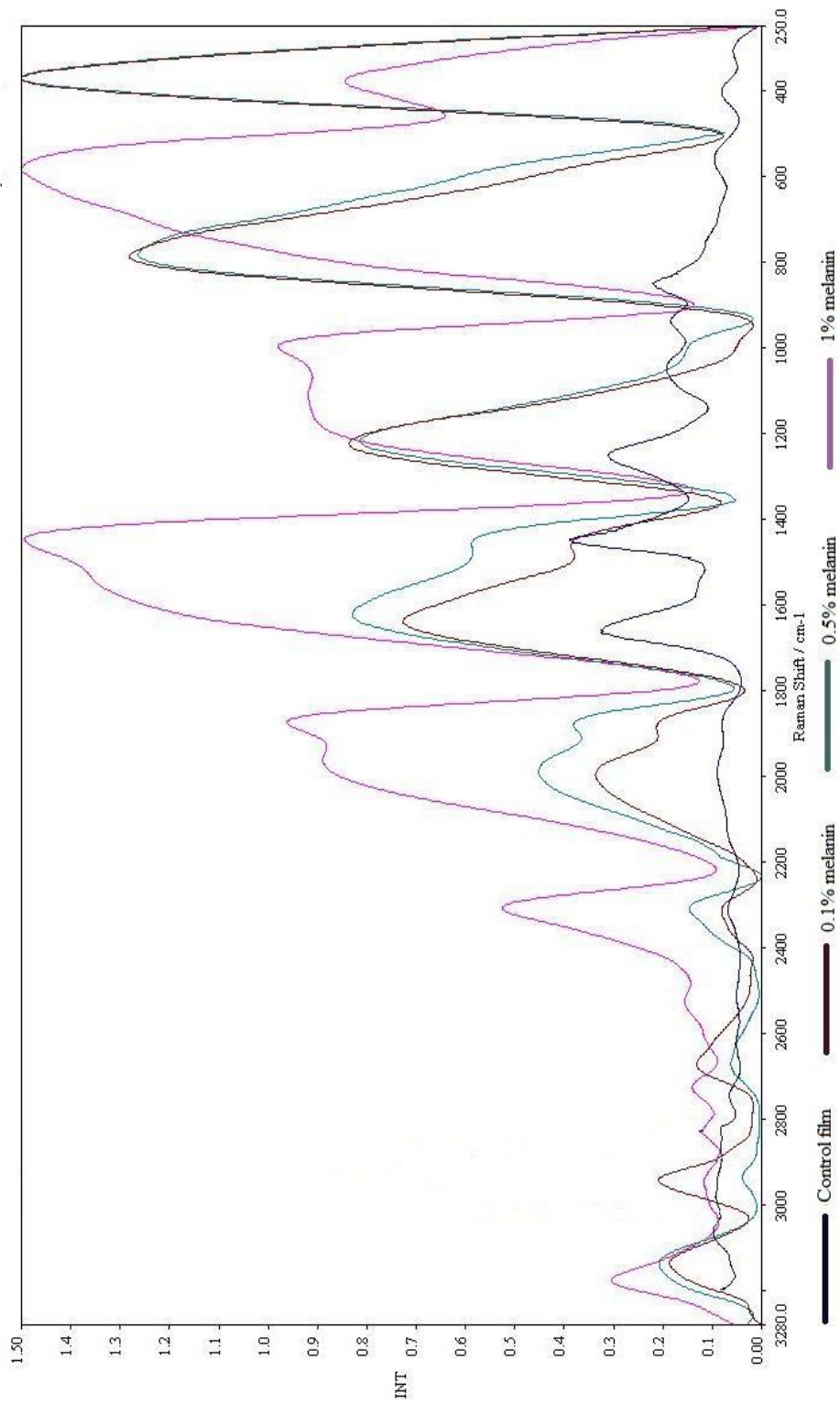


Figure 3. The Raman spectra of pure gelatin and gelatin/melanin films

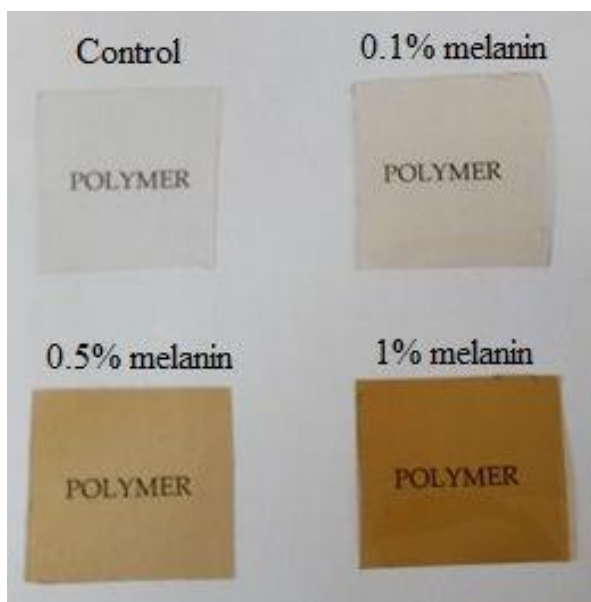


Figure 4. The visual appearance of pure gelatin and gelatin/melanin films

Table 2. Color parameters (L^* , a^* , b^*), chroma (C^*ab), hue angle (h^*ab), ΔE , yellowness index (YI), whitening index (WI) and opacity of pure gelatin and gelatin/melanin modified films.

Sample	L	a	b	C^*ab	h^*ab	ΔE	YI	WI	Opacity
0	96.47 ± 0.01	-0.12 ± 0.00	0.88 ± 0.0	0.89	-1.44	used as standard	1.30	96.36	9.08 ± 0.02
1	94.48 ± 0.00	-0.09 ± 0.00	5.11 ± 0.00	5.11	-1.55	4.67	7.72	92.47	8.40 ± 0.08
2	87.88 ± 0.01	1.03 ± 0.01	17.52 ± 0.00	17.55	1.51	18.76	28.48	78.67	7.23 ± 0.05
3	81.58 ± 0.00	3.23 ± 0.00	29.46 ± 0.01	29.63	1.46	32.39	51.58	65.11	6.71 ± 0.06

3. 7. Opacity

The opacity of pure gelatin and gelatin/melanin modified films is shown in Table 2. The opacity values of modified gelatin/melanin films were lower than the pure gelatin film. The opacity of gelatin/melanin films decreased after the addition of melanin (from 9.08 ± 0.02 of sample “0” to 6.71 ± 0.06 of sample “3”). This may have been due to the color and the content of the melanin. Those differences were statistically significant ($p < 0.05$).

3. 8. Antioxidant Activity

Table 3 presents results of an assessment of the available phenolic groups on the films surface and antioxidant activity of pure gelatin and gelatin/melanin modified films. The total available phenolics were determined to be 0.0134; 0.0199 and 0.0245 $\mu\text{mole GAE/g}$ film for samples “1”, “2” and “3”, respectively. Pure gelatin film (sample “0”) also reacted with Folin-Ciocalteu reagent due to high proline content and showed antioxidant activity (the result was considered as no polyphenolics). The antioxidant activity of modified gelatin/melanin films grew with the increasing content of melanin, reaching $34.95 \pm 0.21\%$. Differences between the modified films and pure gelatin film were statistically significant ($p < 0.05$).

Table 3. The antioxidant activity (AA%) and available phenolic groups (APG) of pure gelatin and gelatin/melanin modified films.

Sample	AA% (%)	APG ($\mu\text{mole GAE/g}$)
0	0.14 ± 0.02	-
1	11.44 ± 0.23	0.0134 ± 0.011
2	17.78 ± 0.18	0.0199 ± 0.021
3	34.95 ± 0.21	0.0245 ± 0.013

3. 9. Antimicrobial activity

Table 4. Antimicrobial activity of the pure gelatin and gelatin/melanin films

Strain	Film sample	Inhibition zone	Number of cells after incubation with films [CFU/mL]
<i>E. faecalis</i>	0	-	3.24×10^7
	1	-	1.98×10^8
	2	-	2.02×10^7
	3	-	2.17×10^7
<i>P. aeruginosa</i>	0	-	3.22×10^7
	1	-	2.56×10^7
	2	-	1.56×10^7
	3	-	3.89×10^7
<i>P. putida</i>	0	-	2.26×10^7
	1	-	1.12×10^8
	2	-	3.34×10^7
	3	-	2.74×10^7

The susceptibility assay of *E. faecalis*, *P. aeruginosa* and *P. putida* with respect to the pure gelatin and modified gelatin/melanin films is shown in Table 4. The results of this research determined that neither pure gelatin nor modified films were found to be active against all strains (no growth inhibition zones). The results of this research demonstrated that pure gelatin and modified films had influence on the growth of *E. faecalis*, *P. aeruginosa* and *P. putida*, a 1-2 log increase of the number of cells was noted when bacterial cells were incubated with the films.

4. DISCUSSION & CONCLUSIONS

The results of this study indicate that melanin isolated from ABW used as an additive for gelatin at various concentrations may influence the properties of modified films, depending on concentration. The increased availability of biopolymers has stimulated increased research and development of activities, which can also be partly attributed to the escalating “green” movement that is encouraging the use of biopolymers. Since the packaging industry plays a dominant role in the short-term use of cheap non-biodegradable petroleum-based materials, their replacement with biopolymers could provide a significant step to eco-friendly solutions [1]. Nowadays, agricultural by-products are usually incinerated or dumped, causing environmental problems such as pollution, soil erosion and decreasing biological activity in soil. The incorporation of agricultural residues into polymer matrices is currently a trending topic in research [4]. Thus, the application of melanin from ABW to modify gelatin matches the current trend for bio-based composite films. Gelatin is unique among hydrocolloids in forming thermo-reversible systems with a melting point close to the body temperature, which is particularly significant in packaging and pharmaceutical applications [67]. Hence, the incorporation of melanin has opened up new avenues to discover its applicability in the packaging industry, such as packaging material for avoiding oxidation of sensitive food, thus expanding the spectrum of its uses.

In order to adequately preserve the quality of food goods, the packaging materials have to provide efficient barriers against light, water vapor, atmospheric gases and volatile organic compounds, preventing food spoilage. When the modified blend film is applied to preserve food, its integrity has to be maintained and all external stress withstood, so these mechanical properties are vitally important characteristics for the film. Gelatin forms a three-dimensional network with zones of intermolecular microcrystalline junctions and the dehydration of this system may produce brittle films. Gelatin films are fragile and susceptible to cracking due to the high cohesive energy density of proteins [50]. So, the addition of a plasticizer is necessary to overcome the brittleness of the films, to reduce inter-chain interactions during the dehydration which can improve flow and flexibility, and to increase toughness and impact resistance of the film coating, to prevent them from cracking during packing and transportation [45,67,68]. The composition, size and form of the plasticizer molecule has an influence on its ability to interact with protein chains and bind with the molecules of water, causing more plasticization owing to the fact that water is an effective film plasticizer based on hydrophilic biopolymers [68]. In this study glycerol was used as a plasticizer, it has hygroscopic character and therefore attracts more water into the structure of the films, thus promoting greater flexibility.

When the modified blend film is applied to preserve food, its integrity has to be maintained and external stress withstood, so these mechanical properties are vitally important characteristics of the film [1]. The mechanical properties of protein films could provide an indication of expected film integrity under conditions of stress that would occur during processing, handling, and storage [3]. Gelatin can be blended with different compounds and/or polymers to obtain bio-composite films and coatings that combine the advantages of each component [4,71-74]. Tensile strength (TS) plays an important role in determining the mechanical properties of edible or packaging films developed for use in many food applications. TS are an indication of film strength, whereas elongation at break is an indicator of the stretchability of films prior to breakage [74]. According to Liang *et al.* stable aromatic ring, may hinder the rotation of intramolecular hydrogen bonds in the films [37]. Melanin has ring structures within its molecules that hinder conformational variations. The observed mechanical properties indicate that the incorporation of melanin is likely to result in the development of a heterogenous film structure, nevertheless the results suggest that melanin did not significantly influence ($p>0.05$) the mechanical properties of the films. Some compounds may enhance the TS of gelatin, which was noted by Liang *et al.*, who used esculin to modify gelatin [37]. These results may be attributed to the supramolecular interactions between gelatin and additives, including bonding between their chemical moieties with gelatin amino acids, as well as hydrophobic or π - π interactions. Ahmad *et al.* noted that moderate amounts of essential oils enhanced the mechanical properties of modified gelatin films, but when the concentration of essential oils was high, the mechanical properties worsened [6]. Tongnuanchan *et al.* observed that the addition of bergamot essential oil decreased the tensile strength of modified films [2], similarly Rawdkuen *et al.*, noted the negative influence of catechin on gelatin film tensile strength [75].

The solubility of edible film in water is an essential asset, and water resistance is typically required for possible commercial applications of these films [9,15]. When a film is placed over the food surface, its solubility largely determines the release of active compounds [75]. The solubility of the melanin-added films decreased as the concentration of melanin increased from 0.1% to 1% ($p<0.05$). In general, high water solubility may indicate lower water resistance, and the lower water solubility of melanin modified films might result from the stronger structure of the film network via strong interactions between the protein and hydroxyl groups of melanin. The incorporation of melanin might be associated with its hydrophobic moieties. Non-polar moieties of melanin interacted favourably with the hydrophobic domains of gelatin, leading to an increase in the hydrophobicity of the resulting film. As a result, the solubility of films was lowered. Similar mechanism has been proposed by Ahmad *et al.* [6]. Another mechanism has been proposed by Nafchi *et al.* [76] who reported that increasing the nanoparticles (ZnO) content of films results in the formation of more hydrogen bonds in the ZnO and the matrix components. Thus, free water molecules do not interact as strongly with nanocomposite films than compared with composite films alone. Our results are in line with the results of Liang *et al.* [37], who observed that the solubility of esculine-incorporated gelatin films was lower than that of the film comprising of gelatin alone. Other authors also noted the influence of some additives on gelatin film solubility, such as tannic acid [42], tannin [77], catechin [75], ribose [74], formaldehyde and glyoxal (as cross-linking agents) [78].

Food products are very susceptible to rancidity caused by the oxidation of lipids that contain unsaturated fatty acids that can be attacked by oxygen free radicals. Antioxidants are

added to foods to intercept and react with these free radicals at a faster rate than the lipid substrate. Nevertheless, the current incorporation of antioxidants throughout the entire food matrix in one large initial dose is not an efficient process due to the oxidation occurring at the surface and high initial doses of antioxidant having a pro-oxidant effect. Therefore, one emerging technology is the use of antioxidant active packaging, where the antioxidant is incorporated to a packaging material with the purpose of being delivered to the food surface during commercialization, at an appropriate rate. Most of the active packaging developments have been based on the mass transportation properties of plastic materials (sorption, migration, and permeation), and the release of the active agents depends on several factors, such as the type of polymer and type of food. However, the presence of synthetic antioxidants in food is questionable, owing to the potential risks. This has been encouraged by strong consumer demand, as synthetic compounds are frequently perceived as undesirable or harmful. Natural antioxidants are preferred to artificial substances, especially by consumers. Moreover, the use of active antioxidant packaging that incorporates natural antioxidants presents important advantages. The addition of a natural compound to the packaging may reduce the need of using synthetic antioxidants in the plastic, reducing the risk of potential toxicity by migration [1]. There are many reports of gelatin modifications by antioxidants known from the literature. A wide spectrum of additives were used, including curcumin and its derivatives [12], essential oils [2,5,8,28-36], esculine [37], butylated hydroxytoluene and α -tocopherol [38], lignin [9,39], liquid smoke [40], tannin [11,42], carvacrol [41], vanillin [43], riboflavin [44], gallic acid [45], aloe vera gel [46,47], tea polyphenols and green tea extracts loaded into chitosan nanoparticles [15,47-50], grapefruit seed extract [51], tomato pulp [52], tomato pomace oil extract [79] and other plant extracts [80]. The control films, devoid of melanin showed radical-scavenging activity to some extent, which may be ascribed to the gelatin, particularly to the peptide fraction with its content of particular amino acids such as glycine and proline [48]. The addition of melanin into gelatin films caused a significant increase in their antioxidant activity ($p < 0.05$). In our study, modified gelatin films have shown good radical scavenging activity that increased with increasing melanin content. This observation is comparable with the results of other authors who also observed that radical scavenging activity is dependent on antioxidant activity concentration [1,11,36,46].

Sensitive components of food such as lipids, flavours, vitamins and pigments may undergo photodegradation reactions. The spectrum and the intensity of the light source, the conditions of light exposure and the degree of packaging material light transmittance are factors that can significantly affect food quality. Thus, packaging plays a pivotal role in the prevention of the photodegradation of food components during storage [1,8]. The design of the packaging for a specific food product involves not only the choice of appropriate packaging material, but also the addition of the right additives or stabilizers to the packaging in order to provide a more efficient UV-Vis light barrier, and thus a significant improvement in the protection of food quality after storage. The absorption and transmission of light by polymers is particularly important in the food packaging industry where the packaged goods are light sensitive. Transparency of a film to some extent is determined by the miscibility of the various components in the film forming solution and hence transparency values can provide information about the regularity of the microstructure of the blends [52]. Thus, films with high transparency values are less prone to damage by UV light owing to limited light penetration into the films [8]. Another issue in fresh food packaging is the effect of irradiation in the package, since ultraviolet light irradiation is a common method used for lowering

microbial population in foods. A food packaging film is required to protect food from the effects of light, especially UV radiation. Therefore the gelatin film enriched with melanin may improve light barrier against UV light, thereby protecting and prolonging the shelf life of the food. The transmission of UV and visible light at a wavelength range of 200-1100 nm of the films were studied. Decreases in light transmission of films modified with melanin were observed at all wavelengths, compared with control film. The result indicated that melanin was able to impede the light transmission through the film. Fig. 1 shows the spectroscopic scans of the films at wavelengths between 200 and 1100 nm. All gelatin films with the melanin addition films showed a pronounced increase in the absorbance level within the UV region than compared to the gelatin films. Although the addition of melanin gelatin led to the films losing their colourless appearance, they still remained transparent. Núñez-Flores *et al.* [39], observed that gelatin films blended with lignin were not transparent. It is well known that the film light transmission depends on many factors such as thickness, the presence of a dispersed phase within the matrix with a particle size bigger than the wavelength of the visible light, as well as the presence of interactions between film components [11]. There are many reports of the addition of some chemical compounds resulted in the improvement of UV-Vis barrier properties in modified films, such as: coconut husk extract [73], catechin [75], lignin [9,39], ferulic, gallic and tannic acids [3,45], tea polyphenol-loaded chitosan nanoparticles [48], vegetable carbon black [23], metal nanoparticles [76] or essential oils [2,8]. There are several proposed mechanisms of influencing the light barrier properties of films, some compounds are able to absorb or reflect the light [73], and some cause light scattering within the polymer matrix, such as essential oil droplets [2,8]. Also, Schiff's base reaction between amine groups and the carbonyl groups of gelatin and additives may occur which can lead to increased barrier property against UV light as well [26]. The chromophoric nature of melanin is known to be well capable of protecting against UV radiation [57-61]. In addition, melanin has been also reported to act as a UV absorber in PLA/melanin composite films or as an additive to coatings for packaging materials [1,63,64].

Films with various amounts of melanin are of a similar pattern, which indicates that there were no major changes in the functional groups of the gelatin films as demonstrated in Figure 2. Addition of melanin triggered noticeable changes in intensity of Amide I band at 1633.38 cm^{-1} , Amide II band at 1538.39 cm^{-1} , and Amide III band at 1237.36 cm^{-1} than compared with pure gelatin film. The amide-I vibration mode is primarily a C=O stretching vibration coupled with the CN stretch, CCN deformation and in plane NH bending modes. The spectral differences between different film samples in amide-I region were largely attributed to the different conformation and orientation of polypeptide chains, affected by the incorporation of melanin. The amide-II vibration modes are attributed to an out-of-plane combination of the NH in plane bend and the CN stretching vibration with smaller contributions from the CO in plane bend and the CC and NC stretching vibrations. The amide-III represents the combination peaks between C-N stretching vibrations and NH deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains of gelatin molecules. In addition, absorption bands at the wavenumbers of 1033.28 cm^{-1} were found. Those bands most likely arose from asymmetric stretching vibrations of -OH groups of glycerol (plasticizer) coupled to the $-\text{CH}_2$ of the amino acid residues of the gelatin molecules. Moreover, amide-A band, arising from the stretching vibration of NH group appeared at wave numbers of 3294.26 cm^{-1} . According to Ahmad *et al.* [6] when the NH group of a peptide is

involved in a hydrogen bond, the position shifted to lower frequencies. Signal intensity lowering in the amide-A region of the modified films in comparison to the control film suggest that gelatin peptide NH groups and melanin functional groups are involved in hydrogen bonds. The amide-B bands were observed at wavenumber 3074.88 cm^{-1} corresponding to an asymmetric stretch vibration of $=\text{C-H}$, as well as $-\text{NH}_3^+$ of peptide fragments of gelatin molecules. Modified gelatin films showed a lower wavenumber at amide-B region, compared to the control film, suggesting an interaction of $-\text{NH}_3^+$ group between peptide chains. In addition, the hydrocarbon chains of melanin give asymmetric and symmetric CH_2 vibrations at 2937.05 cm^{-1} and 2877.50 cm^{-1} , respectively. The most pronounced changes in the films were in the range of $1633\text{--}650\text{ cm}^{-1}$ indicating intrusion caused by melanin in the hydrogen bonding between water and imide residues. Initially, the hydrophobic groups of polyphenol interact with the hydrophobic region of the protein via hydrophobic interaction followed by hydrogen bonding between the phenolic hydroxyl groups of polyphenols and the polar group of the protein. Based upon the above mechanism and FT-IR data, it is tempting to suggest that the hydroxyl and carboxyl group of melanin interact with the amino acids of the gelatin via hydrogen bonding and hydrophobic interaction. Therefore, the incorporation of melanin altered the molecular organisation and intermolecular interaction in the film matrix.

Results of the Raman spectroscopy analysis showed noticeable differences in the obtained spectra. With higher melanin content peaks were observed with greater insensitivity. Similar observations have been made in previous study [1]. The peaks can be interrelated as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C–C bonds within the rings, along with some contributions from the C–H vibrations in the methyl and methylene groups in the melanin molecules [81]. A peak at 2000 cm^{-1} is similar to those obtained by Galvan *et al.* from eumelanin and may be caused by the stretching of three of the six C–C bonds within the melanin aromatic rings [82]. It was noted, that on all modified films, Raman spectra peaks at 395 cm^{-1} are present, which are thought to correspond to peaks obtained from pheomelanin and eumelanin and are caused by an out-of-plane deformation of the phenyl rings. Peaks at 2010 cm^{-1} are also similar to peaks seen in pheomelanin and are probably due to overtone or combination bands [81,82].

The optical properties of films are an important attribute which influences its appearance, marketability, and suitability for various applications. Clear edible films are typically desirable with higher applicability and acceptability in food packaging systems [6,73]. Generally, a clear film is preferable as the appearance of the contents is displayed clearly [46]. Film colour can be affected by the type, nature and concentration of the incorporated additive [46,52]. It is commonly found that the addition of natural extracts alters the original colour of protein-based films to a certain extent and the magnitude of such is determined by the type and concentration of polyphenols which are believed to confer yellow-brown coloration [2,11]. The results are in agreement with Cao *et al.* who reported that gelatin film incorporated with phenolic compounds (tannic acid and ferulic acid) at alkaline pH showed changes in colour [3]. There are several reports that some additives may cause changes in gelatin films, to yellow-brown coloration or influence their lightness such as vanillin [43], catechin [75], lignin [39], seaweed extract [83], gallic acid and silver nanoparticles [84], curcumin [12], some essential oils [2], tannin [11], riboflavin [44], metal nanoparticles [85,86], ferulic and caffeic acids [87], amino acids (histidine and lysine) [50], coconut husk extract [73]. Melanins are known for their dark-brown colouration [57-59], and

there some reports, that their addition into a polymer matrix may influence the colour values [1]. In general, melanins are dark because they do not re-radiate the absorbed visible or invisible light, but transform the energy into rotational and vibrational activity within the molecule and then dissipate it as heat. This phenomenon protects melanised tissues against light-induced damage. In general interaction between natural phenolic compounds and proteins in the presence of O₂ and alkaline conditions leads to the oxidation of the phenolic structure and the formation of a quinon compound. In fact quinon is a dimmer compound, which reacts with amino or polypeptide sulfhydryl chain to a form covalent bond of C-N or C-S. Polyphenol compounds are able to create cross-link bounds between individual protein molecules. Zhang *et al.* [88] found a colour change in bovine gelatin-based film containing caffeic acid from pale yellow to dark brown. These results were found to be in accord with our findings. In addition, all the films were dried at room temperature (25°C), thus eliminating the occurrence of the Maillard reaction which may cause the browning of gelatin [46]. Our results indicate that the increasing addition of melanin influences the colour values than where compared to pure gelatin film, leading to a reduction in lightness (*L*), as well as an increase in the redness (*a*), yellowness (*b*), chroma (*C*ab*) and hue angle (*h*ab*) values. ΔE values ranged from 4.67 to 32.39. $\Delta E > 1$ is considered perceptible to the human eye, so all melanin concentrations caused noticeable colour changes. The yellowness index (YI) increased with increasing melanin amount, while the whitening index (WI) decreased when the melanin content was increased. The yellowness index or a change in the degree of yellowness is a number calculated from spectrophotometric data that describes the change in colour of a test sample from clear or white to yellow. The opacity of gelatin films decreased with the addition of melanin (from 9.08 ± 0.02 of sample "0" to 6.71 ± 0.06 of sample "3"). This was probably due to the colour of the melanin powder. The changes of film colour and opacity as a consequence of the addition of melanin had been reported with PLA films [1]. The difference in opacity among the film samples was perceptible to the human eye and was statistically significant ($p < 0.05$). Gelatin/melanin films in all melanin concentrations still had good transparency, even at high melanin content. This result suggested high gelatin/melanin films transparency, meaning that the packaging film could be transparent, which an important requirement for consumers and would have a clear influence on customer choice.

The water contact angle of the material is associated with its hydrophilicity. In general, the smaller the water contact angle, the higher the hydrophilicity. Gelatin is a kind of hydrophilic material owing to the functional groups in the molecule, such as amino, carboxyl, and hydroxyl. The hydrophilic property has restricted its application in many aspects. Based on this fact, it is necessary to carry out a hydrophobic modification of gelatin [4,89] In our work the contact angle of pure gelatin film was approximately 53.3°. The contact angle of non-modified gelatin films observed by other authors was 52.4° [86], 76.2° [90], 77.8° [89], 89.5° [91], 97.3° [40]. This discrepancy may be a result of the gelatin type and glycerol content. The addition of melanin into gelatin significantly ($p < 0.05$) affected the surface properties of the polymer, increased the contact angle from 53.3° to 72.9°. Shankar *et al.* [86] modified gelatin films with ZnO nanoparticles which increased the water contact angle from 52.4° to 63°. This results are comparable to results of Nafchi *et al.*, who also observed increased hydrophobicity of ZnO amended gelatin films [76]. Yue *et al.* [90] noted that addition of polydimethylsiloxane or glycidol increased the gelatin films contact angle to 112.8°, Wang *et al.* using liquid smoke increased the contact angle of modified films to 111.2° [40], while Wang *et al.* [91] using cellulose nanofibres and palmitic acid achieved

123.7°. The results of this study are quite opposite to the results of our previous study, where melanin particles incorporation into poly(lactic acid) films did not significantly ($p > 0.05$) affected their contact angle [1].

One of the most important properties of bio-based films for the application of packaging is to minimize the moisture transfer from the environment to the packed goods. Water vapor permeability (WVP) is one of the most important properties in food packaging due to the noticeable role water has in deteriorative reactions and microbial growth. For this purpose, the WVP of packaging materials should be as low as possible [1]. However, gelatin films have poor water barrier vapor property, thereby limiting their use as potential packaging. This is due to its hydrophilicity in nature. To tackle this problem, the incorporation of hydrophobic substances such as lipids, fatty acids, waxes and essential oils has been implemented to improve water barrier property [2,5,35]. A possible means to minimize the problem of the moisture content in gelatin films is also their association with some synthetic polymers through blending, such as poly(vinyl alcohol) (PVA) [72]. Polymer blending is a technique widely applied in polymer science to obtain materials with improving properties. On the other hand, some additives may exacerbate the water vapor barrier properties such as ZnO nanoparticles, which is probably due to the discontinuous phase formed between nanoparticles and the polymer matrix, making the nanocomposite film more porous, resulting in an increase in the WVP of the composite films [86]. The pivotal role in WVP of films plays the presence of plasticizer in polymer matrix. Generally, plasticizer e.g., glycerol located between adjacent chains of gelatin molecules decrease the intermolecular forces, thus increasing the free volume of the system and favouring the mobility of polypeptide chains in the film matrix. The increased mobility results in greater free volume and segmental motions, which facilitates the migration of water vapor molecules through the film. The water vapor transfer process in the films also depends on the hydrophilic/hydrophobic ratio of the film constituents [6].

Oxygen is an essential factor for the oxidation of food. The lower oxygen transmission rate of film could better prevent the oxidation of food, gelatin films are known for their oxygen barrier properties, due to their amino acid composition [23,87]. Polyphenolic components can interact with proteins (especially gelatin protein, rich in proline), resulting in the formation of protein–polyphenol complexes and forming hydrogen and covalent bonds with the polar groups of polypeptide gelatin chain [4]. It is speculated that these protein–polyphenol complexes could be responsible for OTR changes, while Ding *et al.* [23] and also Nassiri and Nafchi [25] suggested that more compact structures than the pure gelatin films, are difficult for oxygen to permeate.

Modified gelatin films did not show antimicrobial activity against *E. faecalis*, *P. aeruginosa* and *P. putida*. This data are opposite to the results obtained in a previous study on PLA/melanin modified films which were active against the above-mentioned bacteria species [1]. It is tempting to suggest that another film preparation mechanism (melanin was incorporated into the PLA matrix as particles, whereas in this study melanin was dissolved in alkaline conditions and reacted with melanin) could influence the antimicrobial activity of melanin and resulting modified films. The increased number of bacterial cells incubated with non-modified and modified films in comparison to control samples devoid of any films may result from the utilisation of gelatin as a nutrient source by bacteria. The solution could be the addition of some other antimicrobial compounds. These additives can be obtained from different sources, including plants, animals, bacteria, algae, fungi and by-products generated

during fruit and vegetable processing [4]. Some authors observed the antimicrobial activity of gelatin films modified with essential oils [2,5,8,28-36], chitin nanoparticles [26], lysozyme [92], tomato pulp [52], vanillin [43], tannin [11] and metal nanoparticles, which are known from their excellent antimicrobial activity [4,25,26,76,85,86,93,94].

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