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Effect of Melanin on the Stability of Casein Films Exposed to Artificially Accelerated UV Aging

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Abstract: Petroleum-based polymer food packaging is causing increasing concern. Their biopolymer alternatives should have some added value to compete with them and push them out of the market. This article presents new information related to the effects of melanin on casein films and their protection against artificial UV aging. Casein films were modified with melanin as an active additive and then subjected to artificial aging using UV radiation to evaluate its effect on the preservation of the films' properties. The films were tested for hydrological (moisture content and water solubility), mechanical, barrier against UV-Vis radiation, colorimetric, and antioxidant properties, and the content of free amino acids and sulfhydryl and disulfide groups were checked before and after aging. Melanin influenced the preservation of mechanical properties of the films (elongation at break increased by no more than 20% for melanin-modified samples compared to more than 50% increase for the control sample), better UV barrier properties, increased antioxidant properties (two-fold higher scavenging of DPPH radicals by films modified with the highest melanin content compared to unmodified films before aging, and four times higher scavenging of DPPH radicals after aging). In addition, the presence of melanin had protective properties for sulfhydryl bonds and proteins (the increase in free amino acids after aging for melanin-modified films was not statistically significant), and it also had the effect of increasing the abundance of bands corresponding to oligomers and polymers in electrophoretic separation. The results indicate that melanin has UV-protecting properties on casein films, and it can be assumed that the obtained casein films modified with melanin could potentially find application as food packaging or edible coatings.

Keywords: casein; melanin; food packaging; artificial aging

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1. Introduction

Technological and economic developments, symptomatic of which include the rise of e-commerce and the notorious emergence of new suppliers of goods, including food, directly to the consumer's door, have led to an increase in the use of materials for packaging purposes over the past decade [1]. Food packaging is often designed to be single use only and is often not economically viable to recycle [1,2]. Biodegradable films and edible biopolymer coatings, which are both considered safe for humans and the environment and provide antimicrobial and antioxidant protection for packaged foods, can be an alternative to the synthetic films made from petroleum-based compounds that dominate the packaging industry [3]. Among biopolymer-based films, there are three main groups: lipid-based, protein-based and polysaccharide-based. Protein-based matrix films stand out in terms of mechanical properties and gas barrier properties [4,5] and, in addition, the materials for

their preparation are readily available and the film-forming process is carried out under safe and easily controlled conditions [6]. Protein-based films can also be easily modified with the addition of various active compounds to affect their functional properties [7].

Casein, the largest and constituting the greatest percentage of proteins present in milk, due to its predominant random coil structure, with high molecular flexibility and extensive polar groups, has excellent film-forming properties [8,9]. Due to the presence of hydrophobic and intermolecular hydrogen bonds as well as extensive electrostatic interactions, it is easily possible to form casein-based films [9,10]. Such films are characterized by good mechanical strength and excellent barrier properties to oxygen and lipids; nevertheless, they are susceptible to the absorption and release of water molecules, which deteriorates their functional properties and hinders wide industrial application [11,12]. An additional advantage is the prevention of photo-oxidation of products packaged in casein films due to its potential to absorb UV radiation [13]. The potential functional properties of films and coatings based on neat and modified casein have been demonstrated in the literature [14–18].

Melanins are a high-molecular-weight group of pigments whose different varieties can be found in representatives of all biological species [19,20]. Melanins differ in their source, chemical structure and color (from yellow to red and brown to black) where all three factors are related [21]. Melanins, due to their properties, have found use as additives to polymer-based films. Among other things, they influence the improvement of mechanical properties (due to the strong hydrogen interactions of melanin with the polymer matrix), antioxidant, antimicrobial and UV-protective properties [22]. They have been used to improve the performance of films based on such polymer matrices as agar [23], chitosan [24], polyhydroxy butyrate (PHB) [25], carboxymethyl cellulose (CMC) [26,27], alginate [28], gelatin [29], whey protein concentrate/isolate [30], and carrageenan [31], among others.

The purpose of this study was to provide new information on the effect of melanin addition in different amounts on the performance of casein-based films and the protection of films from artificial UV aging. The films were also subjected to UV light treatment in order to artificially age them and to study the protective effect of melanin on the tested films. For this purpose, their mechanical, barrier, and antioxidant properties were tested. Changes in chemical bonds using FT-IR spectroscopy, the presence of free amino acids and proteins composition were also checked.

2. Materials and Methods

2.1. Materials and Reagents

Methanol, glycerol and ammonia water were delivered by Chempur (Chempur, Piekary Śląskie, Poland). Calcium chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid (TCA), cadmium chloride, 2,2-dihydroxyindane-1,3-dione (ninhydrin), glycine, urea, and 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) were purchased from Merck (Darmstadt, Germany). Casein was purchased from VWR Chemicals (Ballycoolin, Dublin, Ireland). B-mercaptoethanol and tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Aldrich (Darmstadt, Germany). All reagents were of analytical grade.

2.2. Preparation of Casein Films

The melanin used for the modification was obtained from watermelon seeds according to the methodology described elsewhere [32]. Melanin solutions were obtained by adding melanin to 400 mL of distilled water in amounts of 0.008 g, 0.02 g and 0.04 g, respectively. In order to create an alkaline environment for melanin dissolution, 2 mL of ammonia water was added to each mixture. The mixtures in sealed bottles were placed on a shaker at 80 °C for overnight mixing. The solutions, cooled to room temperature, were passed through a paper filter under pressure to separate undissolved residues. Then, 8 g of casein was added to each solution and placed on a magnetic stirrer (300 rpm) overnight for complete

dissolution. In this way, solutions containing successively 0.10%, 0.25% and 0.50% (*w*/*w*) melanin per weight of polymer were obtained. To each solution, glycerol, playing the role of plasticizer, was added at 30% (*w*/*w*) per weight of polymer. After thorough mixing on a magnetic stirrer (300 rpm) for 10 min, the film-forming solutions were poured onto square polystyrene plates (120 mm × 120 mm) at 40 mL per plate and then were dried at 40 °C for 48 h. A control sample containing no melanin was prepared in an analogous manner. All films were prepared in eight replicates.

This procedure resulted in samples sequentially named Cas—a control sample of unmodified casein; 0.10% MEL—a sample containing 0.10% melanin; 0.25% MEL—containing 0.25% melanin; and 0.50% MEL—containing 0.50% melanin by weight of casein used (*w*/*w*).

2.3. Artificial Aging of Films

For artificial aging of the films, rectangular samples (23.5 cm × 7.0 cm) were cut. They were then placed in a Q-SUN accelerated Xenon Test Chamber with 1.5 W/m^2 (Model Xe-2, Q-LAB, Homestead, FL, USA) and exposed to the light treatment for 24 h.

2.4. Biocomposite Film Characterization

2.4.1. Structure and Morphology Analysis of Films

The structure and morphology of obtained materials were analyzed via scanning electron microscopy (SEM) (VEGA3 Tescan, Brno, Czech Republic). An analysis was performed at room temperature with tungsten filament, and an accelerating voltage of 10 kV was used to capture SEM images. All specimens were viewed from above.

2.4.2. Determination of Moisture Content and Water Solubility

Moisture content (MC) was determined by measuring differences in the weight of the films before and after drying at 105 °C for 24 h.

Water solubility was measured by placing pre-weighed film samples in 30 mL of water and measuring the weight of undissolved films after overnight incubation.

2.4.3. Thickness, Mechanical, and Thermal properties of Casein Films

Film thickness was measured using an electronic thickness gauge (Dial Thickness Gauge 7301, Mitoyuto Corporation, Kangagawa, Japan) with an accuracy of 0.001 mm. The thickness of each film was measured at 10 random places, and the results were brought to an average.

The tensile strength and elongation at break of the films were assessed using a Zwick/Roell 2.5 Z static testing machine (Ulm, Germany). The tensile clamp spacing was 25 mm and the head travel speed was 100 mm/min.

Differential scanning calorimetry (DSC) was used to assess thermal properties (DSC 3, Mettler-Toledo LLC, Columbus, OH, USA) using sequential heat–cool–heat cycles over a temperature range of 30–300 °C at ϕ = 10°/min under nitrogen flow (50 mL/min).

2.4.4. Spectral Analysis of Films

The UV-Vis spectra of films were measured using a Thermo Scientific (Waltham, MA, USA) Evolution 220 UV-vis spectrophotometer. Test film samples of equal thickness and dimensions (5.5 cm × 1 cm) corresponding to the dimensions of the quartz cuvette were cut. The films placed on the wall of the quartz cuvette were inserted into the instrument, and the UV-Vis spectrum was measured with a wavelength range of 300–800 nm and a resolution of 1 nm.

The chemical composition of the obtained films was also evaluated using a Perkin Elmer Spectrum 100 FT-IR spectrophotometer (Waltham, MA, USA). The film's fragments were measured directly, in ATR mode (32 scans per sample), and spectra were recorded over a wavelength range of 650–4000 cm^{-1}, with a resolution of 1 cm^{-1}.

2.4.5. Film Color Analysis

The effects of melanin and artificial aging on the color of the films were measured using a colorimeter (CR-5, Konica Minolta, Tokyo, Japan). Each film was tested at 10 randomly selected spots. The results (mean ± standard deviation) were expressed as L*, a* and b* parameters. In addition, ΔE (color difference) and YI (yellowing index) were calculated using the formulas below:

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

$$YI = 142.86 b * L^{-1}$$

2.4.6. Antioxidant Potential of Films

Free radical scavenging activity assay was performed against DPPH radicals. For this purpose, 100 mg of each film was placed in 10 mL of 0.01 mM DPPH solution in methanol and incubated in the dark for 30 min. A polypropylene film of the same size as the sample was used as a negative control, while the prepared DPPH solution without the film was used as a blank control. The mixtures were incubated under identical conditions. Absorbance was measured at 517 nm, and free radical scavenging activity was calculated using the following formula:

$$\text{Free radical scavenging activity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where A_{sample} is the absorbance of the DPPH solution with the addition of the tested films, and $A_{control}$ is the absorbance of the blank DPPH solution.

2.4.7. Free Amino Acids, Sulfhydryl Groups (–SH) and Disulfide Bonds (–S–S–) Content in Films

To measure the amount of free amino acids before and after artificial aging of the films, film sections measuring 1.5 cm × 1.5 cm were cut and placed in 5 mL of ninhydrin-Cd reagent. The reaction mixture was then heated to 84 °C and held for 5 min before being placed in an ice bath. The absorbance of the mixture was measured at 507 nm. Glycine (Gly) was used to prepare a standard curve.

To 180 mg of film samples, 30 mL of Tris-Glycine buffer and 14.414 g of urea were added. The whole mixture was stirred for 30 min. To measure the content of sulfhydryl (–SH) groups, 160 mL of Ellman's reagent was added to 4 mL of mixture. The absorbance was measured at 412 nm.

For the determination of –S–S–, 8 μL of β-mercaptoethanol was added to 4 mL of mixtures obtained by the above procedure. The mixtures were kept at 25 °C for 2 h, after which 10 mL of 12% trichloroacetic acid (TCA) was added. The mixtures were again kept at 25 °C for 1 h and centrifuged at 6000 rpm for 10 min. Precipitates were washed three times with 5 mL of TCA and dissolved in 6 mL of Tris-Glycine buffer. The purified precipitate was combined with 4 mL of Ellman's Reagent, and the absorbance of the samples was measured at 412 nm.

A mixture containing no film samples was used as a control in the two analyses described above. The content of –SH and –S–S– were calculated according to the equations:

$$\text{–SH}(\mu mol/g) = \frac{73.53 \times A142}{C}$$

$$\text{–S–S–}(\mu mol/g) = \frac{Q1 - Q2}{2}$$

where A412 is absorbance at 412 nm; C is the sample concentration (mg of film sample/mL); Q1 stands for the –SH contents before and Q2 stands for the –SH contents after β-mercaptoethanol addition in the mixtures.

2.4.8. Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Prior to electrophoretic separation, 0.05 g of casein film samples and casein powder (casein, VWR Chemicals) were dissolved in 1 mL of lysis buffer (5 M urea, 2 M thiourea, 4% CHAPS, 1% (*w*/*v*) dithiothreitol (DTT)). Next, the total protein concentration in obtained samples was calculated using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer manual. The protein samples were then mixed with the Laemmli buffer (30% *v*/*v* 0.5M Tris (pH 6.8), 10% *w*/*v* SDS, 50% *v*/*v* glycerol, 20% *v*/*v* β-mercaptoethanol and a trace of bromophenol blue) to obtain the samples containing 1 μg of protein in 1 μL of the solution. Then, samples were incubated at 90 °C for 15 min and run in 12% polyacrylamide gels at 40 V for 1.5 h and subsequently at 70 V for 16 h in the electrophoretic chamber (PROTEAN® II xi Cell, Bio-Rad, Hercules, CA, USA). After separation of the proteins in gel, detection with CBB G-250 was performed according to the procedure previously described by Lepczyński et al. [33]. Digitalization of the gel image was performed with the aid of a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA). Densitometric analysis of protein profiles was conducted using ImageLab software (Bio-Rad, Hercules, CA, USA). The calculation of the protein bands molecular weight was performed using Precision Plus Protein™ Unstained Protein Standards (Bio-Rad, Hercules, CA, USA) as a reference.

2.5. Statistical Analyses

All analyses were made at least in triplicate. Statistical analysis was performed using Statistica version 13 software (StatSoft Poland, Krakow, Poland). Differences between means were determined by analysis of variance (ANOVA) followed by Fisher's post hoc LSD test at a significance threshold of $p < 0.05$.

3. Results

3.1. SEM Analysis

The morphology of casein-based films before and after UV artificial aging was analyzed by scanning electron microscopy (SEM), and images are presented in Figure 1. Melanin was completely dissolved in the alkaline environment of the film-forming solution, and there was no precipitation of melanin during film formation, as confirmed by the SEM images taken. As a result, melanin was evenly distributed throughout the film.

Comparing the different pairs of films before and after undergoing artificial UV aging, it can be seen that only in the case of pure casein film (A and A') was there a noticeable difference in film morphology. The neat casein film after artificial UV aging (A') underwent cracking on its surface. In the case of films subjected to melanin modification at different concentrations (B, C, D respectively), comparing them with films after artificial UV aging (B', C', D' respectively), no significant differences could be found in their morphology. This proves that the presence of melanin had a protective effect on the morphology of the obtained films. Moreover, this effect was not dependent on the concentration of melanin in the films.

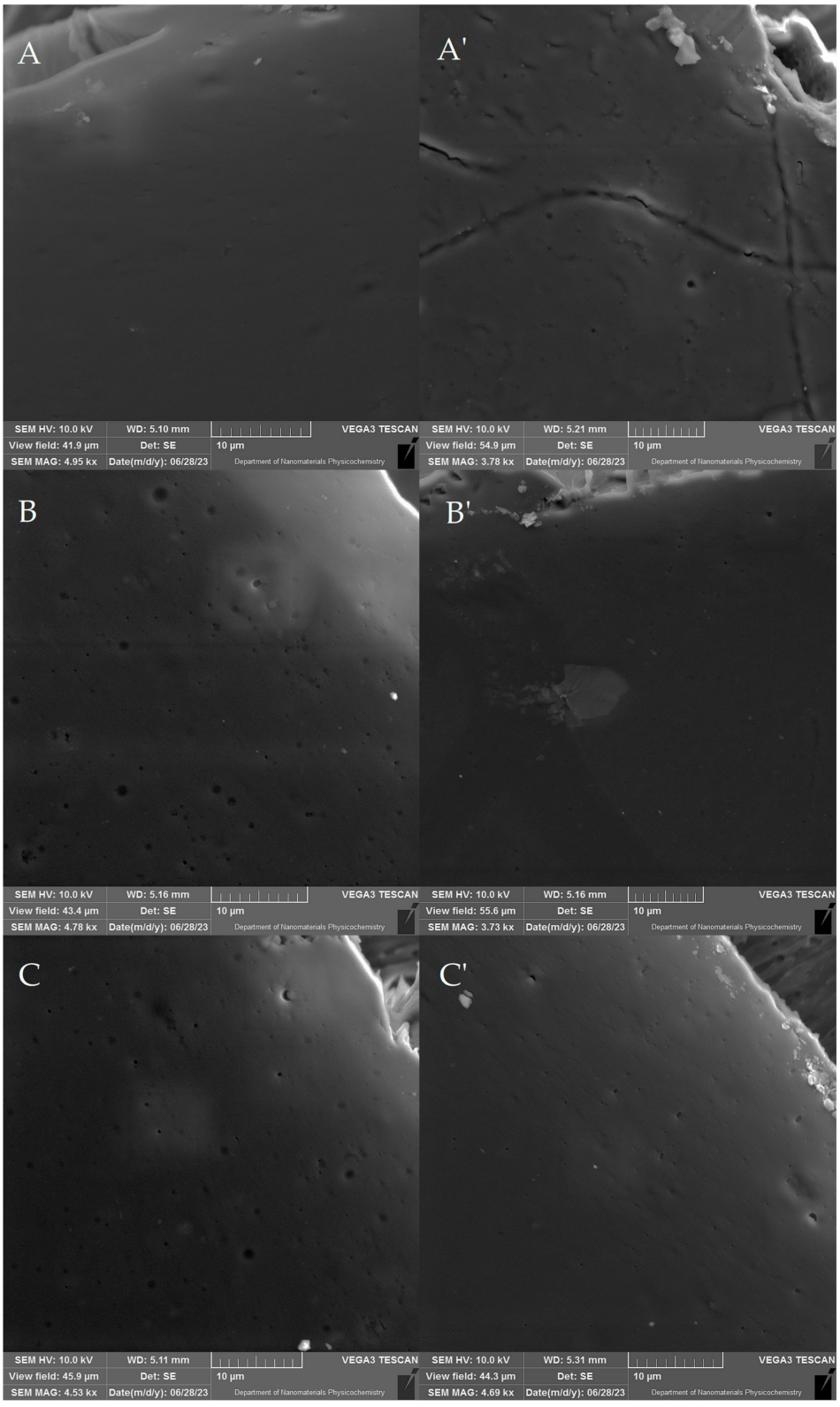

Figure 1. *Cont.*

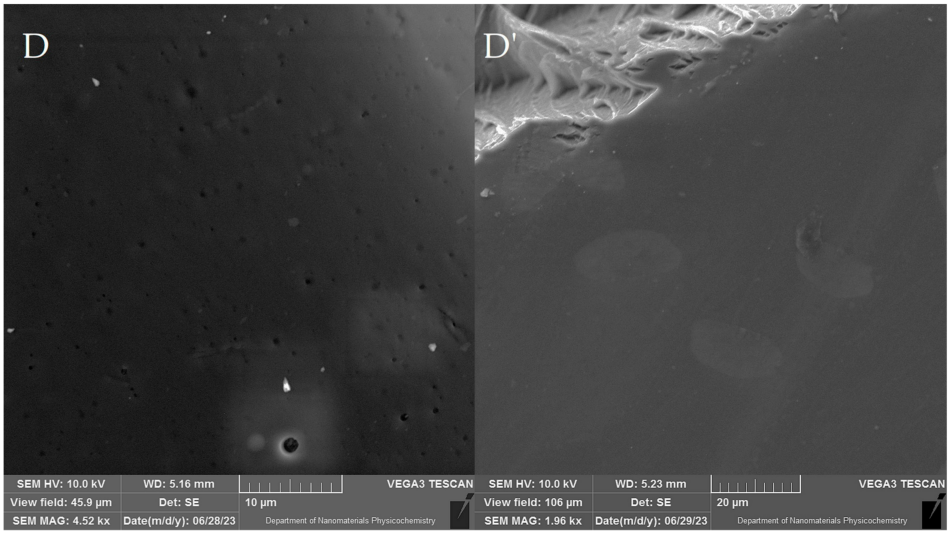

Figure 1. SEM images of Cas unaged (**A**), 0.10% MEL unaged (**B**), 0.25% MEL unaged (**C**), 0.50% MEL unaged (**D**), Cas artificially aged (**A′**), 0.10% artificially aged (**B′**), 0.25% MEL artificially aged (**C′**), 0.50% MEL artificially aged (**D′**) samples.

3.2. Hydrodynamic Properties (Moisture Content and Water Solubility)

In order to determine the effect of melanin addition to the films on hydrodynamic properties, analyses of moisture content and water solubility were performed. The results of the effect of melanin addition to the films on hydrodynamic properties are shown in Table 1.

Table 1. Moisture content (MC) and water solubility (WS) of neat and melanin-modified films.

Sample	MC (%)	WS (%)
A	10.66 ± 0.92^{a}	100.00 ± 0.00^{a}
B	$12.12 \pm 0.83^{a,b}$	100.00 ± 0.00^{a}
C	$11.82 \pm 0.79^{a,b}$	100.00 ± 0.00^{a}
D	13.30 ± 0.73^{b}	100.00 ± 0.00^{a}

Values are means ± standard deviation. Means with different lowercase in the same column are significantly different at $p < 0.05$. Samples names: Cas unaged (A), 0.10% MEL unaged (B), 0.25% MEL unaged (C), 0.50% MEL unaged (D).

The unmodified film showed a moisture content of 10.66 ± 0.92%. The addition of melanin in the lowest and intermediate amounts did not result in a statistically significant difference, but the sample with the highest melanin content showed a statistically higher ($p < 0.05$) moisture content of 13.30 ± 0.73%. These results are in line with previous reports for modified films based on agar [23], alginate [28], whey protein concentrate/isolate (WPC/WPI) [30] or chitosan [34].

In the case of water solubility, all samples were completely dissolved, leaving no undissolved residue. For other biopolymers, there are reports of an effect of melanin addition on reducing the solubility of films in water [28,30], but there are also reports representing the opposite relationship [34]. This may be due to the nature of the polymer matrix used and the interactions created between the matrix (its functional groups) and melanin affecting the amount of free hydroxyl groups capable of binding water [35].

3.3. The Thickness, Mechanical and Thermal Properties

Table 2 presents the thickness, mechanical and thermal characteristics of neat casein films and melanin-modified films before and after artificial aging. There was no significant effect of melanin addition on film thickness. In addition, artificial aging did not significantly affected this parameter. However, it should be noted that deviations from the average often

reach more than 50%, which is due to the uneven evaporation of the solvent and the lack of perfectly even surfaces of the vessels and equipment used. Nevertheless, such a relationship has also been made for other melanin-modified films such as CMC [26,27], whey protein concentrate/isolate [30] or for alginate [28]. However, it should be mentioned that there are also reports of melanin's effect on increasing the thickness of films based on agar [23], gelatin [29], carrageenan [31] or polybutylene adipate terephthalate (PBAT) [36] matrices. The addition of melanin also did not significantly affect changes in tensile strength (TS). For this polymer matrix, which is casein, no such changes were observed as for other melanin-modified polymers: namely, an increase in TS with an increase in the amount of melanin [23,27,29,34,36–38]. Nor did artificial aging have a statistically significant effect on changes in TS. The opposite is true for elongation at break (EB). The presence of melanin here increased the value of this parameter, but no effect of its concentration was observed. Exposure of the samples to the UV light lamp significantly increased EB in the case of the control sample from 38.75 ± 9.40% before exposure to 60.10 ± 10.94 after exposure, which is an increase of more than half. For melanin-modified films, the increase did not exceed 20%. The results of some researchers suggest that melanin can affect the increase in both mechanical parameters (TS and EB), but after reaching a certain critical concentration, the values begin to decrease [31,36,38].

Table 2. Thickness and mechanical characteristics of neat casein films and melanin-modified films before and after artificial aging.

Sample	Thickness (mm) Unaged	TS (MPa) Unaged	EB (%) Unaged	T_m (°C) Unaged	ΔH_m (J/g) Unaged
A	0.020 ± 0.008 ^{a}	14.70 ± 2.33 ^{a,b}	38.75 ± 9.40 ^{b}	117.74	88.69
B	0.019 ± 0.013 ^{a}	15.78 ± 1.92 ^{b}	56.17 ± 13.45 ^{a,b}	111.06	65.33
C	0.022 ± 0.016 ^{a}	13.50 ± 1.23 ^{a}	61.28 ± 8.05 ^{a}	107.19	52.54
D	0.024 ± 0.014 ^{a}	14.64 ± 1.71 ^{a}	71.03 ± 11.07 ^{a}	93.75	45.60
A′	0.022 ± 0.011 ^{a}	14.18 ± 1.37 ^{a}	60.10 ± 10.94 ^{a}	111.23	91.61
B′	0.023 ± 0.009 ^{a}	13.22 ± 1.93 ^{a}	66.55 ± 11.79 ^{a}	107.40	42.20
C′	0.025 ± 0.014 ^{a}	13.05 ± 1.33 ^{a}	66.25 ± 6.43 ^{a}	107.91	42.01
D′	0.024 ± 0.013 ^{a}	12.90 ± 2.08 ^{a}	81.93 ± 13.31 ^{b}	91.74	28.59

Values are means ± standard deviation. Means with different lowercase in the same column are significantly different at $p < 0.05$. Samples names: Cas unaged (A), 0.10% MEL unaged (B), 0.25% MEL unaged (C), 0.50% MEL unaged (D), Cas artificially aged (A′), 0.10% artificially aged (B′), 0.25% MEL artificially aged (C′), 0.50% MEL artificially aged (D′) samples.

In the case of thermal properties of casein films, a decrease in melting point can be observed as the melanin content of the film volume increases. This difference is particularly noticeable for the film with the highest melanin content (0.50% w/w) compared to neat casein films. In addition, melting enthalpies (ΔH_m) decrease as the amount of melanin increases. This stands in opposition to observations previously made for WPI/WPC films modified with melanin [30]. Nevertheless, for melanin-modified alginate films, an inverse relationship between melanin concentration and thermal parameters was also observed [28]. Aging the films had no significant effect on the melting temperature of the films, but in the case of melting enthalpies, the melanin-modified films showed lower values of this parameter after aging.

3.4. UV Barrier Properties

To evaluate the UV-Vis barrier properties, spectrophotometric spectra of the obtained films were analyzed. Figure 2 shows the UV-Vis spectra of unaged and artificially aged casein films modified with different melanin content. Neat casein films showed poor barrier to radiation in the Vis range (400–800 nm). For radiation in the UV range (below 400 nm), the barrier properties began to increase significantly. After melanin was used to modify this polymer, an increase in UV-Vis barrier properties could be observed with an increase in melanin concentration. This is nothing new, as melanin is known for its ability to absorb UV-Vis radiation [39] Melanin's UV-protective properties stem from its ability to absorb and scatter UV radiation. Going further, the energy of UV radiation is converted into thermal energy, which provides protection against the photodegradation of

the material [40]. Similar observations have already been made for many different polymer matrices [23,26–29,31,34,36,38]. The effect of artificial aging significantly weakened the barrier properties of the unmodified casein film. For radiation with a wavelength of 320 nm (the transition wavelength between UV-A and UV-B), a 3-fold decrease was observed. For the 0.10% MEL and 0.25% MEL films, an attenuation of the barrier properties for UV-Vis radiation was also observed, but this was at a maximum of 2-fold attenuation for both films. The 0.50% MEL films showed similar barrier properties both before and after artificial aging. Based on the results, there is an increase in the UV transmittance of the casein film due to artificial aging. At the same time, with an increase in the melanin content of the polymer matrix, lower decreases in barrier properties for radiation in the UV range were observed. Melanin during artificial UV aging acted protectively by scattering radiation, which influenced the better preservation of the original material properties.

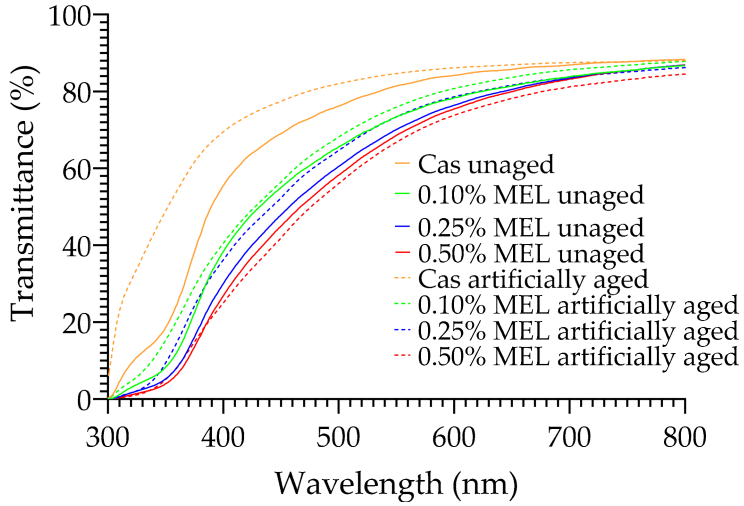

Figure 2. UV-Vis spectra of unaged and artificially aged casein films modified with different melanin content.

3.5. FT-IR Analysis

FT-IR analysis was performed to determine the effect of melanin on the protective properties by photodegradation of the bonds. Figure 3 shows the FT-IR spectra of neat and melanin-modified casein films before and after artificial aging. The addition of melanin had no significant effect on changes in the spectra. Similar results (no effect or little effect) have been previously described by researchers for other melanin-modified polymers [26,28,29,31,35,36]. A slight weakening of the signal is observed in the region of bending vibrations of O–H and N–H (3282 cm^{-1}) and stretching vibrations of $–CH_2$ (2936 cm^{-1}). Artificial aging had no noticeable effect in amide I (C=O stretching) (1638 cm^{-1}) and amide III (C–N and N–H stretching) (1236 cm^{-1}); however, there was a slight signal weakening in the amide II (N–H bending) binding region (1538 cm^{-1}). In addition to changes in signal intensity, no shifts were noted. Potentially, therefore, artificial aging affected the breaking of some of the bonds in the amide (N–H) groups. This result correlates with those obtained by SDS-PAGE presented later in this article.

The FT-IR analysis did not report significant changes within the chemical bonds, but this result is consistent with observations made by other researchers, who, when subjecting other polymers films to artificial UV aging, also reported no significant changes using this analysis [41,42].

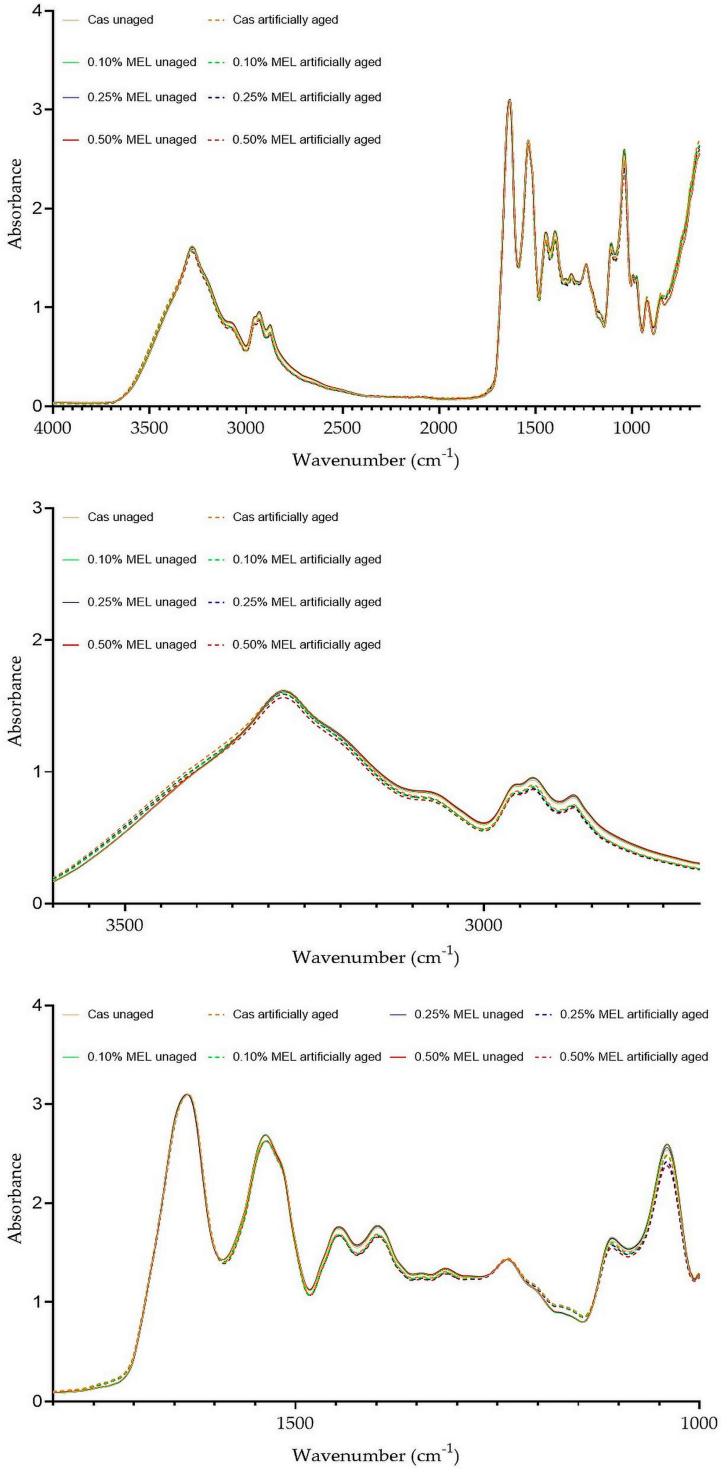

Figure 3. FT-IR spectra of neat and melanin-modified casein films before and after artificial aging. From top, full spectrum, vibrations of O–H, N–H and $–CH_2$ region and amide region (amides I, II and III), respectively.

3.6. Color

In order to study the effects of melanin and artificial aging on the color of the films, chromatic parameter analyses were carried out. Table 3 summarizes the chromatic parameters, total color difference and yellowness index for the tested films. As the melanin content increases, the L* parameter decreases slightly (the films become darker), while the a* and b* parameters increase (the red and yellow components of the overall color hue

increase, respectively ($p < 0.05$)). The yellowness index also increases with an increase in the amount of melanin used ($p < 0.05$). The total color difference for these films is greater than 1 which, according to the literature, accounts for the color difference observable by the human eye [37]. Similar observations have already been made for many different polymer matrices [23,26–29,31,34,36,38].

Table 3. Color (L*, a*, b*), total color difference (ΔE), yellowness index (YI) and transmittance of unaged and artificially aged casein films.

Sample	L*	a*	b*	ΔE	YI	T_{280} (%)	T_{660} (%)
A	47.48 ± 2.17^{a}	-0.19 ± 0.15^{a}	19.62 ± 0.98^{b}	standard	$59.08 \pm 2.94^{a,b}$	57.05 ± 0.45^{d}	$86.11 \pm 0.83^{d,e}$
B	46.63 ± 7.99^{a}	$1.23 \pm 0.55^{a,b,c}$	$23.77 \pm 3.92^{a,b}$	8.19 ± 5.18^{a}	$72.94 \pm 2.58^{b,c}$	40.20 ± 0.31^{b}	$81.93 \pm 0.63^{a,c}$
C	35.37 ± 4.32^{a}	$2.90 \pm 0.59^{c,d,e}$	$24.75 \pm 2.26^{a,b}$	14.00 ± 4.28^{a}	100.54 ± 8.12^{d}	36.33 ± 0.92^{a}	$81.22 \pm 0.77^{a,b}$
D	39.63 ± 4.27^{a}	$3.76 \pm 0.78^{d,e}$	28.14 ± 2.48^{a}	13.40 ± 2.24^{a}	102.12 ± 7.25^{d}	36.45 ± 0.73^{a}	$80.69 \pm 0.68^{a,b}$
A′	75.48 ± 2.94^{b}	$0.73 \pm 0.16^{a,b}$	$29.40 \pm 0.43^{a,c}$	Standard	55.73 ± 2.24^{a}	64.76 ± 0.73^{e}	87.04 ± 0.82^{e}
B′	70.50 ± 1.46^{b}	$2.27 \pm 0.35^{b,c,d}$	$35.71 \pm 1.40^{c,d}$	7.09 ± 1.78^{a}	$63.48 \pm 3.97^{a,b,c}$	40.11 ± 0.36^{b}	$84.16 \pm 1.05^{c,d}$
C′	69.99 ± 2.27^{b}	4.00 ± 0.54^{e}	$38.31 \pm 1.49^{d,e}$	11.05 ± 1.70^{a}	$78.48 \pm 2.76^{c,e}$	37.15 ± 0.85^{a}	$82.15 \pm 0.69^{a,c}$
D′	68.62 ± 1.78^{b}	6.50 ± 0.68^{f}	44.76 ± 1.69^{e}	17.86 ± 1.74^{a}	$93.22 \pm 3.74^{d,e}$	29.65 ± 0.37^{c}	78.90 ± 0.57^{b}

Values are means ± standard deviation. Means with different lowercase in the same column are significantly different at $p < 0.05$. Samples names: Cas unaged (A), 0.10% MEL unaged (B), 0.25% MEL unaged (C), 0.50% MEL unaged (D), Cas artificially aged (A′), 0.10% artificially aged (B′), 0.25% MEL artificially aged (C′), 0.50% MEL artificially aged (D′) samples.

Table 4 also shows the UV barrier properties (T280) and transparency (T660) of neat and modified films before and after artificial aging, which correspond to transmittance at 280 nm and 660 nm, respectively. The unmodified and unaged casein film showed high transparency (86.11 ± 0.83%) and intermediate permeability to UV radiation (57.05 ± 0.45%). The addition of melanin affected a slight decrease in transparency, but it remained at a still high level of more than 80% regardless of the concentration of melanin used. Barriers to UV radiation increased. For the film with the highest melanin content, the amount of UV radiation transmitted was 36.45 ± 0.73%. Similar relationships have been previously demonstrated for films based on alginate [28] or CMC [26] polymer matrices. Treatment of the films with a qSUN lamp did not affect the changes in transparency of the films ($p < 0.05$). Nevertheless, there was a decrease in barrier to UV radiation by the neat casein film (an increase in transmittance to 64.76 ± 0.73%) and an increase in barrier by the film with the highest melanin content tested (a decrease in transmittance to 29.65 ± 0.37%).

Table 4. DPPH radicals scavenging activity of unaged and artificially aged casein films.

Sample	DPPH (%) after 30 min	DPPH (%) after 24 h
A	1.50 ± 1.76^{a}	10.05 ± 1.27^{a}
B	2.76 ± 2.50^{a}	$15.44 \pm 3.38^{a,b}$
C	3.45 ± 0.64^{a}	$15.90 \pm 2.47^{a,b}$
D	3.09 ± 2.01^{a}	$18.89 \pm 3.41^{a,b,c}$
A′	3.81 ± 3.39^{a}	$30.73 \pm 5.18^{c,d}$
B′	3.21 ± 2.25^{a}	32.72 ± 4.06^{d}
C′	$6.38 \pm 1.78^{a,b}$	$27.95 \pm 5.74^{c,d}$
D′	12.37 ± 3.74^{b}	$29.28 \pm 4.27^{d,e}$

Values are means ± standard deviation. Means with different lowercase in the same column are significantly different at $p < 0.05$. Samples names: Cas unaged (A), 0.10% MEL unaged (B), 0.25% MEL unaged (C), 0.50% MEL unaged (D), Cas artificially aged (A′), 0.10% artificially aged (B′), 0.25% MEL artificially aged (C′), 0.50% MEL artificially aged (D′) samples. It is noteworthy that the study of antioxidant properties was performed in the environment of DPPH radicals in methanol. Casein films in such a solution remained in an undissolved form. However, as demonstrated in the section on water solubility, these films undergo rapid, complete dissolution in aqueous medium. Therefore, it is presumed that under aqueous conditions, more melanin would be released from the film volume, and stronger antioxidant properties would be observed.

3.7. Antioxidant Activity

To determine the antioxidant activity, the ability of the tested films to scavenge DPPH radicals was determined over 30 min and 24 h. The results of the antioxidant capacity of neat casein and melanin-modified films before and after radiation with the UV light lamp are shown in Table 4. For both films kept in the reaction solution for 30 min and 24 h, an increase in the ability to scavenge DPPH free radicals was observed as the concentration of melanin in the films increased, but the differences between films were not statistically significant ($p > 0.05$). This is surprising, since melanin is known for its antioxidant properties, which have been demonstrated in many studies where significantly greater differences between samples were achieved [23,27–31,34,37,38]. Once again, this may indicate the influence of the interaction between the polymer matrix and the filler on the properties of the obtained films (their strengthening or weakening). Exposure of the films to artificial aging resulted in an increased ability to scavenge DPPH radicals. For the non-melanin-modified sample, the increase was 2.5-fold, while the 0.50 MEL sample after aging showed four times higher antioxidant properties when incubated for 30 min. At the same time, after aging the films, the differences between the films in free radical scavenging ability increased. Nevertheless, over the period of 24 h, all the aged films achieved a similar free radical scavenging activity. This can be explained by the ability of amino acids and functional residues released during aging to react with free radicals and scavenge them. Nevertheless, the mechanism is still poorly understood, and more analysis is needed in the future to better understand this phenomenon. Similar effects were observed by Shen et al. for tobacco leaves aged with UV-B radiation. They observed an increase in the antioxidant capacity of tobacco leaves after artificial UV aging [43].

3.8. Free Amino Acids, Sulfhydryl Groups (–SH) and Disulfide Bonds (–S–S–) Content

Table 5 shows the contents of free amino acids, sulfhydryl groups (–SH) and disulfide groups (–S–S–) in the analyzed films. In films not subjected to artificial aging, an increase in the amount of free amino acids can be observed as the amount of melanin in the polymer matrix increases. This could potentially be due to the presence of free NH and NH_2 groups on the surface of melanin, as previously showed for melanin from watermelon (*Citrullus lanatus*) seeds [32], the presence of peptide residues or free proteins bound to melanin [44] or interactions between the polymer matrix and melanin. The effect of artificial aging increased the amount of free amino acids, but the increase was not uniform for all samples. The non-melanin-modified casein film after artificial aging showed 25% more free amino acids than before aging (an increase from 11.47 ± 1.03 mg/g to 14.41 ± 0.37 mg/g after aging). This is explained by the destructive effect of UV radiation on peptide bonds. As a result of its action, bonds were broken, resulting in an increase in the amount of free amino acids. As the amount of melanin increased, smaller increases in free amino acids were observed. These increases were 17%, 7% and 6% for the 0.10% MEL, 0.25% MEL and 0.50% MEL films, respectively. A huge increase in the number of sulfhydryl groups was observed after the films were artificially aged. The largest increase was again observed for the neat casein film. The content of sulfhydryl groups increased from 35.44 ± 8.23 to 120.30 ± 13.39 μmol/g and thus by 239%. For melanin-modified films, an increase in the concentration of –SH groups was also observed, but the increase was lower the more melanin was used to modify the polymer matrix. For 0.10% MEL films, the value increased from 26.86 ± 5.92 to 19.10 ± 2.23 μmol/g (a 109% increase), for 0.25% MEL, it increased from 19.10 ± 2.23 to 29.11 ± 4.18 μmol/g (a 52% increase) and for 0.50% MEL, it increased from 9.09 ± 1.88 to 12.77 ± 2.74 μmol/g (a 40% increase). In the case of disulfide bonds, there was a breakage of these bonds as a result of the action of artificial aging with UV light lamp leading to a reduction in their amount, but in neither case were the differences statistically significant ($p > 0.05$). As a result of artificial aging, there has been a reduction in the amount of disulfide bonds while the amount of sulfhydryl groups has increased. It can be speculated that the –S–S– bonds broken by artificial UV aging reacted with dissociated hydrogen ions from water vapor in environment leading to the formation of –SH groups.

Melanin may have affected the binding of proteins in the polymer matrix, leading to changes in the amount of detectable –SH groups and disulfide bridges in unmodified films. As shown in the results from FT-IR (presented earlier) and SDS-PAGE (in the next section), there was no significant degradation of casein, and it was mainly disulfide bridge disruption, potentially leading to changes in protein folding. The melanin-containing films had fewer bond breaks due to melanin's conversion of UV radiation energy into thermal energy. This resulted in fewer –S–S– bond breaks and a smaller increase in –SH groups. This effect was stronger the more melanin was used to modify the films. Melanin's ability to protect disulfide bonds from photodegradation has been previously demonstrated for creatine in human hair, among others [45].

Table 5. The contents of free amino acids, sulfhydryl groups (–SH) and disulfide groups (–S–S–) in the analyzed films.

Sample	Free Amino Acids (mg/g)	–SH (μmol/g)	–S–S– (μmol/g)
A	11.47 ± 1.03^{d}	$35.44 \pm 8.23^{b,c}$	182.14 ± 12.81^{a}
B	$13.83 \pm 1.43^{c,d}$	$26.86 \pm 5.92^{a,b}$	$228.76 \pm 16.25^{b,e}$
C	$15.64 \pm 0.75^{a,b,c}$	$19.10 \pm 2.23^{a,b}$	$270.38 \pm 12.58^{b,c,d}$
D	$16.81 \pm 0.53^{a,b}$	9.09 ± 1.88^{a}	307.91 ± 15.63^{d}
A′	$14.41 \pm 0.37^{a,c}$	120.30 ± 13.39^{d}	174.12 ± 11.73^{a}
B′	$16.21 \pm 0.51^{a,b,c}$	56.17 ± 4.93^{c}	$196.39 \pm 14.30^{a,e}$
C′	$16.78 \pm 0.75^{a,b}$	$29.11 \pm 4.18^{a,b}$	$258.78 \pm 13.72^{b,c}$
D′	17.89 ± 0.84^{b}	12.77 ± 2.74^{a}	$274.12 \pm 10.88^{c,d}$

Values are means ± standard deviation. Means with different lowercase in the same column are significantly different at $p < 0.05$. Samples names: Cas unaged (A), 0.10% MEL unaged (B), 0.25% MEL unaged (C), 0.50% MEL unaged (D), Cas artificially aged (A′), 0.10% artificially aged (B′), 0.25% MEL artificially aged (C′), 0.50% MEL artificially aged (D′) samples.

3.9. SDS-PAGE

In the study, the SDS-PAGE protein separation technique was employed to assess the stability of the proteins that compose casein films to artificial aging with UV light. The separation of the proteins allowed obtaining the protein profiles of casein films that represents bovine milk casein fractions α-casein, β-casein, and κ-casein. Moreover, naturally occurring and artificial caseins polymers and caseins proteolytic degradation products were observed. The protein profiles of bovine milk caseins, as a substrate for films production, and casein films are presented on Figure 4A. The identification of those proteins and peptides resulting from their degradation was performed using previously published results of other authors as a reference [46–48]. The comparison of protein profiles of casein films was performed. It allows us to compare the caseins and their polymerization and degradation products stability after exposure of the films to artificial aging.

The resulted protein profiles and graphs that visualize the relative abundance of protein bands differing the casein films before and after artificial UV are shown in Figures 4B and 4C, respectively. The expression profiles of other analyzed protein bands are given in the Supplementary File (Figure S1).

The analysis of protein profiles revealed the highest abundance of protein band A representing the oligo- and polymers of caseins in the samples representing casein films with 0.25% and 0.50% MEL addition. It may suggest that the addition of melanin as a cofactor for casein-based films increases the natural ability of milk caseins to form high molecular weight polymers. The protein crosslinking properties of melanin were previously confirmed by Lutz, who observed that its addition to the silk solution caused its gelation and increased silk-based hydrogel mechanical properties [49]. It is known that melanin has a strong ability to bind to peptides. Melanin particles are highly negatively charged (through the presence of hydroxyl groups on its surface), so they are capable of binding to cationic groups of peptides/proteins. However, the formation of these bonds is highly dependent on the pH of the environment [44]. The above-described properties of melanin

could potentially explain the observed co-occurrence of high molecular weight casein polymers with an increased number of disulfide (–S–S–) bonds and decreased number of sulfhydryl (–SH) bonds in the films obtained with melanin addition.

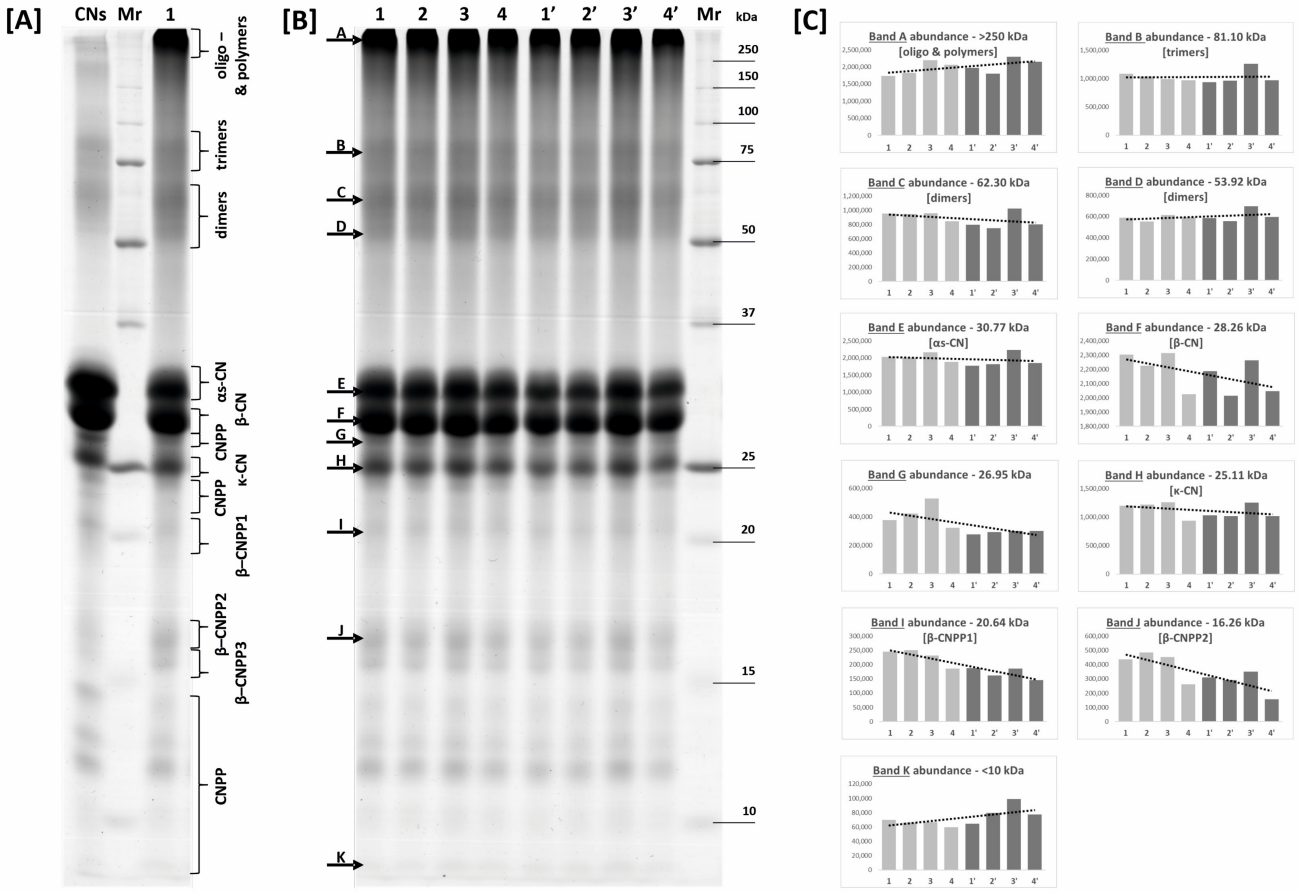

Figure 4. SDS-PAGE electropherograms of casein films. Panel (**A**) presents the protein profile of bovine milk casein fraction (CNs) that serves as a reference for the casein films' protein profiles (1–4; 1′–4′). On line 1, representing the Cas sample protein profile, the known bovine milk caseins and their dimers, trimers and oligo-, polymers as well as known caseins degradation products are marked according to [46–48]. Panel (**B**) represents the casein films protein profiles. Lines 1–4 represent, respectively, unaged Cas, 0.10% MEL, 0.25% MEL and 0.50% MEL sample protein profiles. Analogically, lines 1′–4′ represent, respectively, artificially UV aged Cas, 0.10% MEL, 0.25% MEL and 0.50% MEL sample protein profiles. Lines marked Mr represent the molecular range marker (kDa). Letters from A to K mark the protein bands that represent milk caseins, their -omers and casein proteolysis products. Panel (**C**) presents the graphs with the relative abundance of protein bands in particular lines 1–4 and 1′–4′ with a trend line between the groups. αs-CN—alpha-caseins; β-CN—beta-casein, κ-CN—kappa-casein, CNPP—casein proteolysis product, β-CNPP1–3—beta-casein proteolytic product 1, 2 or 3.

According to the observations of Rossi et al., milk proteins subjected to photo-oxidation are able to crosslink. Based on their results, they observed that oxidized α-casein combined with native κ-casein to form high molecular mass aggregates together [50]. Potentially, this could therefore be the reason why, for unmodified casein films, a decrease in the abundance of these groups and an increase in the abundance of oligo- and polymers were observed. Nevertheless, Rossi et al. used a different source of radiation (visible light, generated by LED diodes) in their study, and thus, the degradation mechanism can be considered different as well.

A comparison of the relative abundances of protein bands representing milk caseins and their di-, tri-, oligo- and polymers (bands A–F, H) of films before and after artificial UV aging shows the highest stability of those bands in the samples representing casein-based films with 0.25% and 0.50% MEL addition. That indicates the protection against the photodegradation of proteins by the highest concentrations of melanin.

Moreover, the most intense degradation as a result of artificial aging was observed for bands representing β-CN and its natural proteolytic products (bands F, I, J) and a protein band with a molecular mass of 26.95 kDa (band G), independent of casein-based films composition. The above may suggest the lowest UV stability of those proteins. We have also observed an increased abundance of protein bands with M_W under 10 kDa (band K), which most probably represents the low molecular weight protein degradation products that resulted from casein films' artificial aging.

4. Conclusions

This work describes the properties of casein films modified with melanin and the protective properties of melanin against the artificial aging of these films. The purpose of the study was to determine how melanin would affect the properties of casein films exposed to UV light radiation intended to correspond to changes in the films over time. Melanin had a protective effect on the preservation of mechanical properties improving barrier properties to UV radiation and improving the ability to scavenge free radicals. Moreover, it increased the abundance of oligo- and polymeric bands in electrophoretic separation. It can be suspected that the developed melanin-modified casein films could find application in the food industry as packaging or edible food wrappers. Nevertheless, further studies on food products would be needed to confirm this supposition.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/coatings13071262/s1, Figure S1: SDS-PAGE electropherograms of casein films. Panel A presents the protein profile of bovine milk casein fraction (CNs) that serves as reference for casein films protein profiles (1–4; 1′–4′). On line 1 representing the Cas sample protein profile the known bovine milk caseins and their dimers, trimers and oligo-, polymers as well es known caseins degradation products are marked according to [45–47]. Panel B represents the casein films protein profiles. Lines 1-4 represents respectively unaged: Cas, 0.10% MEL, 0.25% MEL and 0.50% MEL sample protein profiles. Analogically lines 1′–4′ represents respectively artificially UV aged Cas, 0.10% MEL, 0.25% MEL and 0.50% MEL sample protein profiles. Lines marked Mr represents molecular range marker (kDa). Letters from L to R marks the protein bands that represents milk caseins, and casein proteolysis products that have not differ between aged and unaged films. Panel C presents the graphs with the relative abundance of protein bands in particular lines 1-4 and 1′-4′ with a trend line between the groups. αs-CN—alpha-caseins; β-CN—beta-casein, κ-CN—kappa-casein, CNPP—casein proteolysis product, β-CNPP1-3—beta-casein proteolytic product 1, 2 or 3.

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