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The influence of lingonberry extract on the properties of novel, double-layered biopolymer films based on furcellaran, CMC and a gelatin hydrolysate

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In this paper, the procedure is presented of innovative, biopolymer double-layered film synthesis, based on furcellaran (FUR) (1st layer) and carboxymethyl cellulose (CMC) with a gelatin hydrolysate (HGEL) (2nd layer). The double-layered structure of the films can be clearly observed in SEM images. The water extract from lingonberries was added to FUR to reinforce the antioxidant and antimicrobial activity of the films. The films with the greatest lingonberry extract (LBE) additive demonstrated antimicrobial activity against the tested bacteria and antioxidant activity (from 3.01 to 41.89 mM Trolox/mg - via FRAP method, and from 0 to 20.56% - via DPPH method). The films with 40% of LBE showed the highest thermal stability (improvement by up to ~92%) in comparison to tested films. They were used as active materials in the storage of cherry tomatoes. In the active films, tomatoes lost weight quickly compared to the synthetic ones. However, they were characterised by lower phenol content, which indicated slow ripening. The obtained double-layered films with LBE are an interesting alternative to plastic materials.

1. Introduction

Recently, more and more attention has been focused on finding alternatives to synthetic packaging materials in order to avoid environmental pollution. Biopolymer films, based on polysaccharides and proteins, are becoming an interesting solution because they have the ability to prevent microorganism growth, moisture loss, oxygen penetration or the transportation of active substances. However, biopolymer films have disadvantages such as poor strength parameters and too high water vapour permeability (Jamróz, Janik, et al., 2021). A potential solution to these problems could be implementing double- or multi-layered biopolymer films. The advantage of such packaging is primarily an increase in the strength of barriers preventing gas penetration, as well as giving each layer a specific activity (Jamróz, Kulawik, et al., 2021b).

Among various natural resources, furcellaran and carboxymethyl cellulose have specific benefits in being used as packaging materials. Furcellaran (FUR) is a negatively charged polysaccharide that has been previously been used as a packaging material (Jamróz, Kopeł, et al., 2019; Jancikova, Jamróz, Kulawik, Tkaczewska, & Dordevic, 2019; Pluta-Kubica, Jamróz, Kawecka, Juszcza, & Krzyściak, 2019) and is a carrier of active substances (Milosavljevic et al., 2020; Tkaczewska, Jamróz, Kulawik, Morawska, & Szczurowska, 2019). Carboxymethyl cellulose (CMC) is an anionic derivative of cellulose that is water-soluble...
while showing poor mechanical and moisture barrier properties (Roy & Rhim, 2020). Therefore, in order to improve these properties, it is proposed to produce double-layered biopolymer films. Currently, protein hydrolysates and, in particular, gelatin hydrolysates, are becoming of increasing interest, not only due to reuse of industrial waste from the fish industry, which further contributes to a reduction in the amount of generated post-industrial waste, but also due to their high antioxidant potential (Tkaczewska, 2020). The use of various types of protein hydrolysates in the production of biopolymer films shows promise in improving the rational management of waste from the food industry. Therefore, active biopolymer films enriched with gelatin hydrolysates were developed and used as packaging materials for fish (Jamroz, Kulawik, et al., 2021b; Tkaczewska et al., 2021).

Edible coatings with active ingredients improve the safety, quality and functionality of food products, while inhibiting the growth of undesirable microorganisms during their storage (Valdés, Ramos, Beltrán, Jiménez, & Garrigós, 2017). There are several factors that can affect the effectiveness of antimicrobial packaging, such as the chemical nature of the antimicrobials and food, storage and distribution conditions, the method of application of the coating onto the product, and interactions between the antimicrobials and the polymer (dos Santos Pires et al., 2008). Furthermore, the addition of an antimicrobial agent into packaging may affect the physical properties of packaging materials. Various antimicrobial compounds incorporated into edible films have been investigated (Maizura, Fazilah, Norziah, & Karim, 2007). Incorporation of natural derivatives such as protein hydrolysates and fruit extracts into edible films in order to inhibit the growth of microorganisms has been successfully demonstrated.

The oxidation of lipids in food causes the formation of toxic substances and deterioration of the food products’ nutritional value through the degradation of polyunsaturated fatty acids. One of the directions in development of active packaging is the modification of their surface compatible and thus, promote an even release of the active ingredient over the entire surface of the packed food. In the last decade, various edible coatings containing antioxidants and antimicrobials have been tested as potential active packaging materials (Benbettraib, Debeaufort, & Karbowiak, 2019; Halim, Yusof, & Sarbon, 2016). The potential use of protein hydrolysates and various plant extracts as active ingredients has also been investigated.

Lingonberry (Vaccinium vitis-idaea) is an evergreen shrub that grows in Scandinavia, North America and Europe. The fruits of this plant are extremely rich in active compounds with antioxidant, anti-inflammatory and antioxidant properties. Despite the high content of bioactive compounds, lingonberry is hardly used in the food industry, mainly due to its unpleasant taste of acidity, bitterness and astringency (Marsol-Vall, Kelanne, Nuutinen, Yang, & Laaksonen, 2021).

Thus, an interesting solution to this issue is the incorporation of lingonberry extract into biopolymer films to provide the packaging materials with active properties. This is the first time that the lingonberry extract has been used as an additive to biopolymer films. Our idea was to obtain double-layered films composed of one furcellaran layer enriched in lingonberry extracts at various concentrations, and the second layer consisting of carboxymethyl cellulose and a carp skin gelatin hydrolysate. The obtained films were tested in terms of their physicochemical, mechanical and permeability-related, as well as bioactive (antimicrobial and antioxidant activity) properties. The final objective was to prove potential application of active double-layered films on prolonging the storage period of cherry tomatoes.

2. Hypothesis

It is possible to create double-layered films based on furcellaran (FUR) and carboxymethyl cellulose with a gelatin hydrolysate (CMC + HGEL) that are enriched with a water extract from lingonberry (LBE), and are characterised by appropriate thermal, water and mechanical parameters, as well as water vapour permeability, to be potentially used in the area of food packaging. Moreover, the active films will have a positive effect on the storage quality of cherry tomatoes.

3. Materials and methods

3.1. Materials

Furcellaran (type 7000) was purchased from Est-Agar AS (Karla village, Estonia). Its chemical content (Mw, 2,951 × 10⁵) was carbohydrates totalling 79.61% protein at 1.18%, as well as fat equaling 0.24%. Carboxymethyl cellulose (CAS:9004-32-4) was obtained from POL-AURA (Zabrze, Poland). The lingonberry fruits used in the study were acquired from the Natura Wita company (Płock, Poland). In the case of fruit preservation experiments, cherry tomatoes were procured from a local store (Krakow, Poland). Carp skin gelatin hydrolysates were obtained in accordance with the method described in an earlier study by Tkaczewska, Jamroz, et al. (2019). All of the chemical reagents were applied as upon-arrival and not being subjected to further purification.

3.2. Preparation of lingonberry extract

The lingonberry fruits were blended via a blender (Viva Collection, Phillips). Blended lingonberry fruits in the amount of 20 g were mixed with 200 ml of H₂O. The solution prepared in this way was shaken in a shaker for 30 min at 70 °C. Then, the solution was filtered through Whatman No.1 filter paper several times to obtain a clarified solution.

3.3. Preparation of films

The preparation of individual carboxymethyl cellulose solutions (CMC- 1% w/v, 1 wt% glycerol was added following dissolution – then, the solution was stirred for 12 h at room temperature) and furcellaran was added (FUR-1% w/v, to which, after dissolution, 1 wt% glycerol was added - following, the solution was heated to 130 °C for 20 min.). Into Petri dishes (ø 150 mm), 100 ml of FUR was poured until the solution became gel-like. The gelatin hydrolysate (HGEL), i.e. 0.5 g, was added to 1% v/v CMC. After 20 min, the prepared solution was pipetted onto the 1st layer of FUR. In this manner, the control films were obtained.

To obtain films with LBE, individual FUR and CMC solutions were created analogously. Following, the lingonberry extract was added to the FUR solution, finally, to reach the concentrations of: 10%, 20% and 40% v/w. Each solution was poured onto Petri dishes (ø 150 mm) until a gel was formed. Then, 1% v/v CMC was incorporated with 5 g of the gelatin hydrolysate (HGEL). As soon as the solution achieved a clear state, it was pipetted onto the 1st FUR layer. The film-forming solutions underwent drying at room temperature, which was performed under a frame hood. Following this, when the films were completely dry, they were peeled from the Petri dishes to undergo further testing.

3.4. Scanning electron microscopy (SEM)

FUR/CMC + HGEL film morphology was conducted via the JEOL JSM - 7500F Field Emission Scanning Electron Microscope, which is equipped with a Retractable Backscattered-Electron detector (RBEI) and EDS (energy dispersive spectra) detection system of characteristic X-ray radiation – the INCA PentaFetx3 EDS system. The sample cross-sections were prepared in order to carry out observation of the double-layered film conformation.
3.5. FT-IR analysis

FTIR analysis of the double-layered films was performed with the use of the Nicolet 380 FTIR spectrometer. The infrared spectra were registered in the middle infrared range of 4000–400 cm⁻¹. The samples of single-layered (CMC + HGEL and FUR+20% LBE) and the double-layered films (FUR + x% LBE/CMC + HGEL, where x = 10, 20, 40) were measured with exposition on the 1st FTIR layer. Spectra were obtained by co-addition of 64 scans at a resolution of 4 cm⁻¹. Spectra were analysed using the Thermo Scientific OMNIC™ software package.

3.6. Colour and optical parameters

The film surface colour was evaluated using the method of reflection with the Color i5 spectrophotometer (X-Rite, Grand Rapids MI, USA, illuminant D65) and was later presented as L* (lightness), a* (red-green) and b* (yellow-blue). Additionally, changes in colour differences (∆E) were estimated in the following manner:

\[
\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} 
\]

where, ∆E is the total difference in colour value of FUR/CMC + HGEL films being the standard, and those with regard to FUR/CMC + HGEL films containing LBE.

UV–vis spectra of the films were recorded in the wavelength range (λ) of 350–700 nm using UV–visible spectrophotometer (UV 5500 Metash). To determine the opacity of the film, an ultraviolet spectrophotometer (UV 5500 Metash) was used to measure the transmittance of the composite film from 200 nm to 800 nm. Each sample was analysed in triplicate. The opacity of the tested films was measured according to the method reported by Du et al. (2021), and is as follows:

Opacity = absorbance at 600 nm/film thickness (mm)

(2)

Each sample was analysed in triplicate.

3.7. Water vapour transmission rate (WVTR)

A glass vessel was filled with silica gel, which was then covered with the film under analysis. After that, it was placed in a regulated-microclimat (condition) chamber at a temperature totalling 25 °C and 75% of relative humidity. Following, the vessel was weighed at indicated periods of time. WVTR was assessed on the basis of weight gain. The tested specimens were prepared and then air-conditioned in a normative manner. WVTR was calculated according to the following formula:

\[
\text{WVTR [g/m}^2 \text{· d]} = 240 \times (\text{weight of water} / \text{surface penetration}) \times 24(3)
\]

3.8. Determination of contact angle

The contact angle, with regard to water of the films under study, was determined using the sessile drop method with a video-based measuring system for assessing contact angle (OCA, Dataphysics, Germany). This was performed at room temperature (~23 °C). A droplet (in the amount of 10 μL) of deionised water was gently dripped onto the film surface via a micro-injector and then, the image was obtained. Measurements were carried out for 5 samples.

3.9. Thermal properties

The film sample was weighed (approx. 2 mg), sealed into aluminium pans and then subjected to heating processes from 30 °C to 300 °C, at a rate of 5 °C/min. The reference was implemented in the form of an empty pan. The DSC 204F1 Phoenix differential scanning calorimeter (Netzsch, Germany) was used for testing, while the parameters of observed thermal transitions were estimated using Proteus Analysis software (Netzsch, Germany). Measurements were conducted on the basis of 3 samples.

3.10. Mechanical properties

Mechanical properties: i.e. tensile strength (TS), elongation at break (EAB), max. breaking load (MBL), modulus of elasticity (ME) of the examined films were evaluated using the Shimadzu EZ Testing machine (Kyoto, Japan), and this examination was performed in accordance with the ASTM International testing method No. D882-02. The analyses were carried out in triplicate and on 3 film samples (n = 3 × 3).

The puncture strength (PS) test was conducted via the Shimadzu EZ Testing machine (Kyoto, Japan) equipped with a proper adapter for strength testing (according to the PN-EN 14477:2005 standard). During measurements, a round sample was clamped in the bottom ring-shaped holder (22-mm diameter). From above, the material was pierced using an exchangeable penetrator with a rounded tip (1-mm in diameter). Finally, maximal piercing force of the films was evaluated.

3.11. Antioxidant activity

3.11.1. Antioxidant activity of lingberry extract

The DPPH radical assay was applied to estimate the radical-scavenging ability of the fruit extracts according the method described by (Drozdz, Sezine, & Pyrzyńska, 2017). Lingonberry extract in the amount of 0.1 mL was mixed with 2.4 mL of DPPH radical solution (9 × 10−5 mol/L) in methanol. After 30 min, the change in absorbance at 517 nm was recorded (Spectrophotometer: Helios Gamma, Thermo Fischer Scientific, USA) and then compared with blanks in which the fruit extract had been replaced by distilled water. The results are expressed as % of inhibition.

Ferric Reducing Antioxidant Power (FRAP) was evaluated following the usual procedure but with slight modification (Benzie & Strain, 1996). The lingonberry extract was diluted at 10 mg/mL in a water solution. The solution comprised an acetate buffer (pH 3.6), a ferric chloride solution (20 mM) and a 2,4,6-tripyridyl-s-triazine solution (10 mM TPTZ in 40 mM HCl) at a ratio of 10:1:1 (v/v/v), respectively. Firstly, the FRAP solution was incubated in the dark at a temperature of 37 °C for 5 min, and was then mixed with a film extract at the ratio of 0.4:3.6 (v/v). Measurements were performed on a Helios Gamma UV-1601 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 593 nm after 4 min of incubation.

Total phenolic content of extracts was assessed by using the Folin–Ciocalteu phenol reagent method (Cliff, Fawer, Maier, Takata, & Ritter, 1994). The extract – in the amount of 0.1 mL - was mixed with 0.1 mL of the Folin–Ciocalteu reagent and 9.0 mL of water. After 5 min, 1 mL of 7% (w/v) Na2CO3 and 0.4 mL of water were added. The extracts were mixed and allowed to stand for 30 min before measuring the absorbance on a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 765 nm. A mixture of water and reagents was used as a blank. Total phenolic content was expressed as gallic acid equivalents.

3.11.2. Antioxidant activity of film extract

The film extracts (10 mg/mL) were prepared by adding films in the amount of 100 mg to 10 mL of distilled water, further heated up to 45 °C. Tubes containing the film extracts were put in a 50 °C water bath at a temperature of 50 °C with shaking action for 10 min to ensure complete dissolution of the films. The extracts prepared in such a manner were then used for Ferric Reducing Antioxidant Power Assay (FRAP) and (2,2-diphenyl-1-picryl-hydrazyl-hydrate) evaluation using the free radical method (DPPH radical). In order to determine FRAP, its solution had been freshly prepared just before analysis. The solution comprised an acetate buffer (pH 3.6), a ferric chloride (20 mM) and a 2,4,6-tripyridyl-s-triazine solution (10 mM TPTZ in 40 mM HCl) at a ratio of 10:1:1 (v/v/v), respectively. Firstly, the FRAP solution was incubated in the dark at a
temperature of 37 °C for 30 min, and was then mixed with a film extract at a ratio of 0.4:3.6 (v/v). The solution was incubated once more at 37 °C for 10 min in dark conditions, and following was absorbance measurement at 593 nm via the Helios Gamma UV-1601 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For DPPH assay, the film extract was mixed with 0.1 mL DPPH. This was done in an ethanol solution at a ratio of 0.2:2.8 (v/v). Following, the mixture was incubated in the dark for a period of 30 min. Afterwards, the solution absorbance was evaluated at 517 nm (Helios Gamma, Thermo Fisher Scientific, USA) and then compared with the blanks in which the film extract had been replaced by distilled water. The results are expressed as % of inhibition. The analyses were performed in duplicate for 3 samples of the films (n = 2 × 3).

3.12. Antimicrobial and antifungal measurements

3.12.1. Microorganisms

In this study, 9 microorganisms coming from the American Culture Collection were used and deposited at the Department of Microbiology (UJCM, Cracow, Poland). These were the following: Candida albicans ATCC 10231, Candida krusei ATCC 6258, Aspergillus brasiliensis ATCC 204304, Aspergillus flavus ATCC 16404, Escherichia coli ATCC 25923, Enterococcus faecalis ATCC 29212, Pseudomonas aeruginosa ATCC 27869, Staphylococcus aureus ATCC 25922 and Salmonella enterica BAA664. The microorganisms were taken from the Mycology Department of UJCM in Kraków, Poland.

3.12.2. Antimicrobial properties

On 10 ml of an agar medium, 1-cm x 1-cm film samples were placed aseptically (Mulier Hinton 2 for bacteria and Sabouraud Dextrose Agar for yeasts). As a consequence, a liquefied medium comprising a standard microorganism suspension (100 mL of 0.5 McFahrland inoculum per 10 mL) was poured onto them, which allowed solidification and incubation at 37 °C for a 24-h period. Visual analysis was carried out in order to evaluate microorganism growth in the areas around and over the films. This concrete analysis was carried out in triplicate.

3.13. Evaluation of cherry tomato storage quality

3.13.1. Cherry tomato treatments

Evaluation of active packaging effectiveness was conducted on the example of cherry tomatoes. The tomatoes were wrapped in each type of the examined fruit were analysed on given days.

3.13.2. Weight loss ratio

The weight loss of cherry tomatoes was determined in accordance with the given formula (Eq. 4), in which Wa and Wt signify baseline and cherry tomato weight, respectively and, on a concrete day of storage. Assessment of quality for each sample was examined at the end of every 3rd day.

weight loss (%) = \( \frac{W_0 - W_t}{W_0} \times 100 \)

3.13.3. Determination of total phenolic content

For preparing the extracts, 2.5 g of homogenised tomato samples were weighed, with the addition of 20 mL aqueous ethanol (70%, v/v). All of the sample extracts were kept in an ultrasonic water bath (Polonic, Palczynski, Warsaw, Poland) for a duration of 30 min in order to make the mixtures homogenous and then, to extract polyphenols. Following, the mixture was filtered and poured into volumetric flasks (50 mL), and the volume adjusted using aqueous ethanol. These samples were used for further analyses. The Folin-Ciocalteu method was implemented in order to determine total phenolic content of the tomatoes (Katrci et al., 2020). Clear extracts (0.5 mL) were transferred to test tubes and a Folin-Ciocalteu agent (2.5 mL) was added to each of them. After the passage of 3 min, 2 mL of the Na2CO3 (20%) solution was added, the mixture maintained in the dark for 2 h. Ultimately, absorbances were obtained at a 760-nm wavelength using the Helios Gamma UV-1601 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Gallic acid was applied as a standard for the calibration curve, while TPC was given as milligrams of gallic acid equivalents per litre of the extract (mg GAE/L).

3.13.4. Colour determination of tomatoes

The tested tomato surface colour was evaluated using the CR 200 Minolta chromameter (Konica Minolta, Osaka, Japan), and was further presented as L* (lightness), a* (redness) as well as b* (yellowness). For colour evaluation, 5 cherry tomatoes were selected by performing 3 readings on 2 opposite sides of the equatorial region.

3.14. Statistical analysis

Statistical analyses were carried out using the PQStat 1.8.2.142 statistical package. The Tukey HSD post-hoc test and one-way analysis of variance were carried out on the results of the scales being tested. This was done depending on film type. The Fisher LSD linear trend was also assessed. The test probability at the level of p < 0.05 was considered significant, while at the level of p < 0.01, it was assumed as highly significant.

4. Results

4.1. SEM micrographs

The morphology of completely new, innovative biopolymer films based on furcellaran (FUR) and carboxymethyl cellulose (CMC), prepared with varying concentrations of lingonberry extract (LBE), were evaluated by scanning electron microscopy. The SEM images from the on-top view and the cross-section of the FUR/CMC composite samples confirmed double-layered conformation of the films. In Fig. 1, it is shown that each biopolymer film is composed of a thick furcellaran coating (approx. 150–200 µm), covered by a very thin layer (not exceeding 1 µm), formed by a mixture of carboxymethyl cellulose (CMC) and gelatin hydrolysate (HGEL). Both FUR and the layers enriched with the lingonberry extract (FUR + LBE) were stable, smooth and homogeneous (Jamroz, Kulawik, & Kopel, 2019), which makes them the main platform of the packaging material.

The second CMC + HGEL layer, maintained as the outer part of the film, is continuous, as indicated by the on-top view presented in Fig. 1. The visible platelet aggregates, flakes and delamination of the top layer of the film are the result of sample preparation necessary to determine their presence and thickness. SEM analysis revealed homogeneous distribution of the coating constituents through the thickness of the composite. The addition of LBE to the FUR layer made the films more homogeneous. Interactions between the functional groups of active LBE ingredients and the film components could increase compatibility between the FUR and CMC + HGEL layers. Yao et al. (2021) reached similar conclusions, adding red pitaya flesh extract (RPFE), prickly pear fruit extract (PPFE), red beetroot extract (RBRE), globe amaranth flower extract (GAFE) and red amaranth leaf extract (RALE) to starch/polyvinyl alcohol.

4.2. FT-IR analysis

The FT-IR spectra of single- (FUR + x% LBE and CMC+HGEL) and the double-layered films (FUR + x% LBE/CMC+HGEL, where x = 10, 20, 30, 40, and 50) were obtained using a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The FT-IR spectra were obtained using 32 scans at a resolution of 4 cm⁻¹, and the spectra were transformed by converting the wavenumber data to wave number (cm⁻¹). The obtained spectra were compared with the starting spectra of the individual biopolymers, and the effect of adding the active LBE ingredient was observed. The obtained spectra were also compared with the starting spectra of active LBE and biopolymer layers.
20, 40) are presented in Fig. 2A.

Within the range of 1800–100 cm⁻¹, the spectrum of pure monolayered CMC + HGEL exhibits an intensive complex band with vibration modes at 1680 cm⁻¹, 1615 cm⁻¹, 1475 cm⁻¹, 1430 cm⁻¹, 1335 cm⁻¹, 1130 and 1080 cm⁻¹. The bands at 1475 and 1615 cm⁻¹ are attributed to symmetric and asymmetric vibration of the -COO group present in the CMC constituent of that layer (Esteghlal, Niakousari, & Hosseini, 2018). The adsorption bands at 1680, 1615 cm⁻¹ and 1335 cm⁻¹ correspond to (mainly CO stretch), amide-II (NH bend coupled with CN stretch) and amide-III, respectively in amino acid groups of gelatin hydrolysates (Jancikova et al., 2019).

Deposition of the CMC + HGEL layer onto the FUR film results in the appearance of new bands at 1750 cm⁻¹ and 1280 cm⁻¹ in the spectrum, attributed to the FUR and LBE components. The band at 1280 cm⁻¹, observed both in the FT-IR spectra of FUR+20% LBE and FUR + x% LBE/CMC + HGEL series, is attributed to the stretching vibration of pyran rings, typical of flavonoid compounds (Gutierrez, Ponce, & Alvarez, 2017; Pereira Jr, de Arruda, & Stefani, 2015), and confirm the lingonberry extract in all prepared films.

The double-layered films of FUR + x% LBE/CMC + HGEL exhibit a more complex band composed of several units corresponding to both CMC + HGEL and FUR layers. Therefore, in double-layered films of FUR + x% LBE/CMC + HGEL, the bands at 1750 cm⁻¹, 1680 cm⁻¹, 1615 cm⁻¹, 1475 cm⁻¹ and 1430 cm⁻¹, as well as 1335 cm⁻¹, 1280 cm⁻¹,
1166 cm\(^{-1}\), 1130 cm\(^{-1}\) and 1080 cm\(^{-1}\) are present. This can be attributed to the stretching vibration of C=O, stretching mode of carboxylic groups, C–C stretching due to aromatic ring deformations, COO\(^{-}\) asymmetric stretching, COO\(^{-}\) symmetric stretching and CH\(_2\) symmetric bending vibrations, respectively. These are characteristic groups in the polyphenolic compounds found in berries (Cheikh, Martín-Sampedro, Majdoub, & Darder, 2020; Tuberoso et al., 2010).

Fig. 2. (A) FT-IR spectrum, (B) UV–Vis of active double-layered films.

### Table 1

The physical properties of tested double-layered biopolymer films.

| Characterisation of films | Types of double-layered films |
|---------------------------|-------------------------------|
|                           | FUR/CMC + HGEL | FUR + 10% LBE/CMC + HGEL | FUR + 20% LBE/CMC + HGEL | FUR + 40% LBE/CMC + HGEL |
| **COLOUR PARAMETERS**     |                 |                          |                           |                            |
| L*                        | 90.67 \(\pm\) 0.31 | 83.96 \(\pm\) 0.40       | 77.85 \(\pm\) 0.55       | 65.07 \(\pm\) 0.49        |
| a*                       | \(-0.56\) \(\pm\) 0.05 | 2.80 \(\pm\) 0.25        | 7.34 \(\pm\) 0.45        | 18.41 \(\pm\) 0.39        |
| b*                       | 14.97 \(\pm\) 0.56 | 21.13 \(\pm\) 0.37       | 32.68 \(\pm\) 0.64       |
| \(\Delta E\)             | 19.99        | 40.46                   |                           |                            |
| Appearance                |                 |                          |                           |                            |
| Opacity                   | 0.05 \(\pm\) 0.00 | 0.13 \(\pm\) 0.03        | 0.24 \(\pm\) 0.05        | 0.41 \(\pm\) 0.04        |
| **THERMAL PROPERTIES**    |                 |                          |                           |                            |
| Peak temperature (Tm) (°C) | 193.4 \(\pm\) 0.4 | 201.0 \(\pm\) 0.4        | 204.2 \(\pm\) 0.6        | 198.2 \(\pm\) 0.6        |
| Enthalpy (\(\Delta Hm\)) (J/g) | 85.40 \(\pm\) 0.78 | 110.60 \(\pm\) 2.59      | 123.0 \(\pm\) 5.6        | 164.1 \(\pm\) 9.89      |
| Enthalpy (\(\Delta Hm\)) (J/g) | 85.40 \(\pm\) 0.78 | 110.60 \(\pm\) 2.59      | 123.0 \(\pm\) 5.6        | 164.1 \(\pm\) 9.89      |
| WATER PROPERTIES          |                 |                          |                           |                            |
| Water vapour transition rate (WVTR) (g/m\(^2\)d) | 867.79 \(\pm\) 0.87 | 957.47 \(\pm\) 0.59      | 852.00 \(\pm\) 0.76      | 866.01 \(\pm\) 0.87      |
| Water contact angle (°)    | 82.12 \(\pm\) 1.31 | 78.73 \(\pm\) 2.08        | 75.64 \(\pm\) 1.64       | 69.80 \(\pm\) 0.60       |
| 2nd layer (CMC + HGEL)     | 83.50 \(\pm\) 2.60 | 74.93 \(\pm\) 0.74        | 66.74 \(\pm\) 1.48       | 54.66 \(\pm\) 0.94       |
| **MECHANICAL PROPERTIES**  |                 |                          |                           |                            |
| Max. breaking load (MBL) [N] | 19.41 \(\pm\) 1.14 | 18.19 \(\pm\) 1.97        | 15.29 \(\pm\) 2.31       | 13.67 \(\pm\) 1.33       |
| Tensile strength (TS) [kN/m] | 1.29 \(\pm\) 0.08 | 1.21 \(\pm\) 0.13         | 1.02 \(\pm\) 0.15        | 0.91 \(\pm\) 0.09        |
| Elongation at break (EAB) [%] | 40.67 \(\pm\) 2.03 | 43.86 \(\pm\) 5.35        | 50.46 \(\pm\) 5.32       | 73.03 \(\pm\) 7.48       |
| Modulus of elasticity (ME) [MPa] | 152.87 \(\pm\) 27.37 | 131.27 \(\pm\) 15.17      | 67.87 \(\pm\) 15.72      | 36.25 \(\pm\) 4.74       |
| Puncture Strength (PS) [N] | 1.95 \(\pm\) 0.09 | 2.25 \(\pm\) 0.43         | 2.23 \(\pm\) 0.24        | 1.91 \(\pm\) 0.15        |
| **ANTIOXIDANT ACTIVITY**   |                 |                           |                           |                            |
| FRAP Ferric Reducing Antioxidant Power [mM Trolox/mg] | 3.01 \(\pm\) 0.47 | 14.41 \(\pm\) 2.24        | 22.51 \(\pm\) 0.75       | 41.89 \(\pm\) 2.24       |
| DPPH radical scavenging activity [%] | 0.00 \(\pm\) 0.00 | 24.61 \(\pm\) 2.40        | 18.22 \(\pm\) 1.24       | 20.56 \(\pm\) 0.93       |

*Values are expressed as mean \(\pm\) SD. Different lettering in the same rows indicate significant differences (\(P < 0.05\)).
4.3. UV-vis and color analysis

It has been observed that double-layered films without LBE are a poor barrier to UV light (Fig. 2B), which is in line with expectations as neither FUR nor CMC films have the ability to absorb UV light. In contrast, films with the addition of LBE showed a barrier to UV, and the barrier effect itself can be attributed to the presence of phenolic components (Bonilla, Talón, Atarés, Vargás, & Chiralt, 2013). There were slight shifts between the UV curves of the films with different LBE additive concentrations, which may have resulted from differences in the pH of these films (control films - pH 6.85 and films with 10% LBE - pH 5.70; 20% LBE - 5.18 and 40% LBE - pH 4.51). The colour responses and the corresponding peak shifts may be correlated with the transformation of the anthocyanin structure which is presented in the LBE extract (Kitryte et al., 2020). Different anthocyanins indicate a colour reaction to changes in pH. At a pH of 4-6, anthocyanins are dominated by 4 structural forms: flavylic cation, anhydrous chinoid base, colourless carbolín base and light yellow chalcone. At pHs of 5 and 6, formation of colourless pseudo-base carbolin and chalcone base can be observed (Castañeda-Ovando, Pacheco-Hernández, Pérez-Hernández, Rodríguez, & Galán-Vidal, 2009). Small peak shifts may be related to different pH values of specific LBE-added films. FUR/CMC + Hgel films were characterised by high transparency, while after adding the extract, the films became less transparent and a red colour of varying intensity was obtained depending on the extract content. Visual assessment allows to confirm that with increasing concentration of the lingonberry extract, the colour of the presented films becomes more intense and darker. The lack of transparency does not mean that this type of film can be eliminated from the use of products with a short life-span. The rectangular coordinates (L*, a* and b*) and the total colour difference (ΔE) of films are shown in Table 1. After incorporating the extract, its concentration increased while L* decreased significantly, which is related to darkness of the films. Moreover, with the increase in LBE concentration, parameters a* and b* increased significantly, which indicates a tendency towards redness and yellowing. The results obtained are consistent with the visual observations (Table 1). The effect of adding LBE on the opacity of the FUR/CMC + Hgel films is shown in Table 1. The FUR/CMC + Hgel films were highly transparent and without any other colour; however, change in colour was observed with the addition of LBE onto the 1st layer. As the concentration of LBE increases, the opacity of the FUR/CMC + Hgel film improves the effectiveness of blocking visible light. A higher value of this parameter indicates higher opacity and a lower degree of transparency (Du et al., 2021). Plant extracts can increase the opacity of films due to their colour resulting from the presence of colouring LBE, which significantly contributes to the reduction of light transmission (Munir, Hu, Liu, & Xiong, 2019). Additionally, in plant extracts, selective light absorption of polyphenolic compounds occurs at low wavelengths, which is related to the presence of red colour in films (Fabra, Falco, Randazzo, Sánchez, & López-Rubio, 2018). The natural colour of lingonberry can provide an appropriate barrier against UV radiation in films. Moreover, the greater participation of plant extracts in the food industry eliminates the excess of synthetic dyes. There are many studies in which the effects of adding plant extracts on the colour and transparency of the films are investigated (Moraczewski et al., 2020). Improving colour properties and reducing the tendency of UV transmittance in films with plant extracts would be useful for protecting food against discoloration, unpleasant taste and loss of nutrients (Arafat, Ahmed, Hiremath, Auras, & Joseph, 2017). Too high concentrations of extracts, which are ingredients of films, may change the colour of the coated food product and limit its use as packaging materials for food (Kulawiak, Jamróz, Zając, Guzik, & Tkaczkowska, 2019). In addition, reducing the transparency of the films applied to the product may result in decreased customer acceptance of the films (Zhang, Shu, Chen, Cao, & Jiang, 2019).

4.4. DSC analysis

DSC analysis was used to measure the peak melting point (Tm) and enthalpy parameter (ΔH) of the LBE-added two-layer films (Table 1 and in Suppl. Figure 1). As the concentration of LBE increased, the ΔH values of the films also increased, indicating an increasing tendency in thermal stability (Table 1). The rise in ΔH of the tested films may result from greater interaction between the FUR matrix and the phenolic compounds of the extract (Jamróz, Kulawiak, Krzyściak, Talaga-Cwiertnia, & Juszczyk, 2019). This phenomenon may be due to the formation of covalent bonds that restrict the movement of the films and make it stronger. The reinforced film structure had higher thermal stability, which required more energy to destroy interactions between molecules (Kawprapru, Rungraeng, Osako, & Rawdthuen, 2017). Endothermic transition at a temperature of about 193.4 °C may be related to decomposition of the polymer structure (Hosseini, Rezaei, Zandi, & Parahmandgahi, 2015). Moreover, the peak around 213 °C implied depolymerisation of plant extracts in the composite films (Peng, Wu, & Li, 2019).

4.5. Water behaviour

Compared to FUR films, making double-layered films did not improve the WVTR parameter (Jamróz, Khachatryan, et al., 2020). However, the addition of LBE to the films did not significantly change the WVTR parameter (Table 1). Bonilla and Sobral (2016) and Li et al. (2014) came to similar conclusions by adding plant extracts to biopolymer films. The water contact angle (WCA) of tested films can determine the water-based nature of the film. As shown in Table 1, the value of the contact angle regarding pure FUR exceeded the value of 82.12°. FUR films are hydrophilic (approx. 55°) (Jamróz, Janik, et al., 2021), while covering FUR with CMC + HGel (FUR/CMC + HGel film) slightly increases this value, indicating hydrophilic properties of the films. Thus, double-layered films are characterised by reduced hydrophilicity compared to single-layered ones. Addition of the extract to the FUR layer affected both the 1st and 2nd layers of the film, causing a decrease in the WCA value, which translated into increased hydrophilicity of the film. The reason for such behaviour may be found in the hydrophilic groups of the extract, which improve the interaction of the film with water molecules, resulting in a reduction of the measured contact angles (Sun et al., 2017).

4.6. Mechanical properties

Double-layer films (FUR/CMC + HGel) have better mechanical properties (TS, ME and EAB) than furcellaran films (Jamróz, Janik, et al., 2021; Jamróz, Khachatryan, et al., 2020). In Table 1, results on the mechanical strength of double-layer films are presented. It has been noted that with the increase in the addition of the extract, maximum breaking load (MBL), tensile strength (TS) and modulus of elasticity (ME) values decrease, while the EAB values undergo an increase. This phenomenon can be attributed to the reduction of potential intermolecular interactions between extract components and furcellaran molecules, resulting in a more flexible structure. Kammani and Rhim (2014) reached similar conclusions. These researchers added grapefruit seed extract (GSE) to a carrageenan film and noticed a decrease in TS value, which was motivated by a decrease in molecular interactions between the carrageenan strands and GSE. The addition of green tea extract to agar films also lowered the TS parameter, which may be due to a reduction in intermolecular interaction between the agar polymer strands caused by the incompatible extraneous materials in the polymer matrices (Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013). Reducing the interaction may induce the formation of discontinuities in the structure of the formed layers which, in turn, leads to changes in mechanical resistance results. Thus, it may be suggested that the extract acts as a plasticiser increasing the molecular mobility of
the biopolymer chains (Bajić, Jalsovec, Travan, Novak, & Likozar, 2019). In addition, the mechanical properties of the film depend on the type of biopolymer matrix produced, the type of additives as well as the method and conditions of film production (Jamroz, Kulawik, & Kopeł, 2019).

4.7. Antioxidant activity

Antioxidant packaging is one of the main types of active packaging that can extend the shelf-life of food products. Antioxidant properties of all the films were evaluated using the free radical scavenging test (DPPH) and FRAP assay, the results of which are shown in Table 1. The matrix without the addition of LBE was characterised by relatively good antioxidant properties measured via the FRAP method (3.01 μM Trolox/mg sample) and no free radical scavenging ability. According to our previous results (Tkaczewska, Bukowski, & Mak, 2019), the antioxidant activity of carp skin gelatin hydrolysates (which are one of the film components), measured by DPPH assay and the FRAP method, was quite high (a hydrolysate concentration of 50 mg/ml: 23.76% and 2.65 μM Trolox/mg sample respectively). No free radical scavenging ability of the film matrix may be explained by the interactions established between the film component and the active molecules in the film, which limited their release (Kehou, Jridi, Benbettaieb, Debeaufort, & Narsi, 2020). In our previous research, a reduction was also found in the antioxidant properties of the film with the carp skin gelatin hydrolysate compared to the pure hydrolysate (Jamroz, Kulawik, et al., 2021a).

The extract from lingonberry was characterised by high antioxidant properties. The iron ion reduction capacity for the pure extract was 21.00 ± 0.05 μM Trolox/mg, while the free radical scavenging capacity was 36.21% ± 1.19 (10 mg/1 ml extract concentration). The content of polyphenols in the tested extract at the concentration of 10 mg/ml was 0.458 ± 0.005 gallic acid mg/ml. The addition of LBE to one of the film layers caused a multiple increase in the antioxidant properties of the obtained coatings. The antioxidant activity of lingonberry fruit extract is influenced by derivatives of benzoic acid, flavanol oligomers and anthocyanins. According to the results of radical scavenging, in the reducing and chelating activity of lingonberry phenolic compounds, Raudone et al. (2019) obtained proanthocyanidins, which include epitocatechin units in their structure, and can be characterized by exceptionally strong antioxidant properties.

Along with the increase in LBE concentration of the coatings, an increase in their antioxidant properties measured by the FRAP method was observed (14.41, 22.51 and 41.89 μM Trolox/mg sample, respectively). Such a relationship was not observed when testing the antioxidant properties of coatings using the DPPH method. Surprisingly, the highest ability to scavenge DPPH free radicals was characteristic for coatings with a 10% addition of the lingonberry extract. It can be assumed that this was caused by the different pH of the film with LBE addition. The pH of the FUR+10% LBE/CMC + HGEL film was 5.70, while the pH of the FUR+40% LBE/CMC + HGEL film was characterised by higher acidity and totalled 4.51. According to Dawidowicz, Wia-nowska, and Olszowy (2012), hydrogen ion concentration affects the antioxidant properties of compounds measured by the DPPH method. It should be stressed that the influence of pH on the antioxidant properties of compounds is not yet fully recognised, nonetheless, it can be assumed that the increase in hydrogen ion concentration slows down DPPH/antioxidant reaction kinetics.

The differences in the antioxidant properties of extracts from the same type of plant matrix can result not only from their different pH, but also from differences in the metal ion types and their concentration in the examined extracts. As it results from the literature (Dawidowicz et al., 2012), DPPH as well as numerous compounds exhibiting antioxidant activity are able to form complexes with metal ions. Thus, the increase in metal ion concentration causes deceleration of the DPPH/antioxidant reaction kinetics.

On the basis of the obtained results regarding antioxidant properties of the films, it can be concluded that the lingonberry extract is a good active additive which provides the obtained coatings with antioxidant properties.

4.8. Antimicrobial activity

The antimicrobial activity of all the tested films was examined and the results are presented in Table 2. Control films and films with the addition of 10% and 20% LBE did not show any antimicrobial effects.

The CMC + HGEL/FUR+40% LBE films showed antimicrobial activity against gram-negative and gram-positive bacteria and fungi (Table 2). The performed tests revealed that exceeding a certain threshold value of lingonberry extract in the double-layered film can contribute to antibacterial activity, which is related to the release of the LBE and/or its derivatives from the film matrix, and further diffusion into the agar medium. According to (Holopainen, Jahodar, Seppänen-Laakso, Laakso, & Kauppinen, 1980), this activity is due to the presence of phenolic glycoside-arbutin and methylarbutin. Moreover, lingonberry is rich in benzoic and tannic acids (Puupponen-Pimiä et al., 2001), which demonstrate antimicrobial activity. Kylli et al. (2011) reports that lingonberry fruit extracts mainly contain proanthocyanidins (procyanidin dimer II, procyanidin trimer), and this ingredient may have antimicrobial activity against S. aureus or can inhibit the haemagglutination of E. coli. The tested films did not show any antifungal activity, which may be related to the lack of ellagitannins described as the main antimicrobial compounds against these microorganisms (Nohnyn et al., 2006).

4.9. Application on model food - cherry tomatoes

The usage of coatings during the storage of fruits and vegetables reduces respiration, water loss and oxidative reactions. This has a positive effect on the quality and appearance of the stored fruits. Due to the need to reduce the amount of synthetic packaging, in recent years, a growing interest has been observed with regard to biodegradable, edible coatings for fruit and vegetable storage, which will additionally extend their shelf-life, without adversely affecting their quality (Salehi, 2020).

Weight loss is an important index of post-harvest storage life in fresh products. This is primarily due to the loss of water during metabolic processes such as transpiration or respiration. Both processes are affected by storage environment of the fruit and the loss in weight is an indicator of how the product is handled and stored. For this reason, physiological weight loss appeared to be a major detrimental factor in the storage life and quality of tomato fruits (Abebe & Tola, 2017). Tomatoes covered with biodegradable coatings showed significantly higher weight loss during the entire storage period compared to fruits covered with synthetic films (Fig. 3B). The synthetic film was an effective barrier for the migration of water from the fruit to the external environment, while the designed biopolymer films were water-permeable, which was confirmed via WVT and WCA testing (Table 1). A significant loss of water in the fruit covered with the designed coatings was also noted when assessing their external appearance (Fig. 3A). The tomatoes covered with biopolymer coatings had a more wrinkled surface from day 11 of storage, compared to the fruit covered with synthetic films. Similar results was obtained by Yun et al. (2015). These authors also concluded that tomatoes with coatings having the addition of mustard essential oil had the lowest appearance scores after 21 days of storage.

In this study, a significant difference in total phenolic content of the tomato fruit was observed due to the different coatings applied. After the 3rd day of storage, higher values of total phenolic content were observed in fruits coated with films having the addition of lingonberry extract. The opposite trend was followed after the 11th and 17th days of storage. On those days, tomatoes covered by coatings with 10%, 20% and 40% of the lingonberry extract contained significantly less polyphenols than fruits stored in synthetic films or in coatings without the addition of the
Table 2
The antimicrobial effect of double-layered films based on FUR, CMC, HGEL and LBE.

|                      | FUR/CMC + HGEL | FUR+40% LBE/CMC + HGEL |
|----------------------|----------------|-------------------------|
| **Escherichia coli ATCC 25923** | ![Image](image1) | ![Image](image2) |
|                      | No effect, liquefaction of film | good effect, 90% growth reduction \*visible overgrowth is due to photo taken after 48h |
| **Enterococcus faecalis ATCC 29212** | ![Image](image3) | ![Image](image4) |
|                      | No effect with liquefaction of film | Very good effect with inhibition zone (2 mm from the film border) |
| **Staphylococcus aureus ATCC 25922** | ![Image](image5) | ![Image](image6) |
|                      | No effect with liquefaction of film | Very good effect with inhibition zone (2 mm from the film border) |
| **Salmonella enterica BAA664** | ![Image](image7) | ![Image](image8) |
|                      | No effect with liquefaction of film | Very good effect with inhibition zone (2 mm from the film border) |

(continued on next page)
|                                                        | FUR/CMC + HGEL | FUR + 40% LBE/CMC + HGEL |
|--------------------------------------------------------|----------------|-------------------------|
| **Pseudomonas aeruginosa** ATCC 27859                   | No effect      | Good effect             |
*patches visible on pictures are due bacteriophage infection of test strain
| **Candida albicans** ATCC 90028                         | No effect      | Very good effect        |
| **Candida krusei** ATCC 6258                            | No effect      | No effect               |
|                                                        | No effect      | No effect, slightly reduction of colony number |
| **Aspergillus brasiliensis** ATCC 204304                | No effect      | No effect, alteration of colony morphology |

(continued on next page)
According to the data from literature, fruits could perceive coating materials as a potential abiotic stress, thus resulting in the production of secondary metabolites alike phenols in coated samples (Abebe & Tola, 2017). In previous studies, it was also shown that low O$_2$ and elevated CO$_2$ concentrations increased the production of phenolic compounds during the storage of fresh-cut melons, which was related to oxidative stress on the fruit (Oms-Oliu, Soliva-Fortuny, & Martín-Beloso, 2008). On the basis of the polyphenol content results, it may be assumed that tomatoes covered with synthetic film and the control film without the addition of lingonberry extract matured faster, which resulted in an increase in the amount of polyphenols contained in them, compared to fruits coated with the lingonberry extract. The obtained results are consistent those obtained by Khatri, Panigrahi, Prajapati, and Bariya (2020), who found a lower content of polyphenols and slower maturation of tomatoes stored in chitosan coatings with the addition of A. vera gel, compared to the control samples.

Colour is a very important indicator of ripening as well as a determinant of quality and consumer acceptability of fruit (Abebe & Tola, 2017). There was no significant difference ($p > 0.05$) in the value of the L$^*$ parameter, determining the brightness of the colour on the 1st, 3rd and 6th days of storing tomatoes in different coatings. However, significant differences were found in the brightness of the colour on consecutive days of fruit storage, while on days 11 and 17, a similar trend was observed - the lowest value of the L$^*$ parameter was found for tomatoes stored in synthetic films, while the highest was found for the fruit stored in the control coating without the addition of lingonberry extracts (Table 3). The darker colour of tomatoes covered by the synthetic coating could indicate their higher polyphenol and lycopene content. No observations were noted as to whether the amount of lingonberry extract added to the coatings had any effects on the brightness of the tomatoes stored in these coatings throughout the storage period.

According to the data from literature, the changes in tomato colour are generally reported by parameters a* and b* (da Costa de Quadros et al., 2020). The value of the a* parameter (characterising the intensity of red colour) for tomatoes stored in different coatings was significantly different during the entire period of fruit storage. However, no clear trend concerning changes in this parameter was noted depending on the coating used. The value of the b* parameter (characterising the intensity of the yellow colour) for tomatoes stored in different coatings was significantly different on the 1st, 11th, 14th and 17th days of storage. During the final storage period (days 11, 14, 17), the fruits stored in the synthetic coating had the lowest intensity, while the fruits stored in the control coating without the extract and with 10% addition of lingonberry extract, were the highest. The maturation of tomatoes is predominantly based on colour, with unripe tomatoes being green due to the high chlorophyll content, which degrades to yellow constituents, such as β-carotene and xanthophyll as the tomato matures, finally becoming red due to lycopene (Yang Berbeza, 2020). On the analysis of the colour parameters regarding the tested tomatoes, it may be assumed that the fruit can be obtained in films with a higher degree of maturity after 17 days than in the case of tomatoes coated with biodegradable films.

5. Conclusions

Double-layered films, based on furcellaran (FUR) as well as a mixture of carboxymethyl cellulose (CMC) and gelatin hydrolysates (HGEL), were successfully obtained, which was confirmed by cross-section of SEM micrographs. The 1st layer was enriched with a lingonberry extract, which induced antimicrobial and antioxidant activity of the film. The lingonberry extract caused a reduction in mechanical properties, while positively influencing flexibility of the film. Due to the presence of active ingredients in the extract, an increase in antimicrobial activity against gram-positive and -negative bacteria was observed. Moreover, the films exhibited antioxidant activity as measured by the FRAP and DPPH tests.

Due to their biological properties, double-layered films have been used...
as active packaging materials for cherry tomatoes. Because of high water vapour permeability, tomatoes packed in active films lost a lot of weight during storage. On the basis of the results for polyphenol content, it may be assumed that tomatoes covered by synthetic and control films, without the addition of LBE, matured faster, which further resulted in an increase in the amount of polyphenols contained in them when compared to fruits covered with films having the addition of lingonberry extracts. The obtained results indicate the developmental nature of the work, thus, it is necessary to study the influence of active packaging materials on the storage of other types of food products.

Fig. 3. Visual appearance (A), weight loss (B) and total phenolic content (C) of tomato storage with the different films.

Table 3
The colour parameter of tomato storage with the different films.

| Day   | LDPE | FUR/CMC + HGEL | FUR+10% LBE/CMC + HGEL | FUR+20% LBE/CMC + HGEL | FUR+40% LBE/CMC + HGEL |
|-------|------|-----------------|------------------------|------------------------|------------------------|
| Day 1 | L*   | 33.68±0.15      | 33.07±2.74             | 32.66±0.40             | 33.16±0.36             | 32.93±0.28             |
|       | a*   | 19.94±2.19      | 20.84±1.30             | 18.98±1.26             | 20.11±1.51             | 19.24±2.17             |
|       | b*   | 16.80±1.87      | 17.87±1.60             | 15.60±0.64             | 16.51±0.71             | 15.77±0.83             |
| Day 3 | L*   | 33.55±0.60      | 29.28±10.08            | 32.13±0.27             | 33.14±0.52             | 27.98±9.48             |
|       | a*   | 21.95±1.12      | 20.63±3.05             | 18.48±1.28             | 20.69±1.18             | 19.73±1.97             |
|       | b*   | 16.58±0.63      | 15.56±2.56             | 15.05±0.36             | 16.39±0.82             | 15.95±1.13             |
| Day 6 | L*   | 33.09±0.60      | 32.70±0.75             | 33.27±0.49             | 32.90±0.53             | 33.02±0.74             |
|       | a*   | 20.90±2.03      | 20.32±0.96             | 21.48±0.92             | 21.39±1.26             | 19.36±2.20             |
|       | b*   | 16.37±1.45      | 15.90±0.91             | 16.62±1.00             | 16.68±0.90             | 16.11±1.53             |
| Day 11| L*   | 31.40±1.14      | 33.89±0.72             | 33.38±0.54             | 32.86±1.12             | 32.62±0.58             |
|       | a*   | 17.71±1.24      | 23.30±2.25             | 22.62±1.16             | 22.20±1.55             | 20.46±2.49             |
|       | b*   | 14.14±1.09      | 17.03±1.68             | 16.98±0.82             | 16.69±1.57             | 16.48±0.88             |
| Day 14| L*   | 32.14±0.76      | 33.57±0.72             | 33.69±0.66             | 32.24±0.26             | 33.22±0.55             |
|       | a*   | 19.52±1.22      | 23.11±1.94             | 23.08±2.39             | 22.04±1.29             | 22.43±1.33             |
|       | b*   | 15.19±0.9       | 16.99±1.79             | 17.26±1.52             | 15.29±0.59             | 17.09±1.41             |
| Day 17| L*   | 32.90±0.6       | 34.26±0.74             | 33.84±1.45             | 33.55±0.59             | 33.19±0.85             |
|       | a*   | 21.88±1.81      | 24.92±1.73             | 22.81±2.76             | 22.46±1.37             | 21.47±1.80             |
|       | b*   | 16.07±0.9       | 18.08±1.30             | 17.85±2.47             | 16.58±0.91             | 16.41±1.02             |
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