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CHARACTERISTICS OF EDIBLE CHITOSAN PACKAGING WITH ADDED ELDERBERRY POLLEN

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ABSTRACT

The aim of this study was to characterize properties of edible chitosan packaging with the addition of elderberry pollen. Experimentally produced edible packaging contained 0.075 g; 0.15 g and 0.3 g of elderberry pollen. The results demonstrate the flexibility reduction of the coating from 122.34 ± 10.63% in the control to 90.24 ± 5.08% in the sample with the addition of 0.3 g of pollen. The pollen addition also increased the antioxidant activity the FRAP method it increased from 0.004 µmol Trolox/g in the control to 0.147 µmol Trolox/g in the package with the highest pollen addition, and an increase in values was also observed for the ABTS method. In connection with this, an increase in total polyphenols was also observed. Sensory analysis showed that not a single package was evaluated negatively. Only the aroma affected by the addition of pollen was evaluated as negative. The change in immunoreactivity was not confirmed. The results of measuring the colour characteristics of the image analysis method show that the addition of pollen increases the values of yellowness in relation to the amount of added pollen (7.73 ± 1.28 for control, 25.58 ± 4.72 for the sample with the addition of 0.3 g pollen) ($p < 0.05$). The addition of pollen also affected the size of the particles and their distance. The largest proportion of particles (11.71%) with a size of 800-1200 µm2 was in the sample with the highest addition of pollen.

Keywords: antioxidant; biodegradable film; packaging properties; polyphenol

INTRODUCTION

Food contamination can occur during harvesting, food processing as well as distribution. Packaging is an effective means of protecting food from external impurities and can also help to prevent chemical, physical, and biological changes (deterioration) during storage or even during the preparation of products **(Mousavi Khaneghah *et al.*, 2018)**.
The use of edible packaging materials has a growing potential. The most common materials include those based on starches or other edible polymers (edible films) which have many advantages as they are made of natural materials. Consumption and degradability, and thus minimal environmental impact, are considered to be the main advantages. Edible packaging materials can be divided into coatings and films **(Kadzińska *et al.*, 2019)**.
Edible packaging, due to its composition, can have a large number of desirable properties such as antioxidant, antimicrobial, barrier or intelligent properties, which provide additional information about the food (for example, colour change when the pH of the packaged food changes) **(Kadzińska *et al.*, 2019; Mousavi Khaneghah *et al.*, 2018)**.
Several studies point to the need to evaluate the mechanical (elasticity and stress), thermal, optical (brightness and opacity), morphological, and other properties of edible films, as they create a modified atmosphere that affects gas transfer and further becomes a barrier to aromatic compound transfer. These properties depend on several parameters concerning the composition of the coating and the film, such as preparation conditions (solvent, pH, component concentrations and temperature) and the type of additives added (cross-linking agents, antimicrobials, plasticizers, and emulsifiers) **(Siracusa *et al.*, 2018)**.
The biodegradable chitosan is a suitable naturally occurring polysaccharide with non-toxic and antimicrobial properties. It is a natural polymer obtained by deacetylation of chitin **(Leceta et al., 2013)**. Important properties of chitin include antimicrobial properties. It also has antioxidant properties, and it may be a carrier of various active or functional substances (various natural extracts, browning inhibitors, dyes, flavours), which may prolong the shelf life of food as well as reduce the risk of developing pathogenic microorganisms on the surface of food **(Jamróz *et al.*, 2019; Rößle *et al.*, 2011)**.
Plant pollen is a high-energy material that is collected by insects and stored as a food supply. In traditional medicine, it has been used by humans for religious purposes and as a food supplement **(Tamas *et al.*, 2009)**. Due to its composition (proteins, amino acids, lipids, minerals, carbohydrates, vitamins, and other compounds present, such as flavonoids and phenols), pollen can positively affect the packaged food and the packaging itself **(Salles *et al.*, 2014)**.
Edible packaging has many positive properties, but at the same time, their health impact on consumers and packaged foods is also discussed. In edible packaging containing chitosan, the issue is mainly the content of two basic allergenic proteins from crustacean bodies, namely tropomyosin and arginine kinase **(Xuan *et al.*, 2012)**. Crustaceans contain more than 18 allergenic proteins. Over the years, thermostable proteins ranging in size between 34-38 kDa have been selected, such as the muscle protein called tropomyosin (**Hoffman *et al.*, 1981; Wang *et al.*, 2013)**. Crustacean allergens are among the 10 most common inducers of food allergies. The threshold dose for crustaceans compared to other allergens have not yet been published **(Neethirajan *et al.*, 2018)**. Tropomyosin is also considered to be an allergen causing cross-reactivity within crustaceans or other insect species **(Witteman *et al.*, 1994)**. Most commercial kits and current studies use antibodies to tropomyosin **(Neethirajan *et al.*, 2018; Schubert-Ullrich *et al.*, 2009; Wang *et al.*, 2013)**. The content of tropomyosin in each crustacean specimen varies. It ranges between 0.07-15.5ug/g. In low volumes, it is also present in vertebrates (<0.2) **(Werner *et al.*, 2007)**. Patient reactivity was also confirmed for various molecular fractions (25-200 kDa) **(Broekman *et al.*, 2016)**.
The aim of the work was to evaluate the mechanical, antioxidant, optical, morphological, sensory, and allergenic properties of chitosan packaging with the addition of elderberry pollen.

MATERIAL AND METHODS

Sample Preparation

The first step was the production of edible packaging samples with various additions of elderberry pollen (*Sambucus nigra*, *Viburnaceae*). Chitosan was chosen as the polysaccharide base of the edible packaging, and it was enriched with 3 different concentrations of elderberry pollen. A control sample free of any pollen was prepared as well.
The edible coatings were prepared using the modified method according to **Tauferova *et al.* (2021)**. When preparing the packaging with the addition of elderberry pollen, 1.5 g of low molecular weight chitosan (Sigma-Aldrich, St.

Louis, MO, USA) and the given amount of elderberry pollen (0.075 g; 0.15 g; 0.3 g) were first weighed. Pollen was harvested from elderberry flowers.
A microscope slide was prepared for the evaluation of the coatings under a light microscope, in which 1 mL of the liquid coating was pipetted onto a microscope slide. A square of 22 × 22 mm was drawn using a barrier marker (Elite Mini PAP Pen, USA) in the slide in order to prevent it from spilling over a larger area.
The samples were marked with the following codes: 0.075CHLEP, 0.15CHLEP, 0.3CHLEP (EP – Elderberry pollen), and CTRL (control).

Textural Properties

Strength (MPa) and elasticity (%) were measured using a TA.XT plus texturometer (Godalming, UK)) using the international test method – ASTM D882-02. The produced packages were cut into rectangles measuring 1 x 5 cm and each measurement was performed 5 times according to **Dordevic *et al.* (2021)**.

Antioxidant Properties

FRAP
The FRAP method described by **Behbahani *et al.* (2017)** with minor modifications was performed using TPTZ (2,4,6-Tris(2-pyridyl)-s-triazin, VWR, Stříbrná Skalice, Czech Republic), acetate buffer and FeCl3 (Lachema, Brno, Czech Republic).
0.1 g of the sample, which was extracted in 75% methanol for 30 min, was weighed in an ultrasonic water bath (Bandelin, Berlin, Germany) at room temperature.
The extract was filtered and mixed with a reagent solution (5.0 mL TPTZ + 5.0 mL FeCl3 + 25.0 mL vinegar buffer). After 8 minutes, the absorbance was measured using a spectrophotometer (CE7210 DIET-QUEST, Cambridge, England) at a wavelength of 593 nm. The preparation of the blank sample was identical, but the sample was replaced by distilled water. Trolox was used as a standard.

ABTS
ABTS measurements were carried out according to the methodology published by **Dordevic *et al.* (2021)**.

TPC (Total Polyphenol Content)
The total polyphenol content was determined using Folin – Ciocalteu reagent by method described in the work **Jancikova *et al.* (2020)**. 1.0 g of the sample was mixed with 40.0 ml of distilled water and stirred on a shaker for 10 minutes. Subsequently, the sample was mixed with Folin-Ciocalteu reagent (PENTA, Brno, Czech Republic) (1:10) and 7.5% Na_2CO_3. The mixture was incubated for 30 min at room temperature in the dark. Absorbance was measured at 765 nm with a spectrophotometer (CE7210 DIET-QUEST, Cambridge, England). Gallic acid (PENTA, Brno, Czech Republic) was used as a standard for preparing standard curve.

Sensory Analyses

Sensory analyses were performed at the University of Veterinary Sciences Brno, Czech Republic according to **Tauferova *et al.* (2021)**. The sensory analyses were performed in complete block design. Samples were presented in random order on clear plastic Petri dishes identified by 3-digit numerical codes in a monadic sequential presentation scheme. A quantitative descriptive analysis was performed by a trained panel consisting of 12 evaluators from the Department of Plant Origin Food Sciences. A training session on the selected edible coating descriptors' intensity scale preceded the quantitative descriptive analysis of the samples. For the quantification of the attributes a 9-point category ordinal scale with described extremes from 1 (no perception) to 9 (the highest intensity) was used. The quantitative descriptive analysis was performed in triplicate. Subsequently, hedonic analysis was performed by moderately trained panellists (n = 55) consisting of students and employees of the Faculty of Veterinary Hygiene and Ecology. Attributes were evaluated using the 9-point category ordinal hedonic scale. (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely).

Analysis of allergens

A commercial kit of Veratox® for Crustacea No. 8520 (Neogen, GB) was used to determine the presence of allergens. This test is based on the Sandwich indirect ELISA method. The determination was performed in compliance with the manufacturer's procedure. The detection limit of the method is 2.5 ppm. All samples were measured in triplicate.

Colour Characteristics of Packaging

The colour characterisation of packaging was providing on microscopic slide according to **Bartlová *et al.* (2021).**
The difference between samples and the control (ΔE) was observed **(Jamróz *et al.*, 2019; Luo *et al.*, 2001; Sharma, 2017)**. Colour differences (ΔE) and Chroma (C*) were calculated using the following equations **(Velickova *et al.*, 2015; Vera *et al.*, 2020)**:

$$\Delta E = \sqrt{(L^* - L_0^*) + (a^* - a_0^*) + (b^* - b_0^*)},$$

$$C^* = \sqrt{a^{*2} + b^{*2}},$$

eq. 1

where the L*, a* and b* values were the parameters of pollen-added packaging samples, while the values of L_0^*, a_0^* and b_0^* were the colour parameters of the control sample.
Another parameter was the Whiteness index, which was obtained by the calculation given in **Li *et al.* (2019)**:

$$Whitness\ index = 100 - \sqrt{(100 - L*)^2 + a*^2 + b*^2}$$

eq. 2

In their work**, Saberi *et al.* (2016)** used for their evaluation the parameter of Yellowness index, which is obtained by the following calculation:

$$\text{Yellowness index} = \frac{142{,}86\ b*}{L*}$$

eq. 3

Microscopic Characteristics

From the microscopic methods, the light microscopy and the scanning electron microscopy were used to evaluate the polymer formation.

Light Microscopy
Images obtained by scanning under a light microscope taken during the measurement of colour characteristics, were used to evaluate the resulting particles from the pollen present in the packaging samples. Selected morphological characteristics were measured on the obtained images: particle size, which was formed by combining pollen grains in samples with their addition, their equivalent diameter and the shortest distance to another object (NearestObjDist). NIS-Elements AR 5.20 Programme (Laboratory Imaging, Prague, Czech Republic was used to perform the image analysis.
Area is a principal size criterion. In a non-calibrated system, it expresses the number of pixels; however, in a calibrated system, it expresses the real area.

$$Area = \sum_{\forall\ Pixel \in Object} 1$$

Eq. 4
EqDiameter (Equivalent Diameter) is a size feature derived from the area. It determines the diameter of a circle with the same area as the measured object:

$$\text{Eqdiameter2D} = \sqrt{\frac{4 * Area}{\pi}}$$

Eq. 5
NearestObjDist is the shortest distance to another object (measured between centres of gravity).

Scanning Electron Microscopy
The surface and cracking of the edible packaging were evaluated. The evaluation of the surface of the edible package was performed after forming the gel on a conductive target so as to minimize surface changes caused by bending or cracking during handling like describe **Tauferova *et al.* (2021)**.
For cracking analysis, the edible film was frozen with liquid nitrogen and subsequently mechanically broken. The fragments were applied onto a carbon double-sided adhesive tape.
The analysed samples were gold-coated on a Q150R ES sample coating machine (Quorum Technologies, United Kingdom, Laughton). The samples were gold-coated by 10 nm and subsequently examined with a MIRA3 microscope (TESCAN, CZE, Brno), at a voltage of 5.0 kV. Magnification 1kx, 8kx, 80kx. The samples were examined in triplicates. Surface images were taken at a magnification of 2 kx. Crack size was calculated as total area in nm² (eq. 4) and distance between individual cracks was calculated as the nearest object distance in nm (eq. 5).

Statistical Analysis

The data were processed with the 2021 XLSTAT software (Addinsoft, Paris, France). k means the Kruskal–Wallis test which was used to compare the textural characteristics, antioxidant properties, ELISA results, colour characteristics, and microscopic characteristics. Sensory data were processed with the SensoMineR package for R software (The R Foundation for Statistical Computing, Vienna, Austria) where the Principal Component Analysis (PCA) was used.

RESULTS AND DISCUSSION

Textural Properties

The results of the textural properties of the samples are given in Table 1. The prepared chitosan packages had a high flexibility, which was confirmed by measuring their elasticity. Flexibility decreased with the addition of pollen grains.

A statistically significant difference ($p < 0.05$) for this parameter was found between the samples. **Siripatrawan *et al.* (2016)** report that, for example, in the case of an interaction between phenolic acids and chitosan, the flexibility of the packaging decreases.

Mechanical properties of chitosan films are associated with intermolecular and intramolecular interactions within the chitosan matrix **(Leceta *et al.*, 2013)**. In some cases, literature indicates that the increase in strength may be due to interactions with other components of the packaging which contain, for example, phenolic acids and their esters, such as the addition of propolis. These compounds can react with the hydrophilic groups present in the chitosan matrix, and this interaction can subsequently lead to stronger adhesion between the added extracts and the chitosan molecules. These interactions may increase the strength **(Siripatrawan *et al.*, 2016)**. With some ingredients, the strength increases, in other cases it decreases. In general, the increase is attributed to the strong interaction between the additives and the chitosan chains, which causes an increase in the stiffness of the film. On the contrary, the decrease in strength is due to a reduction in the intermolecular interactions between the chitosan chains and the ingredients present **(Kalaycıoğlu *et al.*, 2017)**. This parameter increased with the addition of pollen, but the amount added did not affect it anymore.

Table 1 Textural properties expressed as strength (MPa) and elasticity (%) (n = 5)

	0.075CHL$_{EP}$	**0.15CHL$_{EP}$**	**0.3CHL$_{EP}$**	**CTRL**
Strength [Mpa]	0.04±0.01	0.04±0.01	0.04±0.01	0.12±0.14
Breaking strain [%]	$109.40±4.67^{bc}$	$101.57±6.47^{ab}$	$90.24±5.08^{a}$	$122.34±10.63^{c}$

Legend: Different letters in rows indicate significant differences between groups, $p < 0.05$

Antioxidant Capacity

The results of the antioxidant properties of edible packaging with added pollen are summarized in Table 2. A slight value of antioxidant activity and TPC was also found in the control sample (CTRL). This may be due to the fact that the packaging is based on chitosan which itself may have antioxidant activity **(Kim *et al.*, 2007)**.

Pollen grains are a very good source of substances with antioxidant properties. Elderberry pollen contains mainly polyphenolic and flavonoid substances **(Marțiș *et al.*, 2021)**. It can be stated that the antioxidant activity of the prepared chitosan packages increased with increasing concentration of elderberry pollen. The concentration of total polyphenols (TPC) also increased.

Table 2 Antioxidant capacity of edible packaging

	0.075CHL$_{EP}$	**0.15CHL$_{EP}$**	**0.3CHL$_{EP}$**	**CTRL**
FRAP [µmol Trolox/g]	$0.044±0.01^{a}$	$0.103±0.01^{b}$	$0.147±0.01^{c}$	$0.004±0.01^{d}$
ABTS [%]	$0.853±0.01^{a}$	$1.831±0.01^{b}$	$2.721±0.01^{c}$	$0.052±0.01^{d}$
TPC [mg Gallic Acid/g]	$0.051±0.01^{a}$	$0.075±0.01^{b}$	$0.132±0.01^{c}$	$0.004±0.01^{d}$

Legend: Different letters in rows indicate significant differences between groups, $p < 0.05$

Sensory Analyses

Quantitative Descriptive Analysis

From the viewpoint of appearance, the colour intensity was clearly affected by the concentration of added pollen. The addition visibly increased the colour intensity when compared with control sample (2.46); samples 0.15CHL$_{EP}$ and 0.3CHL$_{EP}$ were evaluated as statistically significantly ($p < 0.05$) more intense (5.20 and 5.60, respectively) in terms of colour. In terms of aroma intensity, there were no statistically significant difference between the samples with pollen, the mean values had a very narrow range (4.80–4.97), that was statistically significantly higher than in control sample (3.49). As for texture, descriptors of stickiness and surface roughness were monitored. The addition of pollen had not changed the perception of stickiness, values of both, control sample and experimental samples, were characteristic with low stickiness (2.32–3.09). Any clear trend in values of surface roughness related to the concentration of pollen addition was not perceived. The lowest values were detected in 0.3CHL$_{EP}$ sample and in the control sample (3.79 and 4.53), whereas 0.15CHL$_{EP}$ and 0.075CHL$_{EP}$ achieved higher values (6.59 and 7.16, respectively). Low stickiness and smooth surface should be a requirement for the commercial edible coatings, as these are evaluated as more acceptable for the consumer **(Khorram *et al.*, 2017; Santagata *et al.*, 2018)**. Principal component analysis results of the quantitative descriptive analysis data are shown in Fig. 1. Both, Fig. 1 as well as Table 3, clearly demonstrate, that there were statistically significant differences between the individual experimental samples of edible coatings.

Table 3 Matrix with the *p*-values of the Hotelling's T2 tests for each pair of edible film formulations (quantitative descriptive analysis).

	0.075CHL$_{EP}$	0.15CHL$_{EP}$	0.3CHL$_{EP}$	CTRL
0.075CHL$_{EP}$	1	0.0025790	0.0000037130	0.00012830
0.15CHL$_{EP}$	0.0025790	1	0.000000073590	0.0000015240
0.3CHL$_{EP}$	0.0000037130	0.000000073590	1	0.000011380
CTRL	0.00012830	0.0000015240	0.000011380	1

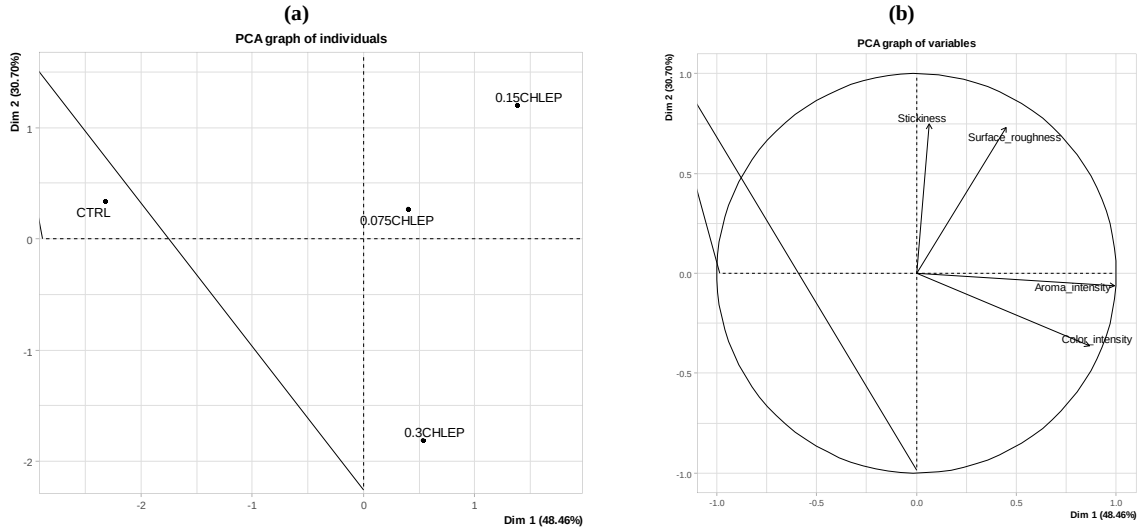

Figure 1 The results of PCA of quantitative descriptive analysis of edible coatings: (**a**) Score plot for the mean points (0.075CHL$_{EP}$, edible coating with 0.075% of elderberry pollen; 0.15CHL$_{EP}$, edible coating with 0.15% of elderberry pollen; 0.3CHL$_{EP}$, edible coating with 0.3% of elderberry pollen; CTRL, control = edible coating without added pollen). (**b**) Variables factor map.

Hedonic Analysis

As can be seen from Fig. 2, results of the principal component analysis explain 99.40% of the variability, whereas the first component accounts for 71.19% and the second for 28.21% of the variability. The factor map of variables shows a close correlation between the overall pleasantness and the pleasantness of appearance. No statistically significant differences ($p < 0.05$) were found between 0.075CHL$_{EP}$ and 0.15CHL$_{EP}$, but there were statistically significant differences found between all other pairs of experimental edible films, as can be clearly seen from Fig. 2. Moreover, Table 4 shows the treated average values of descriptors obtained by the hedonic evaluation. The values highlighted in green represent a statistically significantly better rating ($p < 0.05$); the values highlighted in orange represent a statistically significantly worse rating. The control sample without any added pollen achieved statistically significantly ($p < 0.05$) higher values in the descriptors pleasantness of appearance, pleasantness of texture and overall pleasantness. Paradoxically, 0.3CHL$_{EP}$ sample as the sample with highest percentage of pollen addition achieved the highest values of overall pleasantness both with pleasantness of texture from all experimental samples containing pollen. But as a consequence of higher pollen addition, it was characteristic with more intense aroma and statistically significantly worse rating ($p < 0.05$) in pleasantness of aroma. Elderberry inflorescence with identified three classes of aromatic esters is typical for pleasant strong smell **(Basas-Jaumandreu *et al.*, 2019)**, but although pleasant, it was evaluated as disruptive in case of edible coating, which is preferred to be neutral **(Zhao *et al.*, 2005)**. Nevertheless, none of the experimental samples was considered as unacceptable, as they all achieved ratings higher than 5 (neither like nor dislike) in overall pleasantness, which was considered as the limit for sensory acceptability **(Cortés-Rodríguez *et al.*, 2020)**.

Table 4 Adjusted mean of hedonic evaluation of edible coatings

	Pleasantness of aroma	Pleasantness of texture	Pleasantness of appearance	Overall pleasantness
0.075CHL$_{EP}$	5.632	4.445	4.451	5.191
0.15CHL$_{EP}$	5.518	4.502	4.823	5.382
0.3CHL$_{EP}$	5.003	5.845	5.366	5.725
CTRL	5.889	5.959	6.68	6.239

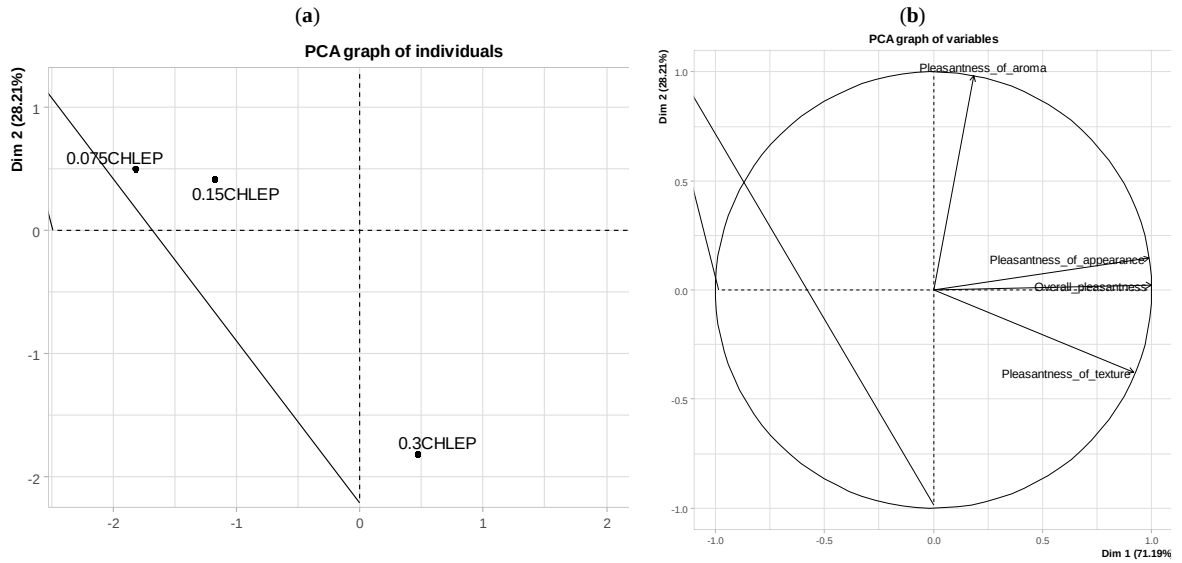

Figure 2 The results of PCA of hedonic analysis of edible coatings: (**a**) Score plot for the mean points (0.075CHL$_{EP}$, edible coating with 0.075% of elderberry pollen; 0.15CHL$_{EP}$, edible coating with 0.15% of elderberry pollen; 0.3CHL$_{EP}$, edible coating with 0.3% of elderberry pollen; CTRL, control = edible coating without added pollen). (**b**) Variables factor map.

Enzyme-Linked ImmunoSorbent Assay

In the analysed samples, the control (CTRL) confirmed the presence of crustacean proteins higher than 0.2 ug/g (2.85 ug/g). Thus, a clean edible package made of chitosan can be considered potentially allergenic. According to the legislation, there is no minimum amount that can be considered allergenic. Available studies mention 10 mg – 100 mg as sufficient to induce an allergic reaction **(Miceli Sopo *et al.*, 2015; Taylor *et al.*, 2014)**.
The addition of elderberry pollen to the packaging samples did not affect the immunoreactivity, the presence of crustacean proteins above 0.2 ug/g was also confirmed.

Colour Characteristics of Packaging

Edible packaging should be almost colourless and as transparent as possible so that the food retains its original colour **(Salama *et al.*, 2019)**. Highly transparent and glossy packaging helps to improve the visual characteristics of food products **(Nawab *et al.*, 2017)**. Table 5 shows the values of colour parameters of the prepared coating samples. The results show that the value of the L* parameter is high, almost approaching the maximum value of 100. This parameter and the a* parameter decreased compared to the control without added pollen and also decreased with increasing addition of pollen. In their work, **Salama *et al.* (2019)** describe a similar influence when monitoring the effect of the frankincense oil addition on edible packaging. In the case of b* parameter, the values increased with the addition of pollen. The same trend for these values is described by **Nawab *et al.* (2017)** who studied the effect of the addition of gums on starch packaging made of mango cores. They argue that an increase in "yellowness" is directly proportional to an increase in b*, while an increase in "darkness" and "greenness" is attributed to a decrease in L* and a*. This observation also follows from our results. With the addition of pollen, the brightness of the packaging decreased, the proportion of green and yellow increased, which is also visible from the photographs of the coatings (Fig. 3). In their study, which addressed the effect of the addition of apple pectin, **Younis *et al.* (2019)** describe the decrease in the L* parameter value.

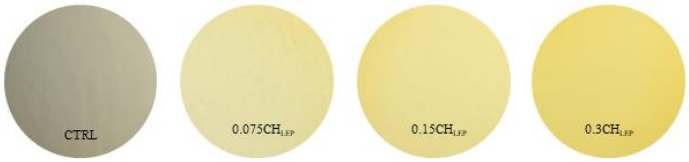

Figure 3 Macro images of the packages

The ΔE00 parameter, which compares the colour characteristics with the control sample, also increased due to the amount of pollen added. Differences between the samples based on the addition of beeswax are described in the study by **Velickova *et al.* (2015)**.
In the case of the Chroma (C*) parameter, we obtained results that had the same course as most other parameters, its value increased with the addition of pollen. **Espino-Díaz *et al.* (2010)** state that the Chroma (C*) parameter can be affected by both, pH and the presence of calcium. The increase in this parameter depending on the amount of the additive is described in the work by **Chana-Thaworn *et al.* (2011), Fabra *et al.* (2018), Vera *et al.* (2020)** and other authors.
Other important parameters influencing the acceptance of the coating include the Whiteness and Yellowness indices. In our case, with increasing addition of pollen, the Whiteness index decreased and, conversely, the Yellowness index increased.

That is consistent with the assumption that the pollen grains were yellow. An increase in the Yellowness index represents a change in the colour of a clear or white sample to yellow. Changes in these parameters are most often caused by the amount of the additive, by non-enzymatic browning, which may occur during drying of the packaging or may be simply absorbed by the packaging **(Cortés-Rodríguez *et al.*, 2020)**. In their work, **Flores *et al.* (2007)** describe that the change in the Yellowness index can also occur during the production of edible packaging, when this index is also affected by the drying conditions of the prepared packaging. These indices can also be affected by various additions to the basic matrix, which is described in the papers by a number of authors.

Table 5 Colour characteristics of edible films with added pollen (mean ± standard deviation, n = 10)

	0.075CHL$_{EP}$	**0.15CHL$_{EP}$**	**0.3CHL$_{EP}$**	**CTRL**
L*	98.94±1.10 ^{b}	98.63±2.86 ^{b}	97.03±1.42^{a}	99.88±0.34^{c}
a*	-2.44±0.68^{c}	-3.37±1.56^{b}	-4.94±1.59^{a}	-2.20±0.26^{c}
b*	7.32±1.35^{b}	11.03±3.19^{c}	17.34±2.99 ^{d}	5.41±0.89^{a}
ΔE	1.87±1.16^{a}	4.53±2.30^{b}	8.24±1.98^{c}	
C*	7.72±1.45^{b}	11.56±3.42^{c}	18.04±3.25^{d}	5.85±0.88^{a}
Whiteness index	92.16±1.61^{c}	88.16±3.89^{b}	81.69±3.41^{a}	94.14±0.88^{d}
Yellowness index	10.58±2.06^{b}	16.06±4.91^{c}	25.58±4.72^{d}	7.73±1.28^{a}

Legend: Different letters in rows indicate significant differences between groups, $p < 0.05$

Microscopic Characteristics

Light Microscopy

In the photographs obtained by light microscopy, the image analysis program measured the area of the particles, their EqDiameter and the Nearest object distance (Table 5). Number of objects larger than 800 µm2 and more was the highest in the sample with the greatest addition of pollen grains. In contrast, particles with an area of less than 400 µm2 were the most abundant in the sample with the lowest addition of pollen, whereas they were mostly individual pollen grains. The same results were obtained by measuring EqDiameter, when the objects with EqDiameter greater than 30 were the most abundant in the sample with the highest addition. In the case of objects with EqDiameter less than 30 µm, these were individual pollen grains, because according to the results of the study by **Wrońska-Pilarek *et al.* (2020)**, the size of *Sambucus nigra* pollen grains ranges from 10.1 to 25 µm. In all three samples, most of the particles were very close to each other. The largest number of particles was spaced in the interval of 0 to 40 µm from each other (49.64%,59.74%,45.67%) and in the interval of 40.0 to 80.0 µm from each other (38.27%, 35.78%, 45.11%).

Table 6 Morphometric properties of particles in samples with added pollen

Number [%]	Area [µm^2]	0-400	400-800	800-1200	1200-1600	1600-2000	2000-2400	2400-2800	2800-INF
	0.075CH$_{LEP}$	69.57	21.61	6.21	1.52	0.64	0.23	0.08	0.13
	0.15CH$_{LEP}$	53.01	24.41	10.06	4.74	3.13	1.62	0.92	2.1
	0.3CH$_{LEP}$	40.71	23.32	11.71	7.51	3.6	3	2.03	8.13
	EqDiameter [µm]	0-15	15-30	30-45	45-60	60-75	75-90	90-105	105-INF
	0.075CH$_{LEP}$	0	88.96	9.93	0.98	0.08	0.03	0.03	0
	0.15CH$_{LEP}$	0	74.24	17.82	5.90	1.44	0.37	0.15	0.09
	0.3CH$_{LEP}$	0	60.15	22.99	8.76	4.28	1.8	0.9	1.13
	NearestObjDist [µm]	0-40	40-80	80-120	120-160	160-200	200-240	240-280	280-INF
	0.075CH$_{LEP}$	49.64	38.27	8.25	2.04	0.88	0.54	0.1	0.28
	0.15CH$_{LEP}$	59.74	35.78	3.3	0.72	0.2	0.07	0.06	0.13
	0.3CH$_{LEP}$	45.76	45.11	6.13	1.53	0.8	0.28	0.23	0.18

The behaviour of particles in edible packaging using light microscopy is described by **Sherwin *et al.* (1998)**. In his study, he researched the effect of the addition of fatty acids (C14 to C22) to the packaging and the subsequent behaviour of the formed crystals. As indicated by their results, depending on the fatty acid addition used, small acicular crystals and different amounts of larger crystals formed depending on the added fatty acid, when the crystals increased with increasing fatty acid chain length.

Fig. 4 shows the results of spectral analysis for individual coatings, both control and packaging with added pollen. The control sample had the lowest absorbance and the largest differences in absorbance were found at the wavelength of 420 nm, where the coatings had less transmittance for the individual colours of the light used. At the end of the spectral characteristic, the effect of the coating manifested itself and interference occurred. The absorbance was evaluated in a study by **Zareie *et al.* (2020)**, where the results show that the sample with 2.5% addition of chitosan had the highest absorbance at 420 nm.

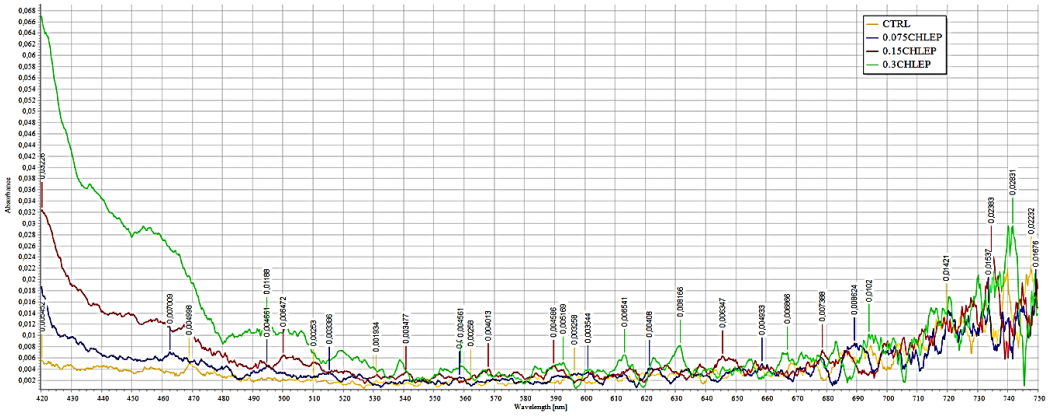

Figure 4 Spectral characteristics of packages containing polled and pollen-free control

Scanning Electron Microscopy

Pollen grains were confirmed in packages with the addition of elderberry pollen, while the increasing concentration was reflected in a larger number of pollen grains, variously prominent on the surface of the package (Figure 5). The presence of surface structures on edible packaging may alter the functional properties of the packaging **(Tripathi *et al.*, 2010)**. Pollen grains are known to adhere to different surfaces **(Elleman *et al.*, 1990)**. The micrographs (Figure 5) show that there is no separation of pollen grains from the edible packaging. At higher concentrations of 0.15 and 0.3 CHLEP, the formation of cracks is also evident due to the different thermodynamic properties of both materials **(Gohargani *et al.*, 2020)**. The number of cracks and the distance between the cracks varied with the varying concentration of elderberry pollen (Table 7). A statistically significant difference was noted between individual groups and the control. These results are corresponded to the study by **Tauferová *et al.* (2021)**. Pollen grains were also observed in the cracks of packages, where their presence was confirmed mainly in the surface layer. Thus, during the gel formation, the pollen grains floated and subsequently stabilized in the upper layer of the coating.

Table 7 Characteristic of surface cracks on formed gel

Sample	Crack size [nm²]	Distance between cracks [nm]
CTRL	626.46±944.27^{a}	58.19±23.38^{a}
0.075CHL$_{EP}$	925.38±1804.10^{b}	46.93±16.97^{b}
0.15CHL$_{EP}$	920.78±1729.48^{c}	52.37±17.47^{c}
0.3CHL$_{EP}$	758.40±2668.66^{d}	43.99±17.31^{d}

Legend: Different letters in individual columns indicate significant differences ($p < 0.05$)

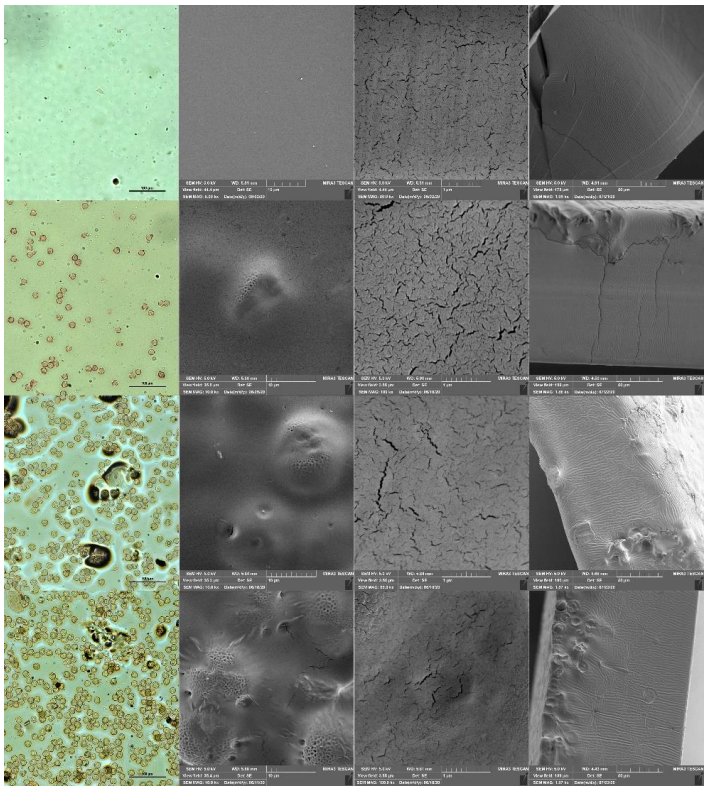

Figure 5 Surface and cracks analysis of coatings with the addition of elderberry pollen, SEM. **Legend:** Row 1 – CTRL; row 2 – 0.075CHLEP, edible coating with 0.075% elderberry pollen; row 3 – 0.15CHLEP, edible coating with 0.15% elderberry pollen; row 4 – 0.3CHLEP, edible coating with 0.3% elderberry pollen; magnification on the left 8000x, in the centre 80 000x, on the right 1800-1900x

CONCLUSION

The study demonstrated the effect of the presence of pollen grains on selected properties of the prepared coatings. Most of the monitored parameters were affected. Some changes had a positive, some a negative effect. The increase in antioxidant potential and the concentration of total polyphenols in proportion to the increasing concentration of pollen in edible packaging can be mentioned as positive. The intensity of the colour was clearly influenced by the concentration of added pollen, which may be desirable in some cases. During the sensory evaluation, the samples with the highest addition were positively evaluated. With the addition of pollen, the brightness of the coating decreased, while the proportion of green and yellow increased. Microscopic evaluation confirmed that the sample with the highest pollen addition had the largest proportion of larger pollen clumps. At higher concentrations, the formation of cracks and a high number of prominent pollen grains on the surface of the packaging were evident. This experimentally produced packaging with the addition of pollen can serve as packaging with added value, especially in terms of antioxidant properties, or due to their ability to better protect the commodity.

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