Edible pH sensitive polysaccharide-anthocyanin complex films for meat freshness monitoring

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Abstract. One of the innovative methods for real-time determination of food freshness is the application of pH-indicator sensors, where the color change can be used for the visual detection of acidic/basic volatile compounds formed during product storage due to microbial growth. The aim of the present study is to develop a pH-responsive freshness indicator based on anthocyanins from chokeberry (*Aronia melanocarpa Elliot*) and black carrot (*Daucus carota ssp. sativus var. atrorubens Alef.*), incorporated into an alginate/pectin/arabic gum composite film. The resulting films show distinct color changes as the pH varies. The color changes from red (pH 2.0 - 3.0) through pink and pale pink (pH 4.0, 5.0 and 6.0) to purple and blue (pH 7.0 - 8.0). The most distinct is the color transition between pH 6.0 and 7.0 for the black carrot extract and the chokeberry: black carrot mixture (1:3). The applicability of the developed pH-indicator films was demonstrated in chicken meat by tracking the changes during its storage at 4°C for 7 d. The observed results show a distinct color change from pink (day 1-3) to violet and blue on day 7. The developed pH-sensitive films have potential for use in a smart packaging system as a sensor for meat freshness monitoring

1 Introduction

One of the most important tasks for the modern production of food products is ensuring safety and extending the shelf life. After the production stage, packaging and storage conditions play a huge role in preserving food quality. The right choice of packaging material and technology has a significant role in the final properties and form of the product when it reaches the consumer.

A promising area in the development of packaging materials is the use of so-called "intelligent" packaging. Intelligent (or smart) packaging materials is a term used to represent a class of packaging materials that can monitor the condition of either the packaged food or the environment inside the package and provide this information to the consumer. Depending on the way information is obtained, intelligent packaging systems can be classified into three categories; radio frequency identification systems (RFID), sensors and indicators [1]. Significant advantages of the indicators are their low price, their simplicity and the possibility for the user to receive information about the current state of the product at any moment of its storage, on the market or at home. Indicator systems provide qualitative information about packaged food, most often through color variations. They can be categorized into 3 groups - freshness indicators; integrity indicators (gas indicators) and time-temperature indicators.

A major focus of research in this area is related to the development of pH-sensitive freshness indicators that change color upon accumulation of microbial metabolites, as microbial growth can induce a change in pH [2, 3]. Meat and meat products contain a high percentage of proteins and free amino acids, which are susceptible to spoilage and deterioration due to the action of microorganisms. As a result, during storage, volatile basic nitrogen compounds are formed and the pH changes.

Currently, most pH-sensitive freshness indicators are made with various types of synthetic dyes that respond to the change in pH with a color change (cresol red, methyl red, bromocresol purple, bromocresol green, bromothymol blue, xylenol, etc.). However, most synthetic dyes are toxic and some are even carcinogenic. Therefore, it is extremely important to find non-toxic and safe dyes for the preparation of pH-sensitive freshness indicators [4].

In search of safe colorants for the production of pH-sensitive freshness indicators, many researchers turn to natural pigments of plant origin. In the last decade, some natural dyes such as curcumin, shikonin and anthocyanins have been the subject of research [1, 5, 6, 7]. Anthocyanins have been widely studied due to their rich color variation over a wide range of pH changes, high safety, great availability, and other functional properties such as antioxidant activity.

Veiga-Santos et al. [8] propose a prototype pH indicator designed as a biodegradable cassava film plasticized with sucrose and invert sugar and containing grape and spinach extracts. Films containing a higher

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concentration of grape extract showed a greater color change at different pH, suggesting that anthocyanins are more effective as pH indicators than chlorophyll or the mixture of the two extracts.

Studies have been conducted to obtain an indicator sensor based on natural anthocyanins extracted from red cabbage and rose, the color of which changes from red to green with increasing pH. Agarose gel and filter paper were used as carriers for the anthocyanin mixture [9]. Other authors suggest a packaging film that can act as a pH indicator and has antibacterial properties. The film contains a matrix of cellulose nanofibrils, anthocyanins from violet sweet potatoes and oregano essential oil as an antibacterial agent [6].

Yoshida et al. [4] developed a pH-colorimetric indicator device based on a chitosan film incorporating anthocyanin extracted from grapes. The advantages of the developed smart packaging material are the fast production process, biodegradability and the use of natural and safe components.

The aim of the current study is to develop and characterize a pH-sensitive freshness indicator based on chokeberry and black carrot anthocyanins incorporated into an edible multilayer alginate/pectin/arabic gum composite film.

2 Material and methods

2.1 Materials

Sodium alginate was supplied by Biosynth AG, high methoxyl apple pectin (DE-61.80%) by Herbstreinth & Fox GmbH, arabic gum by Valerus. Methanol, calcium chloride, sodium acetate and hydrochloric acid were supplied by Merck, ethanol and glycerol by Valerus. All reagents and chemicals used are analytical grade.

2.2 Preparation of anthocyanin extracts

To obtain the anthocyanin extracts, two plant raw materials were used - chokeberry fruit (*Aronia melanocarpa Elliot*) and black carrot root (*Daucus carota ssp. sativus var. atrorubens Alef.*). Chokeberry berries were pre-frozen at temperature (-22°C) and stored in a freezer until processing. Before extraction, the fruits were thawed at room temperature and pressed to separate the juice from the pulp. Pulp extraction was carried out with 75% v/v ethanol acidified with 0.1% HCl and plant material: solvent ratio of 1:2. In the case of the black carrot, the extraction was carried out on the whole raw material, previously ground to dimensions of 0.4 - 0.8 cm. The samples were sonicated for 20 min (Ultrasonic System, M 7652), then left in the dark, at room temperature, for 48 hours. The obtained extracts were filtered and stored in the dark at 4°C.

2.3 Content of anthocyanin pigments in extracts

The content of total monomeric anthocyanins (TMA) in the extracts was determined by the pH-differential method [10]. The absorbance of extract samples in buffers with pH 1.0 and 4.5 and wavelength 520 nm and 700 nm was measured. The calculations were made according to Equation (1):

$$Anthocyanins\ (cydeq\ mg/l) = \frac{A.Mw.DF.10^3}{\varepsilon.l} \quad (1)$$

where A = (A520 nm – A700 nm) pH 1.0 – (A520 nm – A700 nm) pH 4.5; Mw = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF – dilution factor; l- pathlength in cm; ε = 26900 molar extinction coefficient for cyd-3-glu; and 10^3 - factor for conversion from g to mg.

2.4 Film formation

Sodium alginate (3.0% w/v) and high methoxyl pectin (4.0% w/v) were dissolved in distilled water. Film forming composition was prepared by mixing sodium alginate and high methoxyl pectin aqueous solutions at the ratio of 3:1. The mixture was homogenized by magnetic stirring at room temperature and glycerol was used as a plasticizer (0.6 g/g polymers). The prepared film forming solutions (FFS) were poured onto Petri dishes (0.325 g FFS/cm^2) and were dried under vacuum (20 kPa, SPT-200 Vacuum Drier) at 35°C. Dried samples were immersed for 30 min in 0.3 M $CaCl_2$ solution to allow cross-linking, washed with distilled water to remove excess Ca^{2+} and dried at 25°C.

Four variants of indicator solutions were prepared by mixing chokeberry and black carrot extracts in the following ratios: 100 - 0% (AE1), 75 - 25% (AE2), 25 - 75% (AE3), 0 -100% (AE4). A 10% w/v water solution of arabic gum was prepared separately, then 20 ml of each variant indicator solution was mixed with 80 ml of the arabic gum solution and applied to the alginate-pectin film (0.250 g/cm^2). Samples were dried under vacuum at 35°C and obtained pH-indicator films (F-AE1, F-AE2, F-AE3 and F-AE4) were stored at 50 ± 1% RH until analysis.

2.5 Absorption spectra of the extracts

Samples of 1 ml of the obtained chokeberry and black carrot extracts were diluted to 50 ml with distilled water. The light absorption of the solution was measured on a UV-Vis spectrophotometer (Libra S22, Biochrom) in the wavelength range of 400–700nm.

2.6 Test for color change of extracts in different pH

One ml of the respective extract and 49 ml of a 0.1 M buffer solution with pH from 2.0 to 11.0 are placed in glass containers. After homogenization, color changes were observed and recorded.

2.7 Analyses of pH-indicator films

2.7.1 Moisture content

The moisture content (g/100 g) was measured with Sartorius Thermo Control YTC 01L balances.

2.7.2 Film thickness

The thickness of the films was determined with a digital micrometer with an accuracy of 0.01 mm ±5% in five randomly selected sections of the sample.

2.7.3 Color response of pH changes

The color response of indicator films was evaluated by immersion in buffer solutions (pH 2.0 - 10.0). Colour variation was measured using a NR200 Portable Digital Colorimeter (Huanyu). The color parameters (*L*, a*, b**) were determined using white and black patterns. The total difference of color (*ΔE**) was calculated according to eq. (2):

$$\Delta E = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2} \qquad (2)$$

where *L1*, *a1* and *b1* are color parameters of composite indicator films at different pH conditions and *L2*, *a2* and *b2* are color parameters of control film samples.

2.8 Color changes of indicator films for chicken meat spoilage trial

Skinless chicken breast samples (48-50 g) were aseptically placed into sterilized Petri dishes and packed in heat-sealed polyethylene foil packages. The experimental series indicator films (F-AE1, F-AE2, F-AE3 or F-AE4) were cut into 1 × 2 cm pieces and attached on the inside of the package. The meat samples were left at 4 ± 1°C for a period of 14 days. During storage, the color change in the indicator was documented daily with a digital camera. For comparison, an indicator from the relevant test group is placed on the outside of the package. Color change of the indicator was measured instrumentally with a digital colorimeter.

2.9 pH determination of chicken meat

The pH was measured potentiometrically with a pH-meter (Jenway 3300), pre-calibrated at pH 4.0 and 7.0. The measurements were performed on fresh meat in three points of the sample, and the obtained results were averaged.

2.10 Microbiological analysis

The total number of aerobic mesophilic microorganisms (TVC) in chicken meat samples at 0, 7 and 14 d of storage at 4 ± 1°C was determined. Briefly: 10 g of chicken meat samples were cut by sterilized scissor, aseptically transferred into a polyethylene tube containing 90 mL sterile peptone water and homogenized for 2 min. Serial 10-fold dilutions of the homogenate were carried out in sterile physiological saline. Determination of TVC was performed by a horizontal method for enumeration of microorganisms in Plate Count Agar (HiMedia Laboratories Pvt. Ltd.) and incubation at 30°C for 72h, according to ISO 4833-1:2013 [11].

2.11 Statistical analysis

Analyses of anthocyanin extracts and indicator films were performed with 5 replicates. Data are presented as mean ± standard deviation (SD). Microbiological analysis of chicken meat samples was performed with 3 replicates. Statistical evaluation of the results was performed with the Excel 2016 software package. A one-way analysis of variance was performed to determine the differences.

3 Results and discussion

Table 1 presents the results of the analysis of the obtained ethanolic extracts of chokeberry and black carrot. Both extracts have a rich color, respectively purple-red in the case of chokeberry and dark purple in the case of black carrot. The content of total monomeric anthocyanins in chokeberry extract was about 20% higher. According to literature data, anthocyanins in chokeberry fruits are cyanidin glycosides, the main representatives being cyanidin 3-galactoside and cyanidin 3-arabinoside [12]. In black carrot, the main anthocyanidin component is also cyanidin, glycosylated with two or three sugar residues. A large proportion of anthocyanins are acylated with hydroxycinnamic acids, which include sinapic acid, p-coumaric acid and ferulic acid [13, 14].

Absorption maxima of anthocyanin extracts of chokeberry and black carrot were at wavelengths of 520 and 543 nm, respectively.

Table 1. Characterization of the ethanolic extracts of chokeberry and black carrot

Type of extract	Color	TMA content (cyd eq mg/l) ±SD	A max (nm)
Chokeberry	purple-red	1051.84±40.15	520
Black carrot	dark purple	820.10±7.83	543

Analysis of the chokeberry and black carrot extracts showed that they all reacted with a color change when the pH changed (Figs. 1 and 2).

Both extracts showed significant color changes after changing the pH. In general, the color changed from rose-red (pH 2.0 and 3.0) through pink or pale pink (pH 4.0, 5.0 and 6.0) to purple and blue (pH 7.0 and 8.0). At pH above 8.0, the solutions turned violet. Visually, the most distinct color differences were between pH 6.0, 7.0 and 8.0 for samples of black carrot extract (from pink to purple and blue).

The composite films (F-AE1, F-AE2, F-AE3 or F-AE4) obtained after the inclusion of the corresponding

indicator solutions are homogeneous, flexible and pink-red to cherry-red in color. Films were easily peeled from the casting support.

pH 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0

Fig. 1. Changes in the color of chokeberry extract at different pH

pH 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0

Fig. 2. Changes in the color of black carrot extract at different pH

They were characterized by mass per square meter, moisture content (MC) and thickness (Table 2). The type of indicator solution reliably affects the moisture content of the film ($p < 0.01$). MC was highest in F-AE1, which included only chokeberry extract, and lowest in F-AE4, which contained only black carrot extract. Sample F-AE1 also has the largest thickness compared to the others, but the difference is not significant ($p > 0.05$).

Table 2. Mass per square meter, moisture content (MC) and average thickness of composite indicator films. Data are presented as mean ± SD

Film	Mass per square meter, g/m^2	Moisture content, g/100 g	Average thickness, mm
F-AE1	206	10.29±0.14	0.25±0.02
F-AE2	195	8.57±0.21	0.24±0.02
F-AE3	188	8.53±0.34	0.23±0.02
F-AE4	177	7.60±0.16	0.23±0.01

The color response of indicator films was evaluated by immersion in buffer solutions (pH 2.0 - 10.0). The color changes at different pH were visually clearly distinguishable. All indicator film variants became red when submitted to acid pH. At higher pH values, differences in color response were observed among samples. The indicator films with a high percentage of chokeberry extract (F-AE1 and F-AE2) change color from red-brown (neutral pH) to dark green (basic pH). Films with a predominant content of black carrot extract (F-AE3 and F-AE4) changed their color from cherry red (pH 5.0 and 6.0) to purple and blue (neutral pH) and dark violet (basic pH).

The results for color parameters of the indicator films after immersion in buffer media with different pH are presented in Tables 3 - 6.

The lightness (L^*) of all film samples changed as a function of pH value. The lowest value for the parameter L^* was observed, at pH 9.0 and 10.0, indicating that at basic pH the films tend to be darker. The a^* and b^* values varied showing that the films color changed significantly as a function of pH value. A higher value of the parameter a^* indicates the color tendency to red, confirming that anthocyanins in film matrix still keep their property of becoming red at low pH values. A similar trend was also found by Pourjavaher et al. [15] when analyzing the color characteristics of bacterial cellulose films with red cabbage extracts. The parameter b^* values below zero, indicate a color tendency to blue. This trend was particularly noticeable for films F-AE3 and F-AE4, where at pH 8.0 the value of b^* reached (-12.18) and (-13.66), which was in accordance with the visually observed blue color of the samples.

Table 3. Color parameters (L^*, a^*, b^*, ΔE^*) of composite indicator film (F-AE1) at different pH conditions. Data are presented as mean ± SD

pH	L^*	a^*	b^*	ΔE^*
2.0	45.42±0.37	18.21±0.14	4.07±0.04	9.17±0.12
3.0	37.75±0.16	15.17±0.27	6.93±0.25	2.89±0.13
4.0	39.34±0.33	19.00±0.09	9.80±0.13	4.61±0.07
5.0	42.19±0.13	11.55±0.39	5.03±0.02	4.69±0.05
6.0	42.39±0.22	10.80±0.42	3.28±0.13	6.34±0.17
7.0	34.61±0.21	10.70±0.14	3.25±0.11	8.60±0.14
8.0	33.40±0.21	8.34±0.17	1.39±0.07	11.66±0.18
9.0	30.69±0.06	7.78±0.13	-2.06±0.04	15.61±0.20
10.0	28.37±0.36	4.85±0.04	-3.87±0.08	19.63±0.08

Table 4. Color parameters (L^*, a^*, b^*, ΔE^*) of composite indicator film (F-AE2) at different pH conditions. Data are presented as mean ± SD

pH	L^*	a^*	b^*	ΔE^*
2.0	36.07±0.05	21.83±0.23	10.74±0.04	7.90±0.06
3.0	37.38±0.27	23.28±0.13	22.95±0.04	18.09±0.12
4.0	38.23±0.16	22.95±0.04	8.79±0.08	7.40±0.04
5.0	40.36±0.25	19.12±0.08	4.41±0.06	3.90±0.05
6.0	39.30±0.21	18.68±0.13	1.74±0.04	5.46±0.14
7.0	35.50±0.26	13.55±0.04	-1.37±0.05	9.08±0.10
8.0	33.09±0.06	7.72±0.05	-1.63±0.05	13.16±0.18
9.0	30.06±0.11	7.15±0.11	-2.16±0.04	15.45±0.15
10.0	29.42±0.10	6.29±0.01	-3.21±0.01	16.92±0.09

Table 5. Color parameters (L^*, a^*, b^*, ΔE^*) of composite indicator film (F-AE3) at different pH conditions. Data are presented as mean ± SD

pH	L^*	a^*	b^*	ΔE^*
2.0	34.83±0.05	20.84±0.10	7.92±0.06	7.19±0.06
3.0	34.83±0.09	21.8±0.07	6.68±0.06	5.76±0.04
4.0	35.44±0.04	23.26±0.04	3.14±0.10	2.25±0.06
5.0	34.39±0.07	22.89±0.06	-2.51±0.06	3.56±0.03
6.0	33.37±0.05	21.37±0.02	-7.16±0.11	8.45±0.07
7.0	30.52±0.23	12.04±0.04	-9.74±0.04	15.88±0.05
8.0	30.80±0.14	6.21±0.06	-12.18±0.13	21.66±0.12
9.0	30.64±0.18	4.53±0.04	-8.46±0.03	21.10±0.12
10.0	29.68±0.08	3.99±0.01	-5.78±0.01	20.75±0.09

The total color difference *(ΔE^*)* is the most useful color parameter. The ΔE^* values presented in Tables 3-6 were calculated for the indicator films before and after exposure to different pH media. A higher ΔE^* means a greater difference in color, therefore the difference is

more noticeable to the human eye. The *ΔE** values above 5.0 means that the two colors are perceived as different.

The *ΔE** values at pH range of 6.0 to 8.0 were highest in samples F-AE3 and F-AE4. Larger *ΔE** values mean that these indicator films have good visual color variability depending on the pH.

The results of testing the sensitivity of the pH-indicator films during refrigerated storage of chicken meat for a period of up to 14 d are given in Table 7. Table 8 presents data on pH and microbial status of meat in dynamics.

Table 6. Color parameters (*L*, a*, b*, ΔE**) of composite indicator film (F-AE4) at different pH conditions. Data are presented as mean ± SD

pH	*L**	*a**	*b**	*ΔE**
2.0	36.37±0.05	21.59±0.13	8.70±0.07	10.23±0.04
3.0	36.40±0.07	22.86±0.03	8.56±0.04	9.81±0.03
4.0	36.83±0.09	24.98±0.13	3.38±0.06	4.75±0.03
5.0	36.54±0.03	24.06±0.04	-3.44±0.04	2.85±0.04
6.0	34.81±0.15	21.21±0.08	-7.97±0.06	7.69±0.04
7.0	30.74±0.04	13.40±0.28	-9.83±0.04	14.83±0.07
8.0	32.40±0.07	5.79±0.06	-13.66±0.03	22.74±0.13
9.0	30.88±0.06	4.30±0.07	-7.57±0.05	21.65±0.09
10.0	29.89±0.02	4.19±0.04	-4.96±0.05	21.33±0.12

On the 7th and 14th d of meat storage, changes in the color parameters (*L*, a** and *b**) of all indicator film samples were observed. Maximum values for *ΔE** were reported on the 7th (8.04 and 8.45) and 14th (15.57 and 22.70) d in experimental groups F-AE3 and F-AE4, respectively.

Table 7. *ΔE** of experimental groups of pH-indicator films during refrigerated storage of chicken meat

Film	*ΔE**	
	7 days	14 days
F-AE1	6.93	8.04
F-AE2	6.46	13.66
F-AE3	8.04	15.57
F-AE4	8.45	22.70

Table 8. pH value and total microorganism counts (TVC) in chicken meat during refrigerated storage. Data are presented as mean± SD

Time of storage	pH	Total viable count (log CFU/g)
Day 0	6.31±0.09	3.59±0.24
Day 7	6.78±0.08	7.03±0.07
Day 14	7.34±0.14	9.43±0.56

Changes in the color of the indicator films correlate with pH values and total microorganism counts (Table 8). A significant increase in pH was found after 7 and 14 d of storage, 6.78 and 7.34, respectively. TVC increased from 3.59 log CFU/g (day 0) to 9.43 log CFU/g at day 14 of storage.

On the 7 d of storage, the total number of microorganisms was 7.03 log CFU/g. These values reach the permissible limit of 10^7 CFU/g, after which the meat is considered as unacceptable for consumption. Under these conditions, a visible change in the color of the placed pH-indicator films was also observed, which was more pronounced in experimental groups F-AE3 and F-AE4.

4 Conclusion

A series of pH-sensitive indicators, based on chokeberry and black carrot anthocyanins, incorporated into an edible alginate/pectin/gum arabic film was developed. Indicator films change color as a function of changing pH, with significant differences observed depending on the type of extracts included. The total color difference is most pronounced in films containing black carrot extract (F-AE4) and a mixture of chokeberry and black carrot in a ratio of 1:3 (F-AE3). The color transition in these samples varies from pink at acidic pH to purple and blue at neutral pH and dark violet at basic pH. The applicability of the developed pH-indicator films has been demonstrated in refrigerated storage of fresh chicken meat. Changes in the color of the indicator films correlate with pH of meat and total microorganism counts, and the largest values for ΔE on day 7 and 14 were observed in groups F-AE3 and F-AE4. The developed pH-sensitive films have potential for use in smart packaging systems as an indicator for meat freshness monitoring. However, further experiments are needed to increase the sensitivity of the indicator films and to determine the stability of the included anthocyanin extracts.

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