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Bioindicators for the Assessment of Microbiological Purity in Some Alimentary Products

Abstract

A method is presented for the preparation of fluorescence bioindicators in the form of a film. The base film is made of either cellulose acetate or cellulose acetate-covered paperboard prepared from a bio-modified cellulose. The film contains fluorescein acetate (FDA). It has been documented that the bioindicators reveal a colour change in UV light when in contact with esterases produced by microorganisms and accumulated in meat to an amount exceeding the permissible limits. The hue change toward yellow-green proceeds as a consequence of the liberation of free fluorescein by the action of enzymes from the esterase group. The released fluorescein remains in the bioindicator and does not migrate to the tested product. An intensity increase of the excited light (R+G) to the level ≥ 90 units indicates decay of the product.

Key words: bioindicator, fluorescein acetate (FDA), film, food freshness.

Introduction

Food products go bad with the growth of various bacteria, yeast and mould under the destructive action of proteolytic, lipolytic and amylolytic enzymes produced by microorganisms. Conventional identification methods by plate inoculation, commonly applied in the diagnosis of microorganisms, call for laborious procedures. Passing onto consecutive selective media is required, meaning it is a long time before the results are available. A qualitative and quantitative analysis of the microbiological purity of meat and milk by traditional methods usually lasts several days.

Recently, a number of innovative procedures have been prepared that considerably shorten the time required and provide for the selectivity and sensitivity of the analysis, like immunoenzymatic and fluorometric methods, gene probes and biosensors [1-4]. Such modern methods, however, require the use of specialized, high class control and laboratory equipment usually not available in industrial praxis because of the high investment and running costs.

The presently applied methods to assess the amount of microorganisms, such as the estimation of the adenosine triphosphate (ATP) level [5] or the radiation marking of thymidine built in the microorganisms' DNA [6], require highly skilled personnel and are time-consuming in contrast to the simple and fast procedures with FDA [3, 7].

Kramer and Guilbault were the first to report on the use of fluorescein esters for the estimation of lipases' activity [8].

Fluorescein diacetate (FDA) is applied nowadays in the monitoring of the environment, e.g. for the fast estimation of esterase activity in biofilms [9, 10], the estimation of the total microbiological activity of soil by determining the pool of esterases, lipases and proteases in the soil [3, 5] and the determination of esterases in the activated sludge of wastewater treatment plants [11]. Such methods are considered to be simple, sensitive and precise and, therefore, are recommended for the monitoring of bio-ecosystems [12].

The hydrolysis process of FDA is applied as a simple method to determine the total microorganisms' activity. FDA is a colourless substance that undergoes hydrolysis by the action of enzymes (lipases, esterases, proteases) both free and bound with cell membranes. The hydrolysis product – fluorescein – is a colourful fluorescent compound that reveals a maximum light absorption at wavelength $\lambda = 490$ nm and radiation emission at $\lambda = 514$ nm. The release rate of fluorescein is proportional to the enzymes' concentration.

Reports could not be found in the specialized literature on the use of FDA for the monitoring of food freshness.

Alerting to the presence of microbiological impurities is crucial in the quality control of food products to protect consumers. On the other hand, fast analytical methods are indispensable in automated technological processes to safeguard the effective control of the full production lines and the quality of the end product.

Presented below is a method to prepare fluorescent bioindicators in the form of FDA-containing film as freshness sensors designed for a fast and simple assessment of the microbiological contamination of meat.

Materials and research methods

Materials

The following materials were used in the investigations: fluorescein diacetate (FDA) from Sigma Co., cellulose acetate (molecular weight ~ 30 kDa and $\sim 40\%$ substitution) from Aldrich Co. and cellulose pulp from Buckeye Co. The cellulose pulp was bio-modified by means of the cellulolytic enzyme system Ecostone L-300 by AB Enzymes Co. The microbiological contamination testing was carried out with beef.

Methods

Preparation of the bioindicator in the form of film cast on a paperboard carrier (BP)

FDA was dissolved in acetone; next, glycerol (0.75%) and cellulose acetate (2.5%) were added. The FDA concentration in the individual solutions was set at 1.2, 2.4, 4.8, 9.6 and 19.2 mmol/l. A small sheet of paperboard with a surface weight of 100 g/m² was formed from the enzyme-modified pulp [13] in a Rapid-Kothen apparatus. The FDA solution was applied to the paperboard sheet by means of a slot device (slot width 0.1 mm). The paper sheet was left for 2 hours in a well-ventilated room at 20 °C to evaporate acetone. The bioindicator sheet was, after drying, cut into 1×3 cm strips.

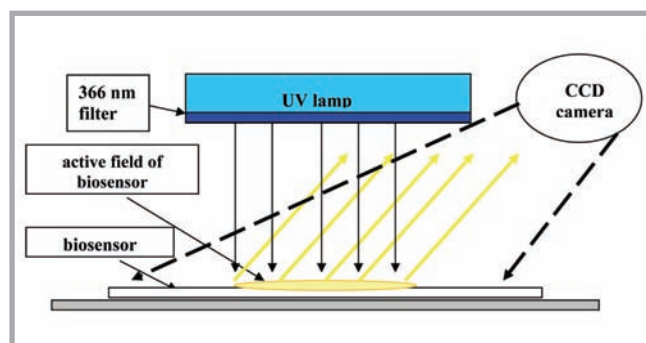


Figure 1. Scheme of the colour detector.

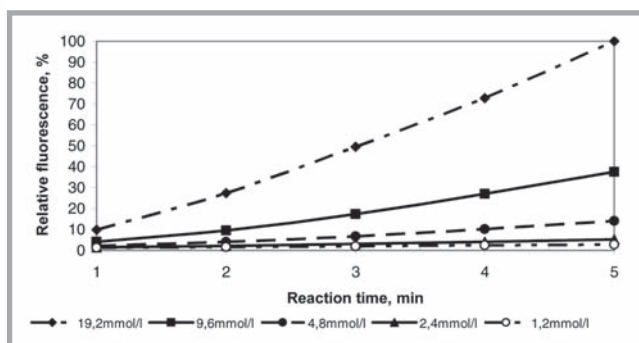


Figure 2. Impact of FDA concentration in the solution and the time of its hydrolysis by the esterases of beef-infecting bacteria upon the relative intensity of fluorescence.

Preparation of the bioindicator in the form of film from cellulose acetate (BF)

FDA was dissolved in acetone; next, glycerol (1.5%) and 2.5 cellulose acetate (5%) were added. The FDA concentration in the individual solutions was set at 0.6, 1.2, 2.4, 4.8, 9.6 and 19.2 mmol/l. The film was prepared by uniform spreading of 1 or 2 ml of the solution on a glass Petri plate with a 6 cm dia. The plate was left for 2 hours in a well-ventilated room at 20 °C to evaporate acetone.

Testing of the film mechanical properties

The testing was carried out on an Instron 5544 machine according to the following standards:

- thickness – PN-EN ISO 4953:1999
- tenacity and elongation – PN-EN ISO 527-3:1998

Estimation of FDA migration from the film to water and methanol

A sheet (surface 12.75 cm²) of the bioindicator was immersed in 3 cm³ of methanol or distilled water and left for 4 hours at an ambient temperature. The FDA concentration in water and methanol was estimated by comparison with calibration curves at a wavelength of $\lambda = 220$ nm.

Estimation of the total number of microorganisms in the meat

Fresh beef was incubated for 1-2 days at 4±1 °C or 22±1 °C. To 10 g of the beef, 90 ml of normal saline was added, which was then shaken for a duration of 30 minutes in a rotary shaker at 100 rpm. A suspension of microorganisms washed out of the meat was filled up to 100 ml. The total number of microorganisms in the tested samples was estimated according to standard PN-EN ISO 4833. The results were reckoned according to the amendment to standard PN-ISO 7218: 1998/A1.

Estimation of esterase activity

The esterase activity in the hydrolysis reaction of p-nitrophenol acetate (substrate) was estimated according to a description to be found elsewhere [14]. To 2.3 ml of the microorganisms suspension washed out of the meat, 0.2 ml of substrate was added and the mixture was incubated at 37 °C for 5-30 minutes. The product (p-nitrophenol) released in the reaction was estimated by comparison with a calibration curve at wavelength $\lambda = 420$ nm. Assumed to be a unit was an activity of the enzyme that causes the hydrolysis of the substrate and delivers 1 μ mol of p-nitrophenol in 1 minute.

Determination of the relative intensity of the fluorescence of FDA hydrolysis products

To 3.8 ml of phosphate buffer at a pH of 7.5, 0.1 ml of acetone FDA solution was added, with a concentration of 1.2, 2.4, 4.8, 9.6 or 19.2 mmol/l as well as 0.1 ml of the microorganism suspension (meat extract). The mixture was for incubated 10 minutes at 22±1 °C. Simultaneously, a control was prepared in which the microorganism suspension was replaced with 0.1 ml of the phosphate buffer. The fluorescence was measured on a Quantech fluorometer at an excitation wavelength of $\lambda = 490$ nm and emission $\lambda = 514$ nm and expressed in % of the highest intensity.

Application of the bioindicators in the testing of meat freshness

The beef that was used for the *in vitro* examination was cut into 10 g pieces and stored at 4 °C and 22 °C. Since the meat surface dries up during storage, it was, prior to the testing, sprinkled with 0.2 ml of sterile 0.1 mol/l phosphate buffer at pH 7.0. The bioindicator was pressed against the meat in the sprinkled area

to allow the microorganisms and their metabolites to saturate the bioindicator surface. The indicator was next placed in a closed glass Petri plate at 22 °C. After a given time (5-60 minutes depending upon the applied FDA concentration), the indicator was put into the detector schematically presented in Figure 1, in which, after exposure to UV light, photos were taken. The change of colour intensity of the indicator was visually observed through an organic glass pane. The digital image in the form of a bit map with 3648×2736 pixel resolution was computer-analysed by means of the MicroScan software. Selected parts of the indicator surface with a yellow-green hue were investigated, located in the region where the indicator was saturated with the bacteria metabolism products (active field) migrating from the meat surface. The intensity level of components R (red) and G (green) were analysed, which, when combined, yield yellow. The intensity of R and G components was expressed on the standard 0-255 scale by the deduction of the R+G level obtained for fresh meat from the sample results.

Results of the investigations

Microflora of the meat and its esterase activity

Food decay is caused mainly by the excessive growth of microorganisms infecting the alimentary products.

Table 1. Content of microorganisms in the examined meat during storage.

Storage temperature	Storage time (days)		
	0	1	2
	Number of microorganisms (cfu/g)		
22 °C	1.39×10 ³	6.75×10 ⁷	5.20×10 ⁹
4 °C	1.39×10 ³	2.54×10 ⁴	8.73×10 ⁶

Table 2. Activity of esterases contained in meat extracts during storage at various temperatures.

Storage temperature	Storage time, days				
	0	1	2	3	4
	Esterase activity				
22 °C	44.68 ¹ 8.93 ²	72.90 ¹ 14.53 ²	92.28 ¹ 18.46 ²	119.41 ¹ 23.88 ²	110.84 ¹ 22.18 ²
4 °C	44.68 ¹ 8.93 ²	47.14 ¹ 9.43 ²	51.72 ¹ 10.34 ²	65.38 ¹ 13.08 ²	57.68 ¹ 11.54 ²

¹ – esterase activity ($\mu\text{mol/ml}\times\text{min}$) calculated on 1 ml of bacteria suspension washed out from a meat sample

² – esterase activity ($\mu\text{mol/ml}\times\text{min}$) calculated on 1 g of meat sample

Table 3. Mechanical properties of the FDA-containing cellulose acetate film.

Film thickness, mm	Thickness coefficient of variation, %	Tenacity, MPa	Tenacity coefficient of variation, %	Elongation, %	Elongation coefficient of variation, %
0.062	6.7	68.4	20.2	42.4	17.3
0.034	4.4	73.8	18.7	22.7	16.2

The food itself, particularly if improperly processed, generates the undesired microflora, the environment being another source of contamination. The microorganisms deliver extracellularly various products of metabolism of which enzymes like esterases and proteases can be harnessed to the monitoring of food freshness.

Table 1 (see page 123) shows the results of the investigation into the number of microorganisms that have their abode on the surface of meat stored at 22 °C and 4 °C. The number increases with time and rising storage temperature. The permissible number of bacteria laid down in the EU Gazette amounts to $5\times 10^5 - 5\times 10^6$ cfu/g [15].

With increasing storage temperature and time, the activity of esterases accumulated by the microorganisms on the meat surface increases (see **Table 2**). There are various microorganisms settled on the meat surface, hence the activity of selected esterases depends not only on the number of microorganisms but also

Table 4. Migration of FDA from the surface of the bioindicator to the model fluid.

Kind of bioindicator	FDA migration to model fluid, $\mu\text{g}/\text{cm}^2$	
	water	methanol
Film of cellulose acetate	0	1.29
Film of cellulose acetate cast on paperboard	0	3.27

on their genus. The same amount of microorganisms taken from different places in the meat may reveal varying esterase activity.

Esterases hydrolyse esters of fluorescein (non-fluorescent) to free fluorescein, which is highly fluorescent. The fluorescence can be seen even at a dilution as high as 1:40 000 000. Its intensity depends upon the activity of esterase and action time and temperature. In the work of Ge et al. [16], it was assumed that the hydrolysis rate of fluorescein esters catalysed by esterases depends upon the chain length of the substituted group considering C_{12} (laurate) as the maximal value. It has been shown in our investigation that the esterases extracted from infected meat hydrolysed fluorescein dilaurate to an insignificant extent while fluorescein diacetate (FDA) underwent hydrolysis with the highest activity. That was the reason why FDA was selected as a marker for the prepared bioindicators.

The esterase-catalysed hydrolysis becomes shorter with increasing FDA concentration. With an FDA concentration amounting to 9.6 and 19.2 mmol/l, the esterases generated by the meat-infecting bacteria effectively liberated fluorescein after only 5 minutes' reaction in a test-tube (**Figure 2**). At a lower FDA concentration, a longer reaction time with the esterases was needed.

Properties of the bioindicator-containing film

The FDA-containing film made of cel-

lulose diacetate is characterized by good mechanical properties (**Table 3**) and transparency, a valuable feature for possible uses of the material in packaging. It was found that fluorescein diacetate does not migrate from the film to a water medium. A small amount migrates to a methanol medium (see **Table 4**).

In vivo assessment of the performance of the fluorescent bioindicators in microbiological purity control of beef

Testing of meat by means of bioindicators prepared on a paperboard carrier (BP)

In the case of fresh beef (bacteria in the amount of 1.39×10^3 cfu/g), the yellow-green hue in the active fields could not be seen with the naked eye after 30 minutes of incubation with FDA concentration in the range of 1.2-9.6 mmol/l. The sensor containing FDA at 19.2 mmol/l concentration revealed a slight hue change with intensity of the (R+G) components equal to 25 (**Figure 4**, curve A).

After 24 hours' shelf time at 22 °C, the meat contained 6.75×10^7 cfu/g, exceeding the permissible limit. The beef did not smell like a stale product, though the analysis with bioindicators revealed that the product was no longer fresh. After 30 minutes, in the active field of the bioindicator with 19.2 mmol/l FDA appeared (naked eye) the distinct yellow-green hue characteristic of delivered fluorescein (**Figure 3b**). The intensity of the components (R+G) was equal to 100 (**Figure 3b**).

When the beef stored for 48 hours at 22 °C containing 5.20×10^9 cfu/g of bacteria was analysed, the distinct yellow-green hue appeared after 20 minutes (**Figure 3c**). For the FDA concentrations 9.6 and 19.2 mmol/l, the intensity of the (R+G) components was 150 and 180, respectively (**Figure 5**, curve B). The reading in the detector after 30 min distinctly deepened the emerged hue of the active fields (**Figure 3d**). After that time, the distinct yellow-green hue was seen in the FDA concentration range 4.8, 9.6 and 19.2 mmol/l, for which the intensity of the components (R+G) was equal to 140, 170 and 210, respectively (**Figure 5**, curve C).

Examination of the meat stored for 48 hours at 4 °C, which contained microorganisms in an amount of 5.24×10^6 cfu/g, showed that it was no longer fresh de-

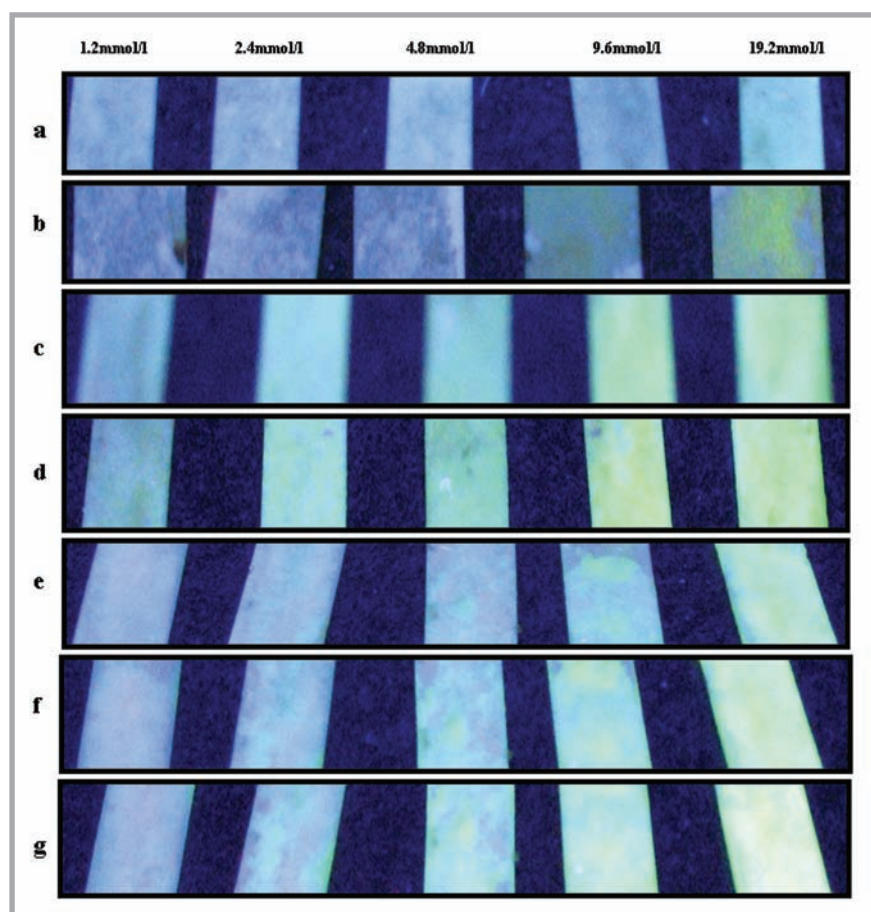


Figure 3. Photos of BP bioindicators taken in the detector in UV light (FDA concentration shown above the images): a) Beef fresh acquired. Reading after 30 min., b) Beef stored for 24 hours at 22 °C. Reading after 30 min., c) Beef stored for 48 hours at 22 °C. Reading after 20 min., d) Beef stored for 48 hours at 22 °C. Reading after 30 min., e) Beef stored for 48 hours at 4 °C. Reading after 15 min., f) Beef stored for 48 hours at 4 °C. Reading after 20 min., g) Beef stored for 48 hours at 4 °C. Reading after 30 min.

spite the absence of an unpleasant smell. After 5 minutes, the bioindicator containing FDA with 19.2 mmol/l concentration changed its hue to yellow-green (Figure 3e) with a 140 intensity of the (R+G) components (Figure 6, curve B). With prolonged action time of the esterases, the yellow-green hue could be seen and measured at a lower FDA concentration. After 20 minutes, a distinct yellow-green hue (Figure 3f) appeared for FDA 9.6 mmol/l concentration with (R+G) = 125 and for 19.2 mmol/l concentration with (R+G) = 165 (Figure 6, curve C). Prolonging the time to 30 minutes resulted in a deeper shade of the arisen hue (Figure 3g), which could be seen at FDA 4.8 mmol/l, 9.6 mmol/l and 19.2 mmol/l with (R+G) = 115, 205 and 230, respectively (Figure 6, curve D).

Testing of the meat by means of bioindicators in the form of cellulose acetate film (BF)

In addition to the bioindicators on paperboard as a carrier, indicators in the

form of a cellulose acetate film were also prepared. The film can also be used as a packaging material for alimentary products. Fresh meat as well as that stored at 4 °C and 22 °C was tested.

The fresh meat contained 1.39×10^3 cfu/g of microorganisms when acquired; after 24 hours' storage at 4 °C, the amount increased to 2.54×10^4 cfu/g. The bioindicator observed in the detector did not reveal the yellow-green hue in the active fields after 60 minutes. The measured intensity of the (R+G) components was very low, in the 5-15 range (Figure 7, curve A).

The meat stored for 24 hours at 22 °C did not emit an odorous smell, though it contained 6.75×10^7 cfu/g of microorganisms. Examination of the product by means of biosensors showed that it was unfit for consumption. Packaging film containing FDA at a concentration of 4.8 mmol/min and more when put into the detector was clearly shining in the yellow-green hue

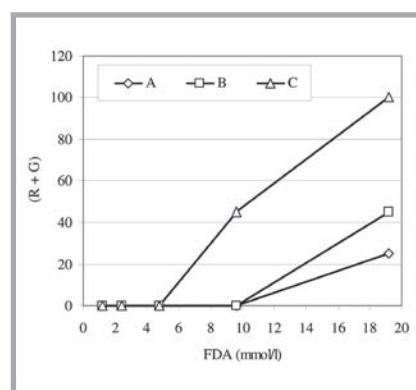


Figure 4. Intensity change of hue components (R+G) of the light excited by UV radiation seen in the detector as yellow-green light. Bioindicator BP. A – Beef freshly acquired, number of microorganisms 1.39×10^3 cfu/g. Reading after 30 min. B – Beef stored for 24 hours at 22 °C, number of microorganisms 6.75×10^7 cfu/g. Reading after 15 min. C – Beef stored for 24 hours at 22 °C, number of microorganisms 6.75×10^7 cfu/g. Reading after 30 min.

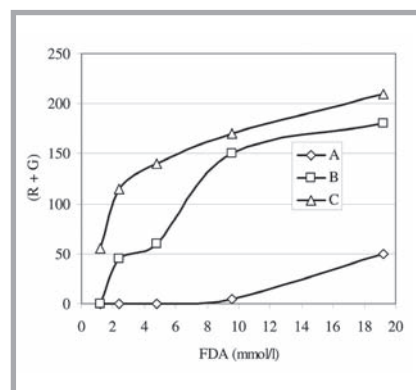


Figure 5. The change of intensity of (R+G) components of light excited by UV radiation observed in the detector as a yellow-green hue. Bioindicator BP. Meat stored for 48 hours at 22 °C, number of microorganisms 5.20×10^6 cfu/g. Reading: A – after 5 min, B – after 20 min, C – after 30 min.

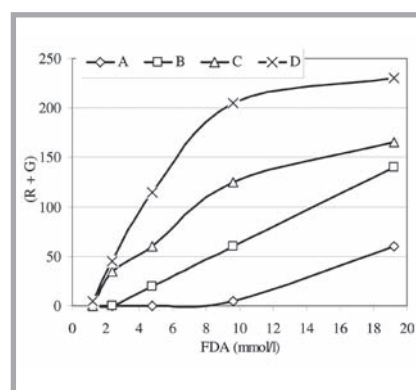


Figure 6. The change of intensity of (R+G) components of light excited by UV radiation observed in the detector as a yellow-green hue. Bioindicator BP. Meat stored for 48 hours at 4 °C, number of microorganisms: 5.24×10^6 cfu/g. Reading A – after 5 min, B – after 15 min, C – after 20 min, D – after 30 min.

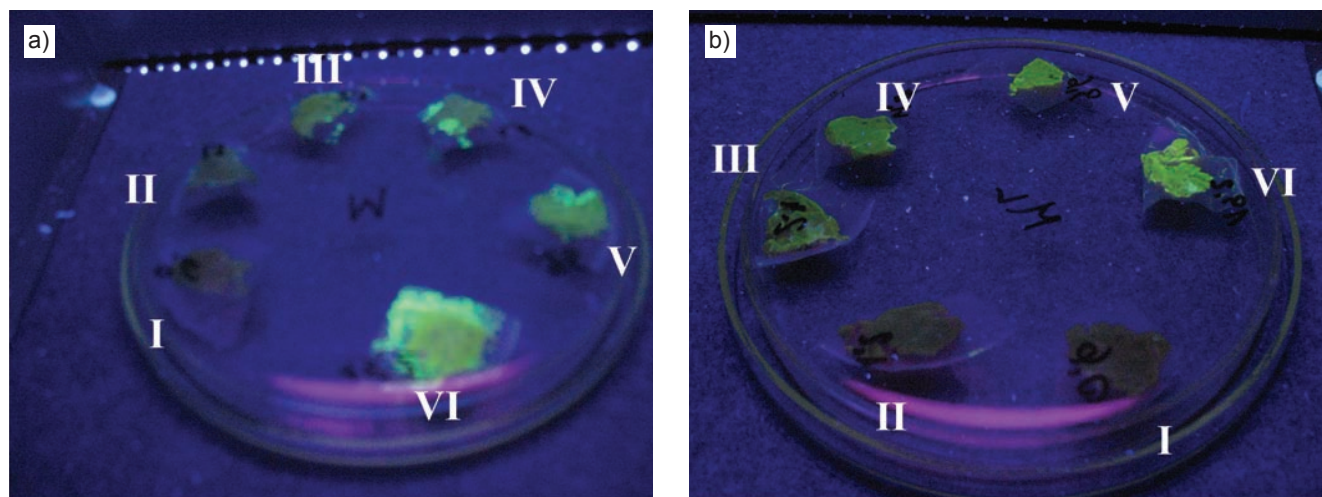


Figure 8. Photos of bioindicators BF taken in the detector in UV light. Reading after 60 min. FDA concentration: I – 1.2 mmol/l; II – 2.4 mmol/l; III – 2.4 mmol/l; IV – 4.8 mmol/l; V – 9.6 mmol/l; VI – 19.2 mmol/l.; a) Beef stored for 24 hours at 22 °C. Number of microorganisms after 24 hours: 6.75×10^7 cfu/g; b) Beef stored for 48 hours at 4 °C. Number of microorganisms after 24 hours: 8.73×10^6 cfu/g.

(Figure 8a). The intensity of the (R+G) components at an FDA concentration of 4.8 mmol/l, 9.6 mmol/l and 19.2 mmol/l amounted to: 90, 140 and 245, respectively (Figure 7, curve B).

The meat stored for 48 hours at 4 °C did not emit any odorous smell, though the measurement of the microorganism amount gave the result of 8.73×10^6 cfu/g. The analysis with film biosensors confirmed that the meat was not fit for consumption. The biosensors put into the detector were clearly shining in a yellow-green hue (Figure 8b). The intensity of the (R+G) components at an FDA concentration of 4.8 mmol/l, 9.6 mmol/l and 19.2 mmol/l amounted to: 90, 110 and 145, respectively (Figure 7, curve C).

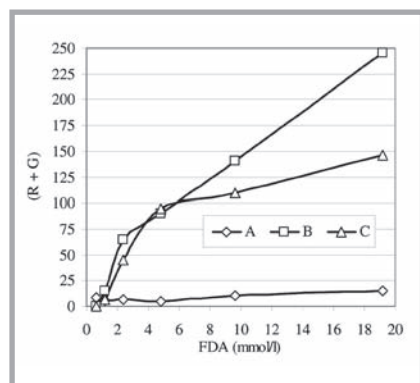


Figure 7. The change of intensity of (R+G) components of light excited by UV radiation seen in the detector as a yellow-green hue. Bioindicator BF. Reading after 60 min. A – Meat stored for 24 hours at 4 °C, number of microorganisms 2.54×10^4 cfu/g. B – Meat stored for 24 hours at 22 °C, number of microorganisms 6.75×10^7 cfu/g. C – Meat stored for 48 hours at 4 °C, number of microorganisms 8.73×10^6 cfu/g.

Summary and conclusions

Quick information signalling the presence of microbiological contamination is crucial in quality control and for the protection of potential consumers. Continuous automated processes in the food industry call for simple and fast analytical methods. The biosensors presented herein are a response to this demand.

It has been shown in this work that a fluorescent bioindicator prepared on a base of paperboard made of enzymatically modified cellulose coated with cellulose acetate containing FDA at a concentration of 9.2 or 19.2 mmol/l allows the reliable estimation of the meat's freshness in as short a time as 30 minutes. A biosensor in the form of cellulose acetate containing FDA features good mechanical properties, thus providing an opportunity for its use as smart packaging material with a built-in meat freshness sensor.

Both kinds of biosensors used in freshness testing present a reliable tool for internal and external quality control of alimentary products. In the course of manufacture, consumer organizations may use the tool for fast and simple freshness control during storage and distribution.

It must, however, be said that the use of the proposed biosensors in routine quality control requires further investigation and testing to prepare a final product for commercial uses.

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