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Development of New Chemiluminescence Biosensors for Determination of Biogenic Amines in Meat

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Abstract

Development of chemiluminescence one-shot biosensors for determination of biogenic amines is described and compared with high-performance liquid chromatography (HPLC) method coupled with pre-column derivatisation.

The biosensors are based on enzymatic oxidation to 4-aminobutyraldehyde with putrescine oxidase or diamine oxidase as catalysts. The lowest measured concentration for the biosensor with putrescine oxidase was 1 mg/L. The detection limit, calculated as 3σ value, was 0.8 mg/L. The biosensor with diamine oxidase had the lowest measured concentration of 1 mg/L of putrescine. Detection limit, calculated as 3σ value, was 1.3 mg/L.

Biosensors were tested on five different meat samples, and the results were compared with HPLC coupled with pre-column derivatization.

Results showed that new biosensors could be used in determination of putrescine concentration in meat samples but improvements, such as sample pretreatment before determination or design of interference free biosensor, are required.

Keywords: biogenic amine, determination, chemiluminescence, biosensor, putrescine, meat freshness

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1. Introduction

Biogenic amines (BA) are synthesized and degraded during metabolism of animals, plants and microorganisms. Histamine, putrescine, cadaverine, tyramine, tryptamine, spermine and spermidine are considered to be some of the most important BA in food and human body (Alonso-Lomillo, Dominguez-Renedo, Matos, Arcos- Martinez, 2010).

The presence and detection of the BA in food is of interest for two reasons: firstly, for toxicological reasons, since high levels of dietary BA can be toxic for certain consumers, and secondly, for their role as possible quality indicators (Ruiz-Capillas, Jimnez-Colmenero, 2005).

Many BA have been studied in scientific literature, however diamines, such as putrescine, histamine and cadaverine are often documented in studies (Den Brinker, Kerr, Rayner, 2002). Putrescine and cadaverine have been confirmed to be useful chemical indicators (Yano, Yokoyama, Tamiya, Karube, I, 1996).

BA are released during storage and processing, if foods are mishandled. Certain proteins within the foods might break down to free amino acids, which may also be naturally present within the food. If the food is contaminated with bacteria containing decarboxylase enzymes, these free amino acids undergo decarboxylation to produce BAs.

Analysis of BAs in food is the subject of numerous scientific studies and articles. The fish products head the list of foods most studied for the presence of BAs. Scombroid poisoning, the most common fish poisoning, occurs by eating spoiled fish. The scombroid poisoning is mainly caused by elevated histamine levels in fish, generated by bacterial enzymatic conversion of free histadine. Many analytical methods were developed for histamine determination. This is the only BA having official limits in fish products (50 mg/kg proposed by the US Food and Drug Administration (FDA), and as 100 mg/kg proposed by the European Commission (EU Directive Regulation EC No 14412007, FDA Fish and Fishery Products Hazards and Controls).

BA are considered as markers of freshness and hygiene during storage of meat and meat products. Tyramine, cadaverine, putrescine and histamine are the most common BAs in meat and meat products. Histamine poisoning is less common for meat products because its concentration is usually quantitatively lower than that found in fish. In the past years, the emphasis in BA studies of meat products has been on processed meat products, because of its higher potential for forming carcinogenic nitrosamines in meat products with high level of BAs and added nitrates and nitrites as preservatives (Ruiz-Capillas, Jimenez-Colmenero, 2004).

1.1. Analytical methods for BAs analysis

The quantification of BAs in food is mainly performed by chromatographic techniques, such as high performance liquid chromatography (HPLC), gas chromatography (GC) and thin layer chromatography (TLC), or by capillary electrophoresis (CE) methods.

Classical reversed-phase HPLC has been generally the most used technique for the determination of BAs in different kinds of food. BAs are extracted from the food matrix before HPLC analysis with acidic solvents (trichloroacetic acid, perchloric acid or hydrochloric acid). Treatment with acids deproteinizes extracts.

Biogenic amines were analyzed with HPLC method in the evaluation of the effect of salt reduction from 6% to 3% in two Portuguese traditional blood dry-cured sausages. Samples were extracted with perchloric acid and derivatised with dansyl chloride in alkaline medium (Laranjo, Gomes, Agulheiro-Santor, Potes, Cabrita, Garcia, Rocha, Roseiro, Fernandes, Fraqueza, Elias, 2016).

The main disadvantage of chromatographic methods in determination of biogenic amines is that they are quite complex and require long analysis times and expensive instrumentation.

An optical sensor microtiterplate for quantitative analysis of the total content of biogenic amines in meat and cheese was developed. A chameleon dye (Py-1) is embedded in a polymeric cocktail which is deposited on the bottom of the

wells in a common microtiterplate. On reaction with biogenic amines (BAs), The fluorescence of Py-1 at 620 nm rapidly delivers a signal in reaction with biogenic amines (Khairy, Azab, El-Korashy, Steiner, Duerkop 2016).

Electrochemical biosensors based on diamine oxidase (DAO) conjugated to magnetic beads (MBs) were developed for the detection of histamine (Hist), putrescine (Put) and cadaverine (Cad). DAO-MBs were immobilised on Co(II)-phthalocyanine/carbon and Prussian Blue/carbon electrodes to obtain mono-enzymatic biosensors, and on Os-wired HRP-modified carbon electrodes to obtain bi-enzymatic biosensors (Leonardo, Campas 2016).

The basic idea for this work was to develop two biosensors for quantitation of putrescine in meat samples and to compare characteristics of new biosensors in comparison with standard method for BA determination (HPLC coupled with pre-column derivatization).

Putrescine is formed in enzymatic reaction of BA with putrescine oxidase or diamine oxidase. Putrescine does not have chemiluminescence properties and thus cannot be determined directly with chemiluminescence reaction. Instead, hydrogen peroxide, a side product of putrescine enzymatic reaction, will produce chemiluminescence light in reaction with luminol. The intensity of the light is equal to the concentration of the hydrogen peroxide. On the other side, concentration of

hydrogen peroxide corresponds to the concentration of putrescine. Since hydrogen peroxide is common reactant in chemiluminescence reaction, it can be used for indirect determination of putrescine in the new biosensors. The determination of hydrogen peroxide concentration relies on its well-known chemiluminescence reaction with chemiluminescent reagent - luminol in the presence of catalyst and surfactant. The intensity of light produced by this reaction is proportional to the concentration of hydrogen peroxide formed in the reaction.

$$\text{Putrescine} + H_2O + O_2 \xrightarrow[\text{diamine oxidase}]{\text{putrescine oxidase}} H_2O_2 + \text{4-aminobutanal} + NH_3$$

$$H_2O_2 + \text{luminol} \xrightarrow[\text{catalyst}]{\text{base}} \text{3-aminophthalate} + \text{light}$$

The Co(II) catalyzed chemiluminescence reaction of luminol with hydrogen peroxide in alkaline solution has been largely used for the determination of hydrogen peroxide (Tahirovic, Copra, Omanovic-Miklicanin, Kalcher, 2007).

2. Materials and methods

2.1 Apparatus

All measurements with new biosensors were performed with microplate luminometer, Lucy1 of Anthos Labtec Instruments GmbH, Lagerhausstrasse 507, A-5071 Wals/Salzburg, Austria. Chromatographic measurements were performed on HPLC equipped with diode array and fluorescence detector, Hewlett Packard (Palo Alto, CA), model 1100.

2.2 Reagents

Luminol, trisodium phosphate, cobalt(II) chloride hexahydrate, sodium laurylsulphate, 1,4-diaminobutane dihydrochloride, sarcosine were obtained from Fluka (Graz, Austria). Diamine oxidase, 3-hydroxytryamine hydrochloride, L-glutamine, dopamine hydrochloride, β-(3,4-dihydroxyphenyl)-ethylamine hydrochloride, trichloroacetic acid, sodium hydroxide, dansyl chloride, ammonium hydroxide were from Sigma Aldrich Chemicals (Graz, Austria). Hydrogen peroxide was from Merck. Putrescine oxidase was obtained from Sorachim Sarl, Paris, France.

2.3. Biosensors preparation

Biosensors were prepared in wells of luminometer microplates, separated on the basis of the applied enzyme, according to the existing procedure (Tahirovic, Copra, Omanovic-Miklicanin, Kalcher, 2007). Two single shot biosensors were prepared with putrescine oxidase and diamine oxidase, respectively.

Hydroxyethyl cellulose (150 mg), weighed into a centrifuge tube, was added with luminol (0.195 μg), sodium phosphate

(8.6 mg) and sodium lauryl sulphate (0.6 mg). All components were mixed and dissolved with water in a solution of 10 mL. Ten microliters of polymer solution was dispensed onto a microscope cover glass (18 mm x 18 mm, Menzel-Gläser, Germany) which was previously rinsed thoroughly with ethanol (96%). The glass was equipped with a self-adhesive reinforcement ring (polypropylene, inner diameter 5.2 mm; Avery Zweckform 5308) in order to obtain a defined area for the membrane formation. The membranes were dried in oven at 70°C for 2 hours and then cooled and stored in desiccators.

The catalyst for luminol reaction, cobalt(II) chloride (10 µL, 0.05 mmol/L) was applied on cooled membranes. The membranes were again dried in oven at 70°C for 2 hours and cooled and stored in desiccators.

The enzyme solution (5 µL) was applied on membrane surface shortly before analysis. The membranes with enzyme solution were dried in desiccators and stored in refrigerator.

2.4. Procedure for biosensors

A biosensor (glass support with membrane) was fixed directly on the photodiode, and then the pipette holder was mounted over it. Recording of the signal was started immediately before dispensing 10 µL of the sample solution onto the membrane, by a 10 µL micropipette. For each measurement, one new biosensor was used (one-shot sensors). Data were evaluated by

summing 100 data points, starting from the beginning of chemiluminescence reaction.

2.5. Sample preparation

2.5.1 Selection of meat samples

Beef, pork, chicken, turkey and fish meat samples were selected as model food for BA analysis. Red and white meat samples were obtained from same butcher's shop, while the fish sample was obtained from fishmongers. Meat samples were stored in refrigerator at 4°C for a period of 8 days and portions of them were analysed for BA content with biosensors and HPLC at day 1, day 3 and day 8 of storage.

2.5.2. Sample preparation for biosensors analysis

All samples (20 g of each) were minced, mixed with 40 mL of water and homogenized in blender. The solutions were then filtrated and used for determination of BA.

2.5.3. Sample preparation for HPLC analysis

Samples for HPLC analysis were prepared as previously described[16]. Minced samples (5 g) were homogenized with 10 mL of 5% trichloroacetic acid (TCA) for 2 min and centrifuged at 3000 g for 10 min (4°C). The supernatant was collected and the residue was extracted again with an equal volume of 5% TCA. Both supernatants were combined and the final volume adjusted to 25 mL with 5% TCA. The extracts were filtered through qualitative filter paper and derivatised by mixing 0.5 mL of each extract or standard solution with 100 µL

of 2 M sodium hydroxide to adjust the pH to about 9.5 and adding 1 mL of 10 mg/mL dansyl chloride (prepared in acetone) at 60°C for 30 min. The reactant was then mixed with 100 µL of 25% ammonium hydroxide and kept at room temperature for 30 min, to remove residual dansyl chloride. After incubation at 60°C for 15 min, to remove the residual acetone, the mixture was adjusted to 2.5 mL with acetonitrile and filtered through a 0.45 µm membrane for HPLC analysis. The analysis was carried out with an HPLC Hewlett-Packard 1100 equipped with a Diode Array Detector (Hewlett-Packard, Palo Alto, CA, USA) and an Agilent Zorbax Extend–C18 column (150 mm x 4.6 mm; 3 µm). The analysis was performed at 30°C, with water (solvent A) and acetonitrile (solvent B) as mobile phase at a flow rate of 1 ml/min. Injection volume was 20 µL and the mobile phase gradient started at 40% B for 1 min, to reach 95% B after 15 min. The compounds were detected at a wavelength of 254 nm with good linearity in the range of concentration considered (R^2>0.999).

3. Results and discussion

3.1. Optimization

Optimization of the membrane composition was done according to the content of enzyme in the membrane. Calibration curves were prepared with optimized concentration of enzymes and used for the evaluation of method performances (limit of quantification, detection limit etc.)

3.2.1.Optimization of putrescine oxidase concentration

Putrescine oxidase was investigated in a concentration range of 5 – 25 mg/L. The biosensors were tested with standard solution of putrescine 500 mg/L

Figure 1.

The results of putrescine oxidase reaction (Figure 1) show an increase of the signal until a concentration of around 10 mg/L . The shape of the curve after 10 mg/L of putrescine indicates that biosensor has reached its optimum for the determination. The higher deviation of signals in this region confirms this assumption.

Therefore, the enzyme concentration of 10 mg/L was used as optimal concentration in further experiments.

An enzyme concentration of 1 mg/L was estimated as the lowest concentration required for the reaction.

3.2.2. Optimization of diamine oxidase concentration

Diamine oxidase was investigated in a concentration range of 10 – 105 mg/L. The biosensors were tested with standard solution of putrescine 500 mg/L.

Figure 2.

The results indicate that chemiluminescence signal increases until 10 mg/L of diamine oxidase (Figure 2). Higher enzyme concentration decreases the signal probably due to quenching of signal.

The concentration of 10 mg/L of enzyme was used for further optimization.

3.3. Calibration curves for biosensors

3.3.1. Calibration curve for biosensor with putrescine oxidase

Putrescine was investigated in the concentration range 0.002–50 mg/L (Figure 3). The calibration curve is linear in the range 1– 2 mg/L. The lowest measured concentration is 1 mg/L and a. The detection limit of 0.8 mg/L was calculated as 3σ value.

Figure 3.

3.3.2. Calibration curve for biosensor with diamine oxidase

The measured concentration for diamine oxidase range was 1 - 25 mg/L (Figure 4). The calibration curve is linear in the range 1-2-mg/L. The lowest measured concentration for putrescine is 1 mg/L and detection limit, calculated as 3σ value, is 1.3 mg/L.

Figure 4.

The results show an increase until 2.5 mg/L of putrescine, followed by a decrease at 5 mg/L. An increase of putrescine concentration above 5 mg/L does not have any significant influence on signal.

The very narrow linear range in both calibration curves is arguably related to the layered design of biosensors and only a layout improvement could enhance their efficiency and extend the dynamic range of the instrumental response

3.4. Interferences

The interference of different amines on the response of the new biosensors was investigated. The investigated amines were: β-(3,4-dihydroxyphenyl)-ethylamine hydrochloride, 1,4-diaminobutane dihydrochloride, sarcosine, L-glutamine, and dopamine hydrochloride. These interferences were investigated at three different concentrations (10 mg/L, 100 mg/L, and 1000 mg/L) (Table 1 and Table 2).

Table 1.

Table 2.

The results show an increase of response between 10% and 60% in presence of the different amines at a concentration of 10 mg/L for both putrescine oxidase biosensor and diamine oxidase biosensor. The interference increased with the concentrations of the amines until values between 40% and 97%. The limitation is related to the structure of the biosensors and further modifications in their layout will be necessary to reduce interferences in presence of other amines and avoid sample pretreatment to remove those interferents.

3.5. Stability of biosensors

To investigate the long term applicability of biosensor, they were stored at room temperature, in refrigerator at 4°C and in deep freeze at -18°C.

The biosensor with putrescine oxidase gave rather stable signal over first 24 hours of storage in refrigerator at 4°C, without significant change in chemiluminescence intensity. Then, the

signal dropped for about 30%. The signal of this biosensor decreased more rapidly when it was stored at room temperature and in deep freeze. The biosensor with diamine oxidase has to be used immediately after preparation since its signal decreased during 24 hours of storage in all conditions.

3.6. Meat samples analysis with biosensors and high performance liquid chromatography

The results obtained with HPLC for putrescine in the different types of meat is reported as indication of the kinetic of formation of BA (Figure 5) (Table 3). It shows an increase during the storage period for all the types of meat, with lower values in beef and pork compared to fish and turkey and a higher production in chicken that reached the maximum value of 12.20 mg/kg already at the third day of storage.

Table 3.

Figure 5.

The comparison between BA content in the five different types of meats, measured with the two biosensors and HPLC, showed similar values (Figure 6). These results indicate a similar behavior in the reaction of BA formed in the different meat sample with putrescine oxidase or diamine oxidase enzymes, independently on the characteristics of the meat. Besides, the values measured with HPLC analysis were consistently lower than those obtained with biosensors. A difference between biosensors and HPLC can be explained by the presence of

others amines in the meat which could interfere with the BA quantification, leading to an overestimation of BA content, as demonstrated in the previous experiment (paragraph 3.4). These interferences can also explain lower repeatability in biosensors' quantification of BA, compared to HPLC. Nevertheless, these results showed a high agreement between the rate of BA production measured with the two biosensors and HPLC, indicating the possibility of their application for the development of fast and simple assays for the quantification of BA formation during meat spoilage.

Figure 6

In order to validate and verified new method, a two-sample t-tests were performed (Table 4 and Table 5) (Two-sample t-test [Online]).

Table 4

Table 5

The outputs obtained from running a t-test is the probability ("p-value") of getting the t-statistic calculated for results. As a general rule, if p-value is less than the critical value of 0.05 it means that the results are significant and therefore support an alternative hypothesis which states that there is a difference in the distributions of two groups of results.

Both t-test, presented in Tables 4 and 5, confirmed that results obtained with biosensors are similar to those obtained with HPLC

4. Conclusions

Two new biosensors, based on putrescine oxidase and diamine oxidase enzymes, were developed for the determination of putrescine as marker of freshness and hygiene of meat products. The biosensors showed linearity for putrescine quantification in the range 1 – 2 mg/L and detection limits of 0.8 mg/L and 1.3 mg/L, respectively. Specific test demonstrated stability of the response of putrescine oxidase biosensor during 24 hours when stored at 4°C, followed by a 30% decrease, while room temperature and -18°C immediately decrease the assay sensitivity. Diamine oxidase biosensor showed low stability at all storage conditions. Interferences from other BA were detected for both biosensors. Analysis of different meat samples during a storage period of 8 days demonstrated the ability of the biosensors of detecting putrescine formation, with results comparable to those obtained with a specific HPLC analysis for the putrescine quantitation. The new biosensors assays represent a valid approach to BA quantification with obvious advantages over traditional HPLC analysis in terms of simplicity and time saving. Nonetheless, further design improvements in biosensors' layout are under study to enhance the linearity range, stability in time and selectivity.

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Figure 1.

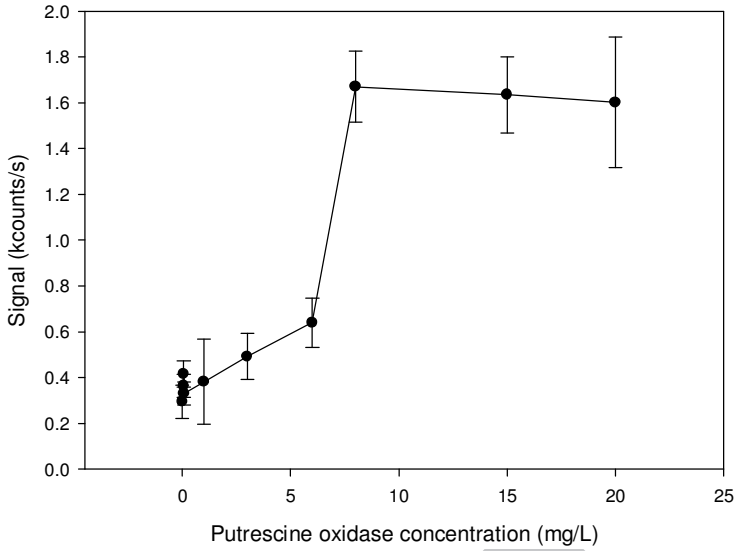

Figure 2.

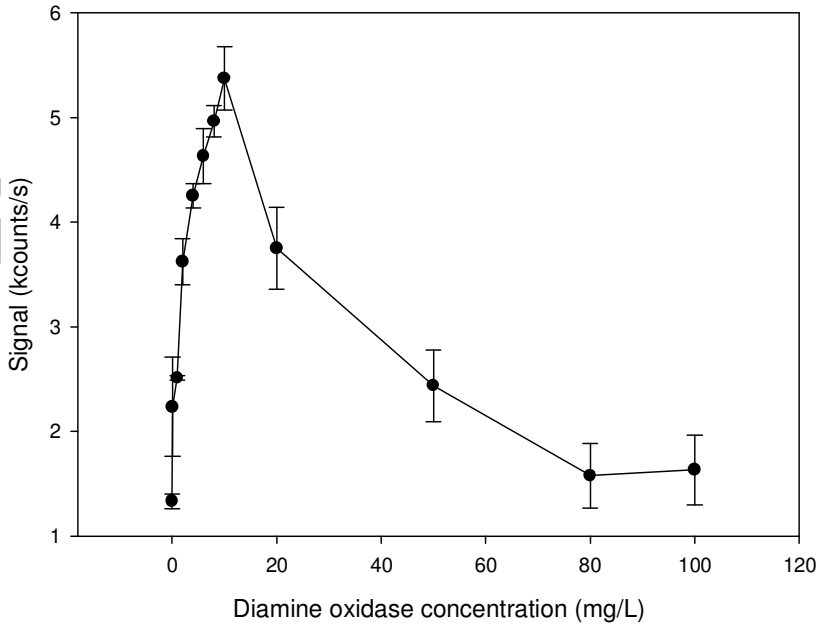

Figure 3.

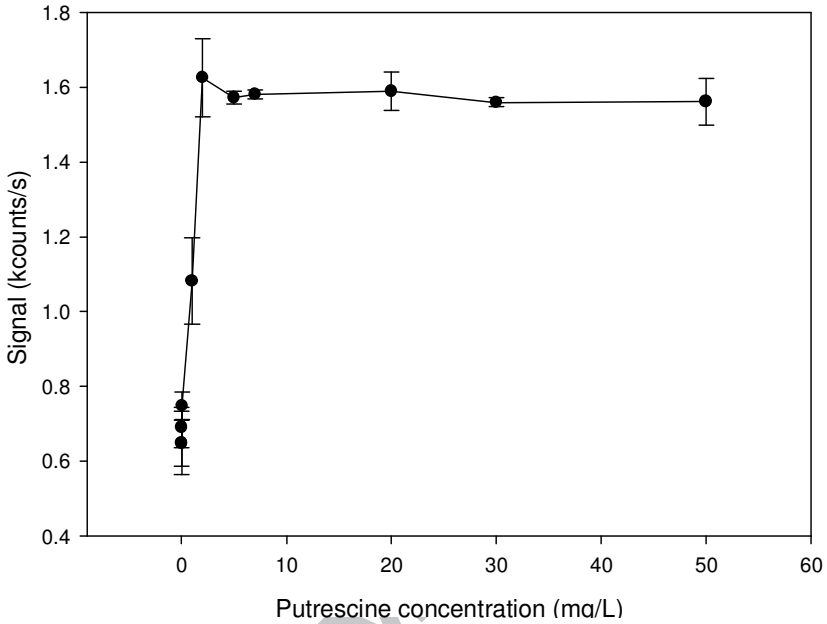

Figure 4.

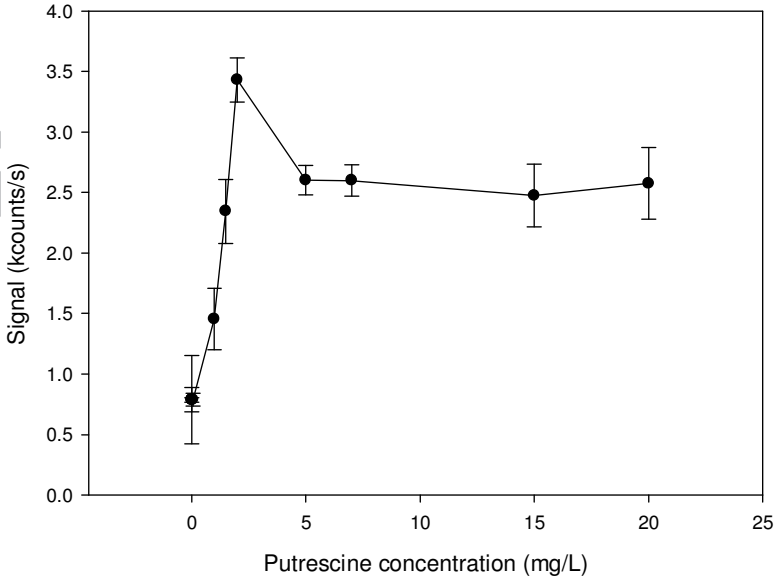

Figure 5.

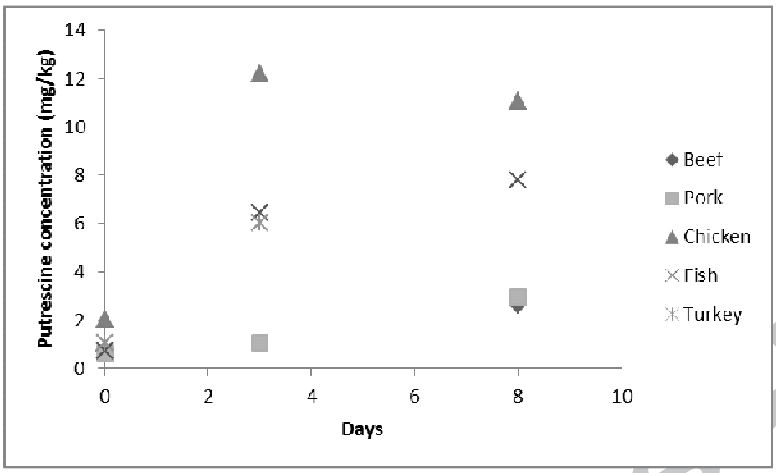

Figure 6.

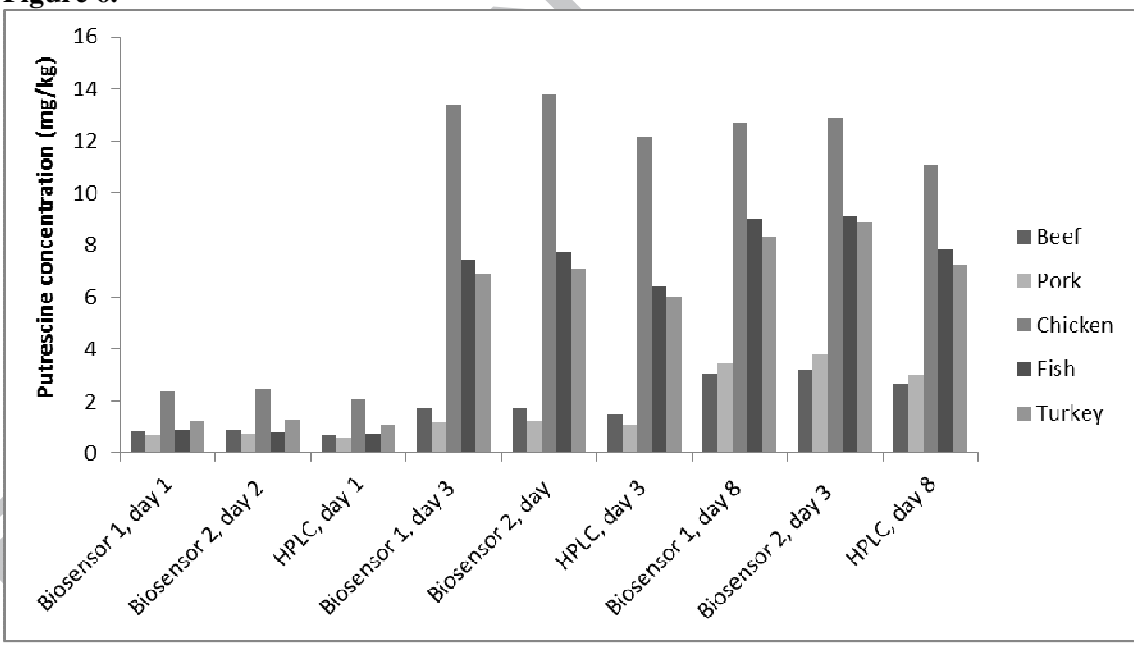

Figure 1. Optimization of putrescine oxidase concentration. Enzyme concentration: 5-25 mg/L. Putrescine concentration: 500 mg/L. The error bars present standard deviation of measurement and three repetitions. Measurements were performed on luminometer Lucy1 of Anthos labtec instruments Ges.m.b.H.

Figure 2. Optimization of diamine oxidase concentration. Enzyme concentration: 10-100 mg/L. Putrescine concentration: 500 mg/L. The error bars present standard deviation of measurement and three repetitions. Measurements were performed on luminometer Lucy1 of Anthos labtec instruments Ges.m.b.H.

Figure 3. Calibration curve for the putrescine oxidase biosensor. Enzyme concentration: 10 mg/L. Putrescine concentration: 0.002 - 60 mg/L. The error bars present standard deviation of measurement and three repetitions. Measurements were performed on luminometer Lucy1 of Anthos labtec instruments Ges.m.b.H.

Figure 4. Calibration curve for the diamine oxidase biosensor. Enzyme concentration: 10 mg/L. Putrescine concentration: 0.005 - 20 mg/L. The error bars present standard deviation of measurement and repetitions. Measurements were performed on luminometer Lucy1 of Anthos labtec instruments Ges.m.b.H.

Figure 5. Time dependence of putrescine concentration in different meat samples. The putrescine concentration was measured by HPLC on day 1, 3, and 8.

Figure 6. The comparison of putrescine concentrations obtained with biosensors and with HPLC. Samples of beef, pork, chicken, fish and turkey were analysed in three repetitions on day 1, 3 and 8 from sample preparation. Biosensor 1 contains putrescine oxidase. Biosensor 2 contains diamine oxidase.

Inteferent compound	10 mg/L	100 mg/L	1000 mg/L
β-(3,4-dihydroxyphenyl)-ethylamine hydrochloride	+59	+80	+97
1,4-diaminobutane dihydrochloride	+30	+45	+67
Sarcosine	+15	+25	+44
L-glutamine	+10	+40	+70
Dopamine hydrochloride	+55	+78	+95

Table 1. Influence of different amines at various concentrations (10 mg/L, 100 mg/L, 1000 mg/L) on the signal obtained with biosensor with putrescine oxidase, expressed as percentage (the signal produced by hydrogen peroxide corresponds to 100 %).

Inteferent compound	10 mg/L	100 mg/L	1000 mg/L
β-(3,4-dihydroxyphenyl)-ethylamine hydrochloride	+32	+70	+90
1,4-diaminobutane dihydrochloride	+20	+43	+75
Sarcosine	+30	+55	+80
L-glutamine	+15	+28	+66
Dopamine hydrochloride	+45	+80	+97

Table 2. Influence of different amines at various concentrations (10 mg/L, 100 mg/L, 1000 mg/L) on the signal obtained with biosensor with diamine oxidase, expressed as percentage (the signal produced by hydrogen peroxide corresponds to 100 %).

a)

Meat	Day 1 biosensor with putrescine oxidase (mg/kg)	Day 1 biosensor with diamine oxidase (mg/kg)	Day 1 HPLC (mg/kg)
Beef	0.84±0.2	0.90±0.3	0.73±0.1
Pork	0.72±0.1	0.78±0.3	0.62±0.2
Chicken	2.36±1.0	2.45±1.1	2.05±0.5
Fish	0.88±0.2	0.82±0.3	0.74±0.1
Turkey	1.24±0.5	1.30±0.6	1.08±0.7

b)

Meat	Day 3 biosensor with putrescine oxidase (mg/kg)	Day 3 biosensor with diamine oxidase (mg/kg)	Day 3 HPLC (mg/kg)
Beef	1.73±0.4	1.77±0.5	1.50±0.3
Pork	1.23±0.3	1.27±0.5	1.07±0.5
Chicken	13.40±2.9	13.78±3.	12.2±2.4
Fish	7.42±1.4	7.75±2.5	6.45±1.9
Turkey	6.94±2.0	7.10±2.5	6.04±1.5

c)

Meat	Day 8 biosensor with putrescine oxidase (mg/kg)	Day 8 biosensor with diamine oxidase (mg/kg)	Day 8 HPLC (mg/kg)
Beef	3.05±0.9	3.23±1.1	2.65±0.9
Pork	3.44±1.0	3.82±1.3	2.99±1.1
Chicken	12.72±2.5	12.90±3.0	11.06±2.3
Fish	8.98±1.7	9.12±2.5	7.81±1.4
Turkey	8.30±1.6	8.85±2.4	7.22±1.6

Table 3. Comparison of BA concentrations determined with biosensors and HPLC. Measurements were done on day 1 (a), day 3 (b) and day (8).

Descriptive Statistics

	Mean	Standard Deviation	n
Group A	4.8833	4.4009	15
Group B	4.2807	3.907	15
Independent Samples t-Test			
t-Statistic	0.3966	**Result**	
Degrees of Freedom	28	Do not reject the null hypothesis.	
Critical Value	2.0484	**Conclusion**	
95% Confidence Interval	[-3.2094, 4.4147]	Group A is not significantly different from Group B, t(28) = 0.3966, p > .05.	

Table 4. Two-sample t-test for applied for validation and verification of the biosensor with putrescine oxidase compared with HPLC method

Descriptive Statistics			
	Mean	**Standard Deviation**	**n**

Group A	5.056	4.5069	15
Group B	4.2807	3.907	15
Independent Samples t-Test			
t-Statistic	0.5034	**Result**	
Degrees of Freedom	28	Do not reject the null hypothesis.	
Critical Value	2.0484	**Conclusion**	
95% Confidence Interval	[-3.0883, 4.639]	Group A is not significantly different from Group B, t(28) = 0.5034, p > .05.	

Table 5. Two-sample t-test for applied for validation and verification of the biosensor with diamine oxidase compared with HPLC method

On the Development of the New Chemiluminescence Biosensors for Determination of Meet Freshness

Highlights:

- Biogenic amines are very important meat freshness indicators
- Recent trends in food safety are focused on miniatiruzation of analytical methods
- Two biosensors for determination of putrescine in meat samples were developed.
- Results obtained with biosensors were compared with HPLC method