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MARGARITA KAGAN

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# Biosensing penicillins' residues in milk flows

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Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia

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# LIST OF ORIGINAL PUBLICATIONS

The present thesis consists of four original research papers and a review, which are referred in the text by Roman numerals I–V.

I. **Kagan, M**., Kivirand, K., Rinken, T. Modulation of enzyme catalytic properties and biosensor calibration parameters with chlorides: Studies with glucose oxidase. Enzyme and Microbial Technology, 2013, 53, 278–282 (DOI: 10.1016/j.enzmictec.2013.02.011.)

II. Kivirand, K., **Kagan, M.**, Rinken T. Calibrating Biosensors in Flow-Through Set-Ups: Studies with Glucose Optrodes. Published in: State of the Art in Biosensors – General Aspects, 2013, 331–351, Intech (DOI: 10.5772/54127)

III. Kivirand, K., Floren, A., **Kagan, M**., Avarmaa, T., Rinken, T., Jaaniso, R. Analyzing the Biosensor Signal in Flows: Studies with Glucose Optrodes. Talanta, 2015, 131, 74–80 (DOI: 10.1016/j.talanta.2014.07.061.)

IV. Kivirand, K., **Kagan, M.**, Rinken, T. Biosensors for the detection of antibiotic residues in milk. Published in: Biosensors – Micro and Nanoscale Applications, 2015, 425–456, Intech (DOI: 10.5772/60464)

V. **Kagan**, **M.,** Printsmann, G., Kivirand, K., Rinken T. Determination of Penicillins in Milk by a Dual Optrode Biosensor. Analytical Letters, 2016, in press (DOI 10.1080/00032719.2016.1202957.)

Author's contribution

Paper I: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

Paper II: Performed the experimental work and calculations.

Paper III: Performed the experimental work and calculations.

Paper IV: Responsible for writing of the paper.

Paper V: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

# ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| $A$ | the total signal change parameter |
| $B$ | the kinetic parameter |
| DOC | dissolved oxygen concentration |
| GOD | glucose oxidase (EC 1.1.3.4) |
| HPLC | high-performance liquid chromatography |
| LOD | limit of detection |
| MIPs | molecularly imprinted polymer sensors |
| MRL | maximum residue limit |
| MS | mass spectrometry |
| PB | phosphate buffer |
| PPB | parts per billion (μg/L) |
| PPM | parts per million (mg/L) |
| SPR | surface plasmon resonance |
| β-gal | β-galactosidase (EC 3.2.1.23) |
| $c$ | dissolved oxygen concentration |
| $s$ | substrate concentration |
| $t$ | time |
| $K_S$ | Michaelis-Menten constant for substrate |
| $K_{O_2}$ | Michaelis-Menten constant for oxygen |
| $V_{MAX}$ | ultimate reaction speed |
| $k_S$ | substrate mass transfer rate between the flowing liquid and sensor surface |
| $k_{O_2}$ | oxygen mass transfer rate between the flowing liquid and sensor surface |
| $k_R$ | reaction rate ( $k_R = \frac{V_{MAX}}{K_S}$ ) |
| $k_{SR}$ | rate parameter describing the reaction and mass transfer of substrate |
| $R^2$ | coefficient of determination |

# INTRODUCTION

Milk and dairy products, generally considered to be healthy and nutritionally balanced natural food comprise essential nutrients for all age groups and are an important part of our everyday diet. However, the use of veterinary drugs, in particular antibiotics for the treatment of food-producing animals generates the risk to human health due to the transmission of the residues and metabolites of these compounds into food chain. In addition, scientists and health experts are also concerned that wide application of antimicrobial agents is contributing to the rise and spread of antibiotic-resistant bacteria. Penicillins, which belong to the group of β-lactam antibiotics, are bactericidal antibiotics and act by disrupting peptidoglycan synthesis in actively multiplying bacteria. Although penicillins have several clinical limitations, as instability in the presence of gastric acids and ineffectiveness against many Gram-negative bacteria, they are still considered to be the best antibiotics to use against most Gram-positive organisms. At present, strict regulations have been established for the levels of antibiotic residues and metabolites in food of animal origin.

Antibiotic residues in food are commonly determined chromatographically and with the help of special tests. The application of biosensors for the detection of antibiotic residues is a good alternative to traditional methods. The benefits of biosensors are their low cost, simplicity and possibility for rapid real-time analysis. Nowadays, the critical problems of biosensing antibiotic residues are the reliability of results and time required for analyses enabling automatic on-line control of production.

The main goal of the present work was to introduce a rapid method for real-time detection of penicillins' residues in milk, to propose a simple and effective model to describe the response of enzymatic bio-optrode operating in flow regime and to study the possibilities of amplifying the biosensor signal to reduce experimental noise and accelerate the acquisition of results. We used a dual-sensor system consisting of glucose and reference oxygen optrodes, enabling to eliminate the fluctuations in the initial dissolved oxygen concentration, temperature and fluidic flow as a robust standard system for model development. The modulation of the catalytic properties of glucose oxidase with different metal cations was studied to amplify the characteristic calibration parameters of glucose and cascaded lactose biosensors based on glucose oxidation in order to accelerate the rate and improve the quality of milk analysis. The effect of sample flow rate on different biosensor calibration parameters, calculated from the biosensor transient phase signal, was also studied. Finally, the dual optrode glucose biosensing system was used for the determination of the effect of penicillins on the biosensor signal in milk and the assessment of substandard milk, collected from cows undergoing treatment with penicillin antibiotics.

# 1. LITERATURE OVERVIEW

## 1.1. The use of antibiotics in dairy farming

The use of antibiotics in dairy farming includes not only the treatment of various inflammatory processes, but also the prophylaxis and addition of sub-therapeutic doses into feed to promote growth and improve production efficiency in some countries [1–3]. The occurrence of antibiotic residues in milk can lead to health problems in humans and risks of developing microbial resistance [2]. In addition, the presence of antibiotics in milk impairs fermentation processes during cheese and yoghurt production [4].

The overall sales of veterinary antibiotics, used in food-producing animals, including dairy farming, were 8 122 tonnes in EU and 13 600 tonnes in U.S., whereas in Estonia veterinary antibiotics sales number was 8.7 tonnes as of pure ingredients in 2013 [5,6]. The highest proportion of the sales constituted penicillins, which belong to the group of β–lactams; and tetracyclines; whereby the use of the first is prevailing in Nordic countries [5–8].

Due to their lipophilic properties, penicillins accumulate in milk. Taking into account the widespread consumption of milk and the high potential of accumulation of antibiotic residues in it, milk is one of the most heavily regulated food products [9]. In EU, the Maximum Residue Limits (MRLs), which are not to be exceeded in milk, are set to 4 μg/L (ppb) for benzylpenicillin, amoxicillin and ampicillin and 30 μg/L (ppb) for cloxacillin [10].

Since the introduction of EU regulations on MRLs of pharmacologically active substances in foodstuff of animal origin (Commision Regulation 37/2010), it has been clear that the concept of regulating MRL values in foods can be implemented successfully only if methods for quantification of these substances are available for on-site use for rapid monitoring and testing of production [10].

## 1.2. Methods for the detection of antibiotics in milk

At present, there are two major methods applied for the assessment of antibiotic residues in milk: various chromatography-based techniques and qualitative milk screening tests [11–14].

High-performance liquid chromatography in combination with mass spectrometry (HPLC/MS) is considered to be the most reliable technique for quantitative detection of antibiotic residues [11,14], and more than 80% of the analyses of veterinary drugs are carried out using this technique [14]. The detection limits for penicillins in milk with HPLC/MS are usually well below 1 ppb [14,15]. However, chromatography based methods require expensive equipment and trained personnel and therefore do not enable to discover the antibiotic residues in milk in farm, prior to the contamination of subsequent food chain with polluted milk. In addition, HPLC techniques demand laborious

pre-treatment of samples like extraction of the analyte from sample matrix and derivatization [11,14]. β-lactams can also degrade if methanol and/or 0.1% formic acid are present in the extraction solvent [15].

Qualitative microbial inhibition tests comprise spores of specific bacteria, sensitive to particular antibiotics on agar gel including nutrients for bacterial growth and a pH indicator. These commercial tests include Delvotest, BetaStar Plus, Charm Farm Test, Eclipse test, Arla test, Copan test, Disc assay, Diffusion test, Valio 101 test, etc [12,14–15]. The main advantages of these tests are their low cost, simple performance, and broad selection towards different antibiotics, e.g. Diffusion test and Delvotest for the determination of β-lactams, Lumac test for the determination of penicillins, Charm Farm Test for the determination of aminoglycosides and tetracyclines [12]. As a rule, the detection limits of these tests for specific compounds are in the range of the established MRL values [11,12,15,16]. Although microbial inhibition tests are considered to be rapid, they take 3 to 24 hours to perform in an incubator [12]. Another problem is that the bacterial strains (*Streptococcus thermophilius*, *Bacillus subtilis*, *Bacillus stearothermophilus* var. *calidolactis*, *Sarcina lutea*, *Escherichia coli*) used in tests should be constantly monitored to ensure that they have not become resistant to the analyzed antibiotics. The interpretation of test results is quite subjective and may lead to false negative or positive results. The presence of natural inhibitors in abnormal milk (e.g. milk of mastitic cows or colostrum) can be the cause of false positive results [11–13]. In addition to microbial inhibition tests; there are different rapid tests, based either on immunoassay or enzymatic operation, available for screening of a number of antibiotic residues in milk [15,17]. These tests can provide preliminary results within 15 minutes, although the reliability of these tests is not very high as several components in mastitic milk (e.g. somatic cells, lactoferrin, lysozyme, microbes, and free fatty acids) may have a major impact on the outcome of the test and cause elevated false non-compliant/compliant results [15,18].

In spite of detection limits in the range of established MRL values, none of the abovementioned technologies are suitable for on-line analytical arrangements.

## 1.3. Biosensors for the detection of antibiotic residues in milk

The application of biosensors for milk analyses is a good alternative to traditional methods, as biosensors enable the development of equipment for real-time analysis in complex matrixes, operating in fully automatic or manual mode. Biosensors are compact devices transferring selective biochemical recognition into a measurable physical signal. This signal can be translated into an indicator of the safety or quality of milk. Due to the large number of different principles used, biosensors can be classified according to the bio-recognition or signal transduction technologies employed [19].

The most common biosensors, developed for the detection of antibiotic residues in milk are immunosensors, based on immunochemical biorecognition reactions and electrochemical and optical signal transduction, the latter most often being a Surface Plasmon Resonance (SPR) biosensor. The application of SPR technology secures low detection limits even below the established MRL values [20–22]. The main drawbacks of SPR biosensors are high cost and time, required for the preparation of specific biorecognition chips and system regeneration [21]. Detection limits of the majority of current immunosensors [IV], which are, designed for the determination of β-lactam antibiotic residues, vary in a broad range: from $10^{-6}$ ppb to 14.6 ppb [22–30]; and the detection limits are in the similar range for other antibiotics [31,32]. Although immunosensors are very selective, the speed of analysis depends on the incubation time required to form antigen/antibody complex, which can be several hours [26,33].

The second most common group of biosensors is receptor or enzyme-based biosensors, where specific receptors or enzymes interact with the antibiotic molecules and the substrates or products of the bio-recognition reaction are determined directly with a suitable transducer [IV]. These biosensors have been designed for the determination of β-lactam residues with the limit of detection (LOD) ranging from $4\times10^{-8}$ ppb to $9\times10^{3}$ ppb [21,34–46]. Receptor or enzyme-based biosensors like immunosensors usually employ optical (mainly SPR) or electrochemical signal detection principles [11,21,34,37,47].

There are a few biosensors for detecting antibiotic residues in milk based on the application of enzymatic activity of microorganisms [48–50]. These microbial biosensors are based on the measurement of the inhibition of bacterial growth due to the presence of antibiotics [11–14]. For instance, systems for the monitoring of β-lactams are based on similar principles as microbiological inhibition tests for milk [12,14] with the difference that the bio-recognition reaction signal is detected quantitatively or semi-quantitatively. Detection limits in both cases are at the MRLs levels [48].

During last years aptamer-based biosensors have been proposed for the detection of antibiotic residues in milk [51–55]. Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule. Specific three-dimensional aptamers, fold into well-defined structures, are produced using Systematic Evolution of Ligands by EXponential enrichment (SELEX) method [56,57]. Aptamers are quite stable and are not affected by reasonable temperature or pH fluctuations, at optimal conditions they can restore their original conformation. Aptamers are smaller in size compared to antibodies enabling to reach previously blocked or intracellular targets [58]. The detection limits of common aptasensors [IV] are varying in the range of $3.5\times10^{-2}$ ppb to 350 ppb for the detection of β-lactam residues [53]. A serious disadvantage using aptamer-based biosensors for milk analyses is the presence of milk proteins and fat; and the non-transparency of the samples, which hamper the application of optical detection methods. Pre-treatment of milk samples is commonly required for the analyses with aptamer-based biosensors [54,55,59–64].

A recent development in the biosensing of antibiotic residues in milk is the application of molecularly imprinted polymer sensors (MIPs) for biorecognition [65–67]. Molecular imprinting is a technique for the creation of synthetic materials containing specific receptor sites having high affinity towards the target molecule [68]. MIPs are effective alternatives to the natural biorecognition compounds in biosensor assays. Unfortunately, so far there are no MIPs for the determination of β-lactam residues. Current MIP sensors have been mostly designed for the detection of aminoglycosides [IV]. These sensors are very sensitive with LOD varying in the range from ≤ 1 ppb to 6 ppb [66,69], which is far below of the allowed MRL values, so dilution of samples is required for their practical applications.

A condensed overview of currently available different biosensor platforms, used for the detection of β-lactam antibiotic residues in milk, is given in Table 1.

**Table 1.** Currently available biosensor platforms for the detection of β-lactam antibiotic residues in milk.

| **Biosensing principle** | **Detection range, ppb** | **Advantages** | **Disadvantages** | **Ref.** |
|---|---|---|---|---|
| Immuno-sensors | $10^{-6}$–14.6 | • High selectivity | • High cost<br>• Time consuming biosensor production<br>• Slow regeneration | [22–30] |
| Receptor or enzyme-based biosensors | $4\times10^{-8}$–$9\times10^{3}$ | • Low LOD | • High cost<br>• Possible non-specific binding<br>• Long time required for analyses | [21,34–46] |
| Microbial biosensors | At MRL levels | • Similar to microbio-logical tests | • Low sensitivity<br>• Semi-quantitative detection<br>• Time of analysis over 120 min | [48–50] |
| Aptamer-based biosensors | $3.5\times10^{-2}$–350 | • High affinity<br>• Stable<br>• Enabling to reach intracellular targets | • Application of optical detection methods is problematic (non-transparency of the samples)<br>• Pre-treatment of the samples is required | [51–55] |

## 1.4. Problems with practical biosensing of antibiotic residues

Concerning the practical application of biosensors, there are several problems to be solved. One of the major problems is time required to get reliable results, especially in case of on-site analyses. The average detection time (excl. pre-treatment) is usually 30–40 minutes or even up to 2 hours if a longer incubation period for bio-recognition is required [29,37,70]. Moreover, some complex biosensing systems require additional pre-treatment of milk samples to remove fat and proteins [54,55,60,62].

The other drawback is that most antibiotics' biosensors have been used for the "proof of concept" and the validation of the proposed technology has been carried out with only spiked milk samples. However, studies using real milk of animals undergoing antibiotic treatment are scarce, although this can be a key factor to indicate the applicability of biosensor technology, as "natural" samples can additionally contain different metabolites of antibiotic compounds, which have a major effect on the measured biosensor signal [15].

## 1.5. Data acquisition for obtaining rapid results

The crucial assignment of on-line measurements is to obtain reliable results as quickly as possible, which requires exact modelling of the processes taking place in the system and smart management of data. Commonly the only information used in obtaining results with biosensing systems, is the steady state output (or the presumed 95% of it) [71]. The main problems with measuring the experimental steady state signal is time, required to reach this state and the imprecision of estimating the attainment of steady state [71–73]. Therefore, for real-time measurements it is unavoidable to develop methods for the determination of the characteristic parameters of the biorecognition reaction from early pre-steady state signal.

Carrying out measurements in flow systems enables further reduction of analysis time allowing high sample throughput and the possibility to work with small volumes of analytes [72,74,75]. Flow arrangements present a wide response range and high sensitivity, but measurements in continuous flows require consideration of the flowing effects, both laminar and turbulent, in the biosensor output signal. Most modelling efforts dealing with biosensor performance in flow regime are made for amperometric signal transducers [76–81]. There is only very few information about detailed signal analyses of optical biosensors, which are working in continuous-flow or flow-through systems. For SPR biosensors, calculation of the two-compartment model had been used in order to determine accurate values of the rate constants and transport coefficients for data that are influenced by flow and diffusion [82]. In comparison with amperometric biosensors, optical biosensors have several advantages as they are resistant to electrical and electrochemical interferences, allow non-contact sensing or transmission of parallel sensor signals over optical fibres, and have relatively long service interval [83].

# 2. EXPERIMENTAL

## 2.1. Dual-optrode biosensor system and flow cell

The system comprises of flow cell, heater, opto-electronic unit, control unit and two oxygen optrodes, one of which is covered with biorecognition layer to form a biooptrode and the other forms a reference optrode [III]. The optrodes (30 mm long optical quarz fibres with diameter of 1 mm, dip-coated with oxygen-sensitive Pd-tetraphenylporphyrine-containing polymethylmethacrylate film) were placed into identical isolated flow channels ($l$=50 mm; $Ø$=3 mm) of the carefully thermostated at 37.0±0.1 °C flow cell. To minimize the accidental forming of microbubbles in flow channels, the inflow was in the bottom and the outflow in the upper part of the cell (Fig. 1).

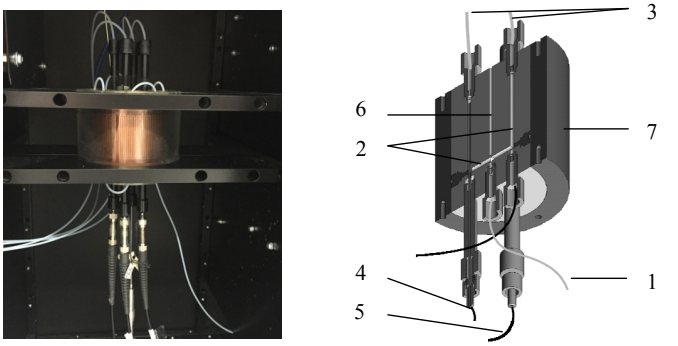



**Figure 1.** The dual-optrode biosensor system set up: 1–sample inflow; 2–flow channels; 3–outflows; 4–glucose bio-optrode; 5–reference oxygen optrode; 6–temperature sensor; 7–cylindrical messing oven for the stabilization of temperature.

The opto-electronic unit provided optical excitation (λ=405 nm) as well as the detection and amplification of luminescence signals (λ= 690 nm). The control unit was connected to personal computer via USB interface and included analog-to-digital signal converter, input/output ports for switching the light-emitting diodes for luminescence excitation, and heater regulator.

## 2.2. Immobilisation of glucose oxidase and preparation of glucose bio-optrode

The glucose bio-optrode was produced by coiling 19 cm of nylon-6,6 thread with covalently immobilized glucose oxidase (GOD, EC1.1.3.4) [III] around the oxygen-sensitive surface of the oxygen optrode, where it formed a bio-recognition layer for glucose [84–86]. The initial catalytic activity of the thread was 0.016 U/cm and this was tested before each series of measurements. If the

activity dropped below critical (80% of its initial value) during measurements [III], the GOD-containing thread was replaced. The reference oxygen optrode was covered with 19 cm of identical "blank" thread to eliminate diffusion effects.

## 2.3. Measurement procedures and data acquisition

### 2.3.1. Measurements in flows

All kinetic studies were carried out in air-saturated solutions at 37 °C varying the flow rate from 0.8 mL/min to 13.0 mL/min for model development [III] and using an optimal flow rate 3.3 mL/min for the studies with milk samples [V].

The output signals of optrodes were recorded with the interval of 1 second. Original software Oxysens 2.0 was developed for the automatic data acquisition and calculation of the dissolved oxygen concentration (DOC) using Stern-Volmer relationship; but also for the automatic control of the system's working parameters. The change of oxygen concentration due to glucose oxidation, catalyzed by glucose oxidase, was calculated as the DOC difference between glucose bio-optrode and reference optrode at every time moment measured, so minimizing the impact of experimental noise; and normalized to bring the data from different sensors onto common scale. Each experimental result was an average of at least 3 identical measurements and consisted of at least 300 data points. After each measurement the system was washed with 0.1 mol/L phosphate buffer (PB, pH 6.50) until the sensors' signals reached their initial values.

### 2.3.2. Assessment of enzyme activity in glass cell

The kinetic measurements for the assessment of modulating and inhibiting effects of different compounds on enzyme activity were performed in an airtight temperature regulated glass cell at constant stirring with an oxygen optrode [I,V]. The oxygen-sensitive film of the optrode was additionally covered with a thin black silicone coating to eliminate the effect of divergent light.

The oxygen optrode was plunged into air-saturated reaction medium, containing substrate or a definite amount of enzyme. The reaction was initiated by injecting soluble enzyme or substrate, initiating the decrease of DOC.

The reduction of DOC in the course of the biorecognition reaction was characterized using 2 independent parameters: the total signal change parameter ($A$) and the kinetic parameter ($B$), calculated from the biosensor transient signal using a modified dynamic biosensor model [87].

The activity of soluble β-galactosidase (β-gal, EC3.2.1.23) was determined also spectrophotometrically, following the formation of optically active product of the hydrolysis of glucoside bond in o-nitrophenol-β-D-galactoside [88].

## 2.4. Milk samples

Milk samples were collected in the Dairy Research Farm of the Estonian University of Life Sciences in Southern Estonia. The collected milk samples were kept under moderate stirring at room temperature. To prepare milk samples spiked with benzylpenicillin, this antibiotic was added to the milk of healthy cows.

The milk samples from two cows undergoing mastitis treatment with penicillin antibiotics (Norbrook® Lactaclox intramammary infusion, a single dose containing 75 mg of ampicillin and 200 mg of cloxacillin; or Bimoxyl intramuscular injections, a single dose containing 150 mg of amoxicillin per 10 kg animal bodyweight) were immediately freezed at –20 °C after collection and melted at +4 °C before testing. The treatment of cows with antibiotics was administered for 3 days followed by a 3-day withdrawal period.

Before injection into the flow cell, milk samples (5 mL) were diluted (1:4) with 0.1 mol/L PB (pH 6.50; 0.01% Tween 20) and aerated with air for 5 minutes at 37 °C.

# 3. RESULTS AND DISCUSSION

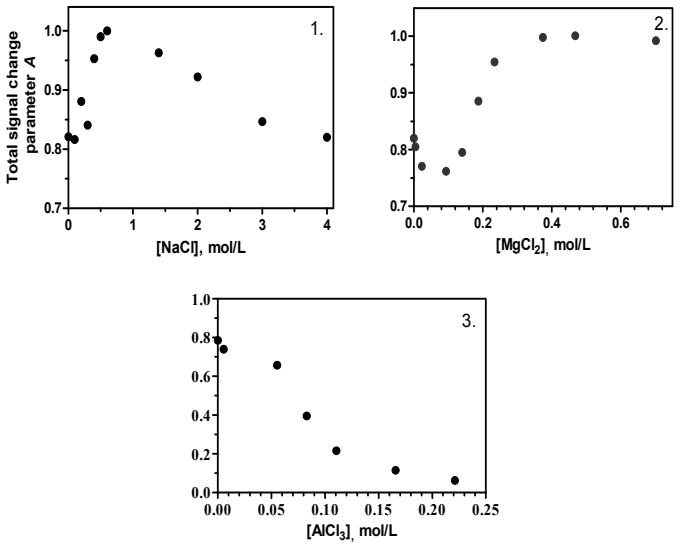
## 3.1. Modulation of the catalytic properties of glucose oxidase

First we studied the possibilities of modulating the glucose oxidase catalytic properties with metal cations: $Na^+$, $Mg^{2+}$ and $Al^{3+}$ [I]. The enzymatic oxidation of glucose was characterized using two different signal parameters, calculated from the transient phase data of the reaction: total signal change parameter and kinetic parameter [89]. The values of these reaction parameters at different concentrations of different chlorides are shown on Figure 2 [I]. NaCl at concentrations below 0.5 mol/L increased the value of the total signal change parameter from its initial value of 0.82 in salt-free solutions to its limiting maximum value 1.00 (Fig. 2(A1)). Magnesium chloride ($MgCl_2$) caused a 1.3-fold essential increase of the total signal change parameter in concentration range from 0.1 to 0.4 mol/L. At lower concentrations, the value of parameter *A* was slightly reduced, and at concentrations above 0.4 mol/L, parameter *A* maintained its maximum value (Fig. 2(A2)). At $MgCl_2$ concentrations greater than 1 mol/L, the glucose oxidation reaction was slow; thus, although the maximum value for the signal change parameter was observed (meaning that at the reaction steady state DOC equals zero), the experimental determination and interpretation of steady state was problematic.

In the presence of aluminium chloride ($AlCl_3$) the total signal change parameter was decreased (Fig. 2(A3)).

As for the practical applications of biosensors, the slope and linear range of the calibration curves, based on biosensor signal parameters, determine the quality of the results – the amplification of the measured values of these parameters enables to increase the accuracy and sensitivity of the analysis. The modulation of the GOD-based biosensor signal parameters with NaCl and $MgCl_2$ at concentrations below 0.5 mol/L increased the calculated total signal change parameter and the slope of the calibration curve by nearly 20% compared to its initial value. At the same time, the kinetic parameter showed decrease in the presence of all three salts (Fig. 2(B1,B2,B3)), so it is not possible to amplify the modulation of this parameter in order to get rapid results with metal cations.

A




B

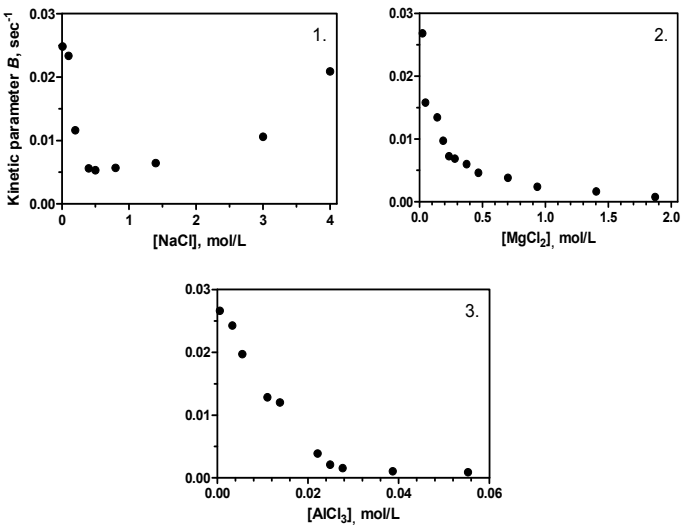



**Figure 2.** The dependence of biosensor signal parameters on the concentrations of different chlorides. (A) Total signal change parameter; (B) kinetic parameter. All measurements were carried out in air-saturated 0.1 mol/L acetic buffer (pH 5.60) at 25 °C; [glucose]=3mmol/L, [glucose oxidase]=1.3 U/mL.

### 3.1.1. Enhanced detection of glucose in cascaded lactose biosensor

Cascaded lactose biosensors usually integrate two consecutive catalytic processes: (1) the hydrolysis of lactose into galactose and glucose, catalyzed by β-gal and (2) the successive oxidation of the produced glucose, catalyzed by GOD [90]. These cascaded biosensors are commonly not applicable for quick analyses due to the relatively low speed of lactose hydrolysis [91]. However, it might be possible to detect smaller quantities of the produced glucose and obtain faster results if the sensitivity of glucose measurements is increased [I].

NaCl caused a similar modulation of the total signal change parameter in lactose cascaded biosensor as in glucose biosensor and as expected, this parameter increased for 20% compared to its initial value. $MgCl_2$ increased values of both parameters *A* and *B*, as $Mg^{2+}$ is supposed to activate also β-gal [92,93]. Reaction parameters *A* and *B* for the detection of lactose were increased by 1.2 and 1.5 times, respectively, at $MgCl_2$ concentrations below 5 mmol/L (Fig. 3).

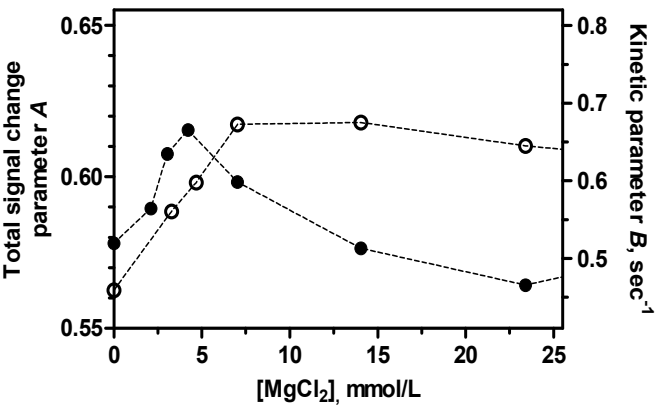



**Figure 3.** Lactose biosensor signal parameters at different $MgCl_2$ concentrations (● Total signal change parameter *A*; ○ Kinetic parameter *B*). All measurements were carried out in air-saturated 0.1 mol/L phosphate buffer (pH 6,50) at 25 °C; [lactose]=0.14 mol/L, [β-galactosidase]=10.08 U/ml, [glucose oxidase]=1.3 U/ml.

The addition of 5 mmol/L $MgCl_2$ was optimal to achieve the highest amplification of the lactose biosensor calibration parameters, hence could be used for the acceleration of lactose analyses. The amplification of the signal parameters is vital for analyses conducted at low temperatures, like in cooled milk, where lactose hydrolysis and glucose production are slow.

## 3.2. Modelling the bio-optrode signal in flows

In order to develop a model, which enables to calculate relevant parameters of the bio-recognition reaction in flows from the pre-steady state biosensor signal and reduce the effect of side processes, we studied the oxidation of glucose by dissolved oxygen, catalyzed by glucose oxidase as a model reaction:

$$\beta\text{ - D - glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \delta - lactone + H_2O_2 \qquad (1)$$

In flow cells, there are two general processes governing oxygen concentration at optrode surface:
1) enzyme-catalyzed reaction in the enzyme containing layer;
2) transport of oxygen and the second substrate into and in the flow cell.

The studied reaction (1) is of ping-pong type [94] and can be described in the quasi-steady state approximation as following:

$$\frac{ds(t)}{dt} = \frac{dc(t)}{dt} = -\frac{V_{MAX}}{1 + \frac{K_S}{s(t)} + \frac{K_{O_2}}{c(t)}}, \qquad (2)$$

where *s(t)* is the substrate concentration and *c(t)* is DOC at time moment *t*; $K_S$ and $K_{O_2}$ are Michaelis' constants for substrate and oxygen, respectively; and $V_{MAX}$ is the ultimate reaction speed.

If the following condition is fulfilled:

$$\frac{K_S}{s} >> 1 + \frac{K_{O_2}}{c}, \qquad (3)$$

Eq. (2) can be simplified to:

$$\frac{ds(t)}{dt} = \frac{dc(t)}{dt} = -k_R s(t), \qquad (4)$$

where

$$k_R = \frac{V_{MAX}}{K_S}. \qquad (5)$$

For the reaction of glucose oxidation $K_S >> K_{O_2}$ [94], and therefore condition (3) is fulfilled in a quite wide range of glucose and oxygen concentrations. The upper limit of oxygen concentration in water solutions is generally determined by its saturation value ($c_0 \sim 0.2$–$0.3$ mmol/L at temperatures 20–40 °C) and the reaction is described by first order kinetics (4).
Regarding the transport processes, the addition of substrate and oxygen into the vicinity of sensor surface is described with linear kinetic terms. Then the overall process can be modelled by two kinetic equations:

$$\frac{ds(t)}{dt} = -k_R s(t) + k_S \left[ s_0 F(t) - s(t) \right] \tag{6}$$

$$\frac{dc(t)}{dt} = -k_R s(t) + k_{O_2} \left[ c_0 - c(t) \right] \tag{7}$$

The rate constants $k_S$ and $k_{O_2}$ characterise the exchange speed of the concentrations of substrate and oxygen, respectively, between the flowing liquid and sensor surface (Fig. 4).

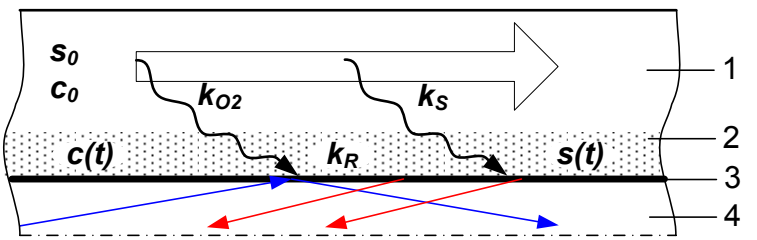



**Figure 4.** Cross-section of the flow cell and scheme of model processes. 1–flowing liquid; 2–flossy coating containing immobilized enzyme molecules; 3–oxygen sensitive coating; 4–core of optical fibre. The arrows inside the fibre represent the light involved in optical oxygen sensing.

Assuming that the oxygen concentration in the flowing liquid entering the cell is always $c_0$, and after passing the initial front, the substrate carried by liquid flow has constant concentration $s_0$. The function *F(t)* describes the arrival front of the substrate into the cell. When the passage of the front is fast compared to the reaction and transport processes, then *F(t)* can be approximated by Heaviside function:

$$F(t) = H(t) = \begin{cases} 0, t < 0 \\ 1, t \geq 0 \end{cases} \tag{8}$$

It is also assumed that there is no substrate present in the cell before $t$=0. Under these conditions the solutions of equations (6) and (7) are:

$$s(t) = s_0 k_S \int_0^t dt' \exp\left[(k_R + k_S)(t' - t)\right], \tag{9}$$

$$c(t) = c_0 - k_R \int_0^t dt' s(t') \exp\left[k_{O_2}(t' - t)\right]. \tag{10}$$

After inserting Eq. (9) into (10) and evaluating the integrals:

$$c(t) = c_0 - s_0 \frac{k_R k_S}{k_{SR} k_{O_2}} \left\{ 1 - \exp(-k_{O_2} t) + \frac{k_{O_2}}{k_{O_2} - k_{SR}} \left[ 1 - \exp(-k_{SR} t) \right] \right\}, \tag{11}$$

where

$$k_{SR} = k_S + k_R . \tag{12}$$

The stationary solution for DOC is:

$$c_f = \lim_{t \to \infty} c(t) = c_0 - s_0 \frac{k_R k_S}{k_{SR} k_{O_2}} \tag{13}$$

The Eq. (11) can be rewritten in a following form:

$$\frac{c(t)}{c_0} = 1 - A \cdot f(t), \tag{14}$$

where

$$f(t) = 1 - \frac{k_{SR}}{k_{SR} - k_{O_2}} \exp(-k_{O_2} t) + \frac{k_{O2}}{k_{SR} - k_{O_2}} \exp(-k_{SR} t) \tag{15}$$

is the normalised function describing the temporal evolution of relative DOC. It is a monotonic function changing from zero to unity, i.e. $f(0) = 0$ and $\lim_{t \to \infty} f(t) = 1$.

Parameter $A$ gives the relative difference between the initial ($c_0$) and final stationary ($c_f$) oxygen concentrations and can be easily determined experimentally as a normalized change of DOC between the initial and final steady states:

$$A = \frac{c_0 - c_f}{c_0} = \frac{s_0 k_R k_S}{c_0 (k_S + k_R) k_{O_2}}. \tag{16}$$

## 3.3. Fitting of the biosensor response to the model

Several series of glucose dual optrode responses were recorded at different glucose concentrations at flow rate 2.7 mL/min in order to compare the biosensor output response with the model [II, III]. Biosensor response at 5 different glucose concentrations (in duplicate) and recovery curves are shown in Figure 5. The arrows mark the time moments, when the substrate was added. The duration of substrate injection was 300 seconds and thereafter the signal was restored in the flow of pure buffer solution to the initial level.

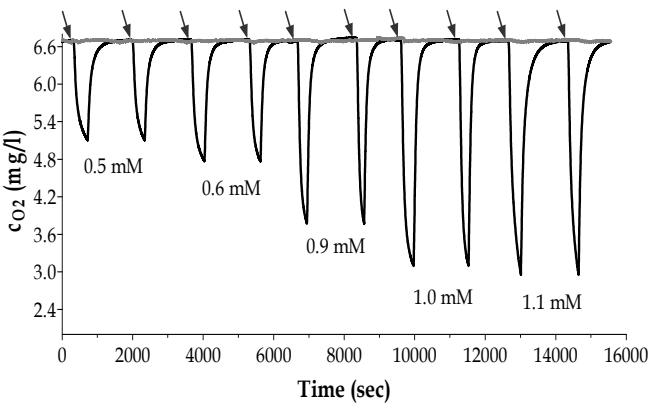



**Figure 5.** Response curves of glucose bio-optrode (black line) and the reference optrode (gray line) of consecutive measurements at different glucose concentrations (two at each concentration). Measurements were carried out at 37 °C in 0.1 mol/L phosphate buffer (pH 6.50) at flow rate 2.7 mL/min. Arrows indicate the time of adding the substrate solution.

The response of the reference sensor at constant saturated DOC was not dependent on glucose concentration. Its signal fluctuations did not exceed 1% of the working range of the sensor at any measured glucose concentration. The response of the glucose biosensor decreased due to consumption of oxygen

according to reaction (1). In order to eliminate all potential experimental noise, the difference between the signals of the reference and glucose sensor responses was used to determine the normalized output of the system.

Normalized response curves at four different glucose concentrations together with the model curves obtained by fitting with equations (14), (15) are shown in Figure 6.

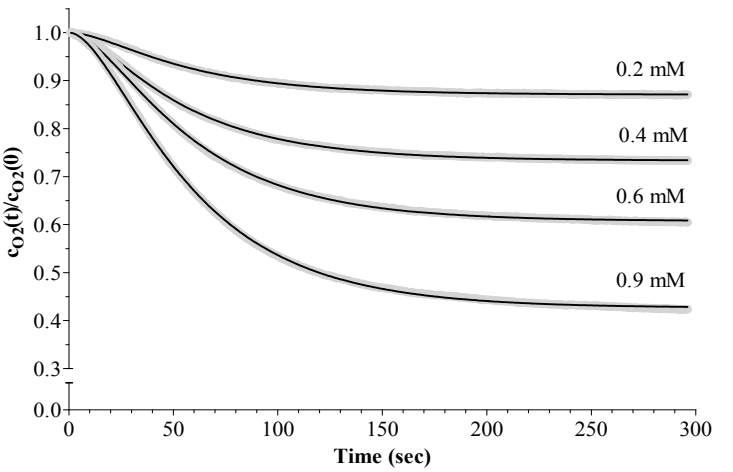



**Figure 6.** Fitting the experimental curves (gray) obtained at different glucose concentrations (0.2 mmol/L (mM); 0.4 mmol/L; 0.6 mmol/L; 0.9 mmol/L) with the proposed model. Theoretical approximations are shown as solid black lines. Measurements were carried out at 37 °C in 0.1 mol/L phosphate buffer (pH 6.50) at flow rate 2.7 mL/min.

Parameter $A$ and two rate constants, $k_{O_2}$ and $k_{SR}$, were determined for each curve. The application of the model for the characterization of the biosensor output signal resulted in good correlation (coefficient of determination–99%, standard deviations from the model–0.00077, $P < 0.0001$). The relatively slow signal decrease at the beginning of the response curves (Fig. 6) is caused by the lack of substrate in the enzyme-containing layer at the initial moment, what is accurately described by the model. The fitting of temporal response curves, as demonstrated in Fig. 6, provides not only more accurate way of determining parameter $A$, but also valuable information included in rate constants.

## 3.4. Practical aspects of model application

The important aspects of practical applications of biosensors are: i) the time required for the acquisition of the results – it should be as short as possible; ii) the repeatability of the experimental measurements; iii) quick regeneration of

bio-sensing system, what is of most importance in real-time analysis; and iv) operational stability and sensitivity of the biosensor system.

In continuous-flow biosensor set-ups the choice of optimal flow rate is an additional presumption of accurate and reliable results. The dependence of parameter $A$ on flow rate at different glucose concentrations was studied in the range of flow rate varying from 0.8 to 13.0 mL/min (or 0.3–5.1 cm/sec) [II,III]. It was not possible to carry out experimental measurements at flow rates under 0.8 mL/min due to the accumulation of air bubbles in the flow cell. The value of parameter $A$ was practically independent on the flow rate in the studied range of glucose concentrations (0.2 to 1.5 mmol/L), and was also indifferent towards the determination of time, at which the analyte front reaches the biosensor, as it is defined as a biosensor total signal change in steady state conditions ($t \rightarrow \infty$) [II,III]. This allows constructing the universal glucose calibration curves, not dependent on the flow rate in the system. The results obtained at a medium flow rate of 2.7 mL/min were used as examples of calibration parameters, as this flow rate offered acceptable response time and sufficient sensitivity [III].

The repeatability of the experimental measurements was studied at glucose concentration of 0.5 mmol/L (15 experiments per day and four days in a row) [II]. Repeatability of the measurements was very good considering that the standard deviation was 0.0051 and the coefficient of determination was 98%. The results showed that the biosensor exhibited a fairly analytical feature of repeatability [II].

Regeneration of bio-sensing continuous-flow system involves passing a background flow of fluid without reactive components through the flowing system. This was controlled by varying the flow rate between 0.8 to 13.0 mL/min. As expected, the washing of the flow system depended on the flow rate and slightly on the substrate concentration [II]. At lower flow rates (0.8 to 2.1 mL/min) the regeneration time of the system increased with the increase of the flow rate and substrate concentration. At higher flow rates the regeneration time did not depend on the substrate concentration and flow rate (because of the diffusion in the flow channels) any more [II]. The rate for GOD-based biosensor system regeneration should be at least 2.1 mL/min. At this flow rate the washing time is approximately 4.5–5 min.

The loss of sensitivity under operational conditions is one of the most serious limits of the practical utility of biosensors. Besides leaching of the bio-selective material, it can be ascribed to inactivation and denaturation of the bioactive compound. The operational stability of the present biosensor system was assessed by a continuous long-term experiment, in which a 0.5 mmol/L glucose solution was repeatedly analyzed [II]. The biosensor system was in everyday exploitation – used for about 15 measurements per day – after what it was washed with 0.1 mol/L PB (pH 6.50) and left overnight at 37°C. The initial activity of the sensor dropped for approximately 20% during the first 3 days; afterwards the biosensor response remained constant for over 35 days operation period with no significant loss of activity [II].

In addition, the actual design of the continuous-flow biosensor enables to prolong the effective lifetime of optrodes. For example, if the enzyme activity and accordingly the reaction rate decreases by 20%, the sensitivity and equally the slope of the calibration curve change just by 1%. Futhermore, the system sensitivity does not drop below 90% of the initial one even in case 30% of the initial enzymatic activity remaining [III].

## 3.5. Time required for data acquisition

The proposed biosensor model describes the sensor responses adequately and allows clear interpretation of underlying processes and relatively easy biosensor calibration from pre-steady state data.

The temporal responses (shown in Fig. 7) were fitted within different time intervals (the curves were fitted between $t$=0 and $t$=$t_F$, whereas $t_F$ was varied from 300 sec to a minimal value of ~ 20 sec) [III]. This allowed simulating the situation, where the sensor output value is determined from shorter and shorter measurements. The results for parameter $A$, determined at three different substrate concentrations, are presented in Figure 7.

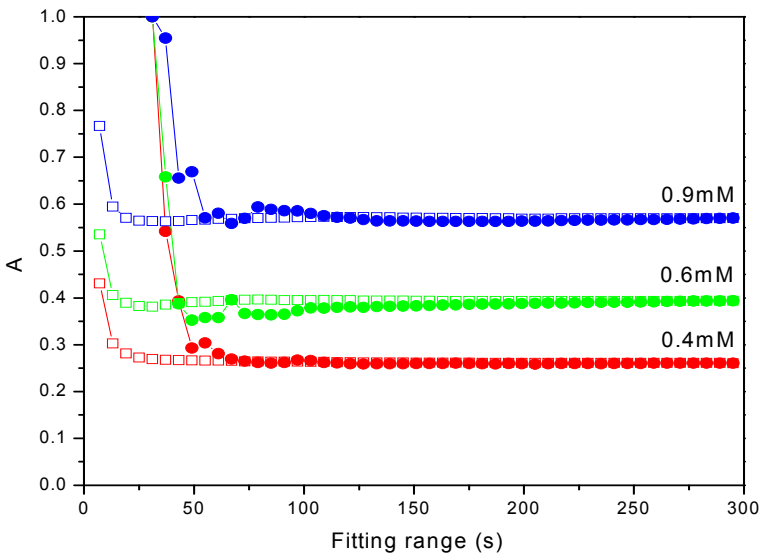



**Figure 7.** The dependence of the total signal change parameter $A$ on the fitting range (measurement time). Measurements were carried out at 37 °C in 0.4 mmol/L, 0.6 mmol/L, and 0.9 mmol/L glucose solutions in 0.1 mol/L phosphate buffer (pH 6.50) at flow rate 2.7 mL/min. At fitting, all three reaction parameters ($A$, $k_{SR}$, and $k_{O2}$; filled dots) or only parameter $A$ (open dots) was freely varied.

In all cases the value of parameter $A$ was practically constant down to the fitting range (measurement time) $t_F \sim 60$ sec. In Figure 7, the data for two fitting procedures are presented: the filled dots present the data obtained when all parameters ($A$, $k_{SR}$, and $k_{O2}$) in Eqs. (14, 15) were freely varied, whereas the empty dots present the data obtained when the pre-calibrated rate parameters were fixed and only parameter $A$ was varied [III]. As a result, using the fixed set of pre-calibrated rate parameters, the measurement time can even be as short as 20 sec. In other words, for obtaining the value of parameter $A$, there is no need to record the signal up to 300 sec, but the recording time can be as short as 20 sec. These concrete time values are related to the parameters of concrete bio-optrodes and may hence vary between different systems, in particular, the optrodes with higher enzyme activity will have higher reaction rate and shorter measurement time as well. If we compare the fitting results in Figure 6 and the signal changes in Figure 7, more general criteria may be formulated. In Figure 7 we can see that the relative signal has dropped at 20 and 60 sec by ~ 20% and ~ 50%, respectively. So, the substrate concentrations can be determined with relatively high accuracy by measuring the signal until it drops only by 20–50% of its maximal (steady state) change, and not waiting until the signal change has reached the 95% level of the presumed final change. This allows significant advantages in biosensor analysis speed, as the measurement time can be shortened more than by one order in magnitude.

## 3.6. Application of glucose dual-optrode biosensor for the determination of penicillins in milk

The main goal of the present work was to study the possibilities of rapid bio-sensing of penicillins' residues in milk and the application of the biosensor for the analyses of real milk samples of mastitic cows.

The excretion of penicillins via milk is dependent on different factors, e.g. an animal's individuality, milking interval etc., but as an average 30–40% of the total amount of penicillins applied are eliminated from the organism with milk as parent compound [15,95].

Milk is a complex colloid of fat globules within a water-based fluid that contains dissolved carbohydrates and protein aggregates with minerals [96]. The major carbohydrate in milk is lactose, which controls milk volume by maintaining its osmolarity [97]. Lactose concentration in raw bovine milk is very stable (around 140 mmol/L), exceeding the typical concentrations of glucose and galactose in milk more than 500 times [98,99]. Diet has no effect on lactose concentration in bovine milk and the regional and seasonal varieties are also very small [98]. As being a highly nutritious food, raw milk contains a diverse and complex microbiota, entering milk from contact with the animal and/or the surrounding environment [100,101]. Over 250 different bacterial species have been found in cows' raw milk [102]. The dominant microbial population in bovine milk are the lactic acid bacteria [103]. In addition, there

are also bacteria carrying the structural genes of *lac* operon, encoding the synthesis of β-gal, which is an enzyme required for the transport and metabolism of lactose and catalyzes the hydrolysis of lactose into glucose and galactose [102]. So, the amounts of soluble glucose and galactose in raw milk are dependent on the activity of β-gal. The presence of antibiotic residues in milk affects the production of β-gal by microorganisms leading to reduced glucose and galactose concentrations compared to high quality milk.

At first the effect of benzylpenicillin on the activity of β-gal was determined with amperometrical and spectrophotometrical methods [V]. It was found, that the catalytic activity of dissolved β-gal (0.1 mol/L PB; pH 6.50) did not change over a 48 hour period (Fig. 8). At the same time, the activity of β-gal decreased for 51% in 48 hours in case $3.3\times10^3$ ppb benzylpenicillin was added to the solution. This benzylpenicillin concentration corresponds to the average minimum concentration of benzylpenicillin in milk of dairy cows during their treatment with procaine-benzylpenicillin, although the actual concentration of benzylpenicillin residues in the milk of treated animals can be as high as $120\times10^3$ ppb [95]. Comparable results were obtained with both amperometrical and spectrophotometric methods [V].

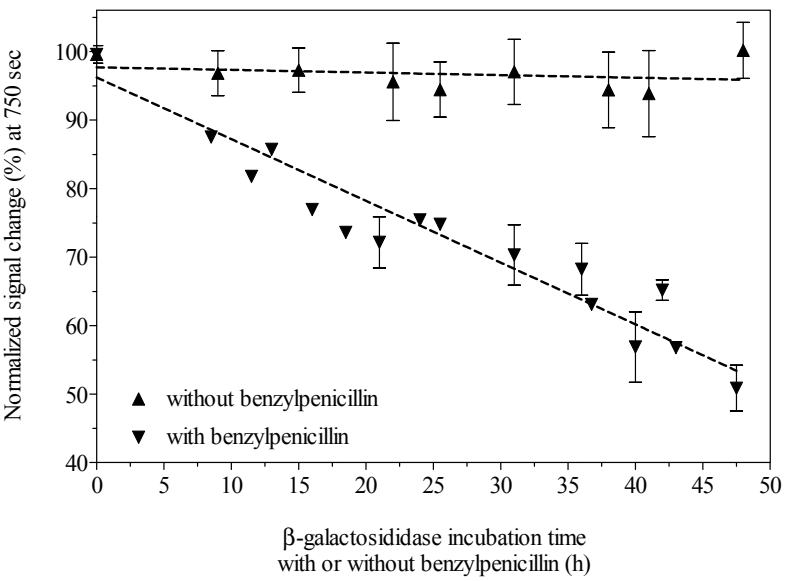



**Figure 8.** Inactivation of β-galactosidase during its incubation with benzylpenicillin. The measurements were carried out under constant stirring at 37 °C in 0.033 U/mL β-galactosidase solutions in 0.1 mol/L phosphate buffer (pH 6.50) with ($3.3\times10^3$ μg/L, ▼) or without benzylpenicillin (▲). Lactose concentration in the system was 140 mmol/L, the lactose incubation time with β-galactosidase was 480 sec and glucose oxidase concentration was 1 U/mL.

In order to imitate the conditions in cow's udder after administration of benzylpenicillin, we first studied the effect of benzylpenicillin on glucose concentration in milk during incubation, both in pure and spiked milk samples. We studied the effect of benzylpenicillin at concentrations 50 ppb and 50 ppm, which are quite high in comparison with MRL levels (4 ppb for benzylpenicillin in milk). These concentrations were chosen because the applied biosensing system enables to analyze the milk of single animals quickly within the early milking process and we could focus on analyzing the milk of the treated animals before it is "diluted" with the milk of other animals in milk collection tank. The determination of antibiotic residues in "undiluted" milk has an additional positive effect on the speed of analyses.

Changes of the glucose levels in milk due to the addition of benzylpenicillin were monitored during 5 hours (Fig. 9).

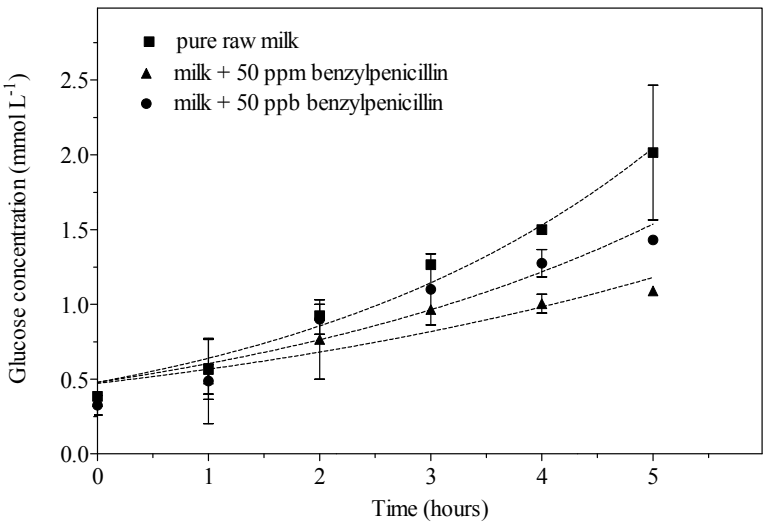



**Figure 9.** The change of glucose concentration in milk samples at different storage times. The measurements were carried out at 37 °C in pure milk (■), in milk spiked with 50 ppm (▲) and with 50 ppb of benzylpenicillin (●). The samples were stored at room temperature under constant stirring.

Immediately after the addition of benzylpenicillin, there was no detectable difference in the measured glucose concentrations (0.35±0.065 mmol/L) in pure and in spiked milk samples (Fig. 9). In time course, the differences in glucose concentrations in spiked and pure raw milk samples increased and after 5 hours, the concentration of glucose had risen almost 7 times from its initial value in pure milk and only 3 times in milk spiked with 50 ppm of benzylpenicillin causing the deceleration of the increase of glucose concentration for approximately 66%. The addition of 50 ppb benzylpenicillin leads to rate deceleration for roughly 38% (Fig. 9).

Finally, the biosensor system was used for the measurement of glucose levels in raw milk of cows, which were administered penicillin antibiotics to cure mastitis, during the treatment and milk withdrawal periods. As expected, the measured glucose concentrations in the milk of the treated animals were considerably lower compared to the milk of healthy animals. The unpaired t-test indicated that this difference was significant ($P$<0,001) (Fig. 10).

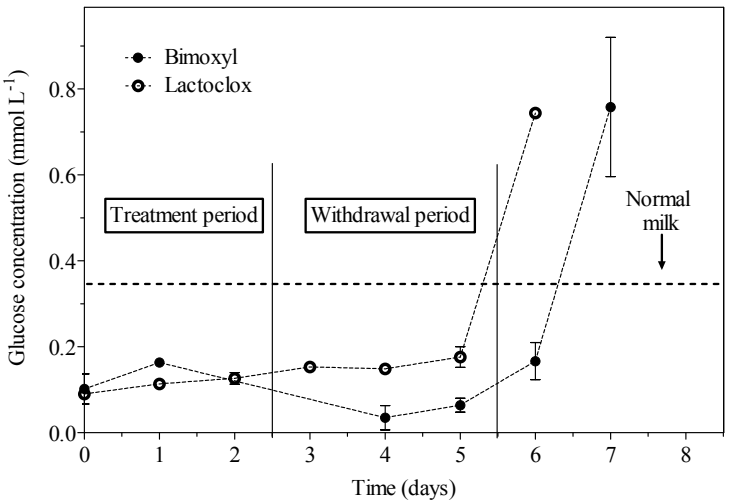



**Figure 10.** Glucose concentrations in the milk of cows, which had conducted mastitis and were treated with penicillin antibiotics. Milk samples were collected in the Dairy Research Farm of the Estonian University of Life Sciences in Southern Estonia. The milk samples from two cows undergoing mastitis treatment with Norbrook® Lactaclox or Bimoxyl LA were immediately freezed at –20 °C right after collection and melted at +4 °C just before testing. The treatment was administered for 3 days followed by a 3-day withdrawal period.

To validate the biosensor results, the presence of penicillin residues in the collected milk samples were analyzed also with Delvotest (DSM Food Specialties B.V.) The Delvotest analyses showed the presence of antibiotic residues above MRL levels in milk samples collected during the treatment and withdrawal periods, where the glucose levels were very low in comparison with glucose levels in pure milk (Fig. 10). Based on these results, the decreased glucose level can well serve as a qualitative indicator of the presence of antibiotic residues in raw milk similarly to Delvotest. The main advantage of using biosensing system for the detection of penicillin residues is time required for analyses – it is only 1 minute compared to 2–3 hours required for testing milk samples with Delvotest.

The output signals of reference oxygen optrode were also analyzed in the milk samples during the measurements, as the decrease of oxygen concentration in time indicates the oxygen demand of the total number of bacteria present in

milk samples. The oxygen demand in milk was low (around 15% of DOC in 5 minutes) and didn't change within days 1–4 counting from the beginning of treatment (slope=0; $P$=0.17171), but it increased by 5% per day from in days 5–9 (slope=0.005; $P$<0.0001). Based on the signal of the reference optrode, the total number of bacteria in samples decreased in the course of treatment and began to rise during the withdrawal period, indicating that the normal microbiota in udder began to recover.

# CONCLUSIONS

As a result, a rapid method and a biosensor set-up have been proposed for the detection of penicillins' residues in milk flows.

A fibre-optical dual-channel glucose biosensor was used as a model set up for studying the processes during bio-recognition in flows on the example of glucose oxidase catalyzed glucose oxidation reaction. A mathematical model involving two-substrate reaction and mass transport of substrates was developed for describing the sensor signal. The model is based on a system of rate equations and has a relatively simple analytical solution. This facilitated the interpretation of the experimental data and allowed to develop a method for determining the biosensor output from early pre-steady state signals. It was demonstrated that the sensor reading could be obtained when the signal had changed only by 20% towards its final steady state value, which reduced the detection time about 10-fold as compared to standard procedures and gave significant advantage in analysis speed. It was found that the sensor parameters did not depend on the flow rate in a relatively wide range (0.8–13 mL/min or 0.3–5.1cm/sec) [II, III]. The application of the model allowed to predict optimal biosensor parameters for obtaining maximal sensitivity and high stability on one hand, and to obtain fast results from the initial phase of the reaction on the other hand [III].

The modulation of the catalytic properties of glucose oxidase with the aim to amplify the characteristic calibration parameters of glucose and cascaded lactose biosensors was performed in the presence of different chlorides in order to accelerate the rate and improve the quality of milk analysis. It was found that sodium and magnesium chlorides at concentrations below 0.5 mol/L increased the total signal change parameter, used for the calibration of glucose biosensors, up to 20%.[I]. The addition of magnesium chloride gave further acceleration to the lactose measurements with bi-enzyme cascaded biosensors, as $Mg^{2+}$ also activates β-galactosidase. The amplification was highest at a magnesium chloride concentration of 5 mmol/L [I].

The glucose dual-optrode biosensor was applied to detect penicillins' residues in the milk of cows with mastitis. The glucose concentration in their milk during treatment and withdrawal periods decreased significantly compared to glucose levels in high quality milk. This decrease was likely due to the decreased activity of β-galactosidase, which catalyzes the hydrolysis of lactose, enabling to use glucose concentration as an indicator of the presence of penicillins' residues in milk [V].

The studied biosensor set-up has a high potential to serve as a system for real-time automatic control of the quality of raw milk in milk production farms. The application of this system allows the separation of substandard milk from the milk flow prior to milk collection tank. The enzymatic oxidation of glucose, going on in the biosensor, can be further accelerated by adding metal chlorides for even more rapid analyses. The system can be upgraded by attaching additional biosensors to build up a more robust biosensor array, where the signals of individual biosensors form a typical pattern of milk sample, which changes in the presence of different antibiotics.

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# SUMMARY IN ESTONIAN

## Penitsilliinide jääkide määramine piimas läbivoolulise biosensorsüsteemi abil

Käesoleva doktoritöö eesmärgiks oli välja töötada biosensorsüsteem penitsilliinide jääkide kiireks määramiseks toorpiimas, samuti pakkuda välja matemaatiline mudel läbivoolulistest mõõtesüstemidest saadud eksperimentaalsete andmete kiireks analüüsiks ja biosensori kalibratsiooniparameetrite arvutamiseks ning uurida võimalusi biosensorites toimuvate äratundmisreaktsioonide kiirendamiseks. Töö praktiliseks eesmärgiks oli uurida võimalusi biosensori kasutamiseks penitsilliinide jääkide kiireks määramiseks antibiootikumiravil olevate lehmade piimas [I–III,V].

Penitsilliinid on beetalaktaamide hulka kuuluvad antibiootikumid, mida kasutatakse peamiselt Gram-positiivsete bakterite poolt tekitatud haiguste raviks. Penitsilliini ja teiste antibiootikumide jäägid piimas tekitavad inimestel allergilisi reaktsioone ning soodustavad resistentsete mikroobitüvede teket, mistõttu on antibiootikumide jääkide lubatud sisaldus toidus rangelt reguleeritud. Penitsilliinide jääkide maksimaalseks lubatud kontsentratsiooniks toorpiimas on bensüülpenitsillini, ampitsillini ja amoksitsillini puhul 4 µg/L ning kloksatsillini puhul 30 µg/L.

Tavaliselt kasutatakse antibiootikumide jääkide määramiseks piimas mitmesuguseid kromatograafial põhinevaid meetodeid ning erinevaid mikroobse inhibeerimise ja immuunoretseptor teste. Üheks võimalikuks alternatiiviks traditsioonilistele analüüsimeetoditele on biosensorite kasutamine. Biosensorite eeliseks on nende lihtsus, suhteline odavus ning kiirus, mis võimaldab nende kasutamist kiireteks analüüsideks reaalajas.

Voolavates süsteemides toimuvaid mõõtmisi iseloomustava mudeli väljatöötamiseks kasutati mõõtesüsteemi, mille kestkeks osaks oli kahe voolukanaliga silindriline mõõterakk, millesse oli paigutatud glükoosi biooptrood ning hapniku võrdlusoptrood. Mõlemale optroodile, mis kujutavad endast hapnikutundliku materjaliga kaetud kvartsfiibrit, oli keritud niit, millele glükoosi optroodi puhul oli immobiliseeritud glükoosi oksüdaas. Mõlemad optroodid kokku moodustasid diferentsiaalse glükoosi biosensori, mis võimaldas eksperimentaalsete mürade mõju vähendamist biosensoris toimuva äratundmisreaktsiooni kirjeldamisel hapniku kontsentratsiooni vähenemise kaudu [III].

Väljapakutud läbivoolu mudeli ja eksperimentaalsete kõverate kokkulangevus oli väga hea ($R^2$=0,99). Leiti, et voolukiiruste vahemikus 0,8–13,0 mL/min signaali kogumuutuse parameetri väärtus voolu kiirusest ei sõltunud [II]. Võrreldes erinevate ajavahemike jooksul saadud andmetest arvutaud reaktsiooni parameetrite väärtusi leiti, et usaldusväärsete tulemuste saamiseks vajalik äratundmisreaktsiooni sügavus on ligikaudu 20%. Seega, uus läbivoolu biosensorite jaoks väljapakutud mudel võimaldab saada reprodutseeritavaid ja usaldusväärseid tulemusi 10-korda kiiremini kui varem [III]. Samuti selgus, et bio-

sensoris oleva ensüümi aktiivsuse vähenemine kuni 20% ei mõjuta oluliselt saadud tulemusi [III].

Piimas tehtavate analüüside kiirendamiseks uuriti ka glükoosi oksüdaasi aktiivsuse modulleerimise võimalusi erinevate metallide katioonide abil nii glükoosi kui ka kaskaadses laktoosi biosensoris [I]. Signaali kogumuutuse parameetri väärtus suurenes kuni 20% naatrium- ja magneesiumioonide mõjul nende kontsentratsioonidel üle 5 mmol/L. Lisaks aktiveeris $Mg^{2+}$ kaskaadses laktoosi biosensoris β-galaktosidaasi, suurendades veelgi mõõdetavat biosensori väljundsignaali [I].

Penitsilliini jääkide määramisel glükoosi biosensoriga selgus, et penitsilliiniga ravitavate lehmade piimas on glükoosi kontsentratsioon võrreldes tervetelt loomadelt saadava puhta piimaga väga madal ning see erinevus on statistiliselt oluline. Glükoosi madal kontsentratsioon on tõenäoliselt tingitud β-galaktosidaasi – ensüümi, mis katalüüsib laktoosi hüdrolüüsi piimas, vähenenud aktiivsusest. Töö tulemusena leiti, et glükoosi tase piimas on heaks indikaatoriks penitsilliini jääkide määramiseks toorpiimas [V].

Kasutatud biosensorsüsteem on rakendatav kiireks penitsillinide jääkide määramiseks toorpiimas piimafarmides. Kiire lüpstava piima analüüs võimaldab mittekvaliteetse piima õigeaegset eraldamist kvaliteetsest toodangust ning kogu toodetava piima kvaliteedi tõstmist. Kasutatud biosensorsüsteemi on erinevate antibiotikumide jääkide määramiseks toorpiimas võimalik tulevikus modifitseerida täiendavate biosensorite lisamisega.

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