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Piezoelectric immunosensors for the detection of individual antibiotics and the total content of penicillin antibiotics in foodstuffs

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ABSTRACT

Piezoelectric immunosensors on the basis of homologous and group-specificantibodies have been developed for detecting penicillin G, ampicillin, and the total content of penicillin antibiotics. The receptor coating of the sensor was obtained by the immobilization of penicillin G or ampicillin hapten–protein conjugates on the polypyrrole film obtained by electropolymerization and activated by glutaraldehyde. The affinity constants and the cross reactivity coefficients have been calculated. This made it possible to estimate the affinity and specificity of the polyclonal and monoclonal antibodies used. The calibration curves are linear in the range of concentrations 2.5–250.0 ng ml^{-1} (penicillin G), 2.5–500.0 ng ml^{-1} (ampicillin), and 1–500 ng ml^{-1} (group of penicillin). The limits of detection are 0.8 ng ml^{-1}, 3.9 ng ml^{-1}, which are lower than MRL, established for penicillin antibiotics. The sensors were tested in detecting penicillins in milk, pork, beef, liver.

1. Introduction

Penicillin antibiotics have been widely used for treating and preventing infectious diseases in veterinary. Penicillins are the least toxic of all antibiotics; nevertheless, owing to their fast removal from an organism, the doses for the treatment of animals manifold exceed the levels of other antibiotics, which can cause high residual concentrations in livestock products. As a result, they may cause allergic reactions, resistance to microorganisms and general lowering of immunity in consumers of meat and dairy products [1].

The risk of polluting food raw materials by antibiotics can be reduced only through effective monitoring of systems at all stages – from production to sales. Thus, severe requirements are established for the techniques of mass control of detrimental compounds in raw materials and animal products: these techniques must provide high sensitivity, selectivity of detection, reliability and reproducibility of the results. The maximum level of penicillin residues is regulated by the legislation of various countries at the level of 4 μg l^{-1}in milk, 0.05 mg kg^{-1} in meat, liver and kidneys [2]. Besides, babyfood must not contain penicillin antibiotics at all.

Physical and chemical methods (chromatographic, electrochemical, spectral, etc.) as well as their combination, e.g. gas chromatography in tandem with mass spectrometry [3–9], are widely applied for the detection of antibiotics. However, despite their high sensitivity, these methods are limited in their application due to long sample pretreatment and the necessity to use expensive equipment. Commercial microbiological tests and strip tests are used for fast and thorough semi-quantitative analyses [10].

Classical immunochemical methods are not widely spread for the detection of penicillin antibiotics [11,12] though the application of immunoreagents provides for carrying out the analysis in structurally complex samples in the presence of compounds whose separation requires physical and chemical methods. At present electrochemical [13,14] and optical immunosensors [15,16] are used for detecting penicillin in foodstuffs. The sensitivity of detection depends on both the nature of the transducer and the properties of the bioreceptor layer. The application of electrochemical immunosensors is complicated by the necessity of introducing additional labels, while the detection limit at MRL level is not always reached with sensors on the basis of surface plasmon resonance because of low affinity of bioreagents or the receptor layer's quality [16]. However, the application of SPR sensors with the receptor layer based on β-lactam receptor proteins makes LOD penicillin G equal to 1.2–1.5 μg kg^{-1}, which is significantly lower than the MRL of antibiotic in milk [15].

The amperometric sensor based on screen-printed devices with immobilized β-lactam specific receptor demonstrates high sensitivity of β-lactam detection in milk at the level of 5–10 μg kg^{-1} [17]. A label-free impedimetric flow injection immunosensor was developed for the direct detection of penicillin G [14]. The receptor layer on the surface of the gold electrode was formed by the immobilization of anti-penicillin G on the self-assembled monolayer of thioctic acid. Real time monitoring of impedance was

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carried out at the optimum frequency of 160 Hz and limit of detection of 3.0×10^{-15}.

Gravimetric sensors on the basis of piezoelectric crystals are applied for the detection of antibiotics of other groups [18,19]; they make it possible to detect drugs at a rather low level and such sensors can be applied for the detection of individual antibiotics and their total content depending on the antibodies used. The detection of low-molecular compounds is usually carried out in the competitive format of the analysis with the preliminary introduction of fixed antibody quantities into the samples, the former binding with analyte into an immunocomplex and participating in the formation of a complex with the albumin conjugate of an antibiotic, immobilized on the surface of the sensor. The analytical signal of sensors is the decrease of quartz frequency (Δf) due to an increase in the weight of the receptor layer (Δm) according to the Sauerbrey equation [20].

The important stage in developing piezoelectric immunosensors is the immobilization stage, which includes the formation of the substrate and the receptor biolayer. Fixation of biomolecules on the substrate created by the electropolymerization method is thought to be promising. While electrogenerating the polymer on the surface of the gold electrode, it is possible to control the mass and thickness of the coating in the process of its obtaining by changing the potential and the scan rate [21]. It is especially important in gravimetric sensors as the large mass of the biolayer can limit the detection range. Electrogeneration of polyelectrolyte films provides strong binding with the surface of a gold electrode, and hence the sensor′s life and its repeated application after the regeneration of the immunoaffine layer. The films obtained by polypyrrole electropolymerization proved to be equally applicable in a piezoelectric sensor.

The aim of this research is to develop a piezoelectric sensor to detect trace quantities of penicillin G, ampicillin and their total content in foodstuffs.

2. The experiment

2.1. Reagents

2.1.1. Immunoreagents

Polyclonal antibodies to penicilline G – Ab-Pen G (supplied by R.A. Abuknesha (King′s College, London, UK)), polyclonal antibodies to ampicillin – Ab-Amp, group-specific monoclonal antibodies to penicillin group –Ab-Pen («Abcam», United Kingdom).

2.1.2. Antibiotics

Penicillin G, bicillin-5, oxamp (ampicillin:oxacillin 1:1), cefazolin, cefotaxime («Synthesis», Russia), ampicillin, streptomycin (Sigma-Aldrich, USA).

Nitric acid, acetone (“Vecton”, Russia), ethanol, glutaraldehyde solution – GA (“Reanal”, Hungary), pyrrole («Vecton», Russia), bovine serum albumin (BSA) (Sigma, USA).

Phosphate buffer, pH 7.2, was prepared by dissolving: 8.0145 g NaCl, 0.2012 g KCl, 2.864 g $Na_2HPO_4 \cdot 12H_2O$, 0.204 g KH_2PO_4 in double distilled water.

2.2. Equipment

The equipment for conducting QCM measuring included a hopper for injecting the sample, a peristaltic pump providing a continuous stream of liquid (“Knauer”, Germany), a digital module “Diskop” (“Bafika”, Russia) and a personal computer for recording the frequency modulation.

The electropolymerization of polymeric coatings was carried out on the potentiostat PI-50-1 (Russia) in the potentiodynamic mode with cyclic potential sweep.

The photos of AFM (atomic force microscopy) were obtained on the scan microscope Solver P47-PRO, produced by Nanotechnology-MDT (Russia), in the semi-contact mode in air with the scan rate of 120–180 kHz.

The hapten–protein conjugate was characterized by the method of infrared-spectroscopy (IR) (IK-40, «Lomo», Russia), comparing the spectrum lines of initial compound (antibiotic, protein) and the final product (hapten–protein conjugate).

The AT-cut 10 MHz resonator with gold electrodes obtained by magnetron deposition was purchased from ELECTRONIC FIRM ETNA COMPANY (Moscow, Russia).

2.3. Sample pretreatment

The milk and meat samples were pretreated for analysis in the following way:

2.3.1. Milk

1 ml of milk и and 1 ml $(NH_4)_2SO_4$ (saturated solution) were added into the centrifuge tube for protein sedimentation, the mixture was then stirred and centrifuged for 10 min at 7000 rpm, the supernatant was then separated and used in the analysis.

2.3.2. Meat

A piece (1 g) of muscular tissue cut from the middle part of the sample was chopped by scissors with the addition of 2 ml of $(NH_4)_2SO_4$, the mixture was carefully stirred and placed in a centrifuge tube. The extraction of antibiotic was carried out for 90 min in a thermostat at $(37 \pm 1)\,^{\circ}C$. The samples were then heated for 30 min on a water bath at $(65 \pm 1)\,^{\circ}C$ and centrifuged at 3000 rpm for 20 min. The supernatant was separated and used in the analysis.

2.4. The formation of the immunoaffinity layer on the basis of polypyrrole coating

Before measuring, the electrode surface was degreased by acetone and then dried in air at room temperature. Since polypyrrole is highly adhesive to the surface of a gold electrode, before the re-synthesis of a polypyrrole film the gold electrode of the sensor was cleaned by concentrated nitric acid.

The receptor layer was formed in several stages. A polypyrrole coating is obtained on the degreased surface of the gold electrode of the sensor in cyclic voltamperometry with a potentiostat (PI-50-1) in the range -0.2 ± 0.8 V versus the silver chloride reference electrode at the scan rate of 10 mV s^{-1} in 0.2 M sodium chloride medium.

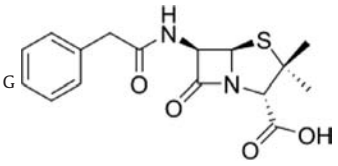
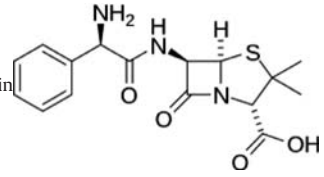
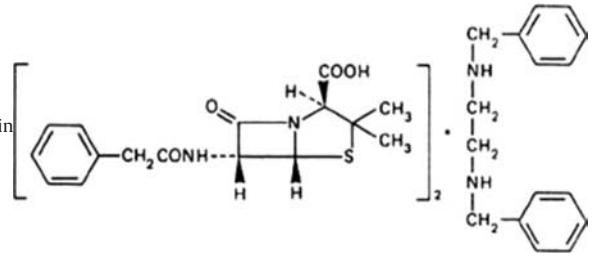
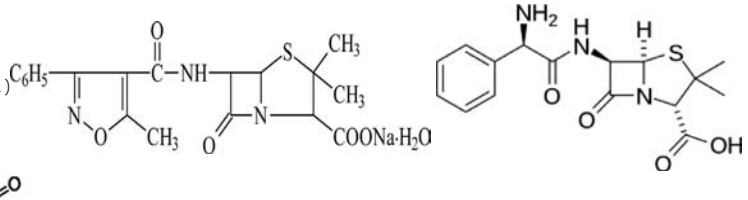
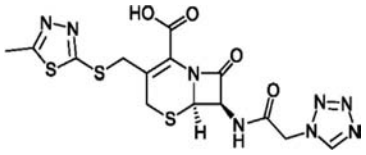
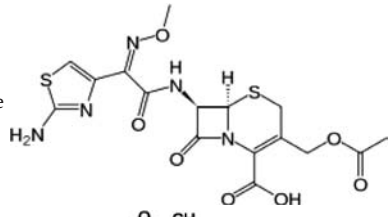
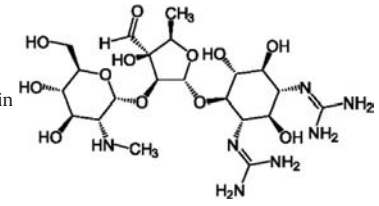
The immunoaffinity layer of the sensor was formed by the activation of the electrogenerated substrate with glutaraldehyde vapours and covalent binding of the hapten–protein conjugate, obtained by covalent binding of penicillin G/ampicillin with bovine serum albumin GA. The polymeric layer of the sensor was dried for 30 min, and the sensor was then placed in a camera with glutaraldehyde vapours for 4 h. After its activation by GA, 10 μl of conjugate was applied onto the polypyrrole substrate and stored in a cold damp camera for 24 h. The increase of mass and roughness on every stage was controlled by QCM and AFM methods respectively.

The immunoaffinity layer is characterized by 10–15 cycles of measurements and high stability to multiregeneration on the basis of the electrosynthesized polypyrrole.

Table 1
Values of kinetic characteristics and affinity constants

Antigen–antibody complex	k_f, $(M^{-1}\,s^{-1})\times 10^{-8}$	k_d, $(s^{-1})\times 10^{2}$	K_{af}, $(M^{-1})\times 10^{-10}$
Ab-PenG–Pen G-BSA	5.1	2.3	2.2
Ab-Amp–Amp-BSA	4.9	2.5	2.0
Ab-Pen–Amp-BSA	1.3	8.5	0.2

Table 2
The values of cross-reactivity coefficients.

Antibiotic	CR_1, %	CR_2, %	CR_3, %
Penicillin G 	100	10	100
Ampicillin 	14	100	94
Bicillin 	5.9	7.3	99.3
Oxamp (ampicillin:oxacillin 1:1) 	6.5	51	87.4
Cefazolin 	1.7	3	9.1
Cefotaxime 	> 1	1,5	5
Streptomycin 	> 1	> 1	1.7

2.5. The synthesis of hapten–protein conjugate

Protein conjugates of penicillin G and ampicillin were pre-synthesized: 34 mg of penicillin G/ampicillin and 68 mg BSA were dissolved in 10 ml mixture of ethanol and PBS (1:1). 40 ml of 25% solution of GA was added and mixed at room temperature for 3 h.

The formation of conjugate was controlled by the infrared-spectroscopy method and it was found that binding between the

amino groups of penicillin G/ampicillin and BSA occurred through GA. Cleaning of a conjugate was carried out by dialysis against PBS for 14 h. Purified conjugate was used to form a biosensitive coating of the piezoelectric sensor.

3. Results and discussion

3.1. The calculation of kinetic characteristics and affinity constants of a reversible immunochemical reaction

The forward rate constant (k_f) and reverse rate constant (k_r) of an immunocomplex were calculated by the method of Scatchard to characterize an immunochemical reaction on the sensor's surface [22]. The affinity constant was calculated as the ratio of the rate constants (Table 1).

High k_f values are characteristic for all studied reactions, which indicate the conformational availability of applied antibodies to be bound with penicillins. Low values of the reverse rate constant indirectly testify to the stability of the obtained immunocomplexes.

Table 3
The choice of the concentration of hapten–protein conjugate.

Concentration of hapten–protein conjugate, M	Ab-PenG–Pen G-BSA		Ab-Amp–Amp-BSA	
	Ab concentration, (μg ml^{-1})	Signal, Δ*f*, (Hz)	Dilution of antibodies	Signal, Δ*f*, Hz
0.1	10	23	1:500	11
	20	14	1:1000	12
0.2	10	45	1:500	22
	20	40	1:1000	20
0.4	10	9	1:500	11
	20	20	1:1000	19

K_{af} values of 10^{-10} indicate the possibility of applying polyclonal and monoclonal antibodies to the selective and highly sensitive detection of penicillin by the piezoelectric immunosensor.

3.2. Estimation of cross-reactivity of antibodies

The cross reactions between polyclonal (Ab-PenG (CR1), Ab-Amp (CR2)) and monoclonal antibodies (Ab-Pen (CR3)) in reference to β-lactams, aminoglycoside (Table 2) were studied in order to eliminate false positive immunochemical reactions of antibodies and antibiotics used in veterinary, and were differing in their structure from penicillin. The cross-reactivity coefficients were calculated by equation:

$$CR, \% = IC_{50}(B) \cdot 100/IC_{50}(A),$$

IC_{50} – concentration corresponding to 50% inhibition of binding penicillin G/ampicillin (A) and its analogue (B).

The values obtained (CR_1, $CR_2 = 100\%$) for the polyclonal antibodies show their high specificity against their own antigens. Insignificant cross reactions ($CR_{1,2} < 3$) were registered for antibiotics of the cephalosporin's group, having common structural fragments with penicillins. The cross reactivity coefficient is equal to 51% as ampicillin is a component of the pharmaceutical medicine «Oxamp».

The monoclonal group-specific antibodies reveal high specificity against all the antibiotics of the penicillin group, having in their structure a β-lactam ring (CR = 88–100%). Therefore the application of Ab-Pen antibodies provides for the determination of the total content of penicillin antibiotics.

3.3. The choice of concentrations of hapten–protein conjugates for immobilization and of antibodies for immunochemical detection

The concentrations of hapten–protein and antibodies, used in the competitive format of the analysis, were chosen to optimize the conditions of detection.

The concentration of hapten–protein conjugate influences the formation of a sufficient number of antigen determinants for

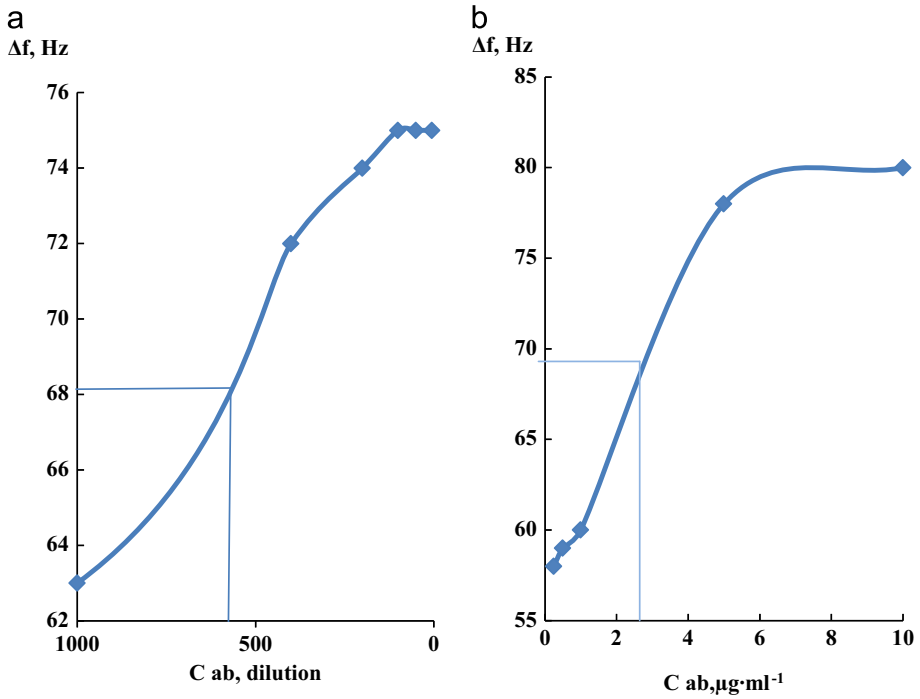


Fig. 1. The choice of the concentration of polyclonal antibodies corresponding to 50%-inhibition to Pen-BSA (a) and AMP-BSA (b)

binding with antibodies on the surface of the sensor's electrode. It was experimentally proved that the maximum analytical signal may be obtained in the application of the conjugate at the immobilization stage with the concentration 0.2 M (Table 3). In the range of lower and higher concentrations of the conjugate the signal of the sensor lowers as a result of insufficient specific concentration of active centres or their steric inaccessibility to bind with antibodies because of their proximity.

The influence of the concentration of polyclonal antibodies introduced in the sample at the pretreatment stage, on the value of the analytical signal, was investigated and the optimal concentration of antibodies corresponding to 50%-inhibition of active centres of the bioreceptor coating was determined (Figs. 1 and 2). The application of the concentrations of antibodies beyond the linear section of the graph causes deviation from the Sayerbrey equation owing to nonspecific binding (in the area of low concentration) or irregularities of linear interconnections Δf and Δm (in the area of high concentration).

The application of the 50%-concentration of antibodies makes it possible to reach an optimum ratio of the number of molecules of antibodies which remain unbound at the stage of the homogeneous immunochemical reaction and the number of active centers on the surface of the immunosensor, which positively affect the detection range of the piezoelectric sensor.

3.4. Drawing the calibration curves

Standard solutions of analytes prepared directly before the analysis and containing a concentration of antibodies corresponding to 50% – inhibition were used for drawing the calibration

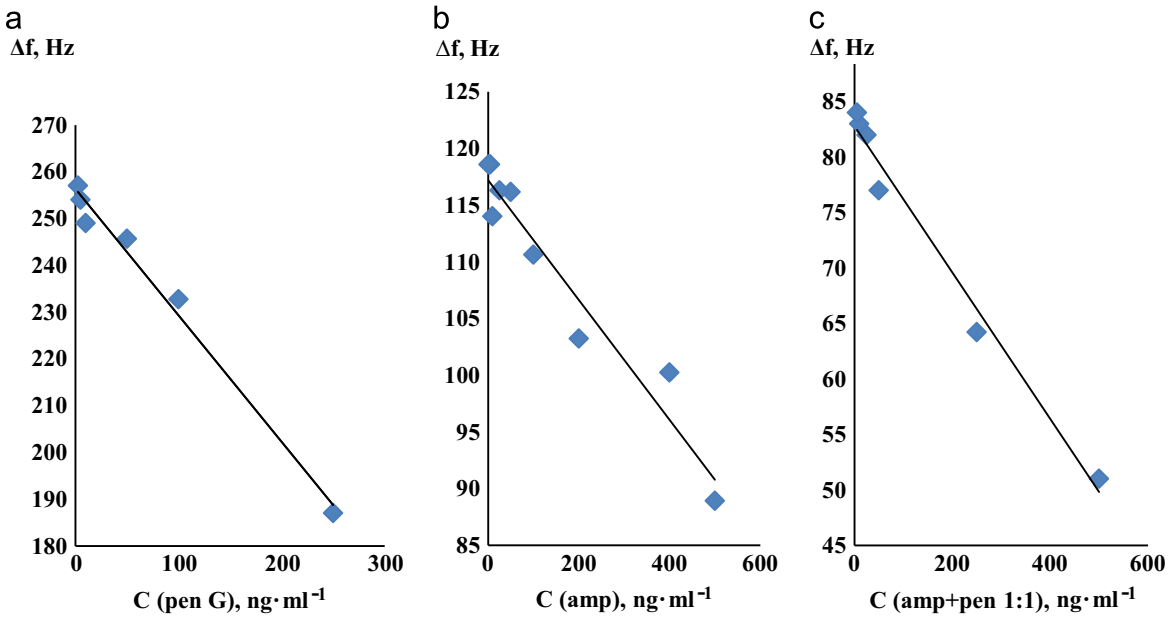


Fig. 2. The calibration curves for the detection of penicillin G (a), ampicillin (b) and the total content of penicillins (c) (according to ampicillin).

Table 4
Analytical features of method for the detection of individual antibiotics and the total content of penicillin antibiotics

Antibiotic	Linear equation (ng ml^{-1})	R^2	Linear range, (ng ml^{-1})	c_{min}, (ng ml^{-1})
Penicillin G	$y=-0.2707x+256.4$	0.987	2.5–250.0	0.8
Ampicillin	$y=-0.0528x+117.2$	0.942	2.5–500.0	3.9
The total content of penicillin antibiotics (according to ampicillin)	$y=-0.0659x+82.8$	0.982	1.0–500.0	1.7

Table 5
Verification of accuracy of penicillin's detection.

Antibiotic	Concentration added, ng ml^{-1}	Concentration found, ng ml^{-1}	Recovery, %	RSD
Penicillin G	10	9.0 ± 0.6	90	0.05
	100	98 ± 3	98	0.02
	200	202 ± 5	101	0.02
Ampicillin	10	8.7 ± 0.5	87	0.06
	100	99 ± 4	99	0.04
	200	203 ± 7	102	0.03
The total content of penicillin antibiotics (according to ampicillin)	10	9.4 ± 0.7	94	0.06
	100	98 ± 4	98	0.04
	200	200 ± 5	100	0.02

Table 6
Penicillin′s detection in foodstuffs by the "added-found" method ($n=3$, $P=0.95$)

Sample	Concentration added, ng g^{-1}	Concentration found, ng g^{-1}	Concentration calculated, ng g^{-1}	RSD
Penicillin G detection				
Milk[a]				
1	10.0	12.6 ± 0.4	2.6 ± 0.4	0.02
Pork				
2	20.0	86 ± 5	66 ± 4	0.03
3	100.0	174 ± 10	74 ± 6	0.02
Beef				
4	20.0	40 ± 4	20 ± 2	0.04
5	100.0	126 ± 10	26 ± 3	0.03
Liver				
6	20.0	31 ± 2	11.0 ± 0.6	0.03
7	100.0	114 ± 8	14 ± 1	0.03
Ampicillin detection				
Milk[a]				
1	10.0	14.8 ± 0.5	4.8 ± 0.4	0.02
Pork				
2	20.0	38 ± 3	18 ± 2	0.03
3	100.0	123 ± 4	23 ± 1	0.01
Beef				
4	20.0	38 ± 1	18 ± 1	0.02
5	100.0	118 ± 7	18 ± 1	0.02
Liver				
6	20.0	44 ± 4	24 ± 2	0.04
7	100.0	124 ± 6	24 ± 1	0.02
Ampicillin and penicillin G detection (1:1)				
Milk[a]				
8	20.0	34 ± 2	14 ± 1	0.03
9	40.0	56 ± 4	16 ± 1	0.03

[a] ng ml^{-1}.

curves. The sensor with the protein conjugate of ampicillin was used for detecting the total content of penicillins, therefore the graduation of the sensor was carried out according to ampicillin. The metrological characteristics of the method are given in table 4.

3.5. The sensor′s application

The verification of accuracy penicillin′s detection was carried out by the "added-found" method with the application of standards solution of antibiotics (Table 5).

The statistical processing of results with the application of Student′s *t*-test did not reveal a systematical measurement error.

The sensors were tested for the detection of penicillin G and ampicillin in animal foodstuffs with the application of the method of additives. A certain quantity of an antibiotic was added to the samples obtained after pretreatment and after the analysis the content of penicillin in samples was then calculated by the difference (Table 6).

The results obtained show that the application of the piezoelectric sensor makes it possible to diagnose antibiotics with high sensitivity.

4. Conclusion

Piezoelectric immunosensors for detecting trace concentrations of penicillin antibiotics in foodstuffs (milk, pork, beef, and liver) were developed. The sensors were tested and the studied samples did not reveal exceeding penicillin concentration.

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