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METHODS OF RESIDUAL QUANTITIES OF ANTIBIOTICS AND INHIBITORS DETECTION IN RAW MILK

The article is devoted to the problems of detection of residual quantities of antibiotics and inhibitors in raw milk. It was analyzed the following microbiological methods: substances diffusion into agar, reductase probe, biocalorimetry and biotesting of microalgae motion. In the publication was shown that methods of biocalorimetry and biotesting of *Euglena gracilis* motion allow to increase sensitivity of antibiotics detection and to decrease time and labour inputs for analyses. These methods may be recommended for practical application at dairy farms and dairy processing enterprises.

Introduction. Modern methods of inhibitors detection in raw milk attract attention of dairy farms and processing enterprises.

Well known that inhibitors in milk disturb biotechnological processes and negatively affect at dairy enterprises producing cultured milk foods, child and clinical nutrition [1].

To inhibitors belong antibiotics, preserving, neutralizing substances; detergents and disinfectants; nitrates, heavy metals etc. The main sources of their appearance in milk are connected with cow care (antibiotics); cattle feeding (pesticides, preserving agent, feed antibiotics, nitrates, heavy metals etc); washing of milking equipment to maintain hygiene and sanitation (detergents, disinfectants neutralizers).

Antibiotics are rated among the most biologically dangerous and frequently detectable types of milk contamination. These substances have high biological activity in low content (maximum permissible concentration MPC 0.01–0.10 mg/kg) and high specificity towards separate vital functions of microorganisms [1].

In the Republic of Belarus residual quantities of antibiotics in milk are tested at three stages:

1) in stockbreeding complexes and at dairy farms;

2) at dairy processing enterprises;

3) in test laboratories and veterinary centres.

The main places of testing inhibitors are dairy farms, but they are not fully equipped. There are no adequate resources and measuring tools for continuous control of alien substances in milk.

Dairy farms and processing enterprises need simple, fast and cheep methods of analysis of residual quantities of antibiotics and inhibitors in raw milk.

Main part. The aim of the work is the analysis of existing methods for detection of residual quantities of antibiotics and inhibitors in raw milk as well as research and development of express methods for their biotesting.

When analyzing problems of residual quantities of xenobiotics in different media it is necessary to resolve three types of tasks: detection of alien substances; their nature determination and quantification.

Not all methods are capable for solving these three problems simultaneously, but in some cases it is not required. For example at dairy farms all employed antibiotics are known and their identification isn't necessary.

Low values of antibiotics' MPC suppose application of extremely sensitive methods of analysis.

For detection of antibiotics and inhibitors in raw milk were employed the following approaches: microbiological methods such as substances diffusion into agar and reductase probe [2, 3]; immunological methods [4]; biotesting of microorganisms motion and biocalorimetry [5, 6].

The most sensitive and highly accurate sustaining method of antibiotics testing in milk is chromatography with mass-spectrometry detection. But it is a very expensive method which requires a high level qualification of staff therefore it has limited application and is used only in major centres for monitoring total situation.

More profitable microbiological methods of analysis which are highly sensitive, cheaper and easy of access. Wide spread occurrence have got microbiological methods of control with help of sensitive strain of microorganisms. They are remarkable for simplicity, high productivity, but the analysis lasts longer and must be carried out purely in microbiological laboratory under sterile conditions. It makes them not accessible for dairy farms.

Reductase probe with lactic-acid bacteria or spore-forming bacteria is a faster and more accessible microbiological method of antibiotic detection in raw milk at dairy processing enterprises [2], but their sensitivity not enough.

The extremely sensitive and most selective analysis techniques can be related with immunological, immunochemical and immune-enzyme methods [4]. In the market there is a wide range of testing systems for antibiotic detection in raw milk. To their deficiency belong high current costs as well as short retention cycle.

The research work was undertaken on water media as well as on milk samples artificially contaminated with antibiotics and inhibitors to the necessary concentration. As antibiotics and inhibitors were used the following substances: benzylpenicillin, streptomycin, tetracycline, erythromycin, chloramphenicol, ampicillin, nisin, salts of heavy metals Fe^{2+}, Pb^{2+}, Cr^{3+}, Cu^{2+}, Cd^{2+}, nitrites and nitrates in concentrations 10^{-3}–10^{-9} M.

The testing studies were directed with cells of microalgae *Euglena gracilis;* 24-hour cultures of mesophilic and thermophilic bacteria borrowed from collection of department of biotechnology and bioecology BSTU. The preparation of milk samples and whey receiving, measuring of cell motion of microalgae *Euglena gracilis* were carried out as in [5].

The influence of inhibitors on cells was expressed by alteration of the quantity B:

$$B = (V_к / V) \cdot 100\%, \quad (1)$$

where $V_к$, V – average speed of cell motion in control and working samples. As detection threshold of antibiotics (C_{min}) served the substance concentration which had altered the quantity B by 20%.

Heat production of microorganisms in milk samples with and without inhibitors was recorded with help of microcalorimeter MKM-C [6]. Content of inhibitors in raw milk was determined by the level of suppression of physiological activity of lactic-acid bacteria which depended on heat production capacity of cells without (q_o) and in the presence (q) of inhibitors or by quantity of heat emitted by them (Q_o и Q). Statistical analysis of obtained data was undertaken by Microsoft Excel.

The results of research of *E. gracilis* motion in whey in the presence of particular inhibitors and antibiotics are summarized in Fig. 1, 2.

As to Fig. 1 the dependence of B value from concentration for inhibitors action is complicated whereas for antibiotics (Fig. 2) it can be expressed:

$$B = -a \cdot \lg C + b, \quad (2)$$

where a, b – experimentally found constants.

In the result of fulfilled work it became possible to calculate values C_{min} of antibiotics (Table).

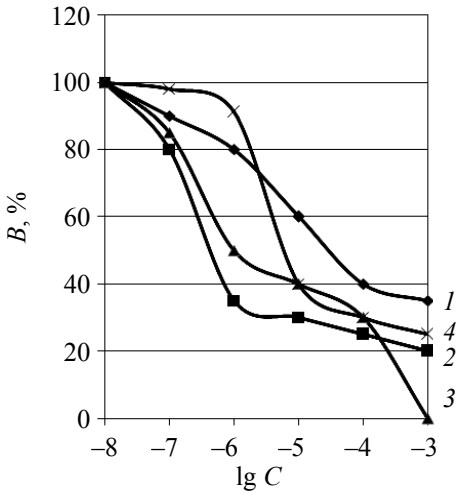

Fig. 1. Relative velocity (B) of *E. gracilis* cells motion from inhibitors concentration (lg*C*). Lozino-Lozinsky medium, 20°C: *1* – phenol; *2* – nitrobenzene ; *3* – Cd^{2+}; *4* – Cr^{6+}

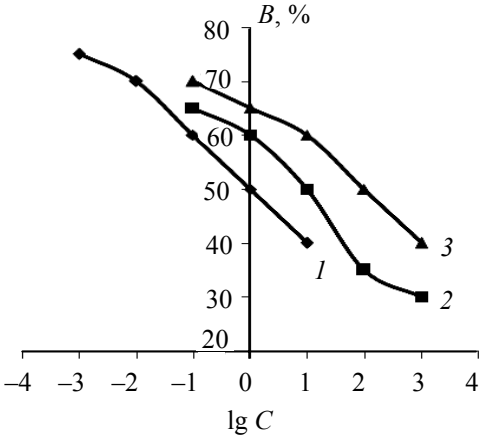

Fig. 2. Relative velocity of *E. gracilis* from antibiotics concentration in Lozino-Lozinsky media, 20°C: *1* – benzyl penicillin; *2* – streptomycin; *3* – tetracycline

The most active were antibiotics erythromycin and benzylpenicillin which have an inhibitory effect on *E. gracilis* movement in concentrations 20–40 times lower than by reductase probe on State Standard 23454–79 and when using the method of substances diffusion into agar on State Standard P 51600–2010.

Comparative analysis of methods for detection of antibiotics availability in raw milk

Antibiotics	C_{min}, mcg/ml				
	Reductase probe State Standard 23454–79	Biocalorimetry		Motion of cells *E. gracilis*	Method of substances diffusion into agar State Standard P 51600–2010
		L. lactis	*Str. thermophilus*		
Benzylpenicillin	0,01	0,042	0,002	0,0005	0,005
Streptomycin	30,0	1,0	5,0	0,003	0,5
Oxytetracycline	1,0	0,2	0,1	0,01	0,1
Erythromycin	0,1	–	–	0,0002	0,05

High sensitivity and simplicity allows to employ cell motion for biotesting residual quantities of antibiotics in raw milk.

Fig. 3, 4 show kinetics of heat emission by bacteria depending on a type and concentration of antimicrobial substances.

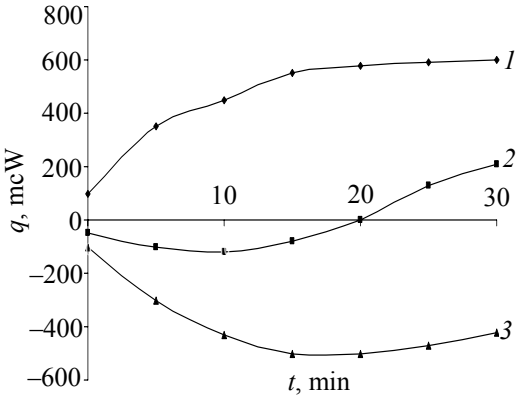

Fig. 3. Kinetics of heat power emission by E. coli cells in presence of antimicrobial substances (T = 30°C, *C* = 0.05%): *1* – control; *2* – gephal; *3* – chlorhexidine

The level of heat emission by microorganisms was decreased and the lag-phase was increased in the result of inhibitors action at metabolic cell activity. Fig. 3 demonstrates that more powerful inhibitory effect among tested substances at the same concentration was for chlorhexidine.

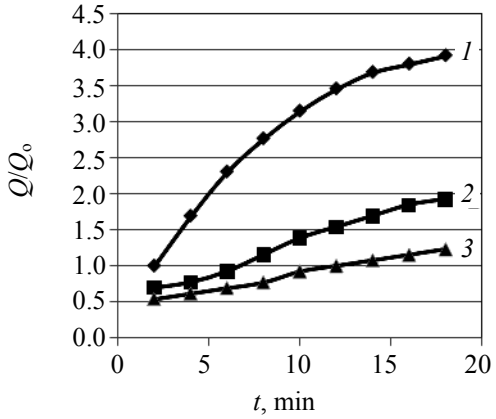

Fig. 4. Kinetics of relative heat emission (Q/Q_o) of lactic acid bacteria in the presence of antibiotic nisin: *1* – control; *2* – 0.05%; *3* – 0.1%

At Fig. 4 is shown decreasing of heat emission by lactic acid bacteria from concentration of antibiotic nisin. It is described by exponential dependence. High sensitivity allows quickly detect the low residual quantities of inhibitors and antibiotics in milk using method of biocalorimetry (Table).

Comparative analysis of employed methods of detection antibiotic availability in raw milk (Table) shows that different microorganisms have their own spectrum of sensitiveness towards particular antibiotics which must be taken into account when choosing test-cultures for analysis.

The sensitiveness of *E. gracilis* cells towards examined antibiotics differed in tens of times but remained considerably higher than for known methods of microbiological analysis. Especially sensitive they were to benzylpenicillin and erythromycin.

The obtained results indicate that behavioral responses of microorganisms based on reception of substances and on chemotaxis are rather sensitive towards antibiotics and inhibitors.

Testing of *E. gracilis* motion is not complicated, highly sensitive; it doesn't require active storage, high qualified staff and can be carried out by simple and cheap microscopes with small magnifying.

Another convenient method of biotesting the antibiotic content in raw milk advisable for dairy enterprises is biocalorimetry. The alteration of heat production of cells in response to action of inhibitors is evident on the early stages of their influence on microorganisms.

The method of biocalorimetry allows to record low concentrations of antibiotics and inhibitors in raw milk and dairy products within 10–20 minutes with relative error not more than 10%.

Conclusion. The paper offers express-methods for detection of residual quantities of antibiotics and inhibitors in raw milk on basis of microalgae *Euglena gracilis* motion and biocalorimetry. They may be recommended for application at dairy farms and dairy processing enterprises.

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CHROMATOGRAPHIC ANALYSIS AND SEPARATION OF HYPERICIN CONTAINING EXTRACT OF ST. JOHN'S WORT

It has been presented qualitative and quantitative chromatographic analysis of hypericin containing extracts of St. John's Wort which could be used for producing drugs for treating of oncological diseases by the method of photodynamic therapy. The optimal conditions of carrying out of TLC and HPLC analysis of ethanol St. John's Wort extracts have been selected. The chromatographic separation of St. John's Wort extract has been carried out assisted by Diaion HP-20 sorbent. This separation gave the opportunity to enrich the initial extract with hypericins more than in 4 times.

Introduction. At present, the important issue in the Republic of Belarus is separation, identification and determination of biologically active compounds from plants of various agro-climatic zones of Belarus. Based on obtained substances it became possible to create medicines, dietary supplements, herbal remedies. According to the influence on the organism the drugs of vegetable origin have several advantages over synthetic analogs, as they exhibit a mildly expressed therapeutic effect.

Photodynamic therapy is a relatively new and promising method of cancer treatment based on the fact that tumor cells are destroyed by the action of reactive oxygen species, which are formed by a chemical reaction that is activated by light energy. For this reaction the photosensitive substance (photo sensitizer) and also a source of light with a wave length corresponding to the maximum absorption of the substance must be present in the target tissue [1].

As photo sensitizer can act only compounds having in their structure chromophore group of atoms that can absorb light in the visible or near-ultraviolet region of the spectrum. These compounds include hypericin. This substance is one of the main bioactive components of St. John's Wort and represents a condensed derivative of anthraquinone. In addition to hypericin in St. John's Wort has been discovered the second potential photo sensitizer – pseudohypericin.

Main part. The aim of this work is a qualitative and quantitative analysis of hypericins in St. John's Wort using chromatographic methods of analysis.

Hypericins from St. John's Wort were isolated by extraction method using Soxhlet and ethanol as a solvent. The qualitative composition of the resulting extract was determined by TLC. For selection of the optimal conditions for the TLC analysis the ethanol extract was separated in elution solvent systems: I) propanol-2 – hexane (7 : 3); II) 2-propanol – water (9 : 1); III) acetate – n-butanol – formic acid – water (5 : 3 : 1 : 1); IV) chloroform – ethyl alcohol (8 : 2); V) ethyl acetate – formic acid (50 : 6).

The substances were detected by viewing the chromatogram under UV lamp with optical filters with a wave length of 254 and 365 nm. Plates were also developed in iodine vapor. In the course of analysis of the chromatograms obtained in the five proposed eluting systems, it was found that the clearest separation of the components of ethanol extract was observed when using the eluting system V. Typical thin-layer chromatogram of the test extract obtained by using eluting system V, is presented in Fig. 1.

Hypericin (Fig. 1, position 7) in the chromatogram was identified by bright red fluorescence and the value R_f = 0.87, which coincides with the value of R_f standard hypericin sample (Carl Roth GmbH, Germany). When using this eluent system pseudohypericin (Fig. 1, position 6) was identified in the chromatogram by bright red fluorescence (in UV light with a wavelength of 365 nm) and by index R_f = 0.83, the value of which corresponds with the R_f, given in the references [2].

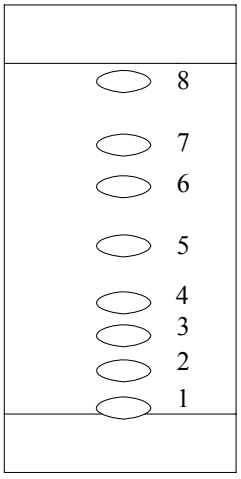

Fig. 1. Typical thin-layer chromatogram of the St. John's Wort extract in eluting system V