MODIFICATION OF MICROBIOLOGICAL DETECTION METHOD OF TETRACYCLINE IN HONEY.

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Tetracycline derivatives are the main chemotherapeutical means against American foul brood of bee brood. In most developed countries the presence of these antibiotics in food products is not possible according to sanitary code. Among other methods of tetracycline detection in food products the most simple is microbiological detection in agar during antibiotic diffusion from lunes with sample solution. According to the information taken from different sources, when using bacteria Bacillus cereus species as a test specimen the concentration of the antibiotic in honey detected by this method is not less than 40 – 90 ppb (Gordon L., 1989)

Sensitivity of the diffusion method is limited by the following factors

- value of minimum inhibitory concentration of tested antibiotic for the used test organism;
- speed balance of germ culture growth and advancement of minimum inhibitory concentration front along the radius from the lune with sample;
- volume of sample in the lune.

To increase susceptibility in the lune it is necessary to put maximum amount of sample and the balance moves to the diffusion speed part by means of growth inhibition with ageing agar with a sample in a frig. Nevertheless the method is absolutely unsuitable for determination of antibiotics concentration that is less than minimum inhibitory typical for the used test organism.

For microbiological detection of tetracycline residues we tried a number of standard method modifications where we made an attempt to take into account physical-chemical factors that influence its sensitivity.

Materials and methods.

As a test specimen Bacillus subtilis ATCC 6633 culture was used. A standard method of tetracycline detection consisted of the following stages:

- cultivation of the test specimen in Petri dishes with nutrient agar (0.1% glucose) during 7 days at 35^0C.
- receiving of spore soliquid by heating dredge of the test specimen at 75^0C during 15 minutes
- preparation of Petri dishes and lunes (8 millimeter diameter) for test solutions of antibiotic and tested samples in agar medium sowed with spores of test specimen.
- building of calibration curve – the graph of relation between inhibition area diameter and antibiotic concentration in the sample.

Citrate buffer pH=5,0-5,2 was used for preparation of tetracycline test solutions and samples growing. Honey with different self antibacterial activity was used in model experiments.

Results and argument.
Method changing turned out to be the most productive when inhibitory front concentration is spread from lune much faster against a background of subliminal antibiotic concentration in agar. For the used test specimen – Bacillus subtilis ATCC 6633 when carrying out a standard analysis, minimum determined concentration of tetracycline in honey was 300ppb (diameter of suppression growth zone 13mm). There was no zone at 150ppb antibiotic content. The amount of liminal background concentration of tetracycline, when only trace growth in agar thickness was observed for the used test organism turned to be equal to 40ppb. Against a background of tetracycline content in agar 30ppb the diameter of deficiency growth zones was 15mm at the equal concentration of antibiotic in the lune.

The usual method of the test specimen inhibition growth is holding of dishes with samples at 4^0C during 1-2 hours. We used the method of predrying in Petri dishes at 50^0C covering them with heavyweight paper. At this temperature the growth of the test specimen is inhibited and the speed of diffusion process increased not less than 16 times in accordance with the general regularity of physical-chemical process double acceleration when increasing temperature per 10^0C. During an hour the sample is noticeably absorbed into agar thickness and it make additional concentration of antibiotic.

For advancing of the default growth zone, the suspension density of the test specimen matters much when antibiotic concentration is close to minimum inhibitory. Independently of reference quantity it is increased fast from the moment of growth onset. In every circular part there is opposition process of antibiotic inflow from the lune and its fixation with target molecule of the microorganism. In case of cells excess a steadier part of the population keeps on growing and the zone moves to antibiotic increasing direction up to equilibrium. And vice versa, in case of light cells density the unbounded with the target molecule antibiotic is spread from the center enlarging the zone of inhibitory concentration. When carry out the experiments the diameter of the inhibition growth zone when testing sensitivity of the test-specimen by a standard disks method fluctuated between 30 and 38 mm depending on bacterial suspension density.

The speed of antibiotic diffusion is determined both its concentration in studied sample (factor 1) and quantity of the sample in the lune (factor 2). The first amount is sought and in this case – minimum. The necessity of honey dissolving in buffer for possibility of accurate volume dosage and lowering its antibacterial activity additionally decreases sensitivity of the method in 2-4 times. The second amount is limited to the size of the lune – its diameter. Moreover the small size of agar antibiotic source (in the limit - point source) the speed of antibiotic diffusion is down because of concentration decrease when moving away from the center is connected with its displacement volume distribution, which is proportional to radius square (factor 3). Expansion of the lune diameter at light antibiotic concentration makes the volume of the sample increase. At the same time if the size of the lune is bigger (comparatively with the inhibitory zone breadth) then the front of antibiotic spread can be considered as flat and the factor 3 does not reduce sensitivity of the method.

When comparing the effectiveness of increasing lunes' volume the evaluation of inhibitory zone diameter we changed into measuring breadth zone from the lune brim by means of eyepiece ruler of a stereoscopic loupe. To smooth inequalities connected with precise estimate, we measured breadth zones in three directions situated at angle of 120^0 to each other calculating average quantity.

Expansion of the lune size in 2 times – from 8mm up to 16mm and correspondingly 400 microliters of the sample instead of 100 microliters increases sensitivity of the method in model experiment in buffered solution of tetracycline four times more.

Table 1 Depending of the lune size on inhibitory zone breadth (mm)

Lune diameter	Tetracycline concentration	
	1,0 microliters/ milliliter	4,0 microliters/ milliliter
8mm	7,5	9,5
16mm	9,0	12

Thus, modification of the standard microbiological detection method taking into account actual physicochemical regularities makes its sensitivity considerably increase.