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Short communication

PERSISTENCE OF TETRACYCLINE AND OXYTETRACYCLINE IN BEE HONEY AFTER IMPROPER APPLICATION ON BEE FAMILIES.

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The problem of drug residues in bee honey takes a central place in the monitoring of the quality and the safety of this product. The usage of drugs in beekeeping leads to a risk of residue accumulation in honey, which may be a cause of allergies and disbacterioses in people who consume it, as well as for development of antibiotic resistance in various microorganisms (Smirnov, 1969).

In the countries of the European Union (EU) the usage of antibiotics against American foulbrood is forbidden (Anonymous, 2000; Bogdanov & Fluri, 2000). This prohibition is based on the absence of data on the maximum residue limits (MRL) of antimicrobial drugs in bee honey, required by the Council Regulation (EEC) No 2377/90 (Anonymous, 1990). Despite all that, about one-third of the honey on the EU market contains antibiotics (Bogdanov et al., 2003). In the world, most commonly applied against Streptococcus pluton and Bacillus larvae (causes of the European and the American foulbrood, respectively in bees) is oxytetracycline, followed by tetracycline, chlortetracycline, and doxycycline (Lehnert & Shimanuki, 1980). In Japan, based on microbiological research, a value of 0.1 ppm was introduced as the allowed residual quantity of tetracycline drugs in honey (Nakazawa et al., 1992).

To determine the residues in bee honey due to non-allowed usage of tetracycline antibiotics, various methods were developed, including microbiological agar-gel diffusion methods (Smirnov, 1969; Simonenkova, 1990; Nakazawa *et al.*, 1992; Petkov & Gechev, 1998; Petkov, 1999; Petkov, 2000). Because of the fact that bee honey has a multi-component structure, including substances with proven antimicrobial properties, some authors (Simonenkova, 1990; Bogdanov, 1997; Bogdanov & Fluri, 2000) consider the results of those methods as tentative.

The aim of the conducted experiment was to monitor the persistence of the active components of tetracycline and oxytetracycline in bee honey resulting from improper usage in bee families, using the microbiological agar-gel diffusion method.

Tetracycline gelatine capsules and Oxytetracycline gelatine capsules (equivalent to 250 mg tetracycline base and oxytetracycline base respectively) were used as well as tetracycline μ oxytetracycline – substances for the preparation of the standard curve.

The experiments included 9 bee families, inhabiting Dadant-Blatt hives, equalized by the method of analogues. In each bee family, 2 empty marked breeding Persstence of tetracycline and oxytetracycline in bee honey after imporper application on bee ...

combs with light colour were put, from which honey for analysis was regularly obtained (three times during honey collection period). Three groups of three bee families were formed: Group I (control) receiving sugar solution (1:1), untreated; group II – receiving sugar solution (1:1) supplemented with tetracycline in a concentration of 500 ppm; group III - receiving sugar solution (1:1) supplemented with oxytetracycline in a concentration of 500 ppm. The feeding was conducted before the period of honey collection, four times, in intervals of 7 days. The contents of 2 capsules tetracycline or 2 capsules oxytetracycline (in group II or III respectively) was preliminarily dissolved in a little bit of cool water, and was added, while continuously stirred, to sugar syrup cooled to 30-40 °C, prepared from 1 part sugar and 1 part water, with a total volume of 1 L. The quantity of the sugar solution and the antibiotic addition was determined by the size of the bee families -120 mL for each frame, completely covered with bees. On the 74th, 99th and 126th day after the last treatment, 10 samples of bee honey were taken from each group, through the method of squeezing the preliminarily unsealed parts of combs.

The antibiotic content in the honey was determined through the agar-gel diffusion method, using *Bacillus subtilis*, variant L_2 (cat. No. 1049, National Bank of Industrial Microorganisms and Cell Cultures, Sofia) as test-microorganism (Arret *et al.*, 1971; Bennett *et al.*, 1966). To establish the presence of the examined antibiotics, 1:1 standard solutions were prepared with honey from the untreated families. The samples from treated families were dissolved similarly.

All bee families were under equal conditions in terms of location and living with the same degree of nectar flow by the plants. The periods and the methods of honey collection were one and the same with both the control and the experimental groups of bee families. Those experimental conditions were chosen with regard of lowering the possibility of high fluctuations of the natural antimicrobial (background) activity of honey, in both the experimental and the control groups. The values of the natural antimicrobial activity of honey in the control group, expressed as tetracycline and oxytetracycline concentrations with the respective equivalent activity, were determined in each of the sampling periods. The close average values of the natural background activity, the low values of the standard deviations (SD), and the coefficient of variation (CV %) were taken into consideration. At equivalent, in terms of tetracycline, activity, the mean \pm SD values and the CV % were respectively 0.2356±0.0378 ppm and 16.04, while the equivalent, in terms of oxytetracycline, values were 0.9808 ± 0.1743 ppm and 17.78 (Table 1). The data, showing relatively low fluctuations in the background activity, were a prerequisite for a lower error of estimations and were used by us as grounds for using the microbiological method.

The concentrations of the samples from treated families were detected on a standard curve, and afterwards the values were recalculated by subtracting the value of background activity. The needed precision was attained by the application of regression analysis. In tetracycline, the range of the standard curve linearity was usually within 250–2400 ppb. That range was 300–4800 ppb for oxytetracycline. The limit of quantification (LOQ) of tetracycline was 300 ppb and the correlation coefficient for the standard curve was $r^2 =$ 0.9797, while for oxytetracycline the respective values were 600 ppb, at $r^2 =$

	Day 74	Day 99	Day 126	Total for the three periods		
Equivalent activity vs tetracycline						
Mean	0.2140	0.2760	0.2086	0.2356		
±SD	0.0267	0.008	0.0254	0.0378		
CV (%)	12.48	2.89	12.17	16.04		
Equivalent	activity vs oxytetra	cycline				
Mean	1.059	0.999	0.82	0.9808		
±SD	0.1531	0.1414	0.1725	0.1743		
CV (%)	14.45	14.15	21.03	17.78		

Table 1. Natural (background) antimicrobial activity of control samples presented as concentrations (in ppm) with respective equivalent activity for each period of collection

Table 2. Concentrations (ppb) of tetracycline and oxytetracycline in bee honey samples from each experimental group of bee families, treated prior to the beginnings of honey collection with tested antibiotics

	Time of honey collection after the last treatment (days)				
	74	99	126		
Tetracycline (n=10)					
Mean	3 310	330 ¹	$30^{1,2}$ (< LOQ n=6)		
±SD	450	260	10		
Oxytetracycline (n=10)					
Mean	250^{3}	210^3 (< LOQ n=7)	240 (< LOQ n=5)		
±SD	240	190	190		

* LOQ – limit of quantification of the antibiotic; ¹statistically significant (at P<0.01) vs the samples from the 74th day; ²statistically significant (at P<0.01) vs the samples from the 99th day; ³statistically significant (at P<0.01) vs the samples from the groups treated with tetracycline at each time interval.

0.9834. The time for reduction of the antimicrobial activity by 50% (elimination half life) was determined via regression analysis. The results of the experiments are presented as mean values and their standard deviations (mean \pm SD). The statistically significant differences were evaluated by ANOVA. The comparison between the data from the 74th, 99th, and 126th day was conducted through the Tukey's test.

The obtained data are presented in Table 2. The concentration of tetracycline in the samples obtained on the 74^{th} day was 3310 ± 450 ppb, on the 99^{th} day – 330 ± 260 ppb, and on the 126^{th} day – 30 ± 10 ppb. A statistically significant (P<0.01) progressive decrease of the va-

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lues in the samples from tetracyclinetreated bee families was discovered, comparing of the samples from the 74th and 99th day, as well as those from the 74th and 126th day. The mean value of elimination half-life of tetracycline was 8.29 ± 1 days. Our results did not confirm the attitude of some authors (Smirnov, 1969), that tetracycline persists for up to 45 days within a bee family.

The presence of oxytetracycline in honey, collected on the 74th day after the last treatment was 250 ± 240 ppb, on the $99^{\text{th}} \text{ day} - 210 \pm 190 \text{ ppb}$, and on the 126^{th} $day - 240 \pm 190$ ppb. There were not any statistically significant differences between the values from the 74th, 99th, and 126th day. The elimination half-life for oxytetracycline could be determined in one of the samples, and it was 38.37 days. The lower values for oxytetracycline contents in honey from the 99th day (210 \pm 190 ppb), and the following increase on the 126^{th} day (240 ± 190 ppb), could be explained by the changes in the intensity of nectar flow (bee pasture), and was probably related to a higher dilution of the honey in combs by the 99th experimental day, due to the more intensive nectar flow during the respective period (Ryahovskii, 1983; Lazarov et al., 1991).

Comparing the elimination half-lives for tetracycline and oxytetracycline, determined in our research, it should be noted that tetracycline was eliminated 4 times as fast as oxytetracycline. The dynamics of oxytetracycline concentration decrease, observed in out study, was different than the *in vitro* reduction determined by Vangelov & Parvanov (1992) at 35–37 °C after adding the antibiotic in the form of powder to polyfloral honey in a concentration of 500000 ppb, and discovered on the 30th, 120th, 210th, and 760th day, in concentrations of 365490 ppb, 2660 ppb, and 1900 ppb, respectively. This proves that there was a significant difference between the decrease in the concentrations of oxytetracycline while treating healthy bee families, receiving sugar solution (1:1) at a concentration of 500 000 ppb, and those while adding the same quantity of antibiotic to gathered pure honey, and storing the honey for a similar period of time (120 days).

Comparing the concentrations of tetracycline and oxytetracycline in bee families treated with equal doses of both antibiotics (Table 2.), it should be noted that there were statistically significant differences (at p<0.01) in the values from the 74th and 99th day. At the same level of significance, there were not any statistically significant differences discovered in the comparison of the samples with tetracycline and oxytetracycline, gathered on the 126th day.

During quantitive analysis of bee honey containing oxytetracycline, through the reverse-phase high-performance liquid chromatography (HPLC) method with UV detection (Argauer & Moats, 1991), it was determined that oxytetracycline breaks up in bee honey for about 5 - 9 weeks into various metabolic products. Those products could not be identified by the microbiological agar-gel diffusion method.

Despite that disadvantage, microbiological methods are applied because they are cheaper, relatively sensitive, easy to perform, and highly productive. That was confirmed by our experiments. Because of that, the microbiological method used in the current research could also be used for preliminary mass screening studies. Through it, increases in the total antimicrobial activity of honey can be found, after which at the establishment of antimicrobial agents presence, the positive samples would have to be studied by other methods. REFERENCES

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