Stability of tetracycline residues in honey

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Abstract: The problem of the availability of veterinary medicines to treat honeybees is discussed extensively worldwide. An uncontrolled administration of antibiotics may lead to contamination of beehive products and contribute to the problem of food safety. In this study, the kinetics of tetracycline (TC) degradation in honey was studied for samples provided by four beekeepers located in the west region of Romania. The samples of honey were stored in the dark at room temperature for 30 days and sub-samples were analyzed every 3 days by the Elisa method. The results of the study revealed that the level of tetracycline decreased with time in all the honey samples. The tetracycline degradation followed a first-order kinetic model with reaction rate constants between 1.2×10^3–2×10^3 days^{-1}. The half-life time, $\tau_{1/2}$, of tetracycline in monofloral honeys: acacia and lime was 251 and 232 days, respectively. Tetracycline degradation in the polyfloral honey was accelerated, exhibiting a $\tau_{1/2}$ of 151 days.

Keywords: honey; antibiotics; storage; degradation; kinetics.

INTRODUCTION

The general aim of the European Union (EU) 2007–2013 Health Strategy is to improve and protect human health. One of the actions under this Strategy is to support safe, innovative and cost-efficient health products and technologies.[1]

Honey is a valuable bee product used frequently as food but also as therapeutic product. From ancient times, the antibacterial effect of honey was perceived due to its ability to stimulate rapid wound healing and the inhibition of wound pathogens. In addition to antibacterial activity, honeys are known to have antioxidant capacities, which may act to modulate the production of free radicals.

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The use of antibiotics, for example tetracyclines, in apiculture for the treatment of bacterial brood diseases, such as American foulbrood (*Paenibacillus larvae*) and European foulbrood (*Melissococcus plutonius*) leads to contamination of honey with drugs.

The presence of tetracycline (TC) and its degradation products in honey may have harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth and gastro–intestinal disturbance due to the selective pressure of antibiotics on the micro flora of human gut.[2]

According to Regulation (EC) No 470/2009[3] and Regulation (EU) No 37//2010,[4] in the European Union, no maximum residue level (MRL) for tetracycline in honey has been set. This means that the presence of tetracycline residues in honey is not allowed. Despite this decision, some countries have established action limits or tolerated levels for tetracycline in honey. For instance, in Belgium, the action limit for the group of tetracycline has been fixed at 20 µg kg^{-1}. France applies a nonconformity limit for tetracycline in honey of 15 µg kg^{-1}; the reporting limit in Great Britain is 50 µg kg^{-1}, while the tolerance level in Switzerland is 20 µg kg^{-1}.[5]

The Community Reference Laboratories proposed 20 µg kg^{-1} as the recommended concentration for screening for tetracyclines in honey.[6] Romania has adopted this action level as an acceptable limit for tetracycline residue detection in honey.

Little information about the stability of tetracycline residues in honey during storage is available in the literature. The first study was published by Landerkin *et al.* in 1957.[7] They reported that after nine months storage at 34 °C, the concentration of tetracycline residues in honey had decreased from the initial concentration of 100 to 2 mg kg^{-1}.

In the study performed by Martel *et al.*,[8] tetracycline was very stable in honey; the half-lives of tetracycline hydrochloride in honey stored at 4, 20 and 35 °C in the dark were 520, 242 and 121 days, respectively. Čuláková *et al.*[9] stored honey fortified with 209.4 mg kg^{-1} of tetracycline in the dark, semi-dark and daylight at a temperature of 25 °C. The half-life of tetracycline in the dark was 210 days and because it is light-sensitive, the half-life decreased to 147 days under semi-dark conditions and to 19.3 days in daylight.

Recently, Tayar *et al.*[10] reported that the residual level of tetracycline in honey was reduced by 76 % after 60-day storage and according to Molino *et al.*[11] after 90 days of storage at 20 °C in the dark, the tetracycline level had decreased by 50 %.

The aim of this study was to determine the contamination of honey samples by tetracycline and to characterize from a kinetic viewpoint the degradation of tetracycline in different types of honey during storage.

EXPERIMENTAL

Honey samples

Three types of honey (two monofloral, acacia and lime, and one polyfloral) were collected in 2009 from four beekeepers located in the west region of Romania.

In all cases, three replicates were analyzed for the presence of tetracycline residues (TC).

Antibacterial residues analyses

A commercially enzyme immunoassay (ELISA) (Ridascreen® tetracycline, R-Biopharm GmbH, Darmstadt, Germany) was used to analyze for tetracycline (TC) in honey. The detection system for the ELISA was a Sunrise Absorbance Microplate Reader from Tecan AG. In order to demonstrate the reliability of the method, the following parameters were investigated according to the European Commission Decision 2002/657/EC: repeatability, reproducibility, recovery, specificity and precision. The values of these parameters are presented in Table I. From the specification of the Ridascreen® kit, chlortetracycline (CTC) could give cross-reactions with tetracycline. Therefore a specificity test was performed for these two compounds and it was proved that the method has the ability to distinguish between TC and CTC.

TABLE I. Parameters showing the reliability of the employed analytic method

Parameter		TC added to the sample, $\mu g \cdot kg^{-1}$			CTC added to the sample, $\mu g \cdot kg^{-1}$
		20	30	40	30
Repeatability[a]	Mean ±SD, $\mu g \cdot kg^{-1}$	20.3±0.67	30.5±0.54	39.8±0.28	–
Recovery[a]	%	101.5	101.6	99.5	–
Reproducibility[a]	Analyst 1 Mean ±SD, $\mu g \cdot kg^{-1}$	20.3±0.67	30.5±0.54	39.8±0.28	–
	Analyst 2 Mean ±SD, $\mu g \cdot kg^{-1}$	19.66±0.33	28.44±0.91	39.85±0.20	–
Precision[b]	Mean ±SD, $\mu g \cdot kg^{-1}$	20.23±0.5	–	–	–
	CV, %	2.47	–	–	–
Specificity[a]	Mean ±SD, $\mu g \cdot kg^{-1}$	–	29.65±0.18	–	ND[c]

[a]Number of replicated samples, $n = 6$; [b]number of replicated samples, $n = 15$; [c]not detectable

Reagent preparation

All the reagents and standards are prepared according to the instructions given by the manufacturer of the test kit.

Sample preparation

A honey sample (1 g) was dissolved with 49 ml 10 mM phosphate buffered saline (PBS buffer, pH 7.4). In order to achieve a better dissolution, the sample solution was vortexed and then kept for 5 min in an ultrasonic bath.

Testing protocol

According to the procedure described for the kit, 50 µl of each standard solution and prepared sample were added in duplicate into different sample wells. Into each well, 50 µl of anti-tetracycline antibody solution was added, mixed gently by shaking the plate manually and incubated for 1 h at room temperature (20–25 °C). Subsequently, the liquid was poured out of the wells and the wells were washed 3 times with 250 µl washing buffer. 100 µl of enzyme

conjugate was added to each well and the plate incubated at room temperature (20–25 °C). After 15 min incubation, the liquid was poured and the washing procedure was repeated. In the next step, 50 µl of substrate and 50 µl of chromogen were added to each well and after mixing, the plate was incubated for 15 min at room temperature (20–25 °C) in the dark. In the last step, 100 µl of the stop solution was added to each well. The absorbance of the sample was read on a plate reader at 450 nm. The limit of detection was 15 µg kg^{-1}.

pH Measurement

The pH of the honey was determined with an InoLab level 1 type pH-meter and a pH electrode for viscous samples.

Storage study

Honey samples were poured into a glass bottle with a metal screw and stored at room temperature (21±1) °C in the dark, for 30 days. Sub-samples from each type of honey were analyzed three times every 3 days by the Elisa method.

RESULTS AND DISCUSSION

The results of the determination of tetracycline residues in the honey samples collected from four beekeepers located in the west region of Romania are summarized in Table II as the mean value of three replicates per sample and the standard deviation (*SD*).

TABLE II. Tetracycline concentrations (mean ± *SD*, µg·kg^{-1}) in the tested honey and the Romania action limit for tetracycline in honey; MRL: 20 ppb

Honey type	1st Apiary	2nd Apiary	3rd Apiary	4th Apiary
Acacia	38.32±0.18	19.14±0.18	27.03±0.16	16.18±0.21
Lime	15.47±0.45	21.27±0.11	18.04±0.26	60.67±0.47
Polyfloral	16.63±0.46	30.72±0.42	15.47±0.45	22.89±0.43

Tetracycline residues were found in 50 % of the examined honey samples. The highest detected tetracycline contamination level was 60.67 µg kg^{-1} for a lime honey sample. For the other types of honey, the maximum tetracycline contents found were 38.32 µg kg^{-1} in acacia honey and 30.72 µg kg^{-1} in polyfloral honey. The corresponding mean pH values of the initial samples were 4.2 for the acacia honey, 4.1 for the lime honey and 3.8 for the polyfloral honey.

In order to study the rate of the degradation of the tetracycline residues, samples with the highest tetracycline concentration from each honey type were placed into Erlenmeyer flasks and stored at laboratory temperature (21±1) °C in the dark. The experiments were performed from the beginning of August 2009 until September 2009.

The results of the effect of storage time on the tetracycline level (expressed as percent of the initial tetracycline concentration in the sample) are summarized in Fig. 1, from which it can be seen that the tetracycline concentration continuously decreased in all the honey samples.
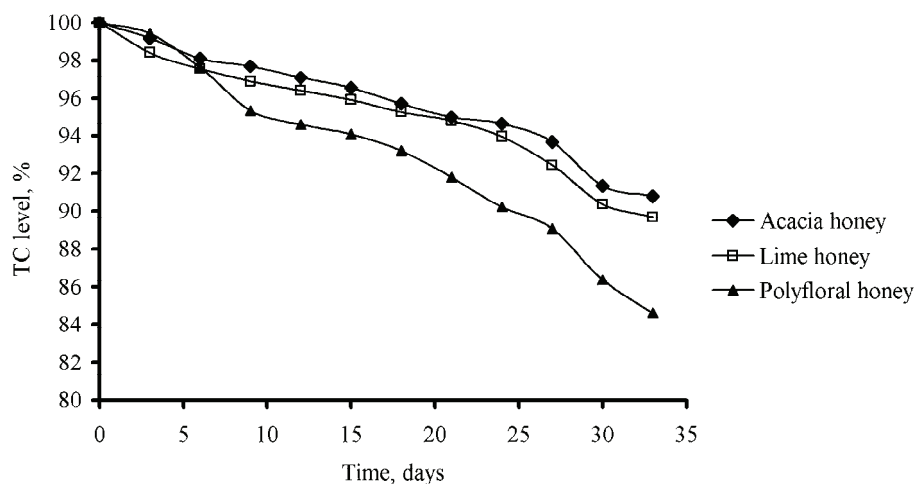
Fig. 1. Decrease in the tetracycline level in different types of honey during storage at room temperature in the dark.

After 3 days of storage, the tetracycline level was reduced by 2.5 % in all three honey samples. This decrease continued but was more pronounced in the polyfloral honey. At day 30, the tetracycline level in the monofloral honeys (acacia and lime) had decreased by 10 % while a 15 % decrease was registered in the polyfloral honey.

Molino *et al.*[11] reported a 21 % decrease of the tetracycline level in honey after 30 days of storage at 20 °C, but the type of honey was not specified. Similar results were obtained by Čuláková *et al.*[9] and Martel *et al.*[8]

In order to describe the degradation of tetracycline in the different types of honey, a first order kinetic model (1) was proposed:

$$c = c_0 e^{-kt} \qquad (1)$$

where c is the concentration of tetracycline in the honey at time t, c_0 is the initial concentration of tetracycline in the honey and k is the first-order kinetic rate constant.

For the determination of the values of the rate constant k, the results of the decrease in the tetracycline concentration in the different types of honey during storage at room temperature and darkness were used. From these data, the values of k were evaluated by plotting $-\ln (c/c_0)$ *vs.* time t (Fig. 2). The plots of $-\ln (c/c_0)$ *vs.* time t gave straight lines, the slopes of which were equal to the respective first order rate constant k.

The half-life of the TC degradation process is independent of the starting concentration and is given by:

$$t_{1/2} = \ln 2 / k \qquad (2)$$

The first-order reaction rate constants (k) and the half-lives ($t_{1/2}$) of the model are given in Table III.
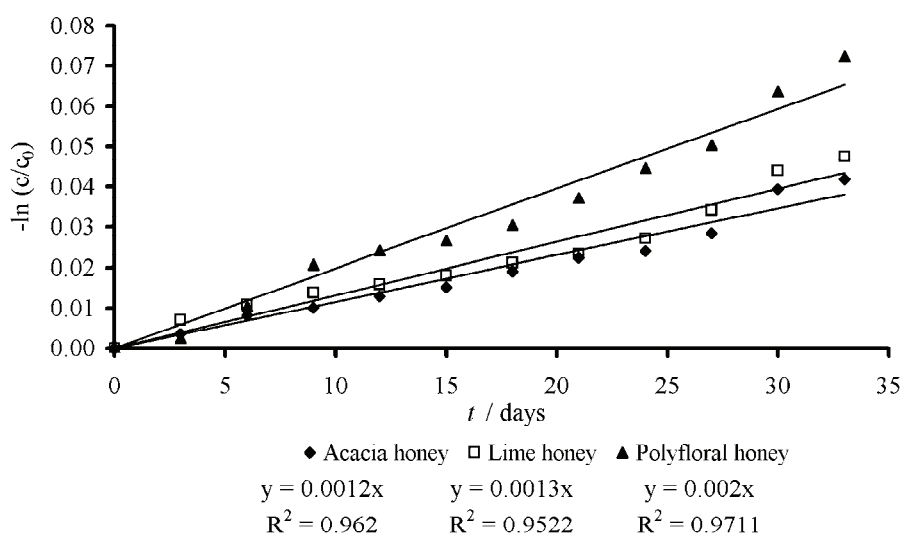

Fig. 2. Determination of the values of the rate constant for tetracycline degradation in different types of honey with time.

TABLE III. Effect of honey type on the k and $t_{1/2}$ values of tetracycline degradation in honey stored at room temperature in the dark

Honey type	$k \times 10^3$ / days^{-1}	$t_{1/2}$ / days	R^2
Acacia	1.2	251	0.9620
Lime	1.3	232	0.9522
Polyfloral	2.0	151	0.9711

In all cases, the R^2 values were higher than 0.95, indicating a good fit of the data to the first-order kinetic model. The k values were almost the same for the two types of monofloral honey, acacia and lime honeys. When comparing the rate constants of tetracycline degradation in the monofloral honeys with that in the polyfloral honey, it can be seen that the degradation in the polyfloral sample was about 1.6 times faster that in the monofloral samples. This can be due to the different qualitative and quantitative composition of the monofloral and poly-floral honey, as well as to the pH of the samples. Tetracycline degradation in the polyfloral honey may also be increased by the presence of enzymes.

The values of half-life time of tetracycline show that it is very stable in honey. In the study of Čuláková *et al.*,[9] the half-life of tetracycline in honey stored at 25 °C in the dark was 210 days. Martel *et al.*[8] reported the half-lives at 20 and 35 °C as 242 and 121 days, respectively. These values are similar to the values obtained in the present study of tetracycline degradation.

On analyzing the initial pH values of the samples and the half-life values of tetracycline in the different types of honeys, it was noticed that the increase in the pH of the samples was accompanied by an increase of the TC half-life. The linear correlation between these two parameters is presented in Fig. 3.
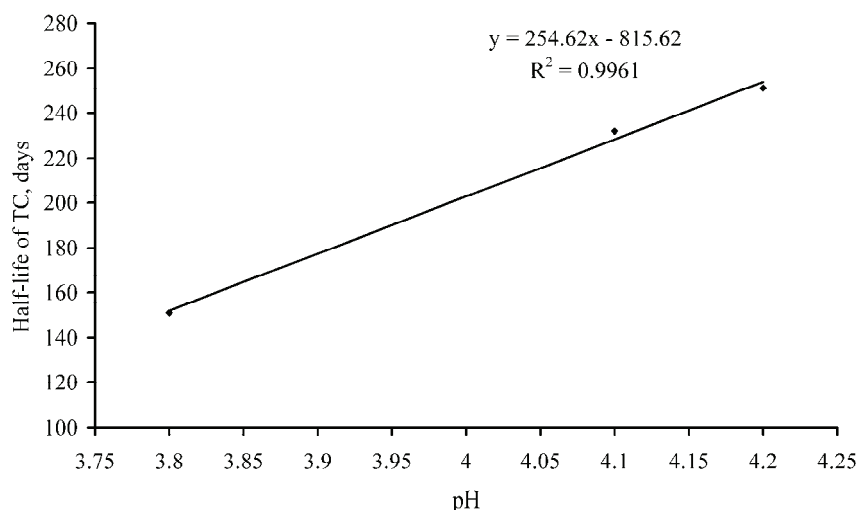

Fig. 3. Variation of the half-life of TC with pH.

The very good linear correlation between the two parameters was confirmed by the correlation coefficient R^2 of 0.9961. The values of pH were also measured at the end of the storage study and the results revealed that the pH of samples were constant during the 30 days of the experiment. These findings are in accordance with the studies of Jimenez *et al.*[12] and Cavia *et al.*[13]

CONCLUSIONS

The results presented in this paper showed that the concentration of tetracycline decreased with time in all the honey samples and that it was more rapidly degraded in the polyfloral honey. Based on the experimental results, it was found that first-order reaction kinetics adequately described the degradation of tetracycline residues in all the types of analyzed honey. The different values of the half-life of tetracycline in the three types of honeys were shown to be due to the pH of the samples.

Although studies of tetracycline degradation in honey are quite rare in the literature, the results presented in this work corresponded with those reported by other authors.

All these emphasize the need to improve the education of beekeepers in order to avoid any use and misuse of antibacterial drugs and to prevent honey contamination.

И З В О Д

СТАБИЛНОСТ ОСТАТАКА ТЕТРАЦИКЛИНА У МЕДУ

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Познато је да постоји проблем у коришћењу ветеринарских медикамената у лечењу медоносних пчела. Неконтролисана примена антибиотика може довести до контаминације пчелињих производа и угрожавања безбедности хране. У овом раду је проучавана кинетика деградације тетрациклина у узорцима меда из западне Румуније. Узорци меда су чувани у мраку, на собној температури, 30 дана и анализирани ELISA методом свака 3 дана. Резултати су показали да концентрација тетрациклина опада с временом. Деградација тетрациклина одговара моделу реакције првог реда, са константама брзине реакције $1,2{\times}10^{-3}$–$2,0{\times}10^{-3}$ дан$^{-1}$. Полуживот тетрациклина у багремовом меду је 251 дан, а у меду од липе 232 дана. Деградација тетрациклина у полифлоралном меду је бржа и полуживот износи 151 дан.

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