Kinetics of degradation of oxytetracycline and tetracycline in honey and in a glucose-fructose model mixture in various storage conditions

VIERA ČULÁKOVÁ – EUGEN KISS – JANKA KUBINCOVÁ – STANISLAV ŠILHÁR

Summary

The kinetics of oxytetracycline (OTC) and tetracycline (TC) degradation were studied in honey and in a glucose-fructose (GF) model mixture spiked with OTC at a level of 209.7 mg.kg^{-1} dry mass and TC at a level of 209.4 mg.kg^{-1} dry mass. The samples of spiked honey were stored in the dark, in the semidark and in the daylight at (25 ± 3) °C. The samples of the spiked GF model mixture were stored only in the daylight. OTC degradation was faster than TC degradation in all conditions. OTC degradation half-time ($\tau_{1/2}$) in the dark in honey was 77.0 days and $\tau_{1/2}$ of TC was 210.0 days. Daylight accelerated the progress of degradation dramatically since $\tau_{1/2}$ of OTC was 3.6 days and $\tau_{1/2}$ of TC was 19.3 days while, at semidark conditions, $\tau_{1/2}$ of OTC was 25.3 days and $\tau_{1/2}$ of TC was 147 days. OTC and TC degraded in honey faster than in the GF model mixture in the same storage conditions.

Keywords

oxytetracycline; tetracycline; honey; degradation; HPLC; kinetics

Hives infected by European Foulbrood (EFB) caused by *Meliscoccus pluton* or American Foulbrood (AFB) caused by *Paenibacillus larvae* are treated with streptomycin, tylosin, sulphonamides and tetracyclines. The presence of tetracyclines in food and in water is undesirable particularly because of their allergenic potential for hypersensitive individuals and because of problems with the development of resistance in microorganisms.

Practical advices with a protocol for application of antibiotics to cure the infected bee colonies were published by MUTINELLI [1]. MARTEL et al. [2] carried out an experiment with the treatment of hives with tetracycline (TC) where two hives were placed close to each other and one of them was treated with TC and the second one was not. The non-treated hive became contaminated with TC residues, too. After 146 days, the concentration of TC in honey was 0.15 mg.kg^{-1} for the untreated hive. This means that the contamination with tetracycline antibiotics concerns both the treated and untreated hives.

In 2004, honey samples gathered from small producers of Slovakia were analysed for the contents of antibiotic residues by The State Veterinary and Food Institute (Dolný Kubín, Slovakia). A number of 95 out of the total of 235 analysed samples were positive for TC residues [3]. Similar analyses were made in Belgium in 2003 where 29 out of 98 analysed samples of imported honey were TC positive [4].

OSTERMANN et al. [5] published a recommendation for administering antibiotics to honey bee colonies. To protect consumers from the consumption of honey contaminated with antibiotic residues and other food contaminants, measures to monitor the antibiotics in animal products were adopted in the European Union there, namely, the Council Regulation (EEC) No 2377/90 [6], Council Directive 96/23/EC, Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC [7]. A new Commission Decision 2005/34/EC [8] was released to preserve consumers from uncontrolled penetration of contaminated food to the EU market. The above legislation facilitates the marketing authorization of animal food products by stating maximum residual limits (MRL). MRL for oxytetracycline (OTC) and tetracycline (TC) for honey is zero [9].

Analytical methods for qualitative and quantitative analysis of antibiotic residues in honey were studied by several authors. The comprehensive

Viera Čuláková, Eugen Kiss, Janka Kubincová, Stanislav Šilhár, VÚP Food Research Institute, Biocentre, Kostolná 7, SK - 900 01 Modra, Slovakia.

Correspondence author:
Viera Čuláková, e-mail: culakova@vup.sk

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residue analysis system CHARM II (scintilation-based detection system for chemical families of drug residues utilizing class specific receptors or an antibody in immunoassay formats) for honey was examined by SALTER [10]. Analytical methods for quantification of antibiotic residues in honey were studied by GALLINA et al. [11]. Advantages and disadvantages of analytical methods for determination of tetracyclines and their quantification in honey were evaluated by MŮNSTED et al. [12]. The current state of analytical methods for detection and quantification of residues of antibiotics in bee products was evaluated by BOGDANOV [13]. In the latter study, screening methods suitable for the selection of positive samples are identified, such as ELISA (Ezyme-linked Immuno Sorbent Assay) or CHARM II, and the quantitative methods, such as HPLC with UV or fluorescence detection, or LC/MS-MS. LC/MS-MS is described as the most specific method with a very low background noise level and with the detection limits (LOD) of 0.5–10 $\mu g.kg^{-1}$. Another method for preliminary screening of tetracyclines in honey, egg, fish and animal tissues was developed by ALFREDSSON et al. [14], who used a dipstick format and confirmation of the results by LC-MS/MS. Authors stated that this combination proved to be suitable for the rapid surveillance of tetracyclines in honey and eggs.

According to our knowledge, only one paper has been published on the stability of antibiotics in honey and in sucrose syrup, namely, a study published by LANDERKIN et al. in 1957 [15]. In this study, beginning with the initial concentration of 100 $mg.kg^{-1}$ of OTC, the authors did not detect any residual concentration of OTC in honey and sucrose syrup stored at 34 °C for nine months. After the same period of time and under the same conditions, the concentration of TC decreased from the initial concentration of 100 $mg.kg^{-1}$ to 2 $mg.kg^{-1}$. The samples were analysed using the microbiological technique (diffusion from a paper disc and a standardized suspension of *Bacillus subtilis* ATCC 6633 spores).

Several papers were devoted to the stability of tetracyclines in other matrices. The stability of TC in a methanolic solution was investigated by UV-spectroscopy, HPLC and TLC methods by LIANG et al. [16]. After dissolution, TC decomposed rapidly under the influence of light and atmospheric oxygen, forming more than fourteen degradation products. A fast and simple flow injection chemiluminometric method for the determination of trace amounts of TC and its major degradation produts epitetracycline, epianhydrotetracycline and anhydrotetracycline was studied by PENA et al. [17]. The determination of TC residues by HPLC with MS detection and OTC degradation products with MS-MS detection were studied by DELÉPÉE et al. [18]. The role of zwitterionic structures at SPE extraction of TC and OTC was investigated by HUQ et al. [19]. The authors found the SPE columns with a weak cation-exchange sorbent the most effective at OTC and TC extraction from honey. Other antibiotics (kanamycine, streptomycine, ampiciline) in honey and in nectar were studied by SOLOMON et al. [20]. POULIQUEN et al. [21] studied the influence of hydrolysis and photolysis on four antibacterial agents, including OTC, in deionized water and sea water. Degradation of tetracyclines in various food matrices was the object of several studies. FEDENIUK et al. [22] observed simultaneous influence of processing temperature and additives (sodium nitrate, sodium phosphate) on the OTC degradation in the pork muscle. These authors concluded that the processing temperature and the effects of additives were important factors influencing the rate of OTC degradation in meat. Degradation of OTC was slower in the pork tissue than in water. In the presence of calcium chloride, degradation was slower both in water and in the pork tissue. The influence of pasteurization on the degradation of some TC antibiotics in milk was studied by LOKSUWAN [23] using a HPLC/UV method. The authors found that a 30 min pasteurization significantly reduced OTC in milk, while the decrease of TC was less significant.

The above literature review illustrates that while several papers have been published on tetracyclines, their analysis and stability in various matrices, as well as their degradation products, insufficient attention has been devoted to degradation of TC and OTC in honey, although this problem is very topical due to the large amounts of honey contaminated by tetracycline antibiotics worldwide. Hence, the aim of this paper was to fill this gap of knowledge and to study the kinetics of elimination of tetracyclines from honey in various storage conditions.

MATERIALS AND METHODS

Samples

Flower honey was obtained from a small local producer (Lakšárska Nová Ves, Slovakia). The honey was harvested in spring 2006.

Chemicals and standards

The following were used: acetonitrile, HPLC gradient grade (Sigma-Aldrich, Steinheim, Ger-

many), methanol HPLC gradient grade (Sigma-Aldrich), oxalic acid p.a. (Lachema, Brno, Czech Republic), oxytetracycline dihydrate ≥ 98% (Fluka Chemie, Buchs, Switzerland), tetracycline ≥ 98% (Fluka), glucose ≥ 99% (Global factory, Pezinok, Slovakia), D(-)-fructose ≥ 98% (Fluka Chemie). McIlvaine buffer pH 3 was prepared in our laboratory. Methanolic stock standard solutions were stored in a refrigerator at 4 °C.

Sample preparation procedure

Honey was spiked with OTC by a thorough homogenization with an OTC aqueous solution to reach the initial concentration of 209.7 mg.kg^{-1}. Separately, honey was spiked with TC in the same way to reach the initial concentration of 209.4 mg.kg^{-1}. GF model mixture was prepared by mixing 64.0 g of glucose and 80.0 g of fructose. The mixture was either spiked with OTC or TC aqueous solution to reach the initial concentration of 102.2 mg.kg^{-1} for OTC or 225.0 mg.kg^{-1} for TC. Afterwards, spiked honey samples were placed into Erlenmeyer flasks and stored at laboratory temperature (25 ± 3) °C in the dark, semidark or in the daylight. The GF model mixture was stored only in the daylight. Experiments took place from the end of August till the end of October.

Solid phase extraction

To an aliquot of 1.0 g of homogenized honey sample, 20 ml of McIlvain buffer solution (pH 3.0) were added and the sample was mixed with a vortex mixer for 2 min. The homogenized sample was filtered through a nylon filter (pore size, 45 μm) and then applied to a Oasis HLB SPE cartridge (Waters, Milford, Massachusetts, USA), previously activated with 3 ml of methanol and 2 ml of water. After sample loading, the cartridge was washed with 2 ml of 5% methanol in water, tetracyclines were eluted with 3 ml of methanol to a 5 ml volumetric flask and the flask was refilled with methanol. An aliquot of 20 μl was injected to the HPLC system.

HPLC

Analyses were carried out by HPLC with a diode array detector using LC 20 AD Prominence (Shimadzu, Kyoto, Japan), detection being carried out at λ = 354 nm. Separations were carried out using the column Purospher STAR RP-8E (particle size, 5 μm) 250 mm × 4.6 mm, coupled with a guard column Purospher STAR RP-8E (particle size, 5 μm) 4 mm × 4.6 mm; (Merck, Darmstadt, Germany) at a flow of 1.0 ml.min^{-1}. The following gradient conditions were used: Mobile phase A – 20 mM oxalic acid in deionized water, mobile phase B – acetonitrile, gradient: 20% B for 0 min, 20–40% B for 0–15 min, 20% B for 15–17 min, 20% B for 17–23 min. Analyses were performed in triplicate.

Recovery of the method was 87.3% for OTC and 84.1% for TC.

Dry mass was determined refractometrically by Abbe refractometer.

RESULTS AND DISCUSSION

Results are summarized in Fig. 1 and Fig. 2. As can be seen, the concentrations of both OTC and TC decreased with time, the decrease being more steep for OTC. The daylight had an accelerating effect on the degradation, the phenomenon being more remarkable for OTC. The dependences of concentration vs. time obeyed the first-order ki-

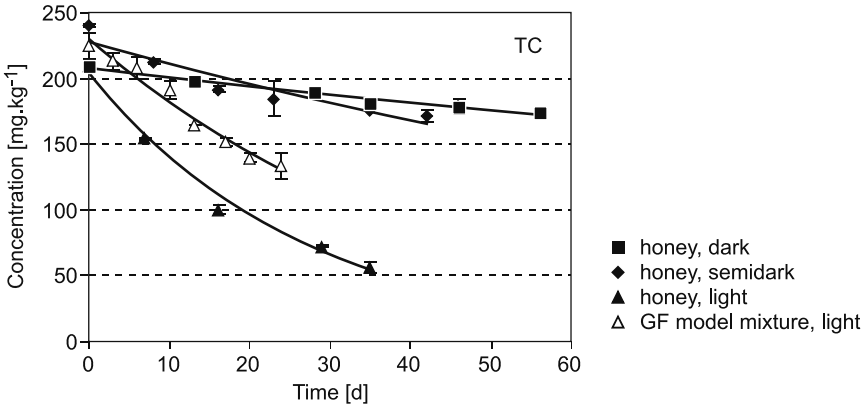

Fig. 1. Degradation of TC in honey and in a GF model mixture in various storage conditions. Concentration is expressed in mg.kg^{-1} of dry matter.

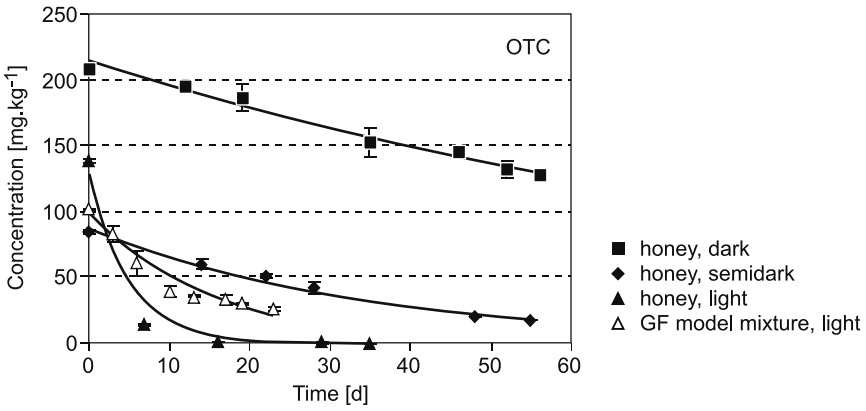

Fig. 2. Degradation of OTC in honey and in a GF model mixture in various storage conditions. Concentration is expressed in mg.kg^{-1} of dry matter.

netics. The results were evaluated using Eq. (1):

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

where c_0 is the initial concentration, c is the concentration at time t and k stands for the rate constant. The rate constants were obtained by minimizing the sum of squares between the experimental points and the calculated dependence given by Eq. (1). The half-time of degradation, $\tau_{1/2}$, was calculated to facilitate a better comparison of the results; Eq. (2) was applied:

$$\tau_{1/2} = \ln 2/k \quad (2)$$

The values of rate constants and half-times are summarized in Tab. 1.

The OTC and TC degradation was the slowest in honey under common storage conditions in the dark. The OTC degradation rate constant was three times higher than that of TC. The rate constant of degradation in honey in semidarkness was six times higher for OTC than for TC. The rate constant of OTC degradation in the daylight in honey compared to the dark was 21-times higher in the case of OTC and 11-times higher in the case of TC.

Tab. 1. Rate constants (k) and half-times ($\tau_{1/2}$) for the degradation of OTC and TC in flower honey and in a GF model mixture.

Storage conditions	Oxytetracycline		Tetracycline	
	$k \times 10^3$ [d^{-1}]	$\tau_{1/2}$ [d]	$k \times 10^3$ [d^{-1}]	$\tau_{1/2}$ [d]
Honey, dark	9.0	77.0	3.3	210
Honey, semidarkness	27.4	25.3	4.7	147
Honey, daylight	192	3.6	36.0	19.3
GF model mix., daylight	59.7	11.6	24.8	27.9

When comparing the rate constants of degradation in honey and in a GF model mixture in the daylight, it can be seen that degradation in honey was about three times faster than in a GF model mixture for OTC and 1.5 times faster for TC. These results are quite surprising since honey is more viscous than the GF model mixture. It is obvious that the matrix viscosity is not the most important factor in TC degradation. A possible explanation of the higher degradation rate in honey comparing to the GF mixture has been formulated by Pouliquen's et al. [21], who studied of the degradation rate of OTC under the influence of light (1700 lux). They reported that the half-time of OTC was 16.0 days in deionized water, 5.0 days in fresh water and 4.0 days in sea water and explained the results by a possible acceleration of OTC and TC degradation by mineral salts. Similar effects might take place also in our study in case of honey. Degradation of TC in honey may be also increased by the present enzymes.

Regarding the OTC degradation, Samuelsen et al. [24] reported the half-times at 5 °C and 15 °C as 7.0 and 5.3 days in sea water after a 24 h illumination of the samples by 40 W fluorescence tube, and 16.3 and 9.8 days in the darkness. Doi et al. [25] determined the half-time for OTC in water in the light as 3.9 days and 14 days in the dark. These values are similar to the value of 3.6 days obtained in our study of OTC degradation in the daylight, and also to the other published values [21, 24, 25]. In the dark, the half-time of OTC degradation was 21-times higher than in the light. These results demonstrate the light sensitivity of OTC.

As can be seen from Tab. 1, the rate constants of TC degradation increased in the following order: dark < semidark < daylight. Similar to OTC, also TC seems to be photosensitive. This result

cannot be compared with the results of other authors since TC degradation studies are very scarce in literature.

CONCLUSIONS

The results indicate that OTC degradation is faster than TC degradation in all conditions. OTC degradation is about three times faster in honey than in a GF model mixture in the daylight conditions. In the dark, degradation of TC in honey was three times slower than OTC degradation. In honey stored in semidarkness, degradation of OTC was almost 6 times faster than that of OTC. In honey, daylight accelerated the degradation of OTC by 21 times and TC by approximately 11 times in comparison with the darkness conditions.

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