Effect of pasteurization on the residues of tetracyclines in milk

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Abstract

The main aim of this work was to determine the effect of high pasteurization of milk (85 °C/3 s) on the residues of tetracycline and oxytetracycline. The samples of raw cow's milk, purchased from a vending machine, were spiked with standard solutions of tetracycline and oxytetracycline. The content of the residues of tetracycline antibiotics was measured before and after heating. Pre-cleaned samples were extracted by a mixed-mode solid phase extraction technique and analysed using high performance liquid chromatography/diode array detection. Whereas the residues of tetracycline decreased only by 5.74% and were not significantly different ($P > 0.05$), the residues of oxytetracycline decreased by 15.3% and this distinction was highly significant ($P \leq 0.01$). Based on the results of our study, the tetracycline antibiotics were proved to have differences in the thermostability of particular substances at pasteurisation temperatures.

Antibiotics, heat treatment, thermostability

Tetracyclines are a group of highly important broad-spectrum antibiotics used in veterinary medicine to treat food-producing animals (Botsoglou and Fletouris 2001; Wang et al. 2012). They are used to treat gastrointestinal, respiratory, genitourinary and skin bacterial infections as well as infectious diseases of the musculoskeletal system and systemic infections, and also in the treatment of cholera and sepsis (Samanidou et al. 2007). However, they have a range of side effects, including disturbances in healthy intestinal microflora, allergic reactions, liver and kidney malfunctions, hypersensitiveness and intense-light related dermatitis. Moreover, nowadays it is necessary to take into account the relatively high probability of acquired tetracycline resistance (Michalova et al. 2004).

In order to protect consumers' health, EU legislation lays down the maximum residue limit (MRL) in food of animal origin for veterinary medical products approved for use in food-producing animals. Legislation establishes the MRL for three tetracycline antibiotics most commonly used in lactating dairy cows. The MRL for tetracycline (TTC), oxytetracycline (OTC) and chlortetracycline (CTC) in cow's milk is 100 $\mu g \cdot kg^{-1}$ (Commission Regulation 37/2010). When heated or exposed to acidic or highly alkaline environments, tetracyclines are subject to chemical transformation processes, such as isomerization and epimerization (Wang et al. 2012). For this reason, when establishing MRLs it is necessary to take into account both the basic compound (tetracycline) and its epimers (the 4-epimer products of TTC, OTC and CTC) (Commission Regulation 37/2010; Spisso et al. 2010).

Information concerning thermal stability of drug residues in food is toxicologically important. Most food of animal origin is not eaten raw, but requires heat treatment: boiling or poaching, frying, roasting or stewing. These culinary processes may lead to protein denaturation, increase in temperature, water and fat loss and changes in pH, which in turn may result in changes in the residues' concentration, chemical structure and chemical reactions as well as to their loss of solubility. Many drugs are chemically unstable to

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some extent and thus prone to degradation during storage and thermal or technological processing (Botsoglou and Fletouris 2001).

One of the basic methods of thermal processing of raw milk is pasteurization. Compared to other food of animal origin, milk is subjected to only a very short heat treatment. The results of many studies show that pasteurization does not lead to full inactivation and degradation of drug residues (Botsoglou and Fletouris 2001).

Even members of the same class of antibiotics with the same structure may exhibit vast differences in thermal stability, depending on different matrix types and conditions of the treatment. When heated, tetracyclines split and degrade into metabolites, which indicate lack of thermal stability among this group of drugs. The degree of instability may vary with temperature. Many other questions are still to be answered. Thermal processing may not only reduce the concentration of drug residues in food, but it can also change their pharmacological and toxic effects (Hsieh et al. 2011). Heat treatment may give rise to new chemical compounds with higher levels of toxicity than that of the parent compound (Botsoglou and Fletouris 2001).

The presence of veterinary drug residues in milk is important from the hygienic point of view as an important quality marker, and also from the technological point of view. Presence of drug residues poses a risk especially for dairy product manufacture, for it may interfere with dairy cultures.

The main objective of this study was to determine the effect of the most basic heat treatment used in dairy industry – pasteurization – on the residues of tetracycline (TTC) and oxytetracycline (OTC) in milk.

Materials and Methods

Milk samples

Raw cow's milk was obtained from a vending machine during July and August 2013. All milk samples came from the Agros Vyškov-Dědice, a.s. farm.

Sample preparation

A preliminary heating of the sample to 40 °C was carried out prior to pasteurization. Then the sample was cooled to ca 20 °C and subjected to a centrifugation process at 2,490 × *g* for 10 min in order to remove fatty constituents. Subsequently, an antibiotic solution was added to create milk samples with concentrations close to 1.5 × MRL (c = 150 μg·l^{-1}). The concentration of tetracyclines in the samples was measured before and after heat treatment (high pasteurization at 85 °C for 3 s).

Pre-analytical processing of the milk samples consisted in precipitation of protein components, which was done by mixing the samples with McIlvaine buffer with ethylenediaminetetraacetic acid (EDTA) and centrifugation at 2490 × *g* for 5 min. The supernatant obtained was adjusted to pH 10 by sodium hydroxide (NaOH) solution (c = 1 mol·l^{-1}). Sample purification was done using a solid phase extraction (SPE) vacuum system and Mixed-Mode Oasis MAX columns (3 cc, 60 mg, Waters, USA). Subsequently, the sample was eluted by a 45:55 mixture of acetonitrile and oxalic acid (c = 7.5 × 10^{-2} mol·l^{-1}). Before analysis the solution was diluted with distilled water to the volume of 1.5 ml.

Chromatographic conditions

Measurements were carried out using a high performance liquid chromatograph (HPLC) Alliance 2695 with photo diode array detector (PDA) 2996 (Waters, USA). Separation was performed on a Nova-Pak C8 column, 4 μm, 3.9 × 150 mm, UV detection at 355 nm. Non-linear gradient elution was used. Mobile phase A consisted of oxalic acid (c = 1.2 × 10^{-2} mol·l^{-1}) and mobile phase B consisted of a mixture of acetonitrile and methanol (1:1) with a flow rate of 0.8 ml/min. Column temperature was 35 °C, sample injection 30 μl.

Validation indicators of HPLC method

Method calibration was carried out by means of matrix samples analysis. Standards of OTC, TTC, and CTC were added to the samples of raw cow's milk to create concentrations ranging from 0.02 to 1.0 mg·l^{-1}, and they were analysed using a standard procedure. The linearity of determination was established from the values of the calibration curve and was verified on the basis of the values of the correlation coefficient R (CTC R = 0.994, TTC R = 0.997, OTC R = 0.988). Recovery and repeatability of the OTC, TTC, and CTC determination was established by means of parallel analysis of the milk samples with the addition of standards of known concentration at levels of 0.5 and 0.1 mg·l^{-1} for each analyte (Table 1).

Table 1. High performance liquid chromatography method parameters.

Parameter	Oxytetracycline	Tetracycline	Chlortetracycline
Recovery (%)	93.30	91.50	88.10
RSD (%)	5.10	11.70	9.90
n	12	12	12
Repeatability			
RSD (%)	9.26	12.18	11.70
n	15	15	15

RSD = relative standard deviation, n = number of measurements

Statistical evaluation

The obtained results were processed and evaluated using a paired *t*-test in STAT Plus (Unistat software ver. 5.1; Unistat Ltd. 1998). This program was also used to establish the basic statistical parameters. Differences were considered significant at $P \leq 0.05$, highly significant at $P \leq 0.01$.

Results

The effect of heat treatment (high pasteurization at 85 °C for 3 s) on OTC and TTC residues in milk is shown in Table 2. Table 2 also reports the results of statistical analysis of measured values. The acquired results confirm the differences in thermostability between the tetracycline analytes. The mean concentration of TTC was 169 ± 15.84 $\mu g \cdot l^{-1}$ in raw milk and 159.84 ± 9.23 $\mu g \cdot l^{-1}$ in pasteurized milk. The pasteurization caused a mean decrease in TTC residues by 5.74%. Paired *t*-test evaluation of both groups (non-heated and heated milk) did not reveal any significant differences ($P > 0.05$). In the case of OTC samples, however, the differences in mean values of raw and pasteurized milk were found to be highly significantly different ($P \leq 0.01$); pasteurization decreased the OTC concentration by 15.3%. The mean concentration of OTC in milk samples dropped from 163.28 ± 13.94 $\mu g \cdot l^{-1}$ to 138.29 ± 11.35 $\mu g \cdot l^{-1}$ during heat treatment. These results indicated that TTC was more stable than OTC. Results suggested that heating under pasteurization conditions could cause only a partial reduction of OTC and TTC residues.

Table 2. Statistical evaluation of oxytetracycline and tetracycline residues content in raw and pasteurized milk (85 °C/3 s).

	Oxytetracycline [$\mu g \cdot l^{-1}$]		Tetracycline [$\mu \cdot gl^{-1}$]	
	Raw milk	Pasteurized milk	Raw milk	Pasteurized milk
n	12	12	12	12
$\bar{x}$	163.28	138.29	169.58	159.84
SD	13.94	11.35	15.84	9.23
Min	135.90	116.10	148.30	143.30
Max	177.10	152.00	201.60	180.00
Paired *t-test*	Highly significant difference ($p = 0.01$)		Non-significant differences ($p > 0.05$)	

$\bar{x}$ - arithmetic mean, n - number of measurements, SD - standard deviation, Min - minimum concentration, Max - maximum concentration

Discussion

Most foods of animal origin are not eaten raw, but require heat treatment before consumption. Heat treatment involving varying combinations of time and temperature depending on the intended use of the starting product – while at the same time ensuring health quality – is an essential part of industrial treatment of raw milk. Along with ultra heat treatment (UHT) and sterilization, pasteurization is one of the basic and most common heat treatment methods. In accordance with current legislation, pasteurization involves either short time heat treatment at very high temperatures (at least 72 °C for 15 s) or long time treatment at low temperatures (at least 63 °C for 30 min) and/or the use of any other combination of time and temperature that has an equivalent effect. Pasteurized milk must show a negative reaction to alkaline phosphatase test (Commission Regulation 1662/2006).

The effect of heat treatment on residues of antimicrobial substances has been studied by a number of researchers (Botsoglou and Fletouris 2001; Hassani et al. 2008; Loksuwan 2002; Hsieh et al. 2011). However, these studies often use matrices other than milk (e.g. meat, aqueous solution, buffer solution) and they also use different temperatures and different methods of thermostability evaluation. Very few studies have dealt with the effects of pasteurization on the concentration of tetracycline residues in milk.

Examples include Loksuwan (2002), who studied the effects of low-temperature long-time (LTLT) pasteurization (63 °C/30 min) on OTC, CTC, and TTC residues in raw milk. The OTC residues were in samples with concentration of 100 $\mu g \cdot l^{-1}$ inactivated to such an extent that they could not be detected; at concentrations of 200 $\mu g \cdot l^{-1}$ and 300 $\mu g \cdot l^{-1}$ the starting OTC concentrations were found to have dropped by 86.7% and 79.36%, respectively. In case of TTC residues, the decrease was distinctively smaller: it amounted to 54.75% in samples with TTC concentration of 100 $\mu g \cdot l^{-1}$ and to 22.97% and 37.45% in concentrations of 200 $\mu g \cdot l^{-1}$ and 300 $\mu g \cdot l^{-1}$, respectively. In contrast, degradation of CTC was very limited: the drug residue concentration decreased by only 9.57% (100 $\mu g \cdot l^{-1}$), 4.88% (200 $\mu g \cdot l^{-1}$), and 3.71% (300 $\mu g \cdot l^{-1}$).

The temperature and time used in our study were different than in the study of Loksuwan (2002). If we compare the decrease in TTC and OTC concentrations in the two studies, it is clear that the heat treatment we chose (85 °C/3 s) was less effective in eliminating tetracycline residues. The temperature we used (85 °C) was higher; however, it appears that the degradation of residues is affected by the heating time as well, which explains the significant difference in the values obtained in the two studies. Both studies show that OTC in milk is more labile when subjected to heat treatment than TTC. Overall, our data confirm the published results which show that pasteurization temperatures lead to the decrease in OTC and TTC residues in milk, but they do not cause complete degradation of the substances.

Pasteurization is not the only technological procedure employed in the dairy industry. Other ways of thermal processing of milk include ultra heat treatment (UHT) and sterilisation, which aim to devitalise the vegetative bacterial forms and spores in raw milk, including enzyme inactivation, in order to extend the shelf life of milk. Both UHT and sterilisation use temperatures above 100 °C. Ultra heat treatment requires temperatures of at least 135 °C for a period of up at least 1 second (usually 3–5 s), which is followed by aseptic filling (Deeth and Datta 2003; Commission Regulation 1662/2006). Sterilization, on the other hand, involves continuous sterilization with aseptic filling and hermetic sealing in final containers, using an effective heat treatment duration; higher temperatures are combined with shorter times (Hinrichs and Atamer 2003).

Hsieh et al. (2011) studied the effects of the above heat treatment on tetracycline thermostability, using double-distilled water as a matrix. They used two different heating temperatures (100 °C, 121 °C) with the same time of exposure (15 min). Their findings show that higher temperatures (121 °C/15 min) cause tetracycline degradation of up

to 99%. At 100 °C the degradation was less extensive, amounting to as little as 54.4%. The results of this study clearly show that the degree of TTC and CTC degradation is temperature-dependent. In OTC samples, however, both temperatures led to the same degree of degradation. In addition to these findings, tetracyclines have been found to have different degradation profiles at 100 °C. The results of this study demonstrate, similarly as ours, that even members of the same group of tetracyclines with the same structure may exhibit vast differences in thermostability. For this reason, the thermostability of drugs cannot be predicted from their membership in a particular group of medicaments.

Hassani et al. (2008) set out to determine the thermostability of OTC and TTC in McIlvaine buffer with varying pH value (pH 7.0, 5.5 and 4.0) and in the same McIlvaine buffer of pH 7 containing sodium chloride (NaCl) at high and ultra high temperatures ranging from 110–140 °C. The results of the study showed that sterilization (118 °C/30 min and 121 °C/20 min) reduced the concentration of TTC and OTC to very small, negligible amounts (less than 0.01%). The UHT, on the other hand, reduced OTC concentrations by more than 40% and TTC concentrations by roughly 30%. At 135 °C/15 s the UHT inactivated OTC residues by 44% and TTC residues by 24%. It follows that while the sterilization process degraded tetracyclines in milk by more than 98%, UHT milk still retained about 50 to 90% of the initial concentrations of tetracyclines. Differences in pH levels and water activity (a_w) had little effect on tetracycline thermostability.

Comparing the results of some of the studies with our own is difficult due to differences in matrix, result verification methods and applied thermal conditions. The degradation and inactivation of antibiotic residues during heat treatment can be influenced by a number of factors: matrix, temperature, chemical structure of the antibiotic and its stability (the formation of active and inactive metabolites from the original substance). Other factors can also impact the results, e.g. the binding capacity of antibiotics to other milk components and the used methods of antibiotic residue measurement (biological or physicochemical methods). The basic method of heat treatment of milk used in the dairy industry – pasteurization – does not ensure a complete degradation of tetracycline antibiotics. From the technological point of view, the decreasing of TTC and OTC in milk during high-temperature pasteurization (85 °C for 3 s) determined in this study is not significant.

Our study confirms that the thermostability of individual drugs varies significantly within the group of tetracycline antibiotics. Compared to TTC, OTC displays lower thermostability during high-temperature pasteurization (85 °C/3 s). The content of OTC residues dropped by 15.3% when subjected to this type of heat treatment. The content of TTC residues, on the other hand, dropped by only 5.7%.

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