Occurrence of Tetracycline, Chlortetracyclin, and Oxytetracycline Residues in Raw Cow's Milk

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Abstract

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The objective of this study was the detection of tetracycline, chlortetracycline and oxytetracycline residues in raw cow's milk. When analysing bulk milk (n = 57) and tanker trailer'a (n = 113) samples, two methods were used simultaneously: a specific rapid test Milk Tetrasensor Kit and high performance liquid chromatography (HPLC) with ultraviolet detection and isocratic elution. For HPLC analysis, Breeze (Waters, USA), a liquid chromatographic system, was used. The samples underwent solid phase extraction before the HPLC analysis. The Nova Pack C8 column (3.9 × 150 mm, 4 µm, Waters) and mobile phase (0.8 ml/min) consisting of acetonitrile, methanol, and 0.05 mol/l of oxalic acid in a 13:13:74 ratio were used. None of the samples analysed with the use of the specific rapid test displayed the presence of tetracycline antibiotics. In all of the samples analysed by means of HPLC, low concentrations of tetracycline antibiotics residues were detected. None of the samples displayed the presence of chlortetracycline. All of the analysed samples displayed residues of tetracycline. Oxytetracycline residues were detected only in 50.6% of analysed samples.

Keywords: residues; tetracyclines; milk

Tetracyclines rank among the antimicrobial substances most frequently used in the animal food production (Schmidt & Rodrick 2003). Tetracyclines display a wide spectrum of antimicrobial action: apart from a stronger action on the gram-positive bacteria and a weaker one on the gram-negative ones, they exercise action also on mycoplasmas, chlamydiae, rickettsias, spirochetes, actinomycetes, and some protozoa (Sundin 2003). The sum of tetracycline action is bacteriostatic. The main goal of the antibacterial action of tetracyclines is proteosynthesis inhibition. They bind to the bacterial 30S ribosomal subunit and present attachment of aminoacyl-tRNA to the ribosomal receptor site (Chopra *et al*. 1992; Roberts 1996). In cattle, tetracyclines are used when treating general, respiratory, urinary, and local infections. A specific indication for administering tetracyclines in cattle is infectious mastitis. A frequent and pervading source of milk contamination is intramammary (intracisternal) administration. Other milk contamination paths

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are percutaneous, intrauterine, subcutaneous, intramuscular, and intravenous administration (Heeschen & Blűthgen 1991). Milk tetracyclines contents reach 50–60% concentrations of those in the blood plasma (Botsoglou & Fletouris 2001). To prevent any harmful health effects on consumers, Food and Agricultural Organization, World Health Organization and European Union (EU) have established the maximum residual limits (MRL) for veterinary drugs (Council Regulation 2377/90/EEC). The maximum residual limit set by the EU legislation for tetracycline (TTC), oxytetracycline (OTC) as well as chlortetracycline (CTC) in raw cow milk is set to 0.1 mg/kg (100 ng/g). The presence of residues at much higher levels in foods may constitute a variety of public health hazards including toxicological, microbiological, immunological, and pharmacological hazards (Heeschen 1993). Most important health aspects which should be taken into account are (Honkanen-Buzalski & Reybroeck 1997; Roberts 1997; Cerniglia & Kotarski 2005): possible impact on the emergence of antimicrobial resistance for antimicrobials administered in human therapy, disorders in the intestinal flora, and possible occurrence of allergic symptoms. Adverse effects on human health after the therapeutic use of tetracyclines are well known. Tetracyclines should not be used by children up to the age of 6–8 years or by pregnant women because of the risk of developing secondary tooth discoloration. Other chronic effects include nephrotoxicity, hepatotoxicity, skin hyperpigmentation in the sun exposed areas, hypersensitivity reactions. Tetracyclines have also been reported to cause hypouricemia, hypokalemia, proximal and distal renal tubular acidosis (Goldfrank *et al*. 2002). The residual presence of tetracyclines just as well as other antimicrobial substances in milk presents technological difficulties in the milk processing industry (Heeschen & Blüthgen 1991).

The widespread use of antimicrobials in dairy cattle management may result in the presence of antibiotic residues in milk. Each country within the European Union has established its own surveillance schemes for controlling the drug residues. General residual surveillance of veterinary drugs is performed using microbiological inhibition methods, which allow their detection and/or semiquantitative determination, and using specific rapid testing (Mitchell *et al*. 1998; Botsoglou & Fletouris 2001). Physicochemical methods are used primarily for the isolation, separation, quantification, and confirmation of the presence of detrimental residues in milk samples. Liquid chromatography has become the most widely used separation technique for determining tetracycline antibiotics in edible animal products (Schenck & Callery 1998; Oka *et al*. 2000; Botsoglou & Fletouris 2001).

The aim of this study was to determine the presence of the following tetracyclines: tetracycline (TTC), chlortetracycline (CTC), and oxytetracycline (OTC) in raw cow's milk. The residues were monitored in milk samples which had been collected from the primary production (bulk milk samples) and at the delivery to the milk processing plant (tanker trailer's samples). Two methods were used simultaneously for the determination of tetracyclines. A specific rapid test was used for the detection purposes (Milk Tetrasensor Kit, Unisensor S.A. Belgium) while high performance liquid chromatography (HPLC) was used for quantitative determination.

MATERIAL AND METHODS

Raw cow's milk samples. The samples of bulk milk (total 57 samples) and those of tanker trailer's milk (total 113 samples) were collected from the milk collection route of a dairy plant manufacturing natural semihard and hard cheeses in July 2007. The milk samples were kept refrigerated (about 4°C) between sampling and analysis.

Screening test. The Milk Tetrasensor Kit (Unisensor, S.A., Angleur, Belgium) as a receptor test was used for a rapid determination of tetracyclines in milk. The sample analysis was carried out based on the producer instructions. The sensitivity limit of the kit was 25 ppb.

HPLC analysis. *Chemicals and material*: Acetonitrile and methanol were of HPLC grade; oxalic acid dihydrate Suprapur and Na_2HPO_4 heptahydrate (Merck Ltd.); ethylene diamine tetraacetic acid (EDTA) disodium salt, citric acid monohydrate (Thermo Fischer Scientific) were of p.a. purity grade. Solid phase extraction (SPE) column Oasis HLB, 3 cc, 60 mg was purchased from Waters (Milford, USA). The vacuum unit for SPE was purchased from Supelco. The other hardware included an analytical balance (Kern, Balingen, Germany), a cooling centrifuge (Mechanika Precyzyjna, Poland), and a rotary vacuum evaporator (Bűchi, Flawil, Switzerland).

McIlvaine buffer preparation: McIlvaine buffer was prepared by combining 400 ml 0.2 mol/l sodium hydrogen phosphate, 600 ml 0.1 mol/l of citric acid and 0.37 g of EDTA (pH 4.1). The solution was stored at 4°C.

Tetracycline antibiotics standard solutions preparation. For the preparation of the standard solutions, antibiotic preparations Tetracycline hydrochloride T 3383, Oxytetracycline hydrochloride O5875, and Chlortetracycline hydrochloride C4881 (Sigma Aldrich, Inc., St. Luis, USA) were purchased. The working solutions in the concentration range of 0.01–10 mg/l were made from the stock solutions of the concentration of approximately 0.1 g/100 ml of the individual antibiotics in methanol.

Sample preparation for HPLC analysis. Before the analysis, the milk samples were heated to 40°C, stirred and cooled to 20°C. Fifteen ml of the milk sample were mixed with 25 ml of McIlvaine buffer. The diluted samples were centrifuged at 4000 rpm and 5°C for 10 minutes. After centrifuging, the upper fat layer was removed. An aliquot part (25 ml) of the supernatant was subjected to SPE on the Oasis HLB columns according to the instructions in the user's manual by the Waters (Milford**,** USA). SPE columns were conditioned with 3.0 ml of methanol, washed out with 2.0 ml of water. Then 25 ml of the supernatant were applied to the column, washed with 1.5 ml 5% methanol in water. The TCAs were eluted with 2.0 ml of methanol and then evaporated to dryness on the rotary vacuum evaporator. The evaporated residues were reconstituted in 1.0 ml of the mobile phase and filtered through 0.2 μm nylon filters for HPLC determination. All samples were analysed soon after the preparation.

Conditions of HPLC analysis. Milk samples were analysed using the liquid chromatography system Breeze (Waters, USA) equipped with a binary gradient pump 1525, dual UV/VIS detector 2487, autosampler 717 plus, and column thermostat. The system is controlled and the data are analysed using the Breeze software. A chromatography column Nova Pack C8, 3.9 mm × 150 mm with the particle size of 4 μm (Waters, USA) was used. UV detection was carried out at the wavelength of 365 nm. The measurements took place in isocratic mode. The mobile phase (flow rate of 0.8 ml/min) was a mixture of acetonitrile, methanol, and 0.05 mol/l of oxalic acid in the ratio of 13:13:74. The sample injection volume was 20 μl, the column temperature was 30°C. For the qualitative and quantitative evaluation, the external standard method was used. Each sample was analysed in duplicates at the least, every series containing a blank sample. Simultaneously, aliquots of the samples with the addition of standard solutions of known concentrations were measured. The detection and quantitation limits were established based on the standard deviation of the blind test and the slopes of the calibration curves, repeatability was based on 20 parallel determinations and the recovery was based on 17 and 7 determinations of the milk sample with the addition of the solution of standards of known concentrations (50 μg/l and 100 μg/l). Basic statistical processing was done using the Unistat software, Version 5.1 (Unistat Ltd. 1998). Student *t*-test was used to compare and determine the differences between the tetracycline and oxytetracycline average levels of two selections (tanker trailer and bulk tank samples).

RESULTS AND DISCUSSION

About 1% of products of animal origin in the USA and in Europe contain antibiotic residues in very low concentrations. The cause for the incidence of the antibiotic residues in milk is in 92% due to their administration in mastitis therapy. In most countries, the most frequently detected antibiotics are β-lactam antibiotics while tetracyclines are detected in milk only rarely (Schmidt & Rodrick 2003). In monitoring the residues, the State Veterinary Administration of the Czech Republic (1999–2007) has not detected tetracycline antibiotics since 1999.

In this study, we focused on the presence of tetracycline antibiotics in raw cow's milk samples from farms (bulk samples) and in raw milk samples from the tanker trailers.

In the processing plant, where the samples were taken, the milk samples from the tanker trailers analysed in the delivery laboratory by a specific rapid test displayed sensitivity only to one group of antibiotics (β-lactam antibiotics). The bulk milk samples were also sent (at least 2 samples monthly) to a licensed laboratory for raw cow's milk quality checks and they were tested for the presence of antimicrobial substance residues using a wide action microbiological inhibition test. The positive samples were sent to the Czech reference laboratory for targeted analysis.

When detecting antibiotic residues in purchased milk, microbiological inhibition methods and specific rapid tests are used most frequently in the Czech Republic. The sensitivity of microbiological inhibition methods to tetracycline antibiotics varies. Commercially produced wide action tests making use of the *Bacillus stearothermophilus* microorganism display a higher sensitivity to β-lactam antibiotics, being less sensitive to tetracyclines which they are capable of detecting at levels much higher than the set MRLs (Botsoglou & Fletouris 2001).

Tetracycline antibiotics determination in milk with HPLC was done by a number of authors (Sokol & Matisova 1994; Brandšteterová *et al*. 1997; Furusava 2003; Fritz & Zuo 2007; Koesukwiwat *et al*. 2007). For OTC, TTC, and CTC determinations, an HPLC method with ultraviolet (UV) detection and isocratic elution was validated. Optimisation of HPLC analysis was carried using standard solutions of tetracycline antibiotics. The repeatability and recovery of the whole analytical process were monitored in 20 samples or more exactly derived from 17 and 7 milk sample determinations with the addition of standard solutions of known concentrations. The overall average recovery of the analytical procedure for TTC, OTC, and CTC was in the range of 88.1–93.5% (Table 1). The established relative standard deviation (RSD) for the addition of approximately 50 and 100 μg/l of the individual analytes in milk was for OTC and TTC < 10% but with CTC it reached 20.6% (Table 1). The detection limits when validating the HPLC method were set from the standard deviation of the blank sample analysis and the slopes of calibration curves and were 5, 5, and 20 μg/l for oxyteracycline, tetracycline, and chlortetracycline, respectively. Kaale *et al*. (2008) developed a rapid reversed phase high performance liquid chromatography method for OTC analysis in raw milk samples. They obtained average recoveries greater than 92% with RSD ranging between 0.8% and 6.6%. Sokol *et al*. (1995) monitored tetracycline antibiotics concentrations in milk after intramammary administration using HPLC. When validating HPLC, they obtained a recovery of 95.41% for CTC, 91.98% for OTC, and 83.44% for TTC, and the determination detection limits at 0.05 ppm (50 ng/g) (Table 1). Brandšteterová *et al*. (1997) compared the separation methods when determining tetracyclines using liquid chromatography. With the method using the extraction on the solid phase SPE, they obtained the detection limits in the range of 15–22 ng/g.

HPLC method did not prove the presence of chlortetracycline in bulk milk nor in tanker trailer samples. This substance was probably contained in concentrations below the detection limits or was not present in the samples at all.

In all of the analysed samples, tetracycline residues were detected (Table 2). Statistical assessment of the tetracycline level in sets of bulk milk and tanker trailer's samples using the Student *t*-test indicated that the differences between the mean

Table 1. Determination recovery of oxyteracycline (OTC), tetracycline (TTC) and chlortetracycline (CTC) for the addition of 50 and 100 μg/l of the individual analytes in McIlvain's buffer and the repeatability for the addition of approx. 50 and 100 μg/l of the individual analytes in buffer and in milk

Parameter	OTC	TTC	CTC
Recovery (%)	93.5	91.5	88.1
n	17	17	7
Repeatability for TC in buffer			
RSD (%)	5.3	4.8	4.9
n	20	20	20
Repeatability for TC in milk			
RSD (%)	6.8	8.0	20.6
n	20	20	20

RSD = relative standard deviation, *n* = number of measurements, TC = tetracyclines

Table 2. Tetracycline residue concentrations in bulk tank and tanker trailer samples of raw cow's milk using HPLC

Tetracycline (μg/l)[b]	Samples with positive finding	Tanker trailer's samples	Bulk milk samples
n_1 (n)	170 (170)	113 (113)	57 (57)
$\bar{x}$	4.63*	5.06[a]	3.79*[a]
Median	3.73	4.17	2.64
Min	0.24	0.53	0.24
Max	24.47	24.47	22.66
SD	3.88	3.89	3.75
CV%	83.79	76.99	98.94

[a]differences between average values have statistical significance $P = 0.05$; [b]detection limit for tetracycline is 5 μg/l
n_1 – number of positive samples; n – total number of examined samples; $\bar{x}$– mean; min and max – minimum and maximum values; SD – standard deviation; *concentration < detection limit; *CV* – coefficient of variation

values were statistically significant ($P = 0.05$). The amounts of tetracycline in the samples were very low, 69.4% samples displayed concentrations of up to 5 μg/l, 22.4% samples between 5 and 10 μg/l, 5.3% between 10–15 μg/l, and 1.8% revealed concentrations of 15–20 μg/l. Only 2 (1.2%) samples displayed concentrations in the interval of 20–25 μg/l. The highest detected concentration of tetracycline was 24.47 μg/l.

Oxytetracycline residues were determined in the total of 86 samples, i.e. in 50.6% of the overall number of the samples collected (Table 3). Similarly as with tetracycline, oxytetracycline concentrations detected in the samples were very low, ranging under the detection limit of the method in most samples (91.9%). Student *t*-test detected a statistically very significant difference ($P = 0.01$) between the average levels in bulk milk- and tanker trailer's samples.

The samples from tanker trailers as well as bulk milk samples of raw cow's milk (a total of 170 samples) were analysed using a specific rapid test called Milk Tetrasensor Kit. In none of the analysed samples did the test detect the presence of tetracycline antibiotics. The sensitivity limit of the kit as advertised by the producer was 25 ppb. Based on this analysis, the samples should not display the presence of tetracycline antibiotics in concentrations higher than 25 μg/l. HPLC did not detect the overall concentration of tetracy-

Table 3. Oxytetracycline residue concentrations in raw cow's milk samples determined by HPLC

Oxytetracycline (μg/l)[b]	Samples with positive finding	Tanker trailer's samples	Bulk milk samples
n_1(n)	86 (170)	59 (113)	27 (57)
$\bar{x}$	2.02*	2.48*[a]	1.01*[a]
Median	1.14	1.76	0.47
Min	0.11*	0.11*	0.18*
Max	9.35	9.35	4.28
SD	2.12	2.33	1.02
CV%	105.15	94.23	100.37

[a]differences between average values indicate high statistical significance $P = 0.01$; [b]detection limit for oxytetracycline is 5 μg/l
n_1 – number of positive samples; n – total number of examined samples, $\bar{x}$– mean; min and max – minimum and maximum values; SD – standard deviation; *concentration < detection limit; *CV* – coefficient of variation

cline antibiotics residues higher than MRL. In one sample, the overall concentration of tetracycline antibiotics (TTC and OTC) residues was 27.44 ± 3.11 μg/l. The presence of tetracyclines was not confirmed by the specific rapid test in the given sample. However, taking into account the SD of the determination, the concentration could have been under the detection limit of the specific test.

This study confirms that tetracycline antibiotics belong in the Czech Republic to frequently used antimicrobial substances even for lactating cows. In all of the samples analysed, HPLC detected tetracycline antibiotics residues in low concentrations. Further processing of milk can bring on the lowering of the concentrations of tetracycline antibiotics. The issues concerning antibiotics inactivation during milk processing are more serious than those with other animal origin foodstuffs which is given by the fact that milk is heat-treated over a very short time interval. Heat stability of the residues depends on the antibiotics type. It has been proved that during the heat treatment of milk, there is only a partial reduction occurrs of tetracycline residues concentrations, total elimination not taking place. For instance, Moats (1999) found that the temperature of 62°C over a period of 30 min caused a reduction in CTC content by 16%, in that of OTC by 23%, while at the temperature of 72°C the reduction was by 27% and 35%, respectively.

In food animal production, a wide range of antibiotics are used. Consumers thus can be exposed to a series of antimicrobial substances when consuming food of animal origin (Dayan 1993). Some authors point to the fact that even low concentrations of these substances in foodstuffs of animal origin can present health risks. The exposure of intestinal microflora to low concentrations of antimicrobial residues contained in food brings the potential risk to human health of compromising the colonisation barrier, leading to pathogenic bacteria overgrowth, or of compromising antimicrobial therapy in humans by exerting a selection pressure on the intestinal microflora thus favouring the growth of microorganisms with natural or acquired resistance (Cerniglia & Kotarski 2005). A notable proportion of the general population has a genuine allergic sensitivity to these substances (up to 7–10% to penicillin due to prior medical treatment). Antimicrobial substances are capable of acting as immunogens either directly or via haptenisation of proteins. The challenge by the oral route may then elicit cutaneous and other allergic reactions, and rarely even anaphylaxis (Dayan 1993).

Because milk and milk products are essential foodstuffs for small children, attention has to be paid to the presence of drug residues in milk. Antimicrobial substance detection in cow's milk samples at the delivery in the dairy should not be focused only on the most frequently used antimicrobial substances (β-lactam antibiotics) but also on other agent groups.

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