

Effect of freezing on stability of oxytetracycline residues in beef from Dodoma region, Tanzania

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SUMMARY

The aim of this study was to determine the effect of freezing on the concentration of oxytetracycline (OTC) residues in beef samples stored at -20 °C (core beef temperature -12 °C and below) for 60 and 120 days. A total of 60 fresh beef samples were randomly obtained from cattle slaughterhouses and butcher shops in districts in Dodoma region in Tanzania. The OTC residues were determined using high performance liquid chromatography (HPLC) with a diode array detector (DAD). Results showed that the mean concentration of OTC residues in 16 positive samples before freezing was 191.71 ± 90.21 ng/g. The mean concentration of OTC after freezing at -20 °C for 60 and 120 days were 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g respectively. These results revealed a significant ($p < 0.05$) reduction of OTC residues of 30% after 60 days and 65% after 120 days of freezing at -20 °C. The percentage reduction of OTC residues was not dependent on the initial concentration or the freezing process but was rather due to unknown time dependent individual beef sample factors. It is concluded that, despite OTC levels in beef decreasing due to non-freezing factors, any residues significantly above Maximum Residues Level (MRL) may not be expected to reduce to acceptable levels as a result of freezing.

Key words: Oxytetracycline, cold storage, HPLC, beef.

INTRODUCTION

The presence of antimicrobial residues (AMRs), in food is a public health concern. The availability of antimicrobial drugs in some countries without effective regulations and with inadequate awareness on appropriate drug use among livestock keepers results in the occurrence of high levels of antimicrobial residues in meat (Nisha, 2008). Some of the effects caused by antimicrobial residues in food include autoimmunity, carcinogenicity, mutagenicity and bone marrow toxicity (Pavlov *et al.*, 2008; Nisha, 2008). Furthermore, AMRs present in meat, milk and other foodstuff can initiate the development of resistant strains of bacteria due to the consumption of sub-therapeutic doses of antimicrobial (Mateu and Martin, 2001; Teale, 2002; Wilson *et al.*, 2003; Hardman and Limbird, 2007).

Several studies have been conducted to determine the levels of AMRs in food products of animal-origin in Tanzania. The prevalence of antimicrobial residues ranges from 2.8% to 100% in beef, chicken meat, milk and eggs in various areas in Tanzania (Mmbando, 2004; Karimuribo *et al.*, 2005; Mdegela *et al.*, 200; Kurwijila *et al.*, 2006). Nonga *et al.* (2010 & 2013); Mgonja *et al.* (2017) reported the prevalence of antimicrobial to be 100%, 76.4%, and 35% in cattle, chicken meat, milk and eggs, respectively.

The destiny of drug residues during heat-treating is still uncertain. Many scientists have been concerned whether antibiotic residues can be destroyed by cooking procedures, pasteurization, or canning processes (Ibrahim and Moats, 1994; Rose *et al.*, 1995; Isidori *et al.*, 2005; Hassani *et al.*, 2008; Hsieh *et al.*, 2011; Mgonja *et al.*, 2016). A study described by EI Atabani *et al.* (2014) reported that out of one hundred local liver samples examined by microbial inhibition test for oxytetracycline (OTC) residues, 5 samples (5%) reacted positive while all the 20 imported frozen liver samples examined were free from OTC residues.

Although freezing is a form of preservation of meat by hindering the development of microorganisms, various researchers have reported many variations in the reduction of antimicrobial residue concentration with time in frozen meat making the reason for the reported reductions to be unclear. The stability of antimicrobials is generally expected to be higher during storage at -20 °C in comparison to storage at 4 °C (Honikel *et al.*, 1978; O'Brien *et al.*, 1981; Pavlor *et al.*, 2005). However, O'Brien *et al.* (1981) reported that the concentration of oxytetracycline decreased by 7.4% and sulphadimidine by 20.1% in meat stored at 4 °C for 6 weeks. Gehad, (2002) reported that there was no antibiotic residue detected in cattle muscle and organs after freezing for three months at -20 °C. It is therefore, hypothesized that cold storage could reduce OTC residues in beef from unacceptable levels to

acceptable values. The aim of this study was to investigate the effect of freezing on OTC residues in beef from selected slaughter facilities and butcher shops in Dodoma region, Tanzania.

Materials and methods

Study site

Sample collection

Beef samples were obtained from cattle slaughter facilities and butcheries in Bahi, Kongwa, and Dodoma Urban and Rural districts. The cattle slaughter facilities and butcheries were selected using a simple random sampling technique. Each sample was transferred in separate sterile and labeled plastic bags in an icebox and transported to Zambia Agricultural Research Institute (ZARI) laboratory for analysis. All samples were analyzed for determination of oxytetracycline residues. The control and test samples were stored in a freezer at -20°C for approximately 1 week. Both control and test samples were thawed at room temperature for four hours before extraction and analysis of OTC residues. Antibiotic-free meat control samples (blank matrix) were collected from the Central Veterinary Research Institute of Zambia. The sixty samples were analyzed by HPLC and found that only 16 samples were positive for OTC. Sixteen beef samples, which were positive for OTC residues were subjected to cold storage at -20°C for 60 and 120 days.

Analytical method validation

The procedures for validation parameter were taken from the guidelines for the Germany Society of Toxicology and Forensic Chemistry GTFCh, (2009).

Samples extraction

The extraction procedures were similar for spiked blank samples and test samples. Samples were removed from the -20°C freezer and thawed for about 1 hour. Approximately 10 g of muscle sample was weighed and mixed with 25 mg (EDTA) per gram sample. The sample and the EDTA were homogenized using a blender for one minute. The blended sample was then further ground using a mortar and pestle. One gram of the homogenized sample was accurately weighed into a 15 mL polypropylene centrifuge tube. To the sample, 50 μL of 50 $\mu\text{g/mL}$ caffeine solution, equivalent to 2500 ng caffeine, were added. Five millilitres acetonitrile was added using a 5 mL volumetric

This study was carried out in Dodoma region in Tanzania. Dodoma Region is centrally positioned in Tanzania, lying at latitudes 4° to 7° South and longitudes 35° to 37° East, and is bordered by four regions namely, Manyara in the North, Morogoro in the East, Iringa in the South and Singida in the West.

pipette and the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube by decantation. Five millilitres acetonitrile was then added to the residue, the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. Both supernatants were combined into a 15 mL centrifuge tube, briefly mixed using a vortex and gently dried under a stream of nitrogen to 2 mL. Thereafter, 0.5 mL of HPLC grade water and 30 μL of formic acid were added, making the mixture 1.2% acidic. Fifteen milligrams of Supelclean ENVI-carb active coal were added; the sample was mixed for 30 seconds using a vortex and centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube and dried to 0.5 mL.

Sample analysis

The reference standard for OTC and Ethylenediaminetetraacetic acid (EDTA) was supplied by Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and methanol were of high performance liquid chromatography (HPLC) grade (Merck Company, Germany). The determination of OTC residues was carried out using HPLC with a diode array detector (DAD) as describe by Mgonja *et al.* (2016). The HPLC apparatus was equipped with a constant flow quad pump at a flow rate of 0.5 mL/min. Elution of OTC from the analyte was done on an Eclipse XDB C-18 column 4.6 x 150 mm, 5 μm I.D with HPLC grade water-acetonitrile containing 0.1% formic acid. A 100 μL of the analyte from each sample was injected to obtain average peak areas of positive samples corresponding to retention times of 5.0 minutes of the reference standard for OTC. The concentrations of OTC residues in the samples were calculated from the linear equation, $Y = 614.8x + 428699$ (where, Y = AUC for sample OTC chromatogram peak, x = concentration of OTC in sample) obtained from the standard curve (Figure 5.1). The Limit of Detection (LOD) was 18.2 ng/g and the Limit of Quantification (LOQ) value was 54.6 ng/g.

Data Analysis

The data was analyzed by using t-test. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

The results revealed that there was reduction in concentration of OTC residues in beef after storage at -20°C for 60 and 120 days by 2%-30% and 11%-65% (Table 1). The mean concentration of OTC after the cold storage days was significantly lower than the mean concentration of OTC before the cold storage (166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g versus 191.71 ± 90.21 ng/g; $p < 0.05$). Only two samples with OTC levels marginally above Codex Alimentarius MRL of 200 ng/g before freezing had their concentration reduced to levels below the MRL during the freezing period. The higher

concentration of OTC was associated with a higher peak. The correlation coefficient associated with the linear regression for the OTC standard concentration with AUC is represented by $R^2 = 0.99$ (Figure 1).

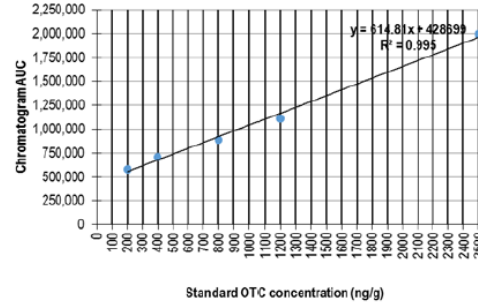


Figure 1. Calibration curve of Oxytetracycline standard

Table 1. Concentration of OTC and percentage reduction after freezing at -20°C for 60 and 120 days

Sample no.	OTC concentration in beef (ng/g)			OTC concentration reduction (%)	
	Before storage	After 60 days at -20°C	After 120 days at -20°C	After 60 days at -20°C	After 120 days at -20°C
1	188.20	131.06	66.20	30	65
2	92.09	82.46	74.35	10	19
3	70.68	57.61	42.02	18	41
4	370.42	357.42	315.66	3	15
5	167.32	145.34	114.00	13	32
6	262.16	233.45	212.07	11	19
7	134.07	128.75	114.73	4	18
8	221.37*	197.27	170.67	11	23
9	318.22	229.7	146.15	28	54
10	228.62	224.01	201.97	2	12
11	143.57	120.71	90.25	28	54
12	322.28	314.33	284.63	2	12
13	132.11	126.6	96.51	30	65
14	108.14	75.25	38.13	4	26
15	207.39*	154.81	108.95	25	47
16	100.77	82.66	59.79	18	41
Range	70.68 - 370.42	57.61 - 357.42	42.02 - 315.66	2 - 30	11 - 65
Mean	191.71 ± 90.21	166.40 ± 86.49	133.50 ± 83.24	13 ± 10	33 ± 22

*Samples with OTC levels above Codex Alimentarius MRL of 200 ng/g in muscle before freezing. Shaded rows show samples with concentrations that reduced to levels below the MRL during the freezing period.

Figure 2 shows that the percentage reduction in OTC residues in frozen beef samples was not dependent on the levels of OTC in the sample before storage but rather on individual field sample

factors that were not investigated in this study. Levels in all samples continued decreasing despite all of them being subjected to the same freezing conditions.

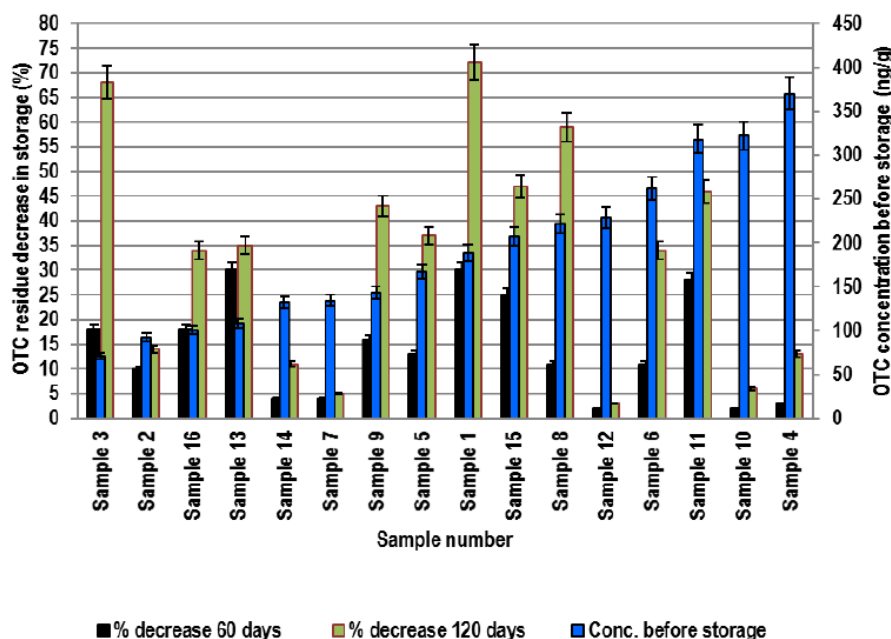


Figure 2. OTC concentration percentage decrease in relation to its increasing concentration before storage

DISCUSSION

Presence of antimicrobial residues in beef can pose hazards to human health. Among them are allergic reaction and imbalance of intestinal microflora, bacterial resistance to antibiotics in microorganisms and losses in the food industry through growth inhibition of food processing microorganisms (Cunha, 2001). Although drug manufactures always recommend withdrawal periods for drugs used in food producing animals, it is common to find oxytetracycline residues at concentrations above Codex Alimentarius Commission MRL in beef readily sold for human consumption (Kaneene and Miller, (1997). Various factors contribute to the presence drug residues in beef including; failure to observe withdrawal periods, age of the animal, disease status (Kaneene and Miller, (1997). This means that sometimes drug residues may be present in beef even when withdrawal period has been observed. Therefore, it is important to understand local factors that may lead to the presence of drug residues in beef, as well as factors that break down these residues to acceptable levels. Pavlov *et al.* (2005) found a decreasing level of tobramycin sulphate from chicken breast and thigh muscle during the period of cold storage. The drug showed initial higher levels in the liver, followed by breast and thigh muscles, with no residues in the muscles on the 30th day. A study by Pavlov *et al.* (1993) showed that freezing at -20 °C caused a lower

degradation than that caused by boiling. So neither boiling nor freezing could be used as reliable methods to get rid of amoxycillin residues in meats.

In this study, HPLC was used to determine the concentration of oxytetracycline (OTC) in beef samples in order to determine whether cold storage has an effect on OTC residues. The mean concentration of OTC before cold storage was $191.71 \pm 90.21.93$ ng/g versus 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g after cold storage at -20 °C for 60 and 120 days. The difference in OTC residue levels before cold storage and after freezing beef (Table 5.1) were statistically significant ($p < 0.05$). The decrease in OTC concentration observed in this study is consistent with other studies in which cold storage decreased the concentration of oxytetracycline and antimicrobial drug residues in foodstuffs.

These results are consistent with a number of other studies that reported reductions in antimicrobial residue concentrations in meat following cold storage. O'Brien *et al.* (1981) used the diameter of growth inhibition zone to establish antibiotic concentrations and observed that the concentration of oxytetracycline decreased by 7.4%, sulphadimidine by 20.1% and ampicillin by 76.05%-100% in meat samples stored at 4 °C for 6 weeks. Usually, the stability of antimicrobials is far higher during storage at -20°C in comparison with

4°C (Honikel *et al.*, 1978; O'Brien *et al.*, 1981; Pavlor *et al.*, 2005).

A study conducted in Turkey indicated that residue levels decreased within days in drugs such as Florfenicol (a fluorinated synthetic analog of thiamphenicol and Florfenicol amine (major metabolite of the antibiotic florfenicol, a fluorinated derivative of chloramphenicol) without any significant difference between storage conditions at -20°C and +4°C (Ayhan *et al.*, 2015). This study is also in line with that by Tansel *et al.*, (2006) which showed that concentrations of gentamicin residues were retained for fourteen days at both refrigerated (+4°C) and room temperatures (15-20 °C), then started to lose strength on day 21 of storage. Another study by Papapanagiotou *et al.* (2005) reported that sulphamethazine (SMZ) residues were stable at -20 °C and -75 °C in all piglet's muscle tissue examined for at least 3 and 5 months, respectively. A study by Alfredsson and Ohlsson (1998) reported that levels of sulphamethazine spiked in beef and frozen at -20 °C for 3 months decreased by 35%.

The level of penicillin G kept in a deep-freezer for 10 days decreased by half in the gluteal muscles, and by 20% in the kidneys (Boison *et al.* 1992). Findings from other studies have shown that freezing of penicillin G, ampicillin, OTC, sulfonamide, quinolones and gentamicin have minor or no effect on the residues levels (Nouws and Ziv, 1976; Boison *et al.* 1992; Verdon *et al.* (2000); Baydan *et al.* (2002) and Sireli *et al.* (2006). The decrease in the quinolones activity in frozen stock solutions stored at -20 °C did not exceed 10%, whereas the levels of β -lactams did not change during 3 months of storage (Okerman *et al.*, 2007).

Although many studies have demonstrated a general decrease of OTC levels during cold storage of beef, our study shows that the decrease was not a result of the process of freezing but was rather due to individual sample factors prevalent at the initial stage of freezing. This is supported by the fact that despite all samples being stored under the same conditions, OTC residues continued degrading at different rates during the whole study period. Only two samples with OTC levels marginally above Codex Alimentarius MRL of 200 ng/g before freezing had their concentration reduce to levels below the MRL during the freezing period. Immediately after slaughter and before grading or freezing, beef must undergo the process of chilling (cold storage at 0 – 4 °C to achieve core beef temperature of 7 °C and below) in order to stop the growth of spoilage microorganism and improve the

quality of meat (FAO 1991). However, variations in the speed of the chilling process can produce meat with varying quality factors such as color, pH and microbial growth (Aalhus *et al.*, 2001) which may have an effect on the stability of drug residues in meat. Therefore, more research is required in order to determine the effects of pre-slaughter and meat chilling factors on OTC residues in meat.

In conclusion, the results of this study show that OTC residues were detectable in frozen beef up to 120 days although on average, there was a significant decrease in concentration. The reduction of OTC residues was not dependent on the freezing process or the initial concentration but was rather due to unknown time dependent individual beef sample factors. Although OTC levels in beef decreased due to non-freezing factors, any residues above MRL may not be expected to reduce to acceptable levels as a result of freezing.

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