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The Effect of Boiling on Stability of Oxytetracycline and Sulfamethazine Residues in Raw Milk using HPLC Method

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ABSTRACT

Two hundred raw milk samples (250 ml of each) were collected from small dairy farms, street peddlers and dairy shops in Mansoura Governorate. These samples were screened using *Bacillus Subtilis* Diffusion Assay for qualitative detection of antibiotics residues; where the percentage of suspected positive samples was 12.5%. High Performance Liquid Chromatography – Ultra Violet detector (HPLC- UV) method was developed and validated to determine the amount of oxytetracycline (OTC) and sulfamethazine (SMZ) residues in raw milk before and after boiling. The results revealed that 8.5 % of the raw milk samples were containing (OTC) residues (6.5 % of them exceed MRL) while, (SMZ) was detected in4 % of the raw milk samples (3 % of them exceed MRL). Upon applying heat treatment, the reduction in the (OTC) content in milk boiled for 2 minutes was 30.5% but boiling for 5 minutes was accompanied with 54.1% reduction. On the other hand, the percentage of (SMZ) reduction was 1.7% and 9.5% in milk boiled for 2 and 5minutes respectively which could be attributed to the low heat stability of (OTC) and high stability of (SMZ).

Keywords: (OTC), (SMZ), (HPLC- UV), Heat treatment.

1. INTRODUCTION

The popularity of raw and natural food is increasing. Raw milk and small- scale dairy products as cheeses are available directly from producers at home. These products have microbiological and chemical adverse effect on the consumer's health, as they could be contaminated with chemicals as: hygienic, agricultural, veterinary and environmental procedures (Claeys*et al* 2013, Khaniki 2007).Veterinary drugs are widely used in pharmaceutical manufacturing field; as a treatment, or for prophylaxis of animal diseases and also to enhance animal growth. Yet they have a major drawback represented in antibiotic residues. This problem results from the bad prolonged usage and the ignorance of drugs withdrawal periods which leads to the appearance of their residues not only in animal tissues, but also in animal by-products as milk (Botsoglou and Fletouris 2001). Exceeding the levels of antimicrobial residues in milk has a deep impact on public health (Heeschen, 1993), where the possible common health risks are antibiotic resistance prescribed for human cure, abnormality in the gut microflora and sensitive reactions (Mitchell et al., 1998). While for the economic stand point, antibiotic residues have a negative effect on the starters used in fermentation process needed for cheese and yoghurt manufacture (Tamime and Robinson, 2007).

(OTC) is considered one of the oldest antibiotics that used routinely in the veterinary practice because of broad spectral activity and its multiple routes of administration. By the end of metabolism, 25-75% of the applied (OTC) are descended into bovine milk. Also, via the entero-hepatic circulation, a small amount of the applied dose may stay in the body for a long period (Botsoglou and Fletouris, 2001). So, if improper a pplication of antibiotic or the withdrawal time not elapsed (OTC) and their metabolites may be found in milk and evoke adverse effects on consumers (Fritz and Zuo, 2007). Sulfonamides are synthetic bacteriostatic compounds which used to cure infections and to improve feed efficiency. Many investigators reported their existence and residues in milk (Brady and Katz, 1988, Charm *et al*., 1988 and Collins-Thompason *et al*., 1988), (SMZ) is considered to be the most carcinogenic member of this group and that is the reason for pushing the codex committee of FAO/WHO through limiting its maximum residue level (MRL) of 25 ppb in milk (WHO, 1989), while in the EC the sulfonamides MRL in milk fixed at 100 ppb (EEC, 1992).

In food safety programs, it is vital to monitor the veterinary drugs residues in raw animal products like milk, eggs and meat to keep public health (Botsoglou and Fletouris 2001and FAO, 2015). Recently, for residue analysis HPLC is used, its important increased day by day, many mobile phases, library of column packing and the variations in the process methods made its importance (Nollet, 1992).Boiling of milk is the most common method of heat treatment specially at home before consumption or processing to decrease the percent of zoonotic pathogens and to increase its validity period (Claeys*et al* 2013). The stability of antibacterial drugs was studied earlier (Moats 1999). So, from the last on-going

two points, this present study seeks the quantitative determination of (OTC) and (SMZ) residues in raw milk samples using HPLC and to focus the light on the effect of boiling process on their residues in raw milk.

2. MATERIALS AND METHODS

2.1. Milk samples

Two hundred raw milk samples (250 ml of each) were collected from small dairy farms, street peddlers and dairy shops in Mansoura Governorate. These samples were received in clean dry sterile sampling bottles and placed in an ice box (4°c) to be sent to the laboratory of Animal Health Research Institute, Dokki, Giza, for the detection of antibiotic residues in comparing with blank milk samples from untreated healthy cows.

2.2. Preparation of milk samples

Each raw milk sample was subjected to Storch test according to (lampert, 1975) as following: 10ml of milk samples, 2 drops of diluted (H2O2) solution (one fraction of H_2O_2, 14fractions of water and 0.1% of H_2SO_4 by volume) then add 2 drops of recently prepared watery solution of 2 % paraphenylene-diamine were added. The contents were mixed thoroughly. Immediate appearance of indigo blue color indicated that the milk was raw.

2.3. Qualitative detection of both antibiotics residues in verified raw milk samples using Screening Bacillus Subtilis Diffusion Assay

Firstly, the antibiotic standard solution; which obtained by preparing different antibiotic concentrations from fortification solution in blank milk samples (0.025, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5 µg/ml. Secondly, the *Bacillus Subtilis* spores suspension preparation (Arret*et al.,* 1971) by sub-culturing the organism on both Nutrient agar slants and plates at 30°C for 24 hours (Oxoid Manual 1998) followed by collecting the growth using sterile saline to be centrifuged at 3000 rpm for 10 min. where the precipitate was suspended in 50 ml of sterile saline and heated for 30 min. at 70°C. then this spore suspension was diluted with sterile saline to obtain density 10^7 spores/ml by using Macfdian tube and the steps were proceeded as follows: One ml of the prepared spores suspension was added to 1000 ml of Nutrient agar medium at 45-50°C to obtain a density 10^4 spores/ml then the medium was well mixed and poured into Petri dishes (1cm depth) in a quantity of 13 ml then the plates were left at room temperature on a horizontal surface till complete solidification after which Sex pores were made on each plate using sterile borer with an outside diameter (8.0 mm) (Carter, 1988)followed by injecting 0.1 ml of each concentration from the prepared antibiotic standard for both (OTC) & (SMZ) antibiotics in the wells of each plate, while control and suspected raw milk samples were injected in the other wells. The plates were then incubated at 30°c for 24 hours and the width of prevention zones was recorded. Interpretation of the obtained results: Inhibition zone < 1mm: negative; Inhibition zone 1-2 mm: suspected: Inhibition zone > 2mm: positive.

2.4. Quantitative estimation of both antibiotics residues in suspected positive raw milk samples using HPLC

2.4.1. HPLC equipment and conditions:

(HPLC unite, chemistry department, Animal Health Research Institute, Dokki, Giza) Agilent series 1200 quaternary gradient pump, USA, An auto sampler plus surveyor, Agilent series 1200, USA, Multi wave detector, Agilent series 1200, USA, Chemstation software (Agilent- Germany) automatic analyser. Analytical column (stationary phase): the stationary phase was done on reversed phase Agilent C18 column (4.6 mm i.d., 250 mm, 5 µm). Surveyor, thermo scientific company, USA.

2.4.2. Determination of (OTC) (Cinquina*et al*., 2003)

Five ml of suspected milk sample was moved into a polypropylene centrifuge tube with t wo ml from 20% trichloroacetic acid (TCA) followed by mixing. 20 ml of McIlvaine buffer was added and the mix centrifuged at 4000 rpm for 20 min. Then supernatant was administrated to a SPE HLB C18 cartridge; which earlier activated with 3 ml of methanol and 2 ml of H2O. After sample loading, the cartridge was cleaned with 2 ml of methanol 5% in H2O. Finally (OTC) was removed with 3 ml of methanol. Then this solvent was dried under a nitrogen stream then residues were soluble in 1 ml of methanol and filtrated with 0.45 µm nylon syringe filter.

2.4.3. Determination of (SMZ) (Weber and Smedley, 2001)

In a separating funnel (125 ml capacity), ten ml of tested milk was transferred along with50 ml of chloroform and a stopper is placed. Then the mixture was vigorously mixed for 1 min; while an excess of pressure was carefully vented through stopper. The funnel was shaken again for 1 min, vented, and the phases were separated for 1 min. The previous step was repeated for another 1 min. and the phases were separated for at least 5 min. after separation, chloroform was drawn off and filtered through a fluted filter paper into a pear-shaped flask (100 ml capacity) then the filter paper was washed twice with 5 ml portions from chloroform and the rinsing solution was received in the same pear-shaped flask. The chloroform solution exposed to evaporation process for dryness on a rotary evaporator at 32°Cfollowed by adding 5 ml hexane to the flask and closure. The residues were dissolved by agitation vigorously on vortex mixer for 1 min. with immediate addition

of 1 ml PDP solution the vigorous agitation on the vortex mixer was repeated for about 1 min, 3 to 4 times over a minimum of 15 min. with subsequent discarding of any hexane. Finally, the aqueous layer from bottom of flask was transferred to an auto injector vial filtrated with 0.45 µm nylon syringe filter.

2.4.4. Heat treatment process (Boiling) (*László et al., 2017*)

The samples of the raw milk that contain residues of antibiotic with a concentrations exceeded the permissible limit were heated at 100.17 °C for different short periods. Heating (boiling) of milk samples were obtained using a stainless-steel pot. All Procedures were designed to stimulate the methods that used commonly in the household practices .The temperature of all heating processes was constantly measured by a thermometer at the exact time. Two sets of heat treatment were conducted. Firstly the samples were heated up to 100.17°C for 2 minutes, secondly milk samples were heated up to 100.17 °C for 5 minutes. The time interval for boiling was measured from the actual boiling (bubbling) of the milk.

3. RESULTS

Upon applying the screening test, twenty-one (21) samples were positive (Inhibition zone > 2mm) and 4 samples were suspected (Inhibition zone 1-2mm). These 25 samples (12.5%) were analysed for the existence of (OTC), (SMZ) residues using (HPLC)with a validation method to detect the quantity the two residues in raw milk and their results were illustrated in (table. 1). While the higher concentrations than permissible limit which heated at 100.17 °C for various short time-intervals (120 and 300 seconds) prevailing in household practice were illustrated in (table 2, 3,4and 5).

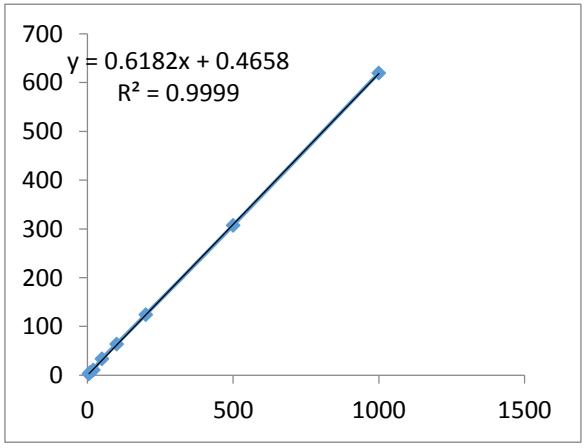

Figure 1. Standard curve of oxytetracycline

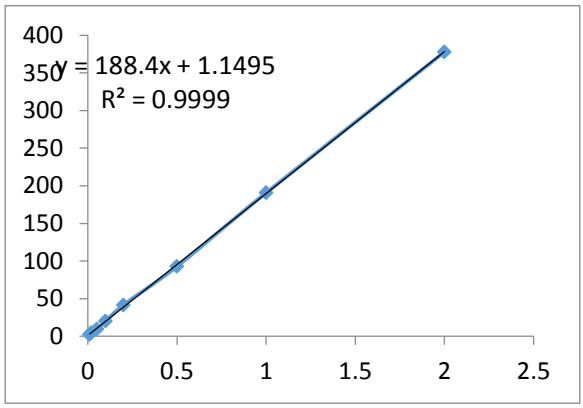

Figure 2. Standard curve of sulfamethazine

Table 1. Results of HPLC analysis for oxytetracycline and sulfamethazine residues.

	Oxytetracycline residues (ng/ml)	Sulfamethazine residues (ng/ml)
1	100*	75
2	146*	140*
3	144*	152*
4	100*	40
5	92	102*
6	50	142*
7	62	116*
8	122*	137*
9	105*	Nd
10	103*	Nd
11	100*	Nd
12	121*	Nd
13	126*	Nd
14	137*	Nd
16	112*	Nd
17	100*	Nd
Mean	119.6	131.5
SE	5.2	7.6

(*) results ≥ MRL

Table 2. Oxytetracycline residues after boiling for 2 and 5 min

	Before (ng/ml)	After boiling for (ng/ml)	
		120 sec.	300 sec
1	100	Nd	Nd
2	146	106	66
3	144	100	63
4	100	75	45
5	122	91	51
6	105	74	44
7	103	74	Nd
8	100	60	Nd
9	121	89	52
10	126	94	56
11	137	98	51
12	112	48	Nd
13	100	63	Nd
Mean	116.6154	81***	53.5***
SE	4.811229	5.014664	2.17377

Table 3. Percentage reduction of Oxytetracycline before and after boiling process

	Raw	Boiling/ 120 sec.	Boiling/ 300 sec.
Mean ± SE	116.6 ± 4.8	81 ± 5	53.5 ± 2.2
Percentage of reduction	100 %	30.5%	54.1%

Table 4. Sulfamethazine residues after boiling for 2 and 5 min

	Before (ng/ml)	After boiling for (ng/ml) 120 sec.	120 sec.
1	140	137	130
2	152	151	121
3	102	102	100
4	142	140	128
5	116	114	110
6	137	132	125
Mean	131.5	129.3333	119
SE	7.623429	7.374129	4.774935

Table 5. Percentage reduction of Sulfamethazine before and after boiling process

	Raw	Boiling/ 120 sec.	Boiling/ 300 sec.
Mean ± SE	131.5 ± 7.6	129.3 ± 7.4	119 ± 4.8
Percentage of reduction	100 %	1.7 %	9.5 %

4. DISCUSSION

Raw and natural food is increasing in popularity, as a part of modern-day dietary habits, raw milk and farmstead cheese are available directly from the producer, and many dairy products possible to be manufactured at home. However, the intake of these products may pose not only microbiological but also chemical adverse effect on the consumer's health. Moreover, milk and dairy products probably polluted with chemicals as, hygienic, agricultural, veterinary and environmental procedures (Claeys*et al*., 2013, Khaniki, 2007).

Veterinary drugs are utilized as a treatment or a prophylaxis for diseases in ediable producers such as beta-lactams and aminoglycosides which utilized in mastitis therapy (Botsoglou&Fletouris 2001). The utilization of those products can't be avoided because of animal health points; despite, it can cause their existence in milk and other eaten tissues, in case of the withdrawal times are not noticed.

Antibacterial residues in milk can lead to in an important health concerns such as allergic symptoms (Dewdney *et al.,* 1991), abnormalities in gut microflora, or affect the presence of resistant bacteria to antimicrobial medication (Mitchell *et al.,* 1998). Also may result in adverse effects on starter cultures utilized to prepare dairy products at home or at industries in fermentation procedures (Tamime and Robinson, 2007).Maximum residue limits (MRLs) and withdrawal times are used to decrease and inhibit the risk and harmful effects of antibiotic residues in food of animal origin (FAO, 2015). Despite with the good notice to withdrawal times that not ensure that milk is residue-free. So the amount of residues might be below the MRLs. The varity of excretion rates between individuals lead tohigher concentrations not be discarded. Moreover, in case of utilization of milk in withdrawal period, results in residues above the permissible limit.

Before processing and consumption of raw milk, it under go heat treatment to decrease the presence of the zoonotic pathogens and to increase its validity period. Various thermal procedures are utilized in the dairy in manufacturing while raw milk is always exposed to boiling at home conditions (László *et al.,* 2018). So, we studied the effect of boiling at 100.17^{0}C on (OTC) and(SMZ)residues in raw milk samples.

At our investigation, two hundred raw milk samples (250 ml of each) were screened for qualitative determination of antibiotic residues using *Bacillus Subtilis* diffusion assay. Twenty-five samples (21 positive and 4 suspected) were examined for presence of (OTC) and (SMZ) residues using high performance liquid chromatography methods (quantitative determination) where the percentage of positive samples were 12.5%, which slightly lower than those identified in Kenya(14%) by both Shitandi and Sternesjo (2004) and Ekuttan *et al.* (2008).

Percent of (SMZ) in the examined milk samples slightly different in this study comparing with that of (OTC). A study by Ahlberg*et al*. (2016)reported sulfonamides and tetracyclines in 0.4% and 2.5%. While Mitema*et al*. (2002) recorded sulfonamides and tetracyclines in24% and 61% respectively.

In Germany, 2007 Kress and other co-authors found sulfonamides in 1.6% of milk samples, which is lower than that reported in Mexico 51.3% (Tolentino *et al.*2005). Recent researches , the presence of tetracyclines, sulfonamides reported to be at limits higher than the permissible limits in milk at various sites of the value chain (Olatoye*et al*., 2016; Chowdhury *et al*., 2015; Layada*et al.,*2016) as 8.5 % of the raw milk samples were containing (OTC) residues (6.5 % of them was exceed MRL) while 4 % of the raw milk samples were containing (SMZ) residues (3 % of them was exceed MRL). Maximum residue limits for both antibiotics were 100 ng/ml according to EMEA, 2012.

In our study, boiling process for 2 and 5 minutes at 100.17 °C significantly reduced (OTC) residues where the reduction percentage was (30.5%) and (54.1%) respectively which can be attributed to the stability of (OTC) that agree with that

have been mentioned by Kuhne*et al.,* 2001 and Hassani*et al.,* 2008 who reported the thermal stability of (OTC), tetracycline and doxycycline in milk and found that conventional thermal procedures (118ºC, 30 min or 121ºC, 20 min) could remove more than 98% of the residues.

These findings were agreed with that studied by László *et al.,* 2017 who discussed the effect of boiling process of raw cow milk with the tetracyclines and sulfonamides and boiled for different short time-intervals common in home. LC-MS/MS measurements are used to determine antibiotic concentrations. Tetracyclines showed low stability in contrast to sulfonamides showed higher heat stability.

The influence of low temperature for long time of exposure (LTLT), pasteurization at 63°Cfor half an hour on CTC,TTC,OTC in raw milk was studied previously by Loksuwan (2002). The OTC residues in content of100 µg/l inactivated to level of no detection, however at content of 200 µg/l and 300 µg/l the initial OTC amount were noticed to be descend by 86.7% and 79.36%, respectively. These results were much greater than our study because the heat treatment we chosed (100.17^0C / 2 and 5 minutes) was less effective in the removal of tetracycline residues. The temperature we used (100.17^0C) was higher, however it appears that the degradation of residues is affected by the heating period that shows the valuable variations in the values recorded in both studies. The two studies show that OTC in milk is more labile when exposed to thermal treatment. From all, our data confirm the published results, it found that boiling temperature because decrease in OTC residues in milk, but not cause a complete degradation of the antibiotic.

Hsieh *et al*. (2011) studied the effects of higher thermal treatments on thermal stability of tetracycline, double-distilled water used as a matrix. They used two different heating temperatures (100 °C) and121°Cwith time of exposure (15 min). Their findings show that higher temperatures (121 °C/15 min) cause tetracycline degradation of up to 99%, while at 100 °C was 54.5%. Beside that results, various degradation patterns showed by tetracyclines at 100.17°C. The results of this study appear similarly as ours. Tetracyclines that have the same structure, and group can show variations in thermal stability. For this reason, the thermo-stability of antibiotics was unable to be predicted from their membership in a special group of medications.

Our results revealed that boiling process for 2 and 5 minutes slightly reduced sulfamethazine residues that were found in raw milk. The data revealed that the reduction in the SMZ content of milk boiled for 2 minutes was 1.7% while for 5 minutes it was 9.5%. These results agree with Roca *et al.,* 2010; who mentioned that sulfonamides have shown high heat stability during thermal treatments utilized in dairy manufacturing .beside that, different degrees of temperatures could not completely destroy antibiotics (72ºC, 15 s; 120ºC, 20 min; 140ºC, 4 s). Roca *et al*. 2013 indicated that sulfonamides were very stable during a process of pasteurization (63ºC, 30 min and 72ºC, 15 s) and UHT sterilization (140ºC, 4 s). Sulfonamides were among the more heat-stable antibiotics and show variations in thermal stability from high-to-intermediate degrees like each other (László *et al.,* 2017).

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