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DETECTION OF TILMICOSIN AND ENROFLOXACIN RESIDUES IN BROILER CHICKEN LIVER AND MEAT SOLED IN MARKETS AT BENHA CITY

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ABSTRACT

Fourty samples of broiler chicken liver and muscles (twenty samples for each) were collected from popular markets of Benha city, Kaluobia governorate, Egypt to detect and analyze of Tilmicosin and Enrofloxacin residues using of High Performance Liquid Chromatography (HPLC), and also to detect their influence by heat processes. Tilmicosin residues were detected in all examined liver samples with a mean concentration of 481.88 ±54.81 ppb while Enrofloxacin residues were detected in only 66.66% of liver samples with a mean concentration of 8.288 ± 1.47 ppb while all examined muscle samples were free from any detectable levels. After boiling and using of autoclave, all residues of liver samples were undetectable. This study emphasized on the importance of strict analyzing of antimicrobial residues prior to marketing and also usefulness of heat cooking with or without pressure, for complete or partial elemination of antibiotic residues.

Key words: *Tilmicosin, Enrofloxacin, Residues, Broiler, Benha City.*

INTRODUCTION

Extensive use of antibiotics leads to potential health problems include allergic reactions, direct toxic effects and a change in the resistance patterns of bacteria exposed to such antibiotics (Weaver, 1992 and Fabrega *et al*., 2008).

Many reports indicated that the resistance of microbes to antibiotics may arise from extensive adminstration of antibiotics, and this resistance may be transferred to human pathogens (Yorke and Froc, 2000). Consumers who consumed Milk, meat and eggs which have antibiotic residues for a long time that can produce bacterial resistance and therapeutic failures among them. Similarly, administration of very low doses of some drugs for a prolonged time produces reproductive and teratogenic effects (Stephen and Sundlof, 1994).

Antibiotic residues in animal derived foods have been extensively recorded in many African countries; these residues have exceeded the WHO maximum residual levels (MRLs) in many cases. (Darwish *et al*., 2013). Animal derived foods must not released for human consumption before expiration of with drawl time of therapeutic antibiotics. The with drawl time (WDT) is the period of time required after completion of treatment needed for tissue concentrations of the drug and/or its metabolites to deplete to less than the established MRLs. The final elimination phase depends on drug pharmaceutical formulation, dose, length of treatment, route and site of administration. According to this, a formulation may require a longer WDT when the drug is slowly depleted from tissues. Otherwise, a shorter WDT can be used when faster depletion is adequately proven (Kukanich *et al*., 2005).

Tilmiscosin is a brood-spectrum macrolide antibiotic. It is synthesized from Tylosin for veterinary use only and has a stronger antimicrobial activity than Tylosin.

It is predominantly effective against *Mycoplasma spp., Pasteurella haemolyticus, P. multocida* and various Gram positive infections in chicken. (Ose, 1987; Prescott, 2000 and Zhang *et al*., 2004). It is advised to be the drug of choice in respiratory infections (CRD) treatment and prevention in broilers derived from positive breeders (Jordan *et al.,* 1999; Abu-Basha *et al.,* 2007 and Amer *et al.,* 2012). Continuous administration of Tilmicosin after 2

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rounds of intensive antibiotic treatment with Chlortetracycline, Doxycycline and Enrofloxacin reduced *Mycoplasma synoviae* shedding from the Ms-positive flocks and eventually eradicated *Ms* from the farm (Hong *et al.,* 2015).

This work was conducted to monitor Tilmicosin and Enrofloxacin residues levels in broiler chicken muscles and liver in Benha city, Kaluobia governorate, "Egypt" and study the effects of some heat treatment processes on them to ensure safety chicken for human consumption.

MATERIALS AND METHODS:

Collection of samples:
Fourty samples of broiler chicken muscles and liver "twenty samples for each" were collected randomly from popular markets of Benhacity, transmitted to the Lab and apply the analytical procedures.

Sample preparation for Tilimicosin analysis.
Broiler chicken liver and muscle samples were minced and homogenized in a homogenizer for 2 min. Five grams of homogenate was accurately weighed into a polypropylene centrifuge tube.

Tilmicosin residues extraction
Extraction of Tilmicosin residues was carried out according to Zhang *et al*. (2004) and Said *et al.* (2016).

Ten ml of acetonitrile were added to the homogenized sample in the centrifuge tube then shaked for 20 min and centrifugated for 10 min. at 3500 rpm were carried out. The supernatant was transferred into a 50ml polypropylene centrifuge tube. Five milliliters of monobasic potassium phosphate buffer and 8ml of acetonitrile were added to the tissue pellet and thorough shaked of the mixture for 20 min. The mixture was centrifugated for 10 min. at 3500 rpm followed by combination of supernatant with 40ml of HPLC water. The mixture solution was subjugated to centrifugation at 3500 rpm for 10 min. The supernatant was introduced to solid phase extraction (SPE) cleanup step. The SPE was conditioned with 10 ml of methanol then 10 ml of deionized water and the sample was applied to the cartridge. The flow rate was not more than 2 drops/s. The cartridge was not allowed to dry at this step, so the cartridge was flushed with 10 ml water then 10 ml of acetonitrile. The SPE cartridge was dried for at least 3 min. under vacuum. Elution was performed successively with 2.5ml ammonium acetate (0.1 mol/L) / methanol / acetonitrile solution. The eluted solution was evaporated until dryness by a nitrogen stream at 30°C in a water bath. The sample was reconstituted by 1ml dipotassium hydrogen phosphate buffer, mixed and filtered through 0.45 um filters before injection into HPLC. Liquid chromatography operating conditions was adjusted for 100 UL injected volume, flow rate, 0.7 ml/min; wave length, 287 nm, column temperature, ambient, stop time: 30 min, post time: 6 min. The mobile phase A was 0.05% trifluoroacetic acid while mobile phase B was acetonitrile (gradient conditions) as at 0 minute 71% from mobile phase A and 29% from B and at 11 min, 54.5% from A and 45.5% from B.50% was taken from mobile phase A and the same from B at 11.5 and 14 minutes. Tilmicosin standard concentrations of 0.05, 0.1, 0.2, 0.5, 1.2 and 5 Ug/ml were preparated after said *et al.* (2016).

Enrofloxacin residues extraction:
The method was carried out according to the technique recommended by Salehzadeh *et al.* (2007).

Sample preparation
The samples were kept at – 20 degrees centigrade until analysis.
2.5 gm of liver or muscle allowed to defrost at room temperature, then 300mg liver and 400 mg muscle tissues were homogenized. The homogenized extract was centrifuged at 4400c for 10 min. at 4°C, repeat extraction step twice and supernatants were pooled. Muscle tissue extract was left at 35°C for 15 min. and then centrifuged at 4400c for 20 min at 4°C and finally filtered by syringe filter.

Sample clean-up by solid-phase extraction:
An SPE cartridge SPE-pakvac 1 cc (100mg) was conditioned with 2.5 ml of methanol and 2.5ml of HPLC – grade water. The final extract (14ml) was applied into the cartridge. When completing the extract loading, the cartridge was washed consecutively with 3ml of HPLC-grade water, 3ml of 0.2M Na_2HP04 (PH9) and 5ml of HPLC-grade water. The cartridge was subsequently dried by air aspiration. Enrofloxacin was eluted with 3.5 ml of MeoH, the elute was evaporated to dry under nitrogen stream. The dry residue was redis solved in 200 ml of 0.2M Na_2HPO4 (PH9). The test tube was vortex-mixed for 30 second and then centrifuged at 4400c for 5 min. at 4°C. The supernatant was transferred to an injection vial and 30 ml was injected into the HPLC system.

Enrofloxacin determination was performed by using a HPLC system consisting of a waters prep Lc4000 and a spectroflow 783 UV-vis detector. The detection wave length was set at 277nm and the mobile phase used was water – CAN – TEA C83:14:0.45 V/V. The flow-rate was 1ml/min.

Preparation of standard stock and working solutions: stock solutions of Enrofloxacin were prepared after Salehzadeh *et al.* (2007).

Effect of Heat treatment on residues.

20 gm of 8 liver samples which contain the maximum levels of Tilmicosin and Enrofloxacin residues (4 for each) were placed in straners and exposed to boiling for 30 minutes and autoclave for 15 minutes under atm. pressure 1.5. (2 samples were exposed to boiling and 2 to autoclave of each antibiotic residue type).

RESULTS

Table 1: Mean concentration of Tilmicosin and Enrofloxacin residues (ppb) in liver and muscle samples (20 for each)

Antibiotic residue	***Examined tissue***	
Tilmicosin	***Liver***	***Muscles***
Mean ± SD (PPb)	481.88 ± 54.81	ND
% of Tilmicosin Positive Samples	100	0
% of Tilmicosin-Positive Samples over MRL	0	0
Enrofloxacin	***Liver***	***Muscles***
Mean ± SD (PPb)	8.288 ± 1.47	ND
% of EnrofloxacinPositive Samples	66.66	0
% of Enrofloxacin-Positive Samples over MRL	0	0

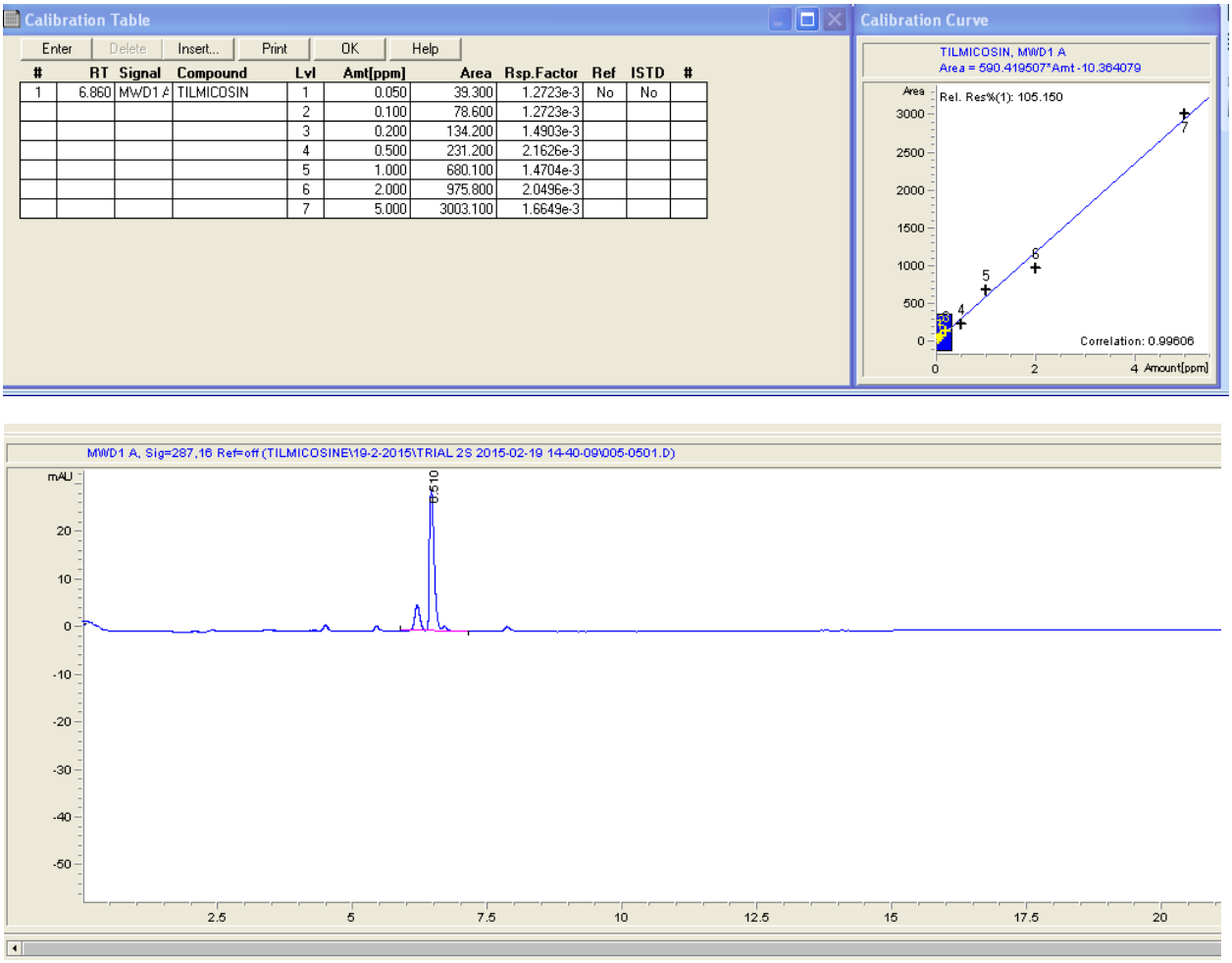

Figure 1: Calibration curve, table and chromatogram of Tilmicosin standard std 0.5 ug /g

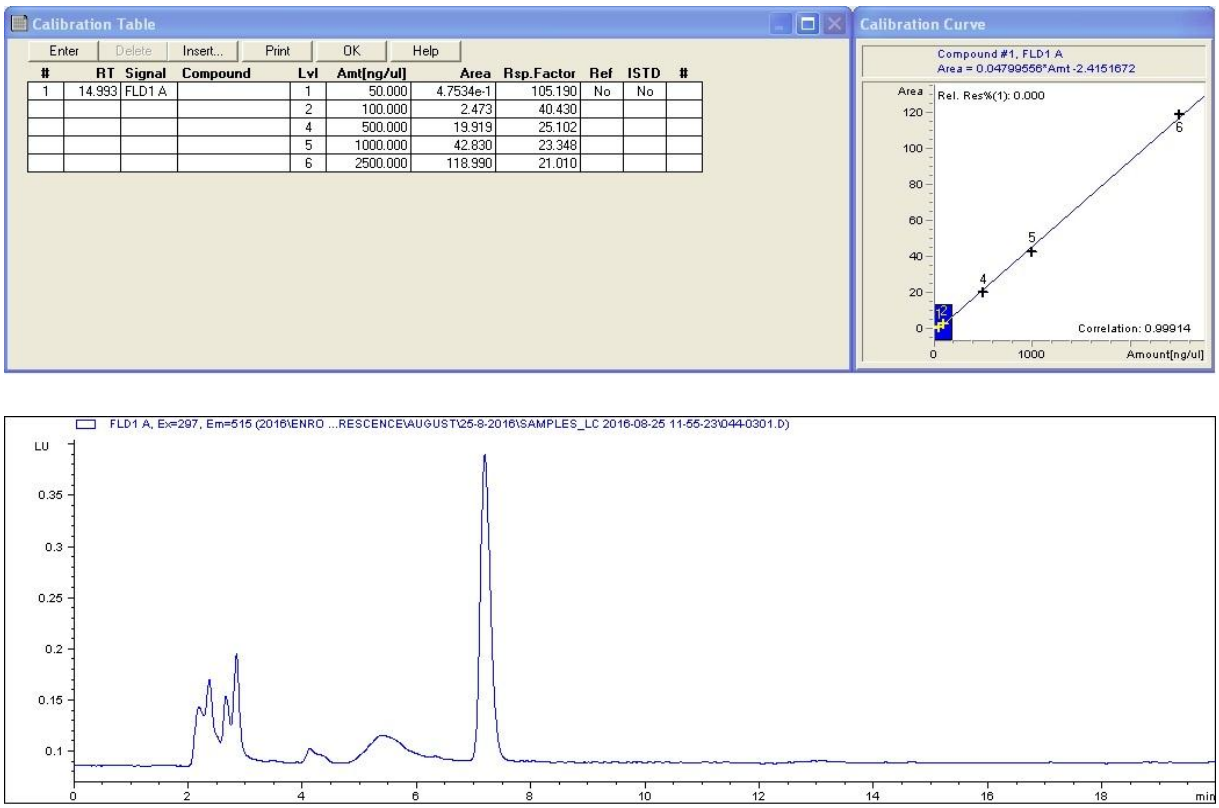

Figure 2: Calibration Curve, table and Chromatogram of Enrofloxacin extract of broiler liver after using of autoclave for 15 min.

The results of this study (Table 1) indicated that Tilmicosin was detected in all liver samples while Enrofloxacin was detected in only 66.66% of liver samples. Tilmicosin and Enrofloxacin redisues were undetectable in all muscle samples. The mean Tilmicosin concentration was 481.88 ± 54.81PPb. while mean Enrofloxacin concentration was 8.288 ± 1.47 PPb in liver samples.

Effect of heat

After heat treatment (boiling for 30 min. and autoclaving for 15 min. under atm. pressure 1.5) of the highest levels positive samples for Tilmicosin and Enrofloxacin residues, all heat treated samples were negative and didn't show any detectable concentrations after analysis with the same procedure.

DISCUSSION

Extensive use of antibiotics during chicken breeding leads to their residues problem. This problem is the focus of public health concern which introduces a serious and novel hazard to the human beings. The residues comprises the non-altered parent compound, metabolites and or conjugates (Haagsma, 1993).

Extensive administration of Tilmicosin not only leads to residues in the chicken tissues but also resistance of the microorganisms. So, Gerchman *et al.* (2011) found that 72% of the strains of *Mycoplasma gallisepticum* isolated from chinical samples since 2006 showed acquired resistance to Tilmicosin.

The problem of antibiotic residues in feedstuffs is currently of some magnitude in different parts of the world, particularly due to associated public health concerns that include hyper sensitivity, hepatotoxicity, teratogenicity and carcinogenicity (Darwish *et al.,* 2013). Also, human exposure to animal products containing significant level of antibiotic residues may proven immunological response in susceptible individuals and cause disorder of intestinal flora Salehzadeh *et al. (*2007). Drug residues mainly caused by improper use or insufficient withdrawal period (WDT). These residues accumulated in edible parts of the food producing animals as in tissues or milk at concentration levels higher than the maximum residual levels (MRL). According to the veterinary drug residue regulations of the Chinese ministry of Agriculture and the European Union, the maximum residue levels (MRLs) of Tilmicosin in broiler chicken muscles, liver and kidney are 0.075, 1.0, and 0.25 Ug/g, respectively and the recommended withdrawal time is 10 days (Zhang *et al.,* 2004). The obtained results in table 1 indicated that all liver samples were contained Tilmicosin residues below the maximum residual levels with a mean value of 481.88 ± 54.81 PPb. This result was in accordance or similar to that of Yamaguchi *et al.* (2015) who

analyzed 28 antibiotic residues using a LC-Ms/Ms screening method and detected Tilmicosin residues at ranges of 150-450 micro g/kg (PPb) in chicken samples, muscle samples were free or contain undetectable levels of Tilmicosin residues.

Enrofloxacin residues also were detected only in liver samples with a mean value of 8.288 ± 1.47 PPb, while muscle samples didn't give any detectable values. The European commission has established MRLs of the sum of Enro and Ciprofloxacin (one of Enrofloxacin metabolites) for chicken muscle and liver tissues with 100 micro g/kg and 200 micro g/kg respectively, while Japan has defined in all chicken tissues MRLs of 10 micro g/kg. (Ministry of Health and welfare, 2005). The fixed MRL for Enrofloxacin by VMD (Veterinary Medicine Directorate) of the European union is 30 ng/g. (Salehzadeh *et al*., 2007).

On the other hand, four days withdrawal period is the allowed time for Enrofloxacin concentration to decrease to an acceptable level (below MRL) in the chicken meat and liver prior to slaughter. (Petrovic *et al.*, 2006).

After four days withdrawal period, most of the metabolites were excreted but residues of Enro and Ciprofloxacin still persisted in tissues at a concentration below the MRL. (Morales-Gutierrez *et al*., 2015).

The obtained results in the present study declared that the mean residual level of broiler chicken liver samples was below the MRLs, while muscle samples were free or may contain undetectable residues.

These results disagreed with Salehzadeh *et al*. (2007) who detected Enrofloxacin residual levels above the MRLs in muscle, liver and kidney.

Absence of the residues from the chicken muscles was in accordance with the result of Amjad *et al*. (2005) who screened quinolones residual antibiotics in broiler muscle, kidney and liver samples and found that Enrofloxacin residues were absent in all samples. These results of the residual levels of Tilmicosin and Enrofloxacin in broiler chicken liver which were below the MRLs may because of slaughtering of the birds at the end of the withdrawal times of both antibiotics.

Absence of Enrofloxacin and Tilmicosin residues in examined muscle samples may be because of not all tissues incorporate antibiotics at the same concentrations. Among poultry tissues, liver had the highest level of antibiotic residues in comparison to other tissues and organs (Sattar *et al*., 2014). They found that Enrofloxacin residues were 40% in liver, 34% in kidneys, 22% in thigh muscles and 18% in breast muscles.

For Tilmicosin, Fricke *et al*. (2008) reported that the highest level of Tilmicosin was found in liver followed by kidney, lungs and muscles. Also, Zhang *et al.* (2004) found that Tilmicosin residual levels were highest in liver and lowest in muscle which suggested that liver should be the targest tissue for Tilmicosin residues in broiler chickens. They mentioned that Tilmicosin residues were eliminated from muscle very quickly but from liver very slowly. They found that Tilmicosin residue in muscle decreased to the approved level 2 days of withdrawal time while in liver after 9 days. So, Said *et al.* (2016) found that Tilmicosin could not be detected at the 9th day after the last dose except in liver.

After heat treatments (Boiling and using of autoclave) all samples were free from the residues. This result was inaccordance with that of Haagsma (1993) who stated that the content of residues of many veterinary drugs decreased as a result of food preparing and processing. Also, inaccordance with khan *et al.* (2015) who found that the maximum depletion of ciprofloxacin occurred after boiling and frying followed by grilling and steaming of broiler meat. The same with Aboul El Nile (2006) who studied the effect of boiling for 30 minutes on Ciprofloxacin residues in chicken tissues and found that boiling process had good effects in decreasing or disappearing of residues. Also, Javadi *et al.* (2011) analyzed chicken samples contained Enrofloxacin residues after various cooking procedures and detected reduction in their concentration. They found that the most reduced residues in the cooked meat samples were related to boiling process and for cooked liver samples were related to roasting process while the highest detectable amount of residues belonged to microwaving process in all cooked samples. They also found that cooking time and temperature could play major roles about antibiotic residues reduction.

For Tilmicosin, the recorded results were similar to that of Heshmati *et al.* (2014) who found that cooking processes load to reduction in Tilmicosinresidues levels and reduction amount might differ according to various cooking methods. They found that in boiling method, Tilmicosin reduction percentage became more by increasedtime. However, it was inversely related to Tilmicosin initial concentration. Also the results were inaccordance with Hassan (1995) who reported that boiling of chicken tissues and organs contain Tylosine residues for 30 minutes completely degraded Tylosine residues in all tissue samples.

CONCLUSION

Great care must be taken to espies antibiotic withdrawal time before slaughtering of the birds and

release them for human consumption. Also, strict legislation must be implemented to minimize the aduse of antibiotics. Heat processes especially boiling for long periods or using cookers with pressure may be profitable for minimizing antibiotic residues.

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الكشف عن متبقيات التلميكوزين والإنروفلوكساسين في كبد ولحوم دجاج التسمين المباعة بالأسواق بمدينة بنها

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تم تجميع ٤٠ عينة من كبد وعضلات دواجن اللحم (٢٠ عينة لكل منها) من الأسواق الشعبية بمدينة بنها (مصر) وذلك لاكتشاف وتحليل متبقيات التلميكوزين والإنروفلوكساسين باستخدام جهاز كروماتوجرافيا السائل عالي الكفاءة (HPLC) وبيان آثار العمليات الحرارية عليها. وقد وجدت متبقيات التلميكوزين في كل عينات الكبد بتركيز متوسط ٤٨١و٨٨ ± ٥٤و٨١ جزء من البليون بينما وجدت متبقيات الإنروفلوكساسين في ٦٦و٦٦% فقط من عينات الكبد بتركيز متوسط ٨و٢٨٨ ± ١و٤٧ جزء من البليون. أما عينات العضلات فكانت كلها خالية من كلا النوعين من المتبقيات. وبعد الغليان لمدة ٣٠ دقيقة واستخدام جهاز الأوتوكلاف لمدة ١٥ دقيقة تحت ضغط جوى ١,٥ كانت كل المتبقيات غير موجودة. وقد أكدت هذه الدراسة على أهمية التحليل الدقيق لمتبقيات مضادات الميكروبات قبل تسويقها وكذلك أكدت جدوى الطبخ الحراري باستخدام الضغط أو بدونه في الاختفاء الكلي أو الجزئي للمتبقيات.