ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Determination of OTC residues in broiler chicken edible tissues by HPLC-UV.

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ABSTRACT

Our work was developed to determine residues of oxytetracycline in in broiler chicken edible tissues and to evaluate the effect of heat treatment on the persistence of these residues. A total of 600 samples of liver, kidney and muscle tissues from broiler chickens were analysed using a microbiological method as a screening method, which revealed a percentage of 37.5%, 30.5% and 23% of positive samples in the liver, kidney and muscle samples, respectively. Chromatographic analysis of the positive samples demonstrated that mean oxytetracycline residues were estimated to be 33071.4643 ± 7.948, 84799.32 ± 4.902 and 4966.55 ± 0.614 µg / kg in the liver, kidney and muscle, respectively. Thus, our results show that the boiling or the reezing of the positive samples causes a significant decrease in the oxytetracycline residues. On the other hand, the frying of the positive samples induces a very significant reduction of the oxytetracycline residues. Hence, methods of cooking (boiling and frying) and freezing induce partial destruction of antibiotic residues. Therefore, those methods can not be used as reliable methods to get rid of antibiotic residues in broiler meat and the only way to preserve consumers' health is to extend the waiting period.

Keywords: Oxytetracycline, *Bacillus Cereus*, HPLC-UV, Boiling, Frying, Freezing.

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ISSN: 0975-8585

INTRODUCTION

Antibiotics are defined as chemical substances produced by microorganisms or representatives of synthetic products which, at very low doses and in a specific manner, have the power to inhibit growth or even kill bacteria, without affecting the host [1]. However, their effectiveness is threatened by excessive and inappropriate use in poultry farming. In veterinary practice, antibiotics are mainly used at therapeutic levels to treat diseases and prevent infections. Also, they are used by poultry farmers at sub-therapeutic levels to increase food efficiency, promote growth and prevent disease during periods of increased vulnerability [2].

Antimicrobial drugs are used by poultry farmers without the control or the intervention of a veterinarian, often ignoring withdrawal periods. These results in the deposition of antimicrobial residues in the tissues of meat and other products derived from animals [3].

Tetracyclines are broad-spectrum bacteriostatic antibiotics with an activity against a wide range of Gram-positive and Gram-negative bacteria [4]. The abuse of tetracyclines by poultry farmers has become a serious public health problem as regards the possibility of contamination of food products by their residues [5], which could induce various toxic effects on the consumer, including gastrointestinal disorders, fetus' teratogenic risk, allergic reactions, bone and dental problems associated with binding to the ion of calcium and disturbance of the intestinal microflora [6]. In addition, low doses of antibiotics in foods consumed for long periods of time may lead to the spread of drug-resistant microorganisms [7].

In many African countries, including Algeria, the use of antibiotics by farmers has been reported. This abuse has led to the spread of illegal auto-medication among farmers. In addition to the appearance of resistant bacteria including Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae and Salmonella enterica which have been found to be increasingly resistant to commonly used antibiotics.

Faced with this misuse of veterinary drugs and the lack of information on antibiotic residues in meat in Algeria. This work aims to detect and to quantify the oxytetracycline residues in the liver, muscle and renal tissues of broiler chickens, as well as the evaluation of the effect of freezing, boiling and frying on the persistence of these residues.

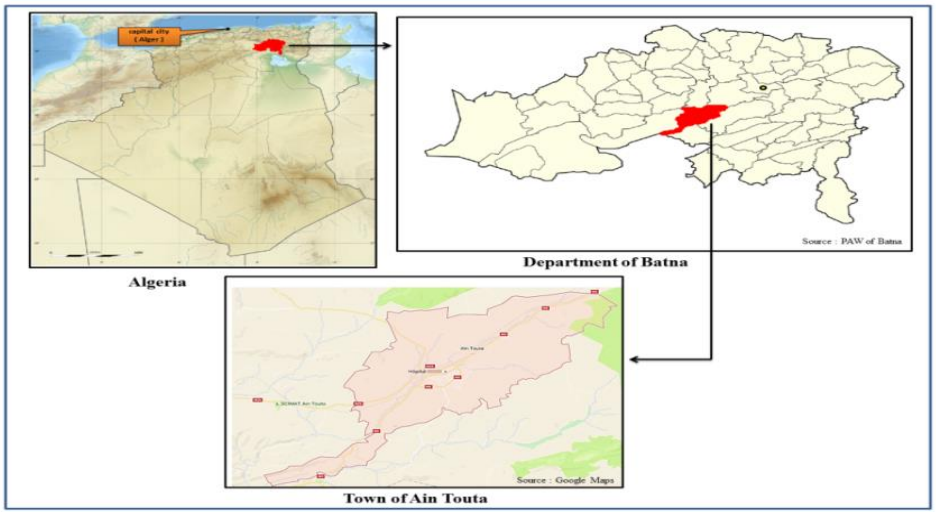

Fig. 01. Location map of the town of Ain Touta

MATERIAL AND METHODS

Sampling

A total of 600 samples of liver, kidney and muscle tissue (200 from each tissue) from broiler chickens collected from farms in the region of Ain Touta (Batna, Algeria) from January 2016 to May 2016, by random sampling methods. The map below shows the plan situation of Ain Touta.

The chickens were kept in closed sheds deprived from daylight and received a diet rich in vitamin D in order to overcome their deficiency caused by the lack of exposure to daylight.

To accelerate chick growth and minimize rearing time, Oxytetracycline is administered as a dietary supplement in the range of 30 mg / kg body weight for 45 days.

These chickens receive a diet in the form of cereal granules or flour in high energy crumbs; rich in protein and containing lysine, methionine, calcium, phosphorus, fats and cellulose with proportions adapted to each rearing period.

After 45 days, 200 chickens of 2500 to 3000 g of body weight were slaughtered and the samples collected were preserved in sterile, labeled amber flasks containing a phosphate buffered saline (PBS) solution and stored at -20 °C until analysis.

Reagents and chemical products

Oxytetracycline hydrochloride (95% purity) was purchased from Sigma Aldrich (St. Louis, Missouri, USA) to be used as an analytical reference standard. Methanol and acetonitrile were HPLC grade and were obtained from Merck (Darmstadt, Germany). The sodium hydrogen phosphate, monohydrate citric acid, Na_2 EDTA and trichloroacetic acid were of analytical grade and were acquired from Sigma Aldrich (St. Louis, Missouri, USA).

Extraction and precipitation procedure

Samples were prepared based on an extraction method developed previously and described by *Yuan & al.* [8] including some changes.

Previously collected samples were ground at 3000 rpm during 3 minutes using an Ultra Turrax mill (IKA, Germany).

A five grams quantity of each homogenized sample was weighed in a centrifuge tube made of polypropylene to which 20 ml of EDTA-McIlvaine buffer solution 0.1M (pH 4.0) was added, consisting of 12.9 g of citric acid monohydrate, 10.9 g of sodium hydrogen phosphate and 37.2 g of Na_2-EDTA in 1 litre of distilled water, the suspension was then blended using a stirrer (IKA, Germany) at 1200 revolutions per minute during 10 minutes. The mix was next shaken in a vortex (IKA, Germany) for 2 minutes at 1600 revolutions per minute (rpm) and then left at room temperature for 3 minutes to ensure a proper distribution of the matrix.

The tubes were consequently centrifuged at 3000 rpm for 10 minutes at a temperature of 10°C using a thermostatic centrifuge 3-16KL Sigma (Germany). The supernatants were carefully collected in new centrifuge tubes.

Two further extractions were carried out but with 20 ml and then 10 ml of EDTA-McIlvaine buffer solution (pH 4.0).

Once the supernatants from all three extractions were collected, they were stirred in vortex devices for 2 minutes with 2 ml of 20% trichloroacetic acid used as a deproteinization agent. After this, they were centrifuged at 3000 rpm for 15 minutes at 10 °C. The supernatants were then filtered through Whatman 541 paper filter (90 mm) for the solid-phase extraction step.

Solid-phase extraction

The extracts previously prepared were loaded into Strata cartridges preconditioned with 5 ml of methanol, to which was added 5 ml of doubly distilled water. After loading the samples, cartridges were cleaned with 10 ml of 5% methanol in water, and then the Oxytetracycline was eluted with 10 ml of methanol containing 0.01 M oxalic acid.

The resulting solutions were filtered through a membrane filter of 0.45 µm, and subsequently transferred to amber bottles, ready for introduction into the LC-UV system.

Microbiological detection

The pre-selection of meat samples for the presence of antibiotic residues was carried out using a microbiological test based on the inhibition of the bacterial growth of the *Bacillus Cereus* strain by any antimicrobial residues present in the samples.

A pure strain of *Bacillus Cereus* was obtained from the microbiology department at the University Hospital of Batna (Algeria).

A volume of 15 ml of nutrient agar were poured into sterile petri dishes (90 mm in diameter). Then, using a sterile swab, a pure strain of *Bacillus Cereus* is taken and the box is inoculated by rubbing the swab over the entire surface of the agar by rotating the box three times by 60 °.

A number of 6 discs of Buvard paper with a diameter of 6.35 mm impregnated with the extracts to be analysed were deposited in the layer of agar after solidification. The discs are at a distance less than 30 mm from one another. After pre-diffusion of about 1 hour, at room temperature, the dishes were incubated at 37 °C for 24 hours.

After incubation, the presence of antibiotic residues in the samples leads to the formation of a transparent zone around the disk known as the zone of inhibition and which corresponds to a complete inhibition of bacterial growth.

The inhibition zones are measured using a digital caliper (HDCD01200, INGCO). Samples with a zone of inhibition greater than or equal to 2 mm in diameter were considered as positive. While the samples with a zone of inhibition totally less than 2 mm in diameter were considered as negative.

Chromatographic confirmation

HPLC and chromatographic conditions

The samples were analysed using a high performance liquid chromatography system, an HPLC Shimadzu LC-10A (Japan) type, equipped with an automatic air vent (DGU-20A5), two high pressure pumps (LC-10 ATvp) , a UV/VIS detector (SPD-10AVvp), an integrator (SCL-10Avp) and an injector provided with an injection loop of 20 µl. The chromatographic separation was performed on an analytical reversed phase column C18 (bonded silica gel) Shimadzu VP-ODS type (4.6 × 250 mm, 5 mm), with isocratic elution of an oxalic acid solution (10 mM) / acetonitrile (pH 2) (80: 20, v / v) used as a mobile phase. The injection volume was 11 µl for all standards and sample extracts with a flow rate of 1.2 ml/min and an analysis time of 6 minutes. Peaks were detected at a wavelength of 355 nm. All samples, solvents and standard solutions have been filtered through a membrane filter SUPELCO type (0.45µm) before use. Data acquisition was controlled by the software Chromatography Workstation Class-VP, Release 6.12, SP1 (Shimadzu Corporation, Japan).

Standard solution

The Oxytetracycline standard solution was prepared by dissolving 10 mg of pure standard solution in 10 ml of methanol to obtain a final concentration of 1 mg/ml. The standard solution was preserved in amber bottles to protect them from photo degradation, and then stored at -20 °C. This solution was considered stable for one month, after which it was replaced with a new fresh solution.

Subsequently, a series of standard solutions 600, 300, 150, 75, 30 and 0.1 µg/ml was prepared to draw the calibration curve and assess linearity.

Quantification

Quantification of Oxytetracycline in the matrices has been based on established standard peak areas which have been used to determine a standard curve and thus linearity of the variation of the peak area as a function of the variation in Oxytetracycline concentrations in different matrices.

Heat treatment and freezing of positive samples

The samples confirmed positive by HPLC were subjected to a heat treatment (boiling and frying), carried out according to the method previously described by **Lolo M. et al. [9]**. Since they were subjected to one-month freezing at -20 °C.

Boiling: 20 g of each of the revealed positive samples were placed in a colander and then immersed in a water bath preheated to 100 °C which was boiled for 30 minutes. Following this, the samples were removed and allowed to cool.

Frying: 20 g of each of the revealed positive samples were fried in a pan containing an appropriate amount of cotton seed oil preheated to 200 °C for 10 minutes and then removed and allowed to cool.

Freezing: 20 g of each of the positive samples were frozen at -20 °C for one month.

Thus, treated samples were analysed again by HPLC-UV.

Statistical analysis

Our results are expressed on average ± SEM. The difference between the results was analysed by the Student test.

RESULTS

Microbiological analysis:

Our results show that 74, 61 and 46 samples from 200 liver, kidney and muscle samples, respectively, demonstrated antimicrobial activity with a percentage of 37.5%, 30.5% and 23%, respectively. These results are given in Table I.

Table 1. Mean zones of inhibition (mm) of antibiotic residues present in the examined samples

Examined Samples	Number of samples	Positive samples		Inhibition zone (mm)	
		Number	Percentage	Average	SEM
Liver	200	74	37.5%	4.5148	0.1591
Kidney	200	61	30.5%	3.6918	0.1216
Pectoral Muscle	200	46	23%	3.2913	0.0447

Therefore, our results revealed that the mean of the inhibition zones recorded in the hepatic tissue samples was significantly higher than the average of the inhibition zones observed in the renal tissue samples (4.5148 ± 0.1591 vs 3.6918 ± 0.1216 mm) and muscular ones (4.5148 ± 0.1591 vs 3.2913 ± 0.0447 mm) ($p \leq 0.05$).

The mean of inhibition area observed in renal tissue was greater than the average of inhibition area observed in muscle tissue samples. This difference was not significant.

Chromatographic analysis:

The relationship between the peak area and the OTC concentration was linear ($r^2 = 0.9993$, n = 6) on a calibration series of 0.1 to 600 µg / ml. The curve was described by equation (Y = 32.04 x + 186.9), where "Y" represents the area of the peak and "x" the concentration in (µg / kg). The correlation coefficient was satisfactory and indicated that this procedure was reliable for quantitative detection (Figure 02).

ISSN: 0975-8585

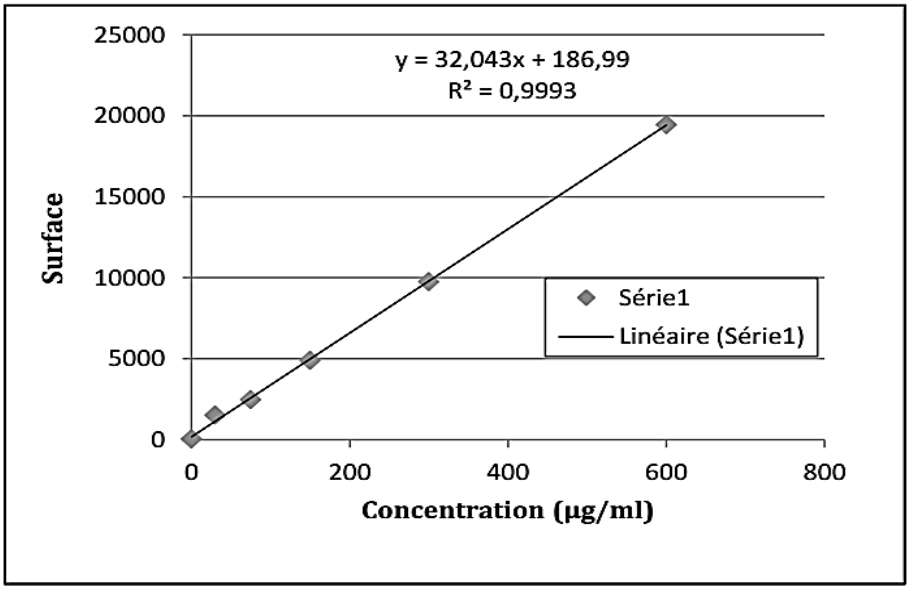

Fig. 02. OTC Calibration Curve

The results obtained show that oxytetracycline was detected in 14, 5 and 2 samples of liver, kidney and muscle, respectively, having previously demonstrated antimicrobial activity. Thus, representing a percentage of 18.91, 8.19% and 4.34%, respectively.

These results demonstrate that in broiler chickens receiving a concentration of 30 mg / kg body weight oxytetracycline by oral route during 45 days of rearing, the mean concentration of oxtetracycline detected by HPLC-UV was 33071.4643 ± 7.948, 8479.32 ± 4.902 and 4966.55 ± 0.614 µg / kg in the liver, kidney and pectoral muscle, respectively.

So, we note that the mean concentration of oxytetracycline exceed the Maximum Residue Limit (MRL) set by the World Health Organization (WHO) which is 600, 1200 and 200 µg / kg for the liver, kidneys and Muscles, respectively.

Following the statistical analysis of the results, we noted that the mean concentration of oxytetarcycline in the liver (33071.4643 ± 7.948 µg / kg) was significantly higher than its concentration in the kidneys (8479.32 ± 4.902 µg / kg) and in the pectoral muscles (4966.55 ± 0.614 µg/kg) ($p \leq 0.05$).

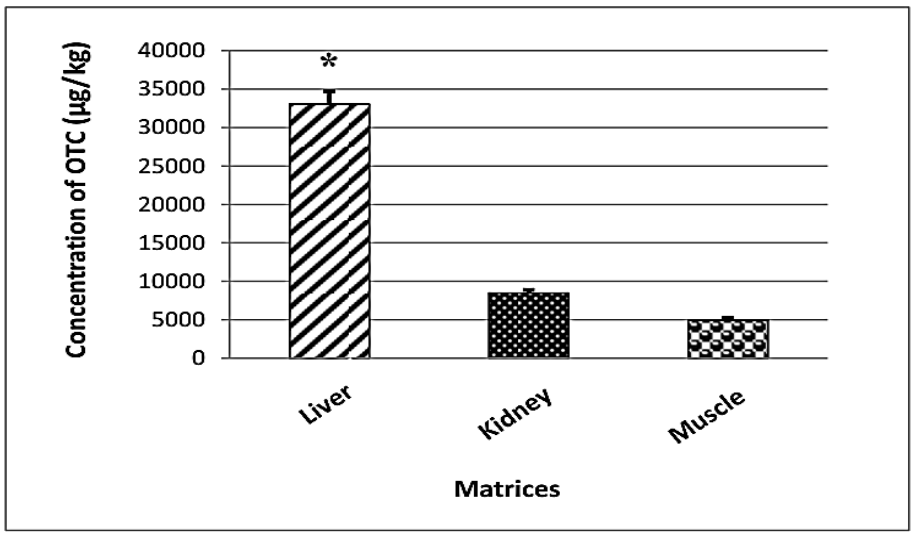

Fig. 03. Concentration of OTC residues in the liver, kidney and pectoral muscle in broiler chickens (mean ± SEM in µg / kg) (*p ≤ 0.05)

ISSN: 0975-8585

The concentration in the kidney samples was higher than the concentration in the muscle. This difference was not significant. These results are shown in figure 03.

Effect of heat treatment and freezing:

Boiling :

After boiling at 100 °C for 30 minutes of samples confirmed positive by chromatographic analysis, it was found that the concentration of oxytetarcycline decreased significantly with a percentage of 65.5%, 51.37% and 67.41% in the liver, kidney and pectoral muscle samples, respectively.

The mean oxytetracycline concentration was reduced from 33071.46 ± 7.948 to 11393.0714 ± 3.910, from 8479.32 ± 4.902 to 4123.663 ± 1.829 and from 4966.55 ± 0.614 to 1618.9 ± 1.136 µg / kg for liver, Kidney and pectoral muscle samples, respectively. However, boiling did not reduce the oxytetarcycline residues concentrationbelow the MRLs recommended by WHO. These results are demonstrated in figure 04.

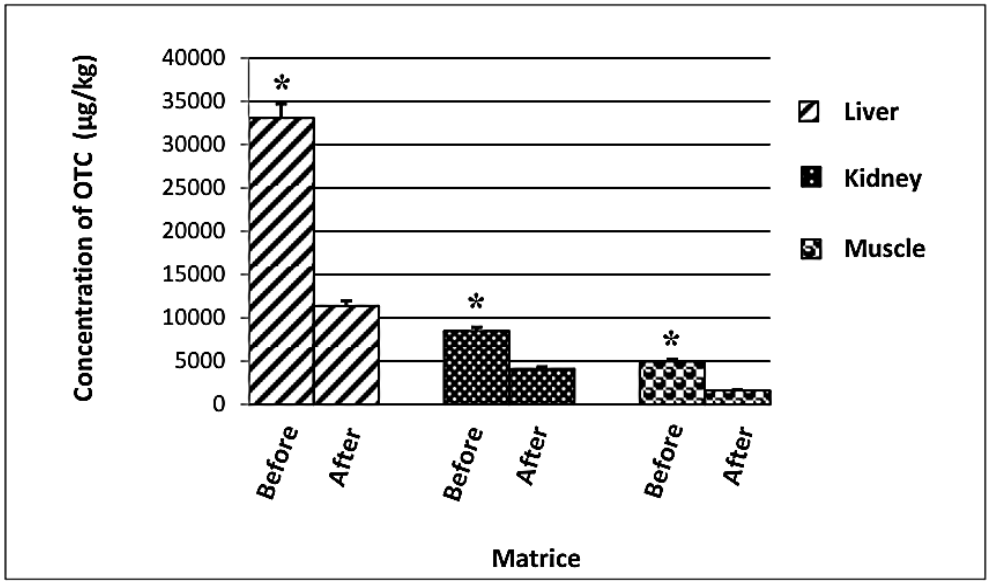

Fig. 04. Effect of boiling at 100 °C for 30 minutes on the concentration of oxytetracycline residues (mean ± SEM in µg / kg) (*p ≤ 0.05)

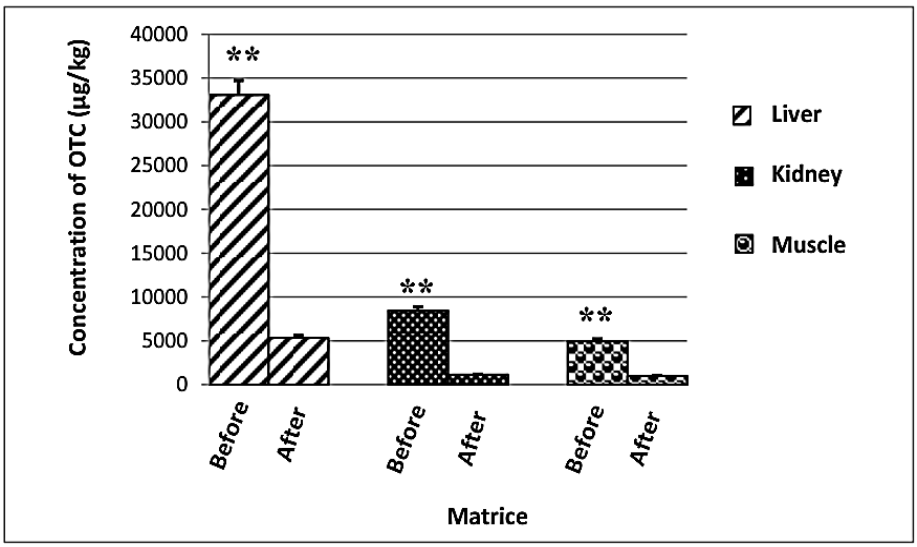

Fig. 05. Effect of frying at 200 °C for 10 minutes on the concentration of oxytetracycline residues (mean ± SEM in µg / kg) (p ≤ 0.01)**

ISSN: 0975-8585

Frying :

After frying the samples in cotton seed oil at 200 °C, oxytetracycline concentration was shown to decrease very significantly with a percentage of 83.84%, 78.09% and 80.17% for liver, kidney and muscle, respectively.

The mean concentration of oxytetracycline residues was reduced from 33071.4643 ± 7.948 to 5345.55 ± 1.499, from 8479.32 ± 4.902 to 1147.9 ± 1.499 and from 8479.32 ± 4.902 to 1147.9 ± 1.280 µg / kg for liver, kidney and muscle samples, respectively. We also note that frying decreased the concentration of OTC below the MRLs recommended for the liver and kidneys but not for the muscle samples. These results are demonstrated in figure 05.

Freezing :

After freezing the samples at -20 °C for a period of one month, It was demonstrated that the mean concentration of oxytetracycline residues decreased significantly with a reduction percentage of 52.28%, 31.46% and 44.63% for liver, kidney and muscle samples, respectively.

The mean concentration of oxytetarcycline residues was reduced from 33071.4643 ± 7.948 to 15783.0071 ± 3.152, from 8479.32 ± 4.902 to 5812.1 ± 3.116 and from 4966.55 ± 0.614 to 2750.4 ± 1.318 µg / kg for liver, kidney and muscle samples, respectively. However, freezing did not reduce the concentration of oxytetarcycline residues below the MRLs recommended by WHO. These results are demonstrated in figure 06.

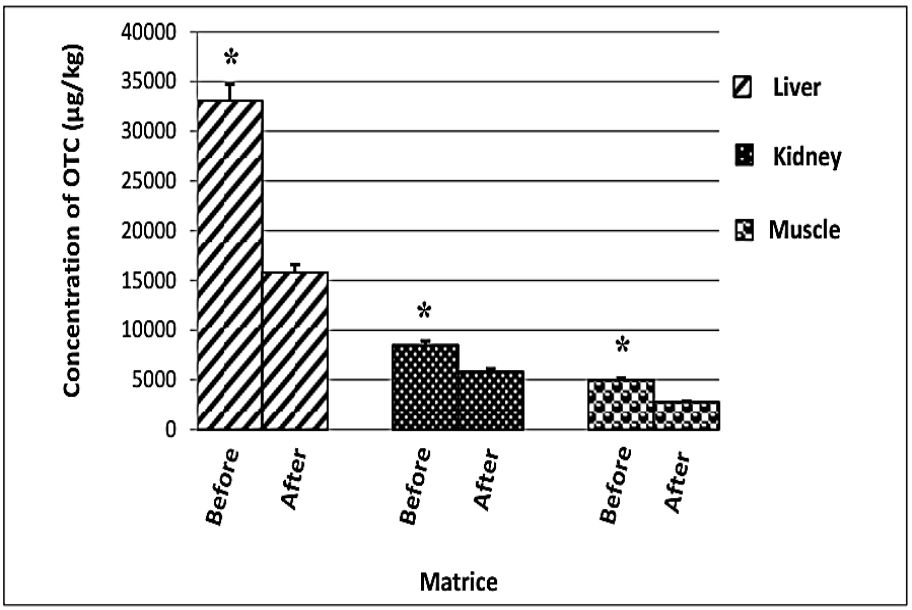

Fig. 06. Effect of freezing at -20 °C for one month on the concentration of oxytetracycline residues (mean ± SEM in µg / kg) (*p ≤ 0.05)

DISCUSSION

Intensive poultry farming, motivated by the continued high demand for poultry, has resulted in a considerable increase in the use of veterinary antibiotics. Thus, the consumer is exposed to residues of antibiotics which may have an adverse effect on his health.

The results obtained during this work show that the oxytetracycline residues were detected at a significantly higher concentration in the liver. These results are in agreement with those obtained by *Sattar & al.* [10], *Olatoye & Basiru* [11] and *Cheong & al.* [12] who reported that the highest concentration of antibiotic residues was present in the liver, they concluded that this could be attributed to the fact that the liver is responsible of the metabolism and detoxification of drugs by microsomal enzymes. Indeed, the liver is the site

of metabolism of oxytetracycline. This residual level could decrease by increasing the waiting period before slaughter.

On the other hand, the oxytetracycline concentration in the kidney samples was higher than its concentration in the muscles, although this difference was not significant. Similar results have been reported by *Villa & al.* [13] and *Morshdy & al.* [14] who reported that high concentrations of oxytetracycline were recorded in kidney samples. This was attributed to the fact that the kidneys are the route of excretion of oxytetracycline.

The mean concentration of oxytetracycline residues detected in the liver, kidneys and muscles in broiler chickens by HPLC was greater than the MRLs recommended by WHO. Although, the daily doses received by these chickens were less than the acceptable daily intake of oxytetracycline. This demonstrates that long-term exposure, even at levels below the acceptable daily intake (ADI), predisposes the consumer to the persistence of antibiotic residues in foods. These results are consistent with those of *Salehzadeh & al.* [15] which indicated that residual levels in chicken meat could constitute a potentially serious threat to the health of consumers. Indeed, exposure of the intestinal microflora to antimicrobial residues could contribute to the colonization of the intestine by resistant bacterial strains, compromising antimicrobial therapy in humans by exerting selection pressure on the intestinal microflora, thus promoting growth of antibiotic-resistant micro-organisms.

Basyoni & Barr [16] stated that the most common causes of the presence of antibiotic residues in food of animal origin are failure to observe the waiting period, overdose of antibiotics and uncontrolled use of antibiotics as growth factors or for prophylaxis.

Most foods of animal origin are cooked to increase digestibility, appetite and duration of the conversation. In recent years several studies have been initiated to evaluate the impact of heat treatment on the persistence of antibiotic residues. The data obtained in our current work, demonstrate that the boiling of the positive samples at a temperature of 100 °C for 30 minutes as well as their freezing at -20 °C, induce a significant decrease in the level of oxytetracycline residues. It was also observed that frying these samples at 200 °C for 10 minutes in cotton seed oil caused a very significant decrease in oxytetracycline residues. These results are in agreement with those obtained by *Nashwa* [17] and *Javedi* [18] which reported that boiling decreased significantly the residual levels of oxytetracycline in turkey meat. *Elkahky & Allam* [19] revealed that frying had a destructive effect on antibiotic residues. *Marouf & Bazalou* [20] concluded that the direct heat effect of frying oil was more effective than boiling water. On the other hand, our results do not agree with those obtained by *Moats* [21] and *Gehan* [22] who mentioned that ordinary methods of cooking meat could not inactivate even the most sensitive compounds such as penicillin and tetracycline.

A subsequent study by *Gergis-Aida* [23] on the effect of heat treatment such as boiling and frying demonstrated that the heat treatment decreased the concentration of antibiotic residues or induced inactivation of the latter depending on the degree of temperature and the time of exposure. In addition, studies conducted by *Pavlov & al.* [24] and *Morshdy & al.* [14] have demonstrated that the freezing of chicken meat could reduce the concentration of antibiotic residues by causing partial degradation of these residues.

In conclusion, although the methods of cooking (boiling and frying) and freezing, induced a decrease in the residual level of antibiotics, they are not reliable methods of getting rid completely of these residues, because they only cause partial degradation of antibiotic residues. In fact, antibiotics must be used with caution and it is imperative and essential to respect the waiting period so that the antibacterial drug is metabolized and excreted by the body. This in order to preserve the health of the consumer and to stop the emergence of resistant bacterial strains.

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