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Effect of cooking methods on tetracycline residues in pig meat

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Tetracyclines (TCs) are widely used for disease control in the livestock and poultry industry due to their broad spectrum of activity and affordability. However, some residues have been found to remain in the animals during slaughter and subsequent consumption. The potential of different cooking methods as strategies to reduce TC residues in pork were therefore investigated. Samples of pig muscles containing oxytetracycline (OTC), TC, chlortetracycline (CTC) and doxycycline (DC) residues were subjected to boiling, deep-frying or microwaving and the residues were extracted in a mixture of McIlvaine buffer-ethylenediaminetetraacetic acid (EDTA)/methanol (75:25, v/v), and then analyzed by high performance liquid chromatography (HPLC)-diode array detection on a XBridge™ C_{18} reverse phase chromatographic column. Results show that TC residues in muscles were reduced by 45.35 to 67.05% after boiling for 9 min, 38.17 to 65.74% after deep-frying for 9 min and 38.17 to 48.47% after microwaving for 1 min. It can therefore be concluded that from a safety and toxicological point of view, reduction of TC residues in pig muscle is an additional advantage of cooking as a food processing method and it is recommended that more studies in other kinds of meats be done.

Key words: Tetracyclines, pig muscle, cooking effects, antibiotics residues.

INTRODUCTION

Tetracycline antibiotics (TCs) have a broad range of activity against variety of gram-positive and gram-negative bacteria and are inexpensive. They are an important group of antibiotics and are widely used in livestock and poultry production. TCs are normally administered via injections, feedstuff or drinking water by veterinarians for therapy, prophylaxis and growth promotion in livestock and poultry (Chopra et al., 2001). The total amount of TCs sold or distributed for use in food-producing animals in the United States in 2009 were 4611892 and 515819 kg for domestic and export markets, respectively (FDA, 2009). The efficacy, availability and affordability of TCs have led to widespread misuse which in turn has led to presence of residues beyond acceptable levels in products of animal origin. Presence of excessive levels of antibiotics and other drug residues in products of animal origin has become a matter of considerable debate in food safety. The residue of these molecules has been correlated to the appearance of allergic reactions, development of bacterial resistances, modifications in intestinal flora, and possible mutagenic and/or carcinogenic effects (Demoly and Romano, 2005). Moreover, the long-term presence of TC residues may generate the evolution of microorganisms with resistance to a wider array of antibiotics (Garcia et al., 2004). To protect consumers from possible health related problems, many countries and economic blocks have set up maximum residue limits (MRLs) of some TCs in animal products. According to the European Union (EU), the MRLs of TC, chlortetracycline (CTC), oxytetracycline (OTC) and

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doxycycline (DC) for all animal species have been set at 100, 300 and 600 ngg^{-1} in muscle, liver and kidney tissues, respectively (Council-Regulation, EEC No. 2377/90/EC of 1990). The corresponding values set by Codex Alimentarius Commission are 200 ngg^{-1} in muscle, 600 ngg^{-1} in liver and 1200 ng g^{-1} in both fat and kidney tissues (FAO/WHO, 2004). The US FDA has set the limit of MRLs of TC, CTC, DC and OTC antibiotic residues at 200, 600 and 400 ngg^{-1} in muscle, liver and milk, respectively (US Code of Federal Regulations, 2003).

Drug residues are procedurally measured on uncooked tissues but most foods of animal origin undergo further processing prior to consumption (thermal or food additive treatments or both) for the purpose of increasing palatability and shelf-life. These treatments are mainly done to ensure safety and quality of the food, since heat will reduce or inhibit microbes in food thus, reducing harm to humans. Several studies have been done to study the effect of heat treatment on antibiotics in meat. Studies have demonstrated the effect of heat treatment on antibiotic residues in meat. Antimicrobial drugs undergo degradation in meat during cooking; while net residues in the cooked muscles have been reported to be reduced up to 40% by boiling, roasting in 12 min each, while by microwaving in 1 min (Furusawa and Hanabusa, 2001). Other studies from different cooking processes reveal a reduction in the concentration of DC residue with some of the residue being excreted from the tissue to the cooking fluid during the boiling process (Javadi, 2011). The biological activity of ampicillin, chloramphenicol and OTC has been found to reduce by 12 to 50% in bovine meat after roasting at 50 to 90°C for 20 min. Factors affecting degradation of antimicrobial drugs include drug formulation and pharmacodynamics, cooking temperature and duration, as well as the shape and thickness of cooked tissues (O'Brien et al., 1981). Rose et al. (1996) reported substantial net reductions of 35 to 94% for OTC in beef and mutton, with temperature during cooking having the largest contribution to this loss. Several studies indicate that thermal treatments may reduce the concentration of veterinary drug residues in food and thus, decrease the potential toxic effects of these compounds on the food consumer (Gratacos Cubarsi et al., 2007). The purpose of this study was to examine the net changes in TC, OTC, CTC and DC residues in pig muscles under boiling, deep-frying and microwaving as cooking methods over different periods of time.

MATERIALS AND METHODS

Chemicals

The chemicals used are acetonitrile and methanol of high performance liquid chromatography (HPLC) grade (Merck Company, Germany), analytical grade oxalic acid, disodium ethylene diaminetetraacetate (Na_2EDTA), and sodium hydroxide (NaOH) and standards of OTC, TC, CTC and DC (Sigma–Aldrich, St. Louis, MO, USA).

Equipment

They include an Agilent Technologies model Series 1200 (Waldbronn, Germany) equipped with a model G1322A online vacuum degasser, a model G1311A quaternary pump system, a model G1367A auto sampler and model G1315B photodiode-array detector. The analytical column was reversed-phase (XBridge™ C_{18} 250 □ 4.6 mm I.D., 5 µm, waters, Ireland). Water purification systems (Mul- 9000 series, USA) online GC 200 expansion vessel for potable water (Global water solution company, USA) and a nano pure Model 7150 (Thermo Scientific). For sample preparation, ULTRA-TURRAX T25 Basic homogenizer (IKA works, Staufen, Germany), Centrifuge Allegra 64R (Beckman coulter, USA), Ultrasonic (KQ-300DE, Kun Shan, China). For cooking processes (C-MAG HP10) (IKA works, Staufen, Germany), Microwave oven (800W, 2450MHz, Galanz, China) were used.

Standard solutions

A stock standard solution of each TC compound was prepared by dissolving 10 mg of the compound in 10 ml of methanol to obtain a final concentration of 1 $mgml^{-1}$. Stock standard solutions were then put in amber glasses to prevent photo-degradation and stored at -20°C and left to stabilize for at least 4 weeks. They were then diluted with methanol to give a series of working standard solutions. Finally, chromatographic solutions for each compound were prepared by dilution of the combined working solution with mobile phase.

Samples

Pork samples were extracted from loin muscles from pigs kept in purely organic farms. All samples were analyzed to ensure that none contained TC residues and stored at -20°C until use.

Cooking operations

Antibiotic free pig muscles were homogenized in a bowl cutter (Model GM 200, Retsch, Germany) and fortified with methanolic stock solution of the different TCs. After this, they were immediately analyzed before further treatment to verify the homogeneity of TC addition in the samples. The mixtures were then made into portions of 10 g pig balls.

Boiling

A 10 g sample (pig ball) was first tempered to an initial temperature of 20 ± 2°C and then immersed in a water bath (Jin Tan, Heng Fing, China) at 100°C for 3, 6 or 9 min.

Deep-frying

A 10 g sample (pig ball) was fried in a non-stick frying pan with sunflower cooking oil at 170°C for 3, 6 or 9 min. No juice was collected.

Microwaving

A 10 g sample (pig ball) was tempered to an initial temperature of 20 ± 2°C and placed at the geometric center of a turntable domestic microwave oven. The sample was cooked under full power (800 W, 2450 MHz) for 0.5, 0.75 and 1 min, respectively. No juice was

collected. After treatment, all samples were immediately placed on an ice bath, and stored at 4°C for subsequent analysis within 1 day. All cooking treatments were done in triplicate.

HPLC analysis

The procedure followed for the extraction of the TCs from the prepared samples had been earlier described (Gratacos Cubarsi, et al., 2007). The procedure for the analysis of OTC, TC, CTC, DC residues in cooked or uncooked pig muscle samples (whole quantity, 10 g) was done as follows: An aliquot of 10 g (accuracy, 0.01 g) of pig muscle sample were cut in small pieces and placed in a glass centrifuge tube. 20 ml of McIlvaine buffer-EDTA/MeOH was added and blended for 3 min with cool homogenizer. Probes were rinsed into centrifuge tubes with 3 ml McIlvaine buffer-EDTA/MeOH solution and the accruing rinsate vortexed for 1 min, and sonicated for 10 min. This was followed by centrifugation at 12100 g for 15 min, then supernatant was collected and the precipitate extracted again after adding 20 ml McIlvaine buffer-EDTA/MeOH as previously described. The combined supernatant was filtered through a filter paper and diluted to a final volume of 50 ml with extracting solution. One (1) ml of the final extract was filtered with a nylon filter (porosity: 0.45 µm) and 100 µl injected into the chromatographic system.

The analytical column was reversed-phase (XBridge^{TM} C_{18} 250 □ 4.6 mm I.D., 5 µm, waters, Ireland) set at a flow rate of 1 ml min^{-1}. The column temperature was 35°C. Mobile phase A was methanol, solvent B was acetonitrile, while mobile phase C was 0.01 M oxalic acid in water. The starting mobile phase composition at 0 min was 10:20:70, methanol/acetonitrile/oxalic acid at 1.0 ml/min. It was switched to 15:20:65 after 10 min and going back to the initial conditions in 5 min. The wavelength of ultraviolet (UV) detector was set at 351 nm.

Calibration curves were prepared daily by injecting chromatographic standard solutions in the range between 50 and 2000 ng injected for each compound and estimates of the amount of the analytes in samples were interpolated from these graphs.

Recovery

Sample recovery was determined with blank pig muscle spiked at 0.5 and 1 µgg^{-1} for the raw samples (10 g) or at 0.5 and 1.0 µgg^{-1} for the samples cooked by boiling for 9 min, deep-frying for 9 min and microwaving for 1 min. The spiked samples were analyzed and the recoveries calculated by comparing the peak area of measured concentration to the peak area of the spiked concentration.

Statistical analysis

A descriptive statistical analysis was applied to the validation data, and the influence of the time or cooking method on concentration of TCs was analyzed by one-way analysis of variance (ANOVA) while means were compared using least significant difference (LSD). All data were analyzed at a significance level of 0.05. All analyses were carried out using the SPSS statistics software version 20.

RESULTS AND DISCUSSION

The procedure employed in this work involved four kinds of TC, all of which were extracted efficiently from both uncooked and cooked samples and accurately measured by HPLC. Typical HPLC profiles of the TCs obtained from the standard solutions and the samples are shown in Figure 1, where the four TCs were well separated by the column with elution times ranging from about 3.5 to 11 min. TCs were identified in the samples by their UV spectrums obtained using the photodiode-array detector and retention times. Linearity was evaluated by calibration curves in the range of 10 to 2000 ng for each compound at five points with triplicate analysis. The obtained results are shown in Table 1. The analysis results further revealed that linear regression coefficients (R^2 > 0.9992) were obtained for all tested analytes. The limit of detection (LOD) is considered to be the quantity yielding a detector response approximately equal to thrice the background noise. The limit of quantitation (LOQ) is the lowest amount that can be analyzed within acceptable precision and accuracy at signal to noise ratio of 10 (Biswas et al., 2007). The data shown in Table 1 demonstrates that LOD for the four TCs ranged from 5 to 13 ng, LOQ was 15, 20, 42 and 31 ng for OTC, TC, CTC and DC, respectively.

The spiked concentration for each TC was set at two levels of 0.5 and 1.0 µgg^{-1} and using standard addition method the recoveries of four TCs in the muscle samples were as shown in Table 1. The recoveries of the four TCs in the spiked pig muscle samples were 79.17 to 89.28%. The relative standard deviation (RSD) of the recovery of the four TCs was less than 7.6%. This means that the applied extraction and purification procedure appeared to be efficient, and the HPLC analysis procedure was applicable to detect TC residues in the pig muscle samples. These results are in clear agreement with previous work (Gratacos Cubarsi et al., 2007). The internal temperature in the centre (calculated geometrically) of the cooked sample for each cooking method was monitored. The internal temperature of the meat did not rise above 100°C in any method, and this temperature was not maintained for more than 9 min in boiling and deep-frying or 1 min for microwaving. The highest achieved temperatures were 96.3, 97.0 and 95.5°C during boiling, deep-frying and microwaving, respectively. At 6 min of boiling, 3 min of deep-frying and 0.5 min of microwaving, the samples had a well-done appearance (the internal temperature in center of sample being ≥ 80°C) (Table 2). In these three cooking methods, decreases in measured sample weights ranging from 16 to 23, 14 to 30 and 15 to 28% of the initial weight of 10 g of raw pig muscle were observed during boiling, deep-frying and microwaving, respectively. The net changes of analyte residues in the samples were then analyzed.

The levels of antibiotic residues in pig muscle before and after cooking were compared. The results for each cooking method are shown in Table 2. The results revealed a decrease of all TCs with boiling reducing the TC residues by 27 to 67% after 9 min (Table 2). All the TC residues decreased gradually after the start of cooking. The reductions in OTC, TC, CTC and DC in pig muscles during deep-frying were 32 to 66% in 9 min

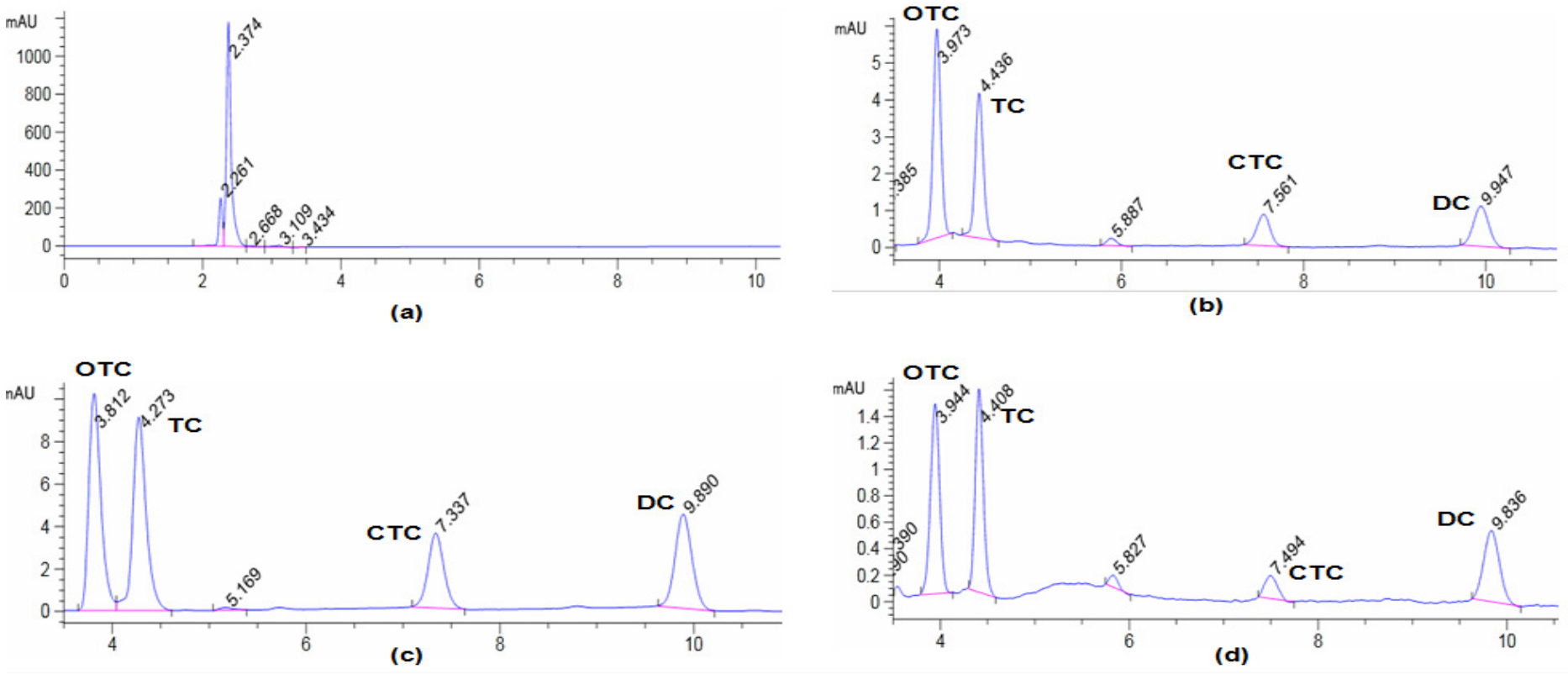

Figure 1. (a), Chromatogram of blank pig muscle samples; (b), chromatogram of spiked pig muscle of each TC; (c), chromatographic standard solution; (d), chromatogram of spiked pig muscle samples of each TC, thermally treated.

(Table 2). Microwaving resulted in 24 to 49% in reduction in TCs levels (Table 2). The statistical analysis (one-way ANOVA) demonstrate a highly significant ($p < 0.05$) difference in the levels of all the TCs between spiked raw and cooked pig muscle samples (Table 2).

The change in TC levels in the tissues depended on the type of TC as well as the method and time of cooking (Table 3). The results showed that CTC had the highest reduction (67, 66 and 48%), while DC showed the lowest reduction (45, 38 and 38%) after cooking by boiling, deep-frying and microwaving, respectively (Table 3). The differences in TC residue under boiling and microwaving were significant for all TCs tested ($p < 0.05$) while OTC was significantly different among all the cooking methods (Table 3).

The results reported here are consistent with those previously reported in studies in which thermal treatments were shown to reduce the concentration of veterinary drug residues in foods, therefore decreasing the possible toxic effects of these compounds to consumers. For example, in investigating the effect of a range of cooking processes including microwaving, boiling, roasting, grilling, braising and frying on OTC residues in animal tissues (Rose et al., 1996) observed substantial net reductions in OTC of 35 to 94%, with temperature during cooking being shown to have the largest impact on the loss. In addition, migration from the tissue into the surrounding liquid or meat juices were observed during the cooking processes. Interestingly, it has been reported that

Table 1. Recoveries (mean, %, n = 5) for raw/cooked TC-fortified pig muscles and linearity, sensitivity, LOD and LOQ of method.

	Cooking method								Linearity, sensitivity, LOD, LOQ method			
	Raw		Boiled		Deep-frying		Microwaving					
TCs added μgg^{-1}	0.5	1	0.5	1	0.5	1	0.5	1	Regression	R^2	LOD ngg^{-1}	LOQ ngg^{-1}
OTC	86.99 (4.91)[A]	87.22 (1.20)	87.72 (1.06)	88.22 (4.95)	87.50 (2.17)	88.82 (1.50)	87.87 (3.48)	89.28 (3.55)	Y = 0.189X - 0.119	0.9996	5	15
TC	81.78 (5.66)	82.68 (2.63)	82.28 (2.55)	84.19 (4.69)	84.93 (4.65)	86.83 (7.56)	84.66 (6.18)	85.62 (5.23)	Y = 0.184X - 0.067	0.9992	7	20
CTC	79.17 (3.69)	81.43 (6.23)	81.59 (1.30)	83.69 (2.29)	82.77 (3.83)	83.88 (6.36)	83.63 (0.80)	85.03 (4.78)	Y = 0.082X - 0.167	0.9992	13	42
DC	82.29 (2.47)	83.38 (2.47)	82.65 (1.84)	84.45 (1.85)	83.17 (4.04)	85.40 (4.71)	83.14 (4.74)	87.10 (4.39)	Y = 0.111X - 0.109	0.9999	10	31

A, Values in parentheses are relative standard deviation of the recovery of the four TCs.

Table 2. Absolute quantities (mean, μg, n = 3) of residual TCs in pig muscles (10 g/raw) cooked by boiling, deep-frying and microwaving.

	Cooking method											
	Boiling				Deep-frying				Microwaving			
Time in cooking (min)	UC*	3	6	9	UC	3	6	9	UC	0.5	0.75	1
Temperature[A] (°C)	20.2	69.5	94	96.3	19.4	93.4	96.5	97.0	19.2	80.4	95.5	93.7
OTC	0.91^{a}	0.61^{b} (33.0)[B]	0.50^{c} (45.4)	0.36^{d} (60.1)	0.91^{a}	0.55^{b} (39.8)[B]	0.45^{c} (50.4)	0.41^{d} (55.1)	0.91^{a}	0.68^{b} (25.2)[B]	0.58^{c} (36.9)	0.47^{d} (48.5)
TC	0.54^{a}	0.37^{b} (31.3)	0.30^{c} (44.2)	0.24^{d} (55.8)	0.55^{a}	0.35^{b} (37.0)	0.31^{c} (44.2)	0.29^{c} (46.7)	0.54^{a}	0.4^{b} (24.8)	0.37^{c} (30.4)	0.32^{d} (41.0)
CTC	0.50^{a}	0.30^{b} (40.4)	0.21^{c} (58.3)	0.17^{d} (66.9)	0.51^{a}	0.33^{b} (35.5)	0.18^{c} (65.1)	0.17^{c} (65.8)	0.51^{a}	0.33^{b} (34.2)	0.32^{b} (37.5)	0.26^{c} (48.0)
DC	0.61^{a}	0.44^{b} (27.3)	0.39^{c} (35.5)	0.33^{d} (45.9)	0.62^{a}	0.42^{b} (31.4)	0.38^{c} (38.4)	0.37^{c} (39.5)	0.58^{a}	0.45^{b} (23.4)	0.44^{b} (25.1)	0.37^{c} (36.0)

A, Internal temperatures in geometric centre of cooked pig muscles; B, values in parentheses are percentages of reduced drug residues from the uncooked samples; [a,b,c,d], values with different letters (a to d) within the same row reduce significantly ($p < 0.05$). *Uncooked meat samples.

Table 3. Effect of different cooking methods on TC residues in pig balls at the end of the heating process.

Cooking process	Temperature/ Power/ time	Percentages of reduced drug residues compared to the uncooked samples			
		OTC	TC	CTC	DC
Raw	Control	100^{a}	100^{a}	100^{a}	100^{a}
Boiling	96.3°C/9	60.16^{b}	55.85^{b}	67.05^{b}	45.35^{b}
Deep-frying	97.0°C /9	55.04^{c}	46.04^{bc}	65.74^{b}	38.17^{c}
Microwaving	93.7°C/800W/1 min	48.47^{d}	41.75^{c}	47.95^{c}	38.17^{c}

[a,b,c,d], Values with different letters (a to d) within the same column reduce significantly ($p < 0.05$).

no individual closely related compound such as 4-epioxytetracycline, α- or β-apooxytetracycline forms a significant proportion of the breakdown products when meat containing TC is cooked by different methods which indicates that the effect of cooking on residues of OTC and other TCs should be considered before data obtained from measurements on raw tissue are used for consumer exposure estimates and dietary intake calculations. In a contrasting report from a study to examine the formation of anhydrotetracycline and 4-epianhydrotetracycline, as toxic degradation products of TC during a heat treatment, it was found that heat treatments led to a significant increase of the amounts of these degradation products (Kuhne et al., 2011) which means that there is need for further studies to provide more insight into the effect of cooking on TCs. Javadi (2011) also reported a great reduction in DC residues in different tissues of broiler chicken under different cooking methods.

A number of studies have demonstrated the formation of 4eATC and ATC in bones containing TCs after a severe thermal treatment Although we did not evaluate the formation of 4eATC and ATC in this study, Gratacos Cubarsi, et al. (2007) have reported that the formation of ATCs is in edible meat samples containing TCs after mild cooking treatments, and they also showed that thermal treatments may reduce the concentration of veterinary drug residues in foods, decreasing the possible toxic effects of these compounds. Microwave heating is the quickest way to reduce TC residues in meat but it also leads to more pronounced production of ATCs probably as a consequence of the higher temperatures quickly reached in meat samples during microwaving compared to other cooking methods. Fedeniuk et al. (1997) showed that some synergy exists when thermal treatments are combined with food additives. They found that additive like sodium nitrite can lead to significant increases in the rate of OTC degradation under thermal treatments. This observation needs further investigation.

Conclusion

When conducting a risk assessment of TC in human health, the presence of these contaminants in meat and other meat products should be considered as a potential source, depending on levels of contaminants and the daily intake of meat. This study was designed to evaluate strategies for reduction of TC in meat before consumption. The results of this study show that the amount of TC residues is significantly affected by cooking whereby both cooking time and methods were found to be important. Though all cooking methods are effective, microwaving exhibited significantly fast reduction of all the four types of TC. Reduction in TC concentrations during boiling and deep-frying was due to migration of the TC from the meat balls to cooking medium (water and oil) while during the microwaving process, reduction was due to juice exuding out from the pig balls. The overall loss of TC residues was due to denaturation of protein - TC compounds. From the safety and toxicological point of view, these findings show an additional advantage of cooking as a food processing method. The cooking methods used in the present study are similar to those widely applied to meat during household cooking. Therefore, the observed findings may be helpful in confirming and selecting the ideal method for cooking so as to effectively reduce TC and probably other antibiotic residues in meat prior to consumption.

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