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Formation of Tetracycline Degradation Products in Chicken and Pig Meat under Different Thermal Processing Conditions

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Tetracycline (TC) and 4-epitetracycline (4eTC) degradation, as well as anhydrotetracycline (ATC) and 4-epianhydrotetracycline (4eATC) formation, has been evaluated in thermally treated chicken breast, pig loin, and pig loin with added back-fat. Samples containing TC and 4eTC residues were submitted to microwave or boiling heating, extracted with a mixture of McIlvaine buffer/methanol (75:25), and analyzed by high-performance liquid chromatography−diode array detection on a phenyl-hexyl reverse phase chromatographic column. The formation of ATC and 4eATC, as well as of two unidentified compounds, was described for the first time in edible meat samples submitted to mild thermal treatments, similar to those applied at home to cook foods. Degradation of TC and 4eTC and formation of ATC and 4eATC versus time of treatment fitted satisfactorily a first-order kinetic. Even if the potential toxic effects of these breakdown compounds should be further investigated, their formation in cooked meat should be taken into account when maximum residue limits are established.

KEYWORDS: Tetracycline; anhydrotetracycline; HPLC; meat; thermal degradation

INTRODUCTION

Veterinary drug residues in foods of animal origin are a matter of great concern for the final consumer, due to their potential negative effects on human health. These food safety issues are commonly managed by public authorities with integrated approaches based on toxicological evaluation and risk analysis. The final result of this process is the establishment of maximum residue levels (MRLs), or tolerable residue levels, for active substances in edible tissues. In some cases the use of the relevant substance is prohibited. In the European Union (EU), the evaluation procedure is described in Council Regulation (EC) 2377/90 of June 26, 1990 (*1*).

Tetracyclines are a wide family of antibiotics used in human and veterinary medicine that are active against Gram-positive and Gram-negative bacteria including some anaerobes (*2*). Tetracycline (TC), oxytetracycline, and chlortetracycline are registered in the EU as therapeutic agents for food-producing animals. Tetracyclines seem to undergo a limited metabolism in living animals because they are extensively excreted in urine and feces (*3*). Anyway, desmethyl-tetracycline derivatives have been reported in hen plasma and eggs (*4*).

The chemical stability of tetracyclines is strongly affected by several factors such as light, temperature, and pH (*5*−*11*). Under alkaline conditions, tautomerization, desmethylation, and the formation of terranoic acid and iso forms have been described (*6*). Under acidic conditions, epimerization reactions of tetracyclines take place (*5*, *8*) to give 4-epimeric, anhydrous, α/β apo, and ter forms (*3*, *6*).

The EU authorities partially take into account these degradation products because the MRLs for tetracyclines, ranging from 600 to 100 μg/kg, are expressed as the sum of parent drugs and their 4-epimers (*1*, *12*).

Recently, several studies emphasized the rapid formation of anhydrous derivatives in chicken and pig bones containing TC after high-temperature and strong acidic treatments (*8*, *9*). From the toxicological point of view, anhydrotetracycline (ATC) and 4-epianhydrotetracycline (4eATC) are relevant compounds, because they have been related to Fanconi-type syndrome, a reversible renal dysfunction (*13*).

This evidence clearly shows that information on the stability of veterinary drug residues under different thermal treatments, which are common in industrial and household preparation (cooking, boiling, or frying), would be rather useful for a better assessment of the actual risk for consumers' health.

At the same time the current trend in food processing is the reduction of the thermal impact to improve the quality of foods. Therefore, technologies providing high heating rates, such as

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microwave heating, have been proposed for food preservation as well as for household preparations (*14*).

Even though in microwave processing the thermal effects on microorganisms, enzymes, and nutrients seem to predominate, enhanced thermal and nonthermal effects have been also described (*15*, *16*). These effects are usually considered to be positive, because they improve the thermal effects and increase the safety of microwave-treated foods.

Several published papers described the effect of different thermal treatments on residual tetracyclines in foods (*17*−*21*), but in these studies only the degradation of the parent drug was evaluated, whereas the accumulation of potentially toxic breakdown products was not studied.

The aim of this study was to evaluate the degradation of TC and 4-epitetracycline (4eTC) as well as the accumulation of ATC and 4eATC in pork and chicken meats, under different thermal processing conditions (boiling and microwave heating), which reproduce common mild industrial and household thermal treatments.

Effects of the tissue type and heating conditions were considered and discussed as well as the possible consequences of the presence of new breakdown-related compounds in cooked foods.

MATERIALS AND METHODS

Chemicals. Acetonitrile (ACN) and methanol (MeOH), both of liquid chromatography grade, were from J. T. Baker (Mallinckrodt Baker, Deventer, Holland). Tetracycline hydrochloride (TC, purity > 95% by HPLC; CAS Registry No. 64-75-5), oxalic acid dihydrate, ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, anhydrous NaH_2PO_4, and Na_2HPO_4 were purchased from Sigma-Aldrich (Madrid, Spain). Anhydrotetracycline hydrochloride (ATC, purity > 95% by HPLC; CAS Registry No. 13803-65-1), 4-epi-tetracycline hydrochloride (4eTC, purity > 95% by HPLC; CAS Registry No. 23313-80-6), and 4-epi-anhydrotetracycline hydrochloride (4eATC, purity > 95% by HPLC; CAS Registry No. 4465-65-0) were purchased from ACROS Organics (Morris Plains, NJ).

Single stock solutions for each compound were prepared by dissolving 125 mg of pure standard in 25 mL of methanol (final concentration = 5 g/L). Then, a combined working solution containing 1 g/L of TC, 4eTC, ATC, and 4eATC was obtained by a suitable dilution of the stock solutions with methanol.

Finally, chromatographic solutions (0.1, 1, 5, 10, and 20 mg/L, for each compound) were prepared by dilution of the combined working solution with mobile phase.

Meat Sample Preparation. Chicken breast, pig muscle (M. longissimus dorsi), and subcutaneous pig backfat (F) were obtained from local stores.

Chicken muscle (CM), pig muscle (PM), and pig muscle plus subcutaneous fat (85:15, w/w) (PM+F) samples (500 g each) were homogenized in a bowl cutter Mixer R2 (Robot Coupe USA, Inc., Jackson, MS).

To aliquots of 250 g for each meat type was added 5 mL of the TC methanolic stock solution (5 g/L) to reach a final concentration of 100 mg/kg of TC in the tissue (contaminated samples).

Blank samples (not spiked with TC) and contaminated samples were used to prepare 10 g (±0.1 g) cylindrical hamburgers in a 38 mm diameter × 10 mm height plastic mold.

Blank and contaminated hamburgers ($n = 3 + 3$ for each type of meat) were immediately analyzed (untreated samples) to verify the absence of detectable levels of tetracyclines in blank samples as well as the homogeneity of TC addition in contaminated samples.

Contaminated hamburgers were packed in aluminum bags for boiling treatment ($n = 9$ for each type of meat) or commercial food grade polypropylene trays (155 × 115 × 25 mm, W × D × H) for microwave treatment ($n = 9$ for each type of meat). Samples were sealed under vacuum, refrigerated (4 °C), and treated within 2 h.

Thermal Treatments. For the boiling treatments, hamburgers were first tempered to an initial temperature of 17 ± 2.5 °C and immersed in a water bath (Precisterm, JP Selecta SA, Barcelona, Spain) at 100 °C during 2, 4, or 14 min (Tb_1, Tb_2, Tb_3). After treatment, samples were immediately refrigerated in an ice bath, stored at −20 °C, and analyzed within 1 day.

For the microwave treatment, samples were tempered to an initial temperature of 17 ± 2.5 °C and placed at the geometric center of a turntable domestic microwave oven (NNT251W Inverter, Panasonic, Germany) with a frequency of 2450 MHz.

Before the microwave treatment, the plastic cover of each tray was perforated to avoid water vapor overpressure.

Three different treatments were applied as follows: 440 W for 45 s (Tm_1); 440 W for 45 s and then 100 W for 120 s (Tm_2); 440 W for 45 s and then 100 W for 360 s (Tm_3).

After treatment, samples were immediately refrigerated in an ice bath, stored at −20 °C, and analyzed within 1 day.

On-line Temperature Measurement. In a series of preliminary assays, the core temperature of the samples was recorded every second during both microwave and boiling treatments described above. The acquisition data system (Microwave Workstation; FISO Technologies Inc., Quebec, Canada) included a computer interface, a fiber optic slip-ring (OSR) for on-line temperature measurements, the Workstation Commander Control, and the software for data collection. The OSR system was coupled to inside-optical fiber temperature probes (FOT-L/2.5 m; FISO Technologies Inc.) with an accuracy of ±0.5 °C.

Recorded data were used to calculate the maximum temperature in core sample (T_{max}) reached during the process and, using eq 1, the cooking values (C_{V1}, which corresponds to 6.75 min of total heating time, at the end of the microwave treatment and C_{V2}, which corresponds to 14 min of total heating time, at the end of the boiling treatment)

$$C_V = \int_0^t 10^{((T-100)/33)}\,dt \quad (1)$$

where T is the temperature of the sample at time t (*22*).

The temperature increase rate between 25 and 80 °C (TIR_{25-80}) was calculated as $dT/dt_{25-80°C}$.

HPLC Analysis. Extraction of TC and its derivatives from treated and untreated samples was optimized by taking into account the conditions proposed by MacNeil et al. (*23*). Initially three different conditions of extraction were evaluated: (i) MeOH; (ii) McIlvaine buffer-EDTA; and (iii) McIlvaine buffer-EDTA/MeOH (75:25 v/v).

The 10 g hamburgers were cut in small pieces, and 20 mL of the extractant solution was added; the liquid phase formed during the thermal treatment was also collected and added to the extract.

Extracts were centrifuged at 12100*g* for 15 min with a centrifuge Beckman J2-MC at 4 °C. The extraction was repeated twice; the supernatants were mixed, filtered through a paper filter, and diluted to a final volume of 50 mL with extractant solution. One milliliter of the final extract was filtered with a nylon filter (porosity = 0.45 μm) and injected (100 μL) into the chromatographic system, an Agilent Technlogies 1100 series quaternary pump equipped with a diode array detector, and a Chemstation Data Analysis System (Agilent, Palo Alto, CA).

The chromatographic separation was performed at room temperature on a Luna Phenyl-Hexyl 5 μm (250 mm × 4.6 mm i.d.) analytical column (Phenomenex, Torrance, CA).

Elution at a flow of 1 mL/min was realized with a linear gradient between solvent A (0.01 M oxalic acid/MeOH/ACN, 80:10:10 v/v/v) and solvent B (0.01 M oxalic acid/ACN, 55:45 v/v), from 92% A and 8% B (initial conditions) to 20% A and 80% B at 15 min.

Peak identification was made by comparing both retention time and spectra (collected between 220 and 500 nm) with those of the corresponding pure standard solutions. Peak purity was also checked with the Chemstation software utility considering a threshold value of 990/1000.

TC and 4eTC were quantified at 360 nm (16 nm bandwidth), whereas ATC and 4eATC were quantified at 274 nm (12 nm bandwidth). Data acquisition was carried out with a reference signal at 650 nm (100 nm bandwidth). External standard calibration curves were created for 4eTC,

Table 1. Mean Recovery (Percent), at a Spiking Level of 25 mg/kg, for Each Compound Under Different Extracting Conditions and Linearity Data for the Calibration Curves (Y = Nanograms Injected, X = Peak Area)

	recovery studies						
	MeOH	McIlvaine-EDTA	McIlvaine-EDTA/ MeOH (75:25) v/v			calibration curves	
	CM (**mean**, *SD*)	CM (**mean**, *SD*)	CM (**mean**, *SD*)	PM (**mean**, *SD*)	PM+F (**mean**, *SD*)	equation	R^2
4eTC	**14.9**, *1.5*	**57.8**, *2.6*	**60.5**, *4.2*	**48.2**, *4.6*	**52.5**, *5.5*	$Y = 0.5323X - 0.4780$	0.9997
TC	**24.6**, *3.2*	**77.8**, *5.0*	**88.5**, *5.2*	**64.8**, *4.2*	**89.6**, *8.5*	$Y = 0.6314X - 0.9470$	0.9999
4eATC	**8.4**, *0.7*	**15.0**, *0.8*	**27.9**, *2.7*	**26.9**, *2.9*	**29.0**, *1.0*	$Y = 0.1920X + 0.7141$	0.9999
ATC	**10.8**, *0.9*	**20.5**, *1.2*	**39.2**, *3.8*	**37.2**, *2.3*	**50.4**, *1.5*	$Y = 0.1602X + 0.3347$	0.9996

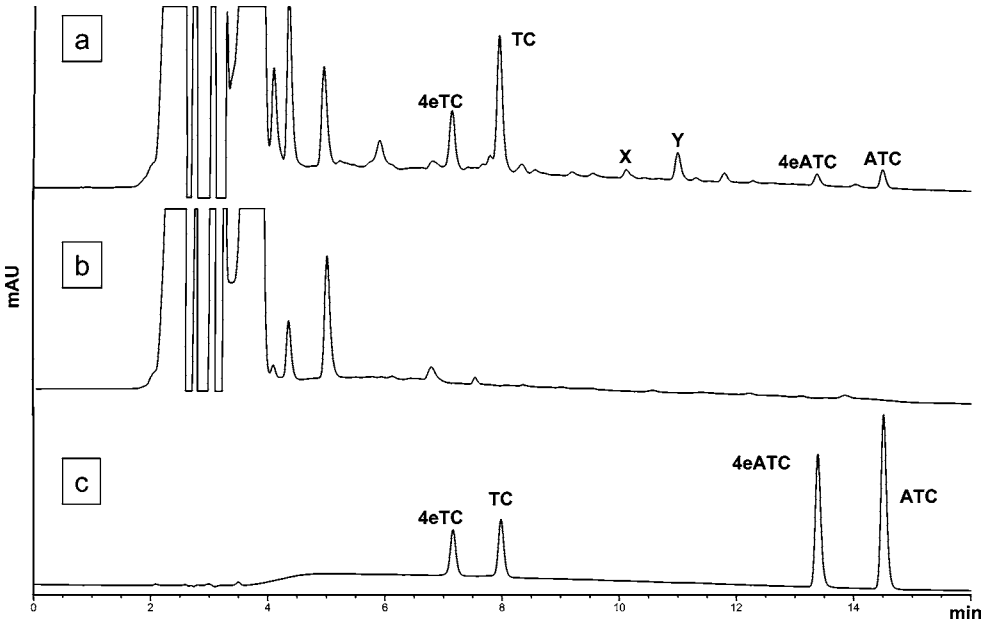


Figure 1. Typical chromatogram profiles (λ = 274 nm) of (**a**) microwave-treated chicken sample (Tm_2); (**b**) blank chicken sample, thermally treated; and (**c**) chromatographic standard solution.

TC, 4eATC, and ATC by injecting chromatographic standard solutions in the range between 10 and 2000 ng injected for each compound (n = 7). Residual concentration in the samples were expressed as the sum of the corresponding epimeric forms (TCs = TC + 4eTC and ATCs = ATC + 4eATC). Two unidentified peaks (peaks X and Y) were quantified as TC equivalents, considering their absorption at 270 nm (12 nm bandwidth) and the response of TC at 270 nm.

Two blank hamburgers for each thermal process and meat type (n = 12) were thermally treated (Tb_2, Tm_2) and then fortified at 25 mg/kg with the combined work solution containing 1 g/L of each compound. Samples were extracted as above, and recovery was evaluated in each matrix. The limit of detection (LOD) was calculated by considering the concentration of pure standard giving a signal to noise ratio (S/N) = 3.

Physicochemical Analyses. Physicochemical analyses were performed on two samples for each type of edible tissue. Protein content (percent) and total fat content (percent) were determined by near-infrared rransmittance with an Infratec 1265 meat analyzer (Tecator AB, Sweden) as described previously (*24*). Ashes and moisture were analyzed according to the official methods (*25*).

Water loss was calculated as $[(H_i - H_{T3})/H_i \times 100]$, where H_i was the initial moisture of the sample and H_{T3} the moisture at the end of the treatment.

Statistics. A two-way multiple analysis of variance (MANOVA) was carried out for the T_3 samples, considering treatments (microwave, boiling) and meat type (CM, PM, PM+F) as independent variable and TCs, ATCs, and peak X and Y concentrations as dependent variables.

RESULTS AND DISCUSSION

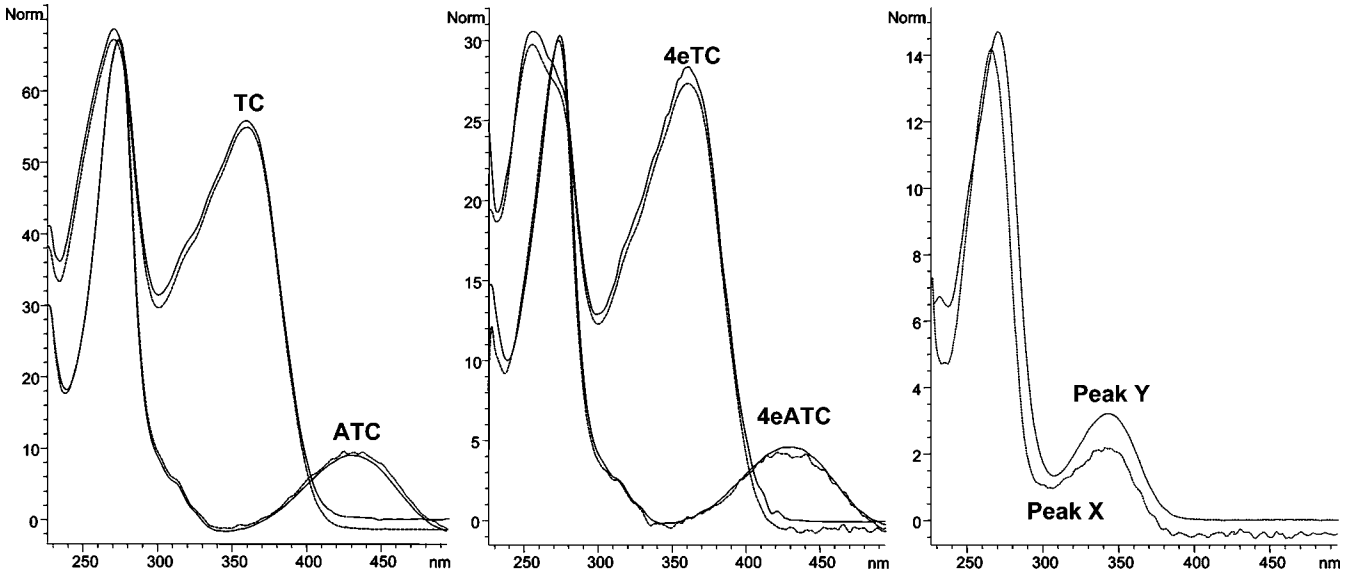
HPLC Analyses. A preliminary study was carried out to evaluate the extraction and analysis of TC and its derivatives in treated chicken tissues. The use of methanol as extracting solution provided low recoveries (**Table 1**). On the contrary, the use of both McIlvaine buffer-EDTA and McIlvaine buffer-EDTA/MeOH (75:25 v/v) increased the recovery values up to 60−80% for TC and 4eTC. These results were very similar to those reported by McNeil et al. (*23*) using the McIlvaine-EDTA solution in pork tissues.

Low recoveries were obtained for ATC and 4eATC (**Table 1**), even if with a satisfactory reproducibility.

Previously, Kuhne et al. (*8*) obtained recoveries between 63 and 71% for TC and ATC by analyzing bone-derived feed, whereas Loke et al. (*26*) observed low recovery values for oxytetracycline derivatives in manure. No other published data are available for the recovery of ATC derivatives in meat, but it can be hypothesized that the different polarity, as reflected in the chromatographic separation, could affect the interaction with matrix components and their extraction. Finally, the combination of McIlvaine buffer-EDTA and MeOH 75:25 (v/v) was selected because that mixture offered the better recoveries for all analyzed compounds (**Table 1**), including the anhydrous forms (recovery between 27 and 50%).

The use of a phenyl-hexyl stationary phase provided a suitable separation of the four compounds with good efficiency (number of theoretical plates N/L = 3610 cm^{-1} for 200 ng TC injected) and peak shapes (peak symmetry between 0.839 and 0.904) (**Figure 1**).

A complete chromatographic analysis needed approximately 20 min, with a good resolution between the epi-isomeric forms and no significant interfering peak in meat extracts. These results



	t_R (min)	λ_{max1} (nm)	λ_{max2} (nm)
4eTC	7.16	256.4	360.4
TC	7.94	270.0	359.2
peak X	10.06	264.4	341.2
peak Y	10.90	268.4	341.2
4eATC	13.41	274.0	427.6
ATC	14.55	273.2	432.0

Figure 2. UV–vis spectra (220–500 nm) of TC, ATC, 4eTC, 4eATC, and peaks X and Y corresponding to chromatographic standard solutions (continuous lines) and extracts obtained from thermally treated sample (dotted lines).

were better than those previously obtained with a monolithic column (*27*) and could be related to the limited −OH interaction between tetracycline and the free silanol groups of the stationary phase, a common problem that affects the separation of tetracyclines (*5*).

The use of two different wavelengths of detection (274 and 360 nm) allowed a suitable detection of the four compounds (4eTC, TC, 4eATC, ATC), with limits of detection ($S/N > 3$) always better than 65 μg kg^{-1} for all of the compounds. Linearity in the range of the calibration set was satisfactory with an R^2 better than 0.9995 for all of the compounds (**Table 1**). Mean RSD values ($n = 12$) for separated determinations on spiked samples were under 9%.

Figure 2 shows the spectra of the four identified compounds and of two unidentified peaks (X and Y), which were observed in treated samples but not in the untreated ones. Both compounds possess the region of the spectra between 220 and 300 nm very similar to that of TC and ATC, whereas retention times were between those of TC and ATC. Further studies should be performed to elucidate their structure, but it is reasonable to hypothesize that the unidentified compounds are intermediate degradation products formed during thermal treatments of edible tissues containing TC residues.

Tetracycline Degradation during Thermal Treatments. Temperature profiles measured in the center of the PM+F samples are reported in **Figure 3**, whereas heating parameters are detailed in **Table 2**.

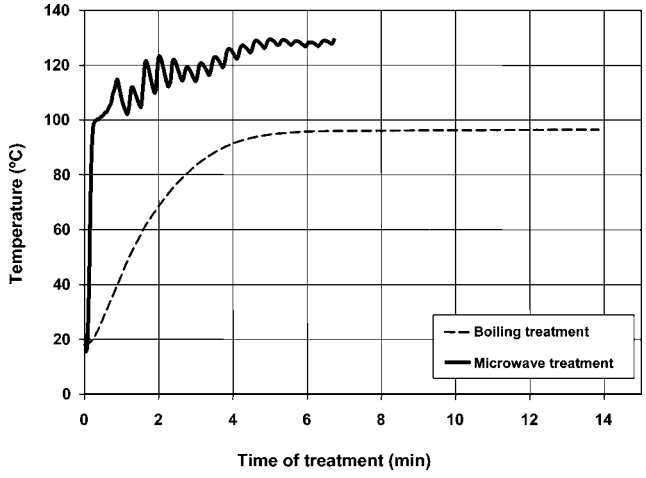


Figure 3. Typical temperature profiles in the sample core for boiling and microwave treatments (PM+F samples).

As expected, the microwave treatment caused a rapid increase of the temperature in the sample (TIR_{25-80} between 6.5 and 12.1 °C/s), with a final temperature of approximately 130 °C (**Table 2**) at the end of the process.

On the contrary, the boiled samples reached an average maximum temperature of 97.6 °C, with a TIR_{25-80} of just around 0.4 °C/s for all meat types.

Calculated C_{V1} values for boiled samples were lower than the microwave-treated ones. Only after 14 min of treatment did

Table 2. Heating Parameters (Mean, *Mean Squared Error*) Obtained from the Temperature Profiles for the Three Types of Meat and Two Treatments ($n = 2$ for Each Combination of Tissue × Treatment)

	microwave treatment			boiling treatment			
sample	T_{max} (°C), *MSE*	C_{V1} (au), *MSE*	TIR_{25-80} (°C/s), *MSE*	T_{max} (°C), *MSE*	C_{V1} (au), *MSE*	C_{V2} (au), *MSE*	TIR_{25-80} (°C/s), *MSE*
CM	114.2, *4.1*	6.1, *0.3*	6.5, *0.8*	97.5, *0.8*	3.4, *0.1*	9.2, *0.4*	0.4, *0.1*
PM	123.3, *13.2*	14.4, *6.1*	9.8, *1.6*	98.7, *0.1*	3.2, *0.1*	9.6, *0.2*	0.4, *0.1*
PM+F	129.7, *4.2*	31.6, *7.5*	12.1, *1.8*	96.5, *1.2*	2.8, *0.2*	8.1, *0.7*	0.4, *0.1*

Table 3. Physicochemical Parameters for Different Meat Samples

					water loss (%)	
sample	water (%, w/w)	total fat (%, w/w)	total protein (%, w/w)	ash (%, w/w)	microwave	boiling
CM	76.0	1.1	22.4	1.3	80.2	30.0
PM	67.6	11.7	20.3	1.1	88.7	37.9
PM+F	60.3	22.6	16.5	1.0	93.7	40.3

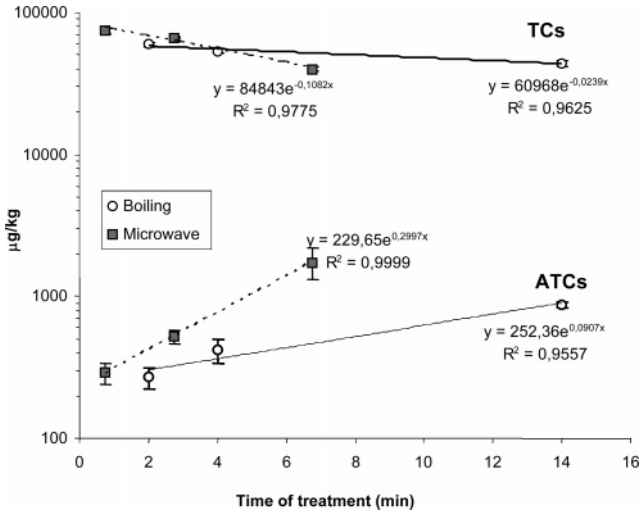


Figure 4. Time course evolution of TCs and ATCs in chicken meat samples (CM) during microwave and boiling treatments.

the boiled samples reach cooked values (C_{V2}) comparable to those obtained with the 6.75 min microwave treatment.

The quantity of fat in the meat sample influenced the efficiency of the microwave process. Under the same microwave treatment, an increase in all of the heating parameters was observed in the meat samples with the higher fat content (**Table 3**). These results are in clear agreement with previous works (*28*, *29*). On the contrary, under our experimental conditions, the heating parameters registered for boiled samples did not show any significant difference between the meat samples of different composition.

The microwave-treated samples suffered higher water losses (**Table 3**) than the corresponding boiled samples, probably as a consequence of the differences between thermal treatments, sample nature, and packaging type. The higher values were registered for PM+F samples treated with microwaves (**Table 3**), which could explain the continuous increase of the temperature registered in the samples during the microwave treatment.

The thermal treatments decreased TC residues in all meat samples, whereas residues of ATCs, peak X, and peak Y increased. The evolution of compound concentration versus time of treatment is represented graphically in **Figures 4**−**6**. A first-order curve fitted well the degradation kinetic of TCs as well as the formation of ATCs in the last part of the treatments (T_1, T_2, T_3), with satisfactory R^2 values for all samples.

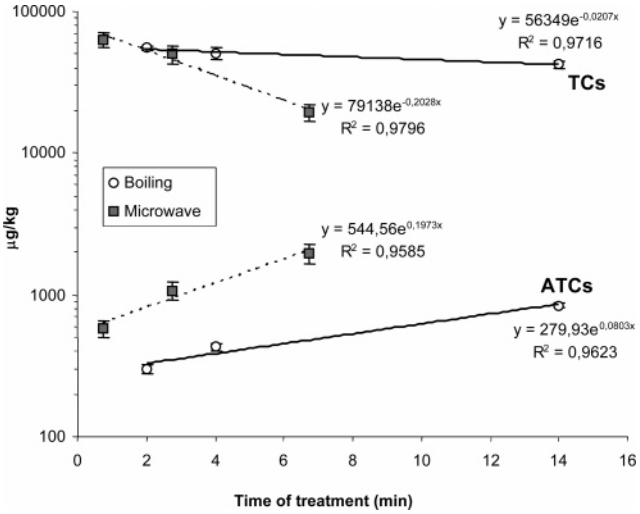


Figure 5. Time course evolution of TCs and ATCs in lean pork samples (PM) during microwave and boiling treatments.

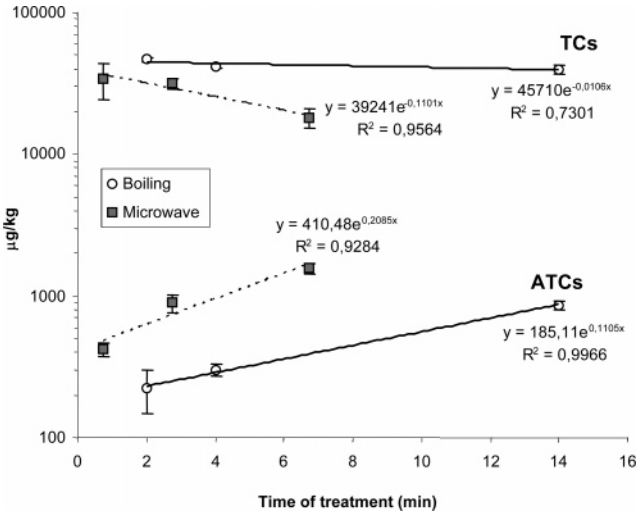


Figure 6. Time course evolution of TCs and ATCs in a mixture of pork meat and back fat (PM+F) during microwave and boiling treatments.

A similar behavior was observed for the accumulation of compounds corresponding to unidentified peaks X and Y, expressed as TC equivalents.

The residual concentrations of identified and unidentified compounds at the end of the different treatments (Tb_3, Tm_3; **Table 4**) were submitted to a MANOVA to evaluate the effects of meat type and thermal treatment.

The type of heating treatment significantly affected the TC degradation as well as the formation of ATCs ($p < 0.001$). These results seem to be mainly related to thermal effects (runaway heating, T_{max}), even if under our experimental conditions "nonthermal" effects cannot be exactly evaluated.

At the same time, the type of meat influenced significantly the residual concentration of TCs ($p < 0.05$). On the contrary,

Table 4. Concentrations of TCs and TC Derivatives in Meat Samples at the End of the Heating Process (T_3)

sample		TCs [C] (μg/kg), *SD*	ATCs [C] (μg/kg), *SD*	peak X [C] (μg/kg), *SD*	peak Y [C] (μg/kg), *SD*
boiling treatment	CM	43898, *2362*	878, *42*	711, *35*	2076, *67*
	PM	42332, *2881*	846, *21*	883, *13*	2753, *24*
	PM+F	39715, *2633*	863, *71*	785, *43*	2386, *97*
microwave treatment	CM	40111, *13979*	1741, *437*	897, *114*	2983, *468*
	PM	19463, *2652*	1968, *320*	802, *121*	2560, *325*
	PM+F	18169, *2964*	1568, *121*	794, *91*	2060, *207*

no significant effect of matrix or heating treatment was observed for compounds X and Y. This could support the hypothesis that these compounds are intermediate derivatives in equilibrium between the precursors (TCs) and the final degradation products (ATCs).

Previous studies demonstrated the formation of 4eATC and ATC in bones containing TCs (*8*) after a severe thermal treatment (100−133 °C for up to 45 min), although no other degradation products were taken into account. In this study, for the first time, the formation of ATCs is reported in edible meat samples containing TCs after mild cooking treatments, similar to those used by consumers to heat food at home.

Microwave and boiling treatments reduced the initial concentration of TCs (between 56 and 82%). Our results are in good agreement with other studies (*8*, *9*, *17*−*21*, *30*) and confirm that thermal treatments may reduce the concentration of veterinary drug residues in foods, decreasing the possible toxic effects of these compounds. At the same time, the thermal treatments caused the formation of the corresponding ATCs in all treated meat samples.

Both phenomena were more pronounced with the microwave heating, probably as a consequence of the higher temperatures reached in those samples under our experimental conditions. The quantities of ATCs found in meat samples after the thermal treatments were between 0.8 and 2.0% of the initial TC content.

Furthermore, the accumulation of two unidentified compounds (X and Y) was also observed in all thermally processed samples, up to approximately the 3.0% of the initial TC content (**Table 4**).

Our study indicates that relatively mild thermal processes, similar to those applied to meat during household cooking, may provoke the thermal breakdown of the original drug residue (TCs) and the formation of ATCs and two unidentified degradation products.

Additional studies are currently in progress to elucidate the structure of these unknown compounds as well as to confirm the formation of all breakdown compounds in meat when the initial TC residual concentration is near the MRL.

We suggest that the toxicological characteristics of these TC thermal breakdown products should be evaluated and, maybe, taken into account when the safety residual levels for veterinary drugs in foods are established.

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