Oxytetracycline Degradation in Thermally Processed Farmed Salmon

David D. Kitts,*,† Connie W. Y. Yu,† Ron G. Aoyama,‡ Helen M. Burt,‡ and Keith M. McErlane‡

Department of Food Science and Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

Oxytetracycline (OTC) thermal degradation time (TDT) studies were performed in both model buffer and salmon muscle samples for the purpose of determining OTC thermal stability. Thermal stability of OTC was pH dependent and different between model buffer (pH 3.0 and pH 6.9) systems. OTC decimal reduction (*D*) values were higher at pH 3.0 than at pH 6.9 in buffer. Corresponding OTC *D* values from salmon muscle indicated greater stability than when present in a similar model pH buffer. OTC *z* values in muscle (30.1 °C) were lower than in the pH 6.9 buffer (42.6 °C). Retention of OTC under normal frying cooking conditions was 30.1 ± 6.8%, which agreed with results from the TDT studies. It is concluded that OTC is more heat stable in salmon tissue than in simple buffer systems.

INTRODUCTION

The administration of antimicrobial agents to farmed fin-fish for the prevention and treatment of bacterial diseases is an important management practice in the aquaculture industry. There is concern about the presence of trace amounts of antimicrobial residues in fish at harvesting which may result from excessive use of antibiotics or a lack of adherence in regulation withdrawal times. The presence of certain antimicrobials in muscle foods poses a potentially serious health threat to the consumer, especially hypersensitive individuals that may experience a variety of allergic-type reactions to specific antibiotics (Schindel, 1965; Black, 1984).

Oxytetracycline (OTC) is a primary antibiotic used in the aquaculture industry for the treatment of bacterial kidney disease (BKD) and vibriosis. The antibiotic is usually administered in a form of medicated feed; however, vaccines have also been used (Strasdine and McBride, 1979). Detection limits for OTC in fin-fish muscle have been reported at levels of 0.05 ppm (Aoyama et al., 1991).

The thermal stability of antibiotics in liquid media, such as buffers, water, milk, and meat extracts, has been investigated by many workers (Shahani, 1957; Pilet et al., 1969; Konecny, 1978). Other studies have monitored antibiotic activity in thermally processed solid foods, including animal and shellfish tissue, and eggs (Bernarde, 1957; Escanilla et al., 1959; Yonova, 1971). An important factor to recognize in the risk assessment of antibiotic residues in fish is that most fish are consumed after a thermal treatment, such as cooking or conventional thermal processing methods. While cooking temperatures involve baking (180 °C for 20 min), boiling (10 min), or frying (100 °C for 15 min), conventional canning and retorting (>100 °C) are also possible thermal treatment for processed salmon. Earlier studies with thermally treated fish have shown that cooking significantly reduces the concentration of organochlorine compounds (Zabid et al., 1979; Stachiw et al., 1988). On the basis of previous studies using other muscle food systems (Moats, 1988), it is of interest to determine if thermal processing will reduce antibiotics to negligible or near zero levels in the event that detectable levels of antibiotics are present in raw farmed salmon.

* Author to whom correspondence should be addressed.
† Department of Food Science.
‡ Faculty of Pharmaceutical Sciences.

Thermal death time (TDT) and Arrhenius methods have been successfully used to determine the kinetics of heat-labile microorganisms, nutrients, and food quality factors following a thermal process (Bigelow and Esty, 1920; Ramaswamy et al., 1989). With the TDT method, the decimal reduction time (*D* values) describes the slope index of the logarithmic reaction rate curve derived at specific temperatures and the *z* value is the temperature change required to alter the rate of inactivation of the nutrient or microorganism by a factor of 10 (Ramaswamy et al., 1989). These parameters are useful for generating kinetic data suitable for use in calculating retention of nutrients and biological components during a thermal process. The objectives of the present study were to determine the kinetics of OTC thermal destruction by measuring the *D* and *z* values of this antibiotic in both buffer and salmon muscle systems. The retention of OTC residues in fish muscle following a typical cooking procedure was also investigated.

MATERIALS AND METHODS

Materials. (*a*) *Reagents.* Oxytetracycline hydrochloride and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (St. Louis, MO). Epitetracycline hydrochloride was obtained from the European Pharmacopoeia (Strasbourg, France). Disodium hydrogen orthophosphate dihydrate, potassium dihydrogen orthophosphate, citric acid solution, and phosphoric acid (85%) were obtained from BDH Chemicals (Toronto, ON, Canada). HPLC grade methanol was obtained from Fisher Chemicals (Vancouver, BC, Canada). Purified water was produced using a Milli-Q water purification system (Millipore Canada Ltd., Mississauga, ON, Canada).

(*b*) *Fish.* Chinook salmon were housed in circular flowing (40–60 L/min) seawater tanks at the West Vancouver laboratory of the Department of Fisheries and Oceans (West Vancouver, BC, Canada). The specific conditions used for administration of OTC-medicated feed have been detailed previously by Aoyama et al. (1991). Briefly, the dosage of OTC administered to fish was 80 mg (kg of biomass)$^{-1}$ day^{-1} for 10 days. The 12 fish used in this study were collected on days 8–14 following the last dose of medicated feed. Fish were gutted and whole fillets collected, pooled, and homogenized to produce a uniform sample. Fish samples were frozen at −25 °C until required for use.

Methods. (*a*) *Thermal Destruction Time (TDT) Studies: Model Buffer Study.* The thermal stability of OTC was examined at 60, 80, 90, and 100 °C in buffer solutions at pH 3.0 (sodium citrate buffer) and 6.9 (potassium phosphate buffer). OTC buffered stock solutions (5.0 ppm) were dispensed into Supelco screw-cap glass vials (2 mL) to perform these TDT experiments. In each experiment, two additional vials were fitted with a copper–

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constantan thermocouple which was inserted through the vial cap for the measurement of solution temperature in the center of the ampule, during both thermal lag and actual heating times. Temperatures in the vials were monitored with a Doric datalogger (Minitrend 205) equipped with T-type thermocouples. A water bath was used to conduct the 60 and 80 °C TDT experiments, and studies performed at 90 and 100 °C were conducted in a mineral oil bath. Reaction vials were immersed in the constant-temperature baths and removed at intervals between 1 and 65 min, cooled immediately in ice water, and stored at −25 °C until used for analysis.

(*b*) *Thermal Destruction Time (TDT) Studies: Fish Muscle Study.* Frozen fish samples were thawed and thoroughly mixed immediately prior to the TDT study. A sample was removed for composition analysis. Moisture content was determined using a vacuum oven at 70 °C. Ash and fat content were determined according to standard AOAC methods, while protein was determined according to the Kjeldhal method (Corcon and Soltess, 1973). The pH of salmon tissue was also measured by a surface electrode prior to the TDT experiments. Samples of salmon tissue (25 g) were dispensed into a series of retort pouches (ACF Felexible Inc.; Scarsbourgh, ON, Canada) and vacuum sealed. For each heating temperature, two additional pouches containing salmon were prepared containing thermocouples inserted into the center of the sample to monitor the thermal lag period and cooking temperature. Thermal treatments were conducted in water baths (60 and 80 °C) and a mineral oil (90 and 100 °C) bath. Samples were removed at specified time intervals and immediately immersed in ice water. All samples were stored at −25 °C until required for HPLC analysis.

In addition to TDT studies, OTC degradation was also examined in salmon (100 g; 8 × 2 cm) patties that were fried in an electric frying pan for 15 min at 100 °C to simulate a home-cooking procedure. Copper–constantan thermocouples were inserted into the center of the salmon patties during frying, and temperatures were recorded by a datalogger.

(*c*) *HPLC Analysis.* The HPLC system consisted of a Beckman Model 110A pump, a Shimadzu Model SIL-9A autoinjector, set to deliver a 20-μL sample volume, a Shimadzu Model SPD-6A variable-wavelength detector set at 365 nm, a Shimadzu C-R3A chart integrator, and an Ultrasphere octadecylsilane reversed-phase column (5-μm particle size, 4.6 mm i.d. × 25 cm). The mobile phase was methanol/0.02 M phosphate buffer (45/190) adjusted to a pH of 2.25 using concentrated phosphoric acid and delivered isocratically at a flow rate of 1.0 mL/min.

In the model study, OTC stock solutions prepared for calibration were diluted with buffer (either pH 3.0 or 6.9) to standard concentrations ranging from 0.5 to 10 ppm. Calibration curves were constructed by plotting OTC peak area vs standard OTC concentrations. A calibration curve was also prepared for the fish muscle samples by adding 0.5 mL of internal standard solution (epitetracycline hydrochloride, 0.2 mg/mL) and 0.5 mL of OTC standard solution to 5 g of salmon muscle tissue, yielding OTC concentrations ranging from 0.1 to 3.0 ppm. Calibration curves of the ratios of peak areas of internal standard to those of the peak areas of OTC vs standard OTC concentrations were constructed. All OTC solutions were prepared immediately before use.

The extraction of OTC from salmon was conducted in a 50-mL polypropylene centrifuge tube containing 5 g of salmon tissue, 15 mL of 0.02 M phosphate buffer, 1.0 mL of 50% TCA, and 0.5 mL of internal standard solution (Aoyama et al., 1991). Samples were homogenized, vortex-mixed, and centrifuged (30000*g*, 10 min). Supernatants were stored in the dark at 4 °C in a 50-mL Erlenmeyer flask. The remaining tissue pellet was resuspended with 15 mL of buffer and 1.0 mL of TCA, and the homogenization and centrifugation steps were repeated. The combined supernatants were passed through a Bakerbond Sephadex disposable 6-mL column that had been conditioned with phosphate buffer. Eluants were collected under vacuum in a 50-mL Erlenmeyer flask and passed through an activated J. T. Baker C-18 disposable (6 mL) column. The OTC and internal standard were eluted from the C-18 column with methanol and evaporated under nitrogen at 39 °C to dryness. Samples were reconstituted in 1.0 mL of mobile phase and aliquots (20 μL) injected onto the HPLC column. The mean OTC recovery from Chinook salmon muscle tissue using this procedure is 74.4%.

Table I. Composition of Raw and Cooked Salmon Muscle[a]

parameter	raw muscle	cooked muscle
moisture, %	74.5 ± 0.1	62.8 ● 0.2
fat, g/100 g	6.7 ± 0.23	6.3 ± 0.1
protein, g/100 g	14.8 ± 0.32	14.4 ± 0.22
ash, g/100 g	2.1 ± 0.02	2.2 ± 0.01
OTC, ppm	1.19 ± 0.02	0.47 ± 0.08
pH	6.60	6.50

[a] Cooked muscle represents 100 °C for 15 min. Values represent mean ± SEM of four analyses.

(*d*) *Analysis.* Decimal reduction times were calculated from the linear regression equation for each thermal destruction time curve value where D = −1/slope = (Δ time of heating)/(log Δ concentration). z values for the destruction rate of OTC in the two buffers were calculated from the linear regression equation obtained from the negative reciprocal slope of the D-value curves, where z = −1/slope of the TDT curve (Ramaswamy et al., 1989; Lund, 1988).

RESULTS

The nutrient and OTC compositions of the raw and cooked composite fish sample used in this study are presented in Table I. The moisture, protein, and fat contents in the raw fish muscle correspond to those of typical fin-fish (Exler, 1987), thus precluding the possibility that the storage and homogenization procedures adversely altered overall composition. The pH values of 6.60 and 6.55 for the unprocessed and cooked salmon, respectively, were slightly lower than the pH 6.9 used in the model buffer study. Following the thermal treatment of fish muscle by frying at 100 °C, a decline in moisture and OTC occurred. The rationale for using a composite fish sample rather than individual fish was based on reducing error associated with individual fish OTC residue levels. Considerable potential for large differences in tissue concentrations of antibiotics (Walisser et al., 1990; Aoyama et al., 1991) and other contaminants (Zabik et al., 1979) between fish has been reported and could influence the accuracy of derived OTC degradation estimates. The OTC concentration of the composite salmon muscle sample was 1.19 ± 0.02 ppm (derived from individual fish muscle samples which ranged from 1.0 to 1.33 ppm).

Linear calibration curves were obtained for OTC over a concentration range of 0.5–10 ppm in pH 3.0 (r^2 = 0.973) and 6.9 buffer (r^2 = 0.989), respectively. Temperature come-up times and thermal destruction parameter data of OTC at 60, 80, 90, and 100 °C in pH 3.0 and 6.9 buffers are shown in Table II. For the temperature range tested, OTC destruction followed pseudo-first-order kinetics. OTC was more heat stable at pH 3.0 than at pH 6.9, as evidenced by the higher D values at pH 3.0 (Table II). Thus, longer heating times at a specific temperature would be required to reduce OTC by 1 order of magnitude at pH 3.0 compared to those at pH 6.9. The lower z value at pH 3 indicates that thermal destruction of OTC is more temperature dependent at pH 3.0 than at pH 6.9, as shown by the greater temperature change required to result in a 10-fold change in the D value at pH 6.9.

In salmon muscle tissue, a linear (y = 0.7663x +0.045, r^2 = 0.996) calibration curve for OTC was obtained over a concentration range of 0.1–3.0 ppm. No significant changes in OTC were found in salmon muscle heated at 60 °C, compared to 80–100 °C (Figure 1). As a result, only TDT curves for OTC in salmon at 80–100 °C are shown in Figure 2. Corresponding D values and computed z value for OTC in salmon muscle are presented in Table II.

Table II. Oxytetracycline *D* and *z* Values in Model Buffer and Fish Muscle[a]

medium	temp, °C	C.U.T., min	*D* value, min	r^2	*z* value, °C	r^2
pH 3.0 buffer	60	3.0	645.2	0.904		
	80	5.0	50.0	0.999	26.9	0.964
	90	5.8	48.0	0.994		
	100	7.0	18.7	0.976		
pH 6.9 buffer	60	3.0	111.7	0.980		
	80	5.0	22.1	0.970	41.6	0.965
	90	5.8	16.5	0.971		
	100	7.0	12.7	0.981		
fish muscle	60	3.3	ND[b]	ND		
	80	6.0	143.5	0.879		
	90	6.7	75.8	0.986	30.1	0.995
	100	7.0	31.0	0.770		

[a] C.U.T., come-up time. ND, not determined.

Although more scattering of data was observed with OTC degradation in salmon muscle, the r^2 values were significant at the $P \leq 0.01$ confidence level, thus indicating first-order kinetics. A comparison of fish tissue *D* values with *D* values obtained in the model pH 6.9 buffer indicated that OTC is more heat stable in salmon tissue than the corresponding (pH 6.9) buffer. Similarly, the calculated *z* value for fish tissue (30.1 °C) was smaller than the corresponding *z* value calculated from the pH 6.9 buffer (41.6 °C), indicating that OTC degradation is more temperature dependent in salmon tissue than in buffer media.

In the practical cooking experiment with salmon muscle tissue, the reduction of OTC was 60.88 ± 6.8% ($n = 4$) of the original (1.19 ppm) OTC concentration in the salmon patties (data not shown). The resulting OTC residual amount of 0.47 ± 0.08 ppm (equivalent to a 32.12 ± 6.8% reduction) after 15 min of cooking at an average temperature of 99.4 °C corresponded to a similar amount of residual OTC (equivalent to a 35.2 ± 2.8% reduction) found in the same salmon heated for 15 min at 100 °C in the TDT study. The results of the TDT studies with salmon homogenate thus correctly characterized the thermal destruction of OTC during conventional cooking.

DISCUSSION

The thermal destruction kinetics of OTC and other antibiotic activities in typical animal food products such as milk, eggs, and beef and poultry muscle systems have been the subject of previous studies (Shahani, 1957, 1958; Konecny, 1978) and a review (Moats, 1988). In contrast, there is a paucity of information concerning the thermal stability of antibiotics such as OTC in farmed fish, expressed as a function of absolute concentration rather than antimicrobial activity. Taken together, the lack of information on OTC stability in fin-fish may be attributed to its relatively recent introduction in aquaculture. Moreover, the assessment of a risk from using antibiotics in salmonid fish culture has only recently been made possible with the development of sensitive quantitative HPLC methods for detecting specific antibiotics in farmed salmon (Walisser et al., 1990; Reimer and Young, 1990; Aoyama et al., 1991).

The thermal stability of OTC in liquid media, as indexed by the antimicrobial activity retained following heating (at 71 °C) has been shown to be much less than that of other antibiotics, namely streptomycin and penicillin B (Shahani, 1957, 1958). OTC possesses limited stability in aqueous solutions and, from the pH–rate profile, shows specific acid and base catalysis, with overall hydrolysis

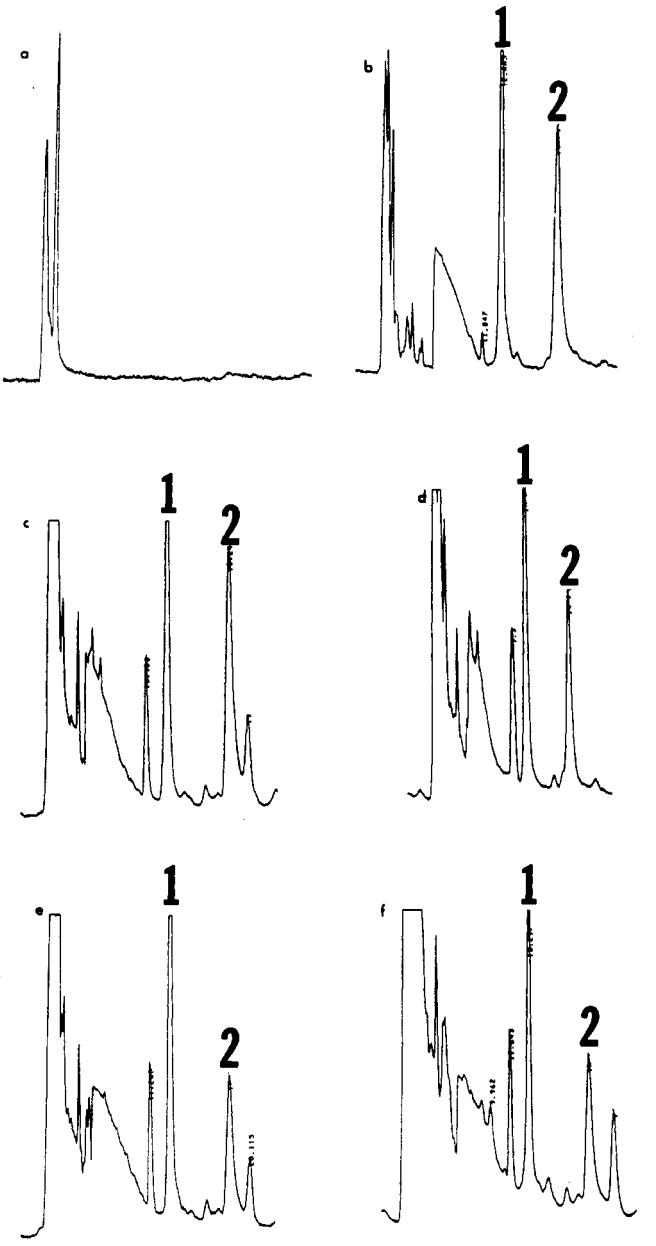

Figure 1. Representative chromatograms of internal standard, epitetracycline HCL (1), oxytetracycline (2), and an unidentified compound (3) derived from (a) blank salmon tissue extract, (b) fish muscle heated at 60 °C for 15 min, (c) fish muscle heated at 80 °C for 15 min, (d) fish muscle heated at 90 °C for 15 min, (e) fish muscle heated at 100 °C for 15 min, and (f) fish muscle fried at 100 °C for 15 min. Chromatograms b–e represent the TDT study, and chromatogram f represents a standard cooking procedure.

observed to follow pseudo-first-order kinetics (Conners et al., 1986). The maximum stability of OTC is at pH 2, and the optimum stability ranges from pH 1 to 3 (Conners et al., 1980), which accounts for the present finding that OTC has greater heat stability at pH 3.0 than at pH 6.9. A similar example of a pH effect on thermal stability of penicillin G has been observed in buffered meat homogenates at pH 5 and 7, respectively (Tropilo, 1985).

The results of the OTC thermostability experiments in solid fish tissue are more representative of actual cooking and processing conditions than those in liquid media (Moats, 1988). The length of time at a particular temperature to which antibiotic residues are subjected in muscle tissue is relatively more difficult to determine, because heat penetration will be slower, uneven, and more difficult to control. These factors could explain the greater

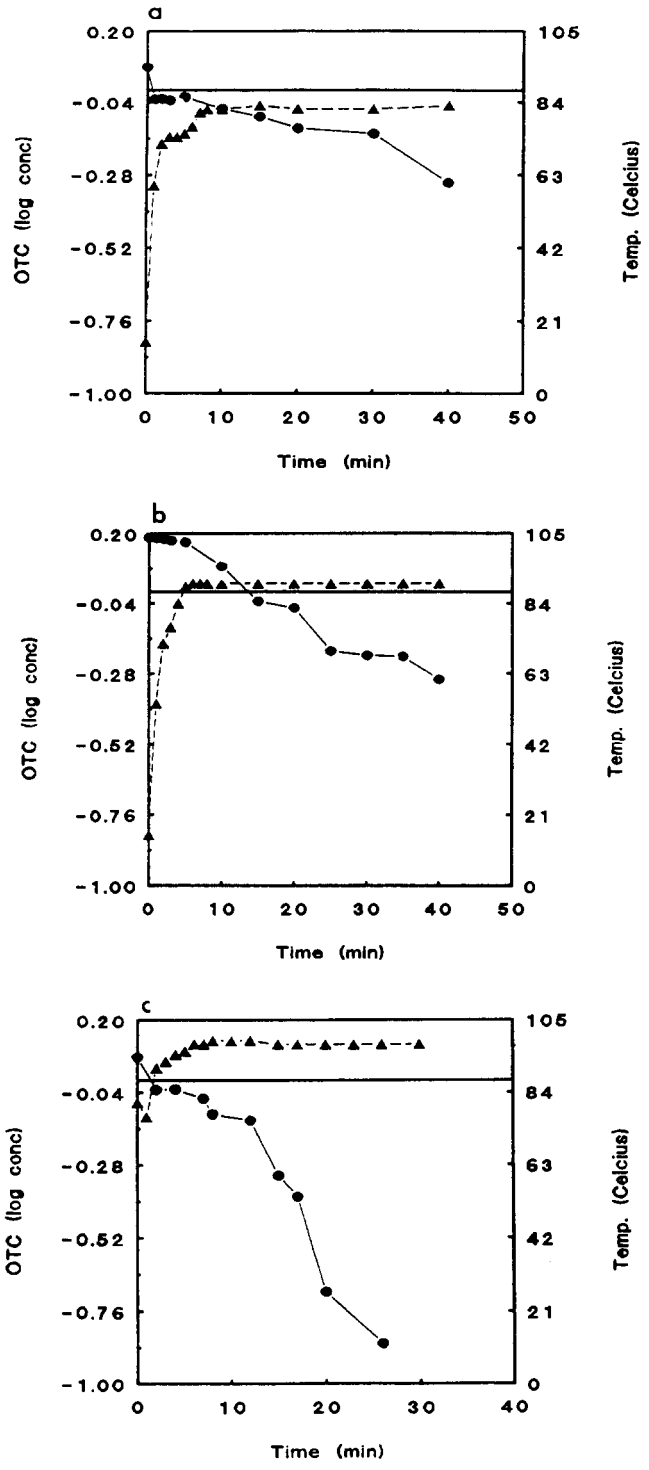

Figure 2. Oxytetracycline thermal destruction rate profile (●) and come-up temperature profile (▲) in composite salmon muscle sample derived from experiments conducted at 80 (a), 90 (b) and 100 °C (c).

heat stability of OTC in salmon tissue compared to that in the model system used in this study. Therefore, thermocouples were implanted into the center of the muscle sample and reaction vials, enabling both temperature come-up time and variation to be monitored. In all cases, temperature lag times were the same in both the buffer and fish muscle systems and did not contribute significantly to the TDT curves. The different OTC D values from similar pH buffer and salmonid muscle matrices were consistent with previous findings that have shown characteristically different thermostabilities of OTC activity in various media (Pilet et al., 1969). For example, OTC is more stable in milk than chicken or beef muscle extract when heated at 100 °C (Pilet et al., 1969).

Confirmation that the results of the TDT experiment with OTC in salmon tissue represented an actual cooking procedure was done by cooking fish patties at 100 °C for 15 min. The residual amount of OTC in the patties corresponds to previous work conducted with chlorotetracycline in similarly sized ground beef patties and frankfurters (40% retained) that were cooked at 136 °C for 10 min (Escanilla et al., 1959) but is far greater than that reported for OTC in breaded oysters (3.6% retained) or crab cakes (7.5% retained) (Bernarde, 1957). The differences in these studies may be due to the greater specificity of HPLC in quantitating differences in absolute OTC concentration vs OTC antimicrobial activity. An alternative explanation for the differences in these studies is the variation in relative weights of the muscle foods tested, which in turn would influence center point temperature and thus the degree of exposure of the drug to heat. Results obtained in poultry meats and eggs, which completely inactivated OTC at similar thermal processing temperatures used in this study, were conducted for longer time (40 min) periods (Yonova, 1971). O'Brien et al. (1981) examined the effect of roasting temperatures on antibiotic stabilities and reported only partial inactivation with oxytetracycline. Scheibner (1972a,b) showed complete inactivation of OTC with canning in glass and tin containers but very little loss of OTC activity with smoke processing or typical scalding procedures. Thus, our OTC degradation results in cooked salmon muscle support the conclusions of Moats (1988), drawn from studies with meat, milk, and eggs, that ordinary cooking conditions cannot be relied on to totally inactivate even the more heat sensitive antibiotics, such as OTC.

Finally, the finding that the D value and corresponding z value for OTC were comparable with other nutrients and toxins but different from most microorganisms potentially present in food at a common temperature (Lund, 1988; Gill et al., 1985) indicates that OTC is much more heat stable and its stability less temperature dependent than some common food-borne bacteria or spores of public health concern. The OTC Arrhenius activation energy constant calculated in salmon muscle (E_a = 20.4 kcal/mol) in this study is common to the inactivation of many nutrients and sensory (texture, colour) parameters which exhibit a similar temperature dependency (E_a = 20–30 kcal/mol; Lund, 1988) but is different from some bacterial spores and heat-labile enzymes that have a thermal destruction pattern which is more temperature dependent (E_a = 50–200 kcal/mol). Thus, it can be concluded from our study that the total destruction of OTC residues in fish muscle by thermal processing would result in the loss of nutritional and sensory quality factors that govern acceptance for consumption.

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