Oxytetracycline Degradation in Model Meat Processing Systems

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Ricky Wayne Fedeniuk

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ABSTRACT

With the advent of the near ubiquitous use of antibiotics and other xenobiotics in food production, the potential for traces of these compounds to reside in foodstuffs exists. Oxytetracycline (OTC), an antibiotic from the tetracycline family, is widely used in animal husbandry and is one of the most frequently found residues in foods of animal origin. OTC does not undergo metabolic transformation *in vivo*, but it does exhibit a certain degree of lability at temperatures used in meat processing. The determination of the rate of OTC breakdown under conditions commonly used in meat processing would allow the appropriate authorities to estimate with greater confidence the probable intake of this compound and its degradation compounds in the diet.

To date, few reliable techniques for the separation and quantification of mixtures of OTC and its common degradation products exist. A reverse-phase liquid chromatographic technique was developed that successfully quantified mixtures of OTC, 4-epioxytetracycline (4-epiOTC), α- and β-apooxytetracycline (α- and β-apoOTC) down to 40, 20, 50 and 140 ng/ml, respectively, at ambient temperature in less than 35 minutes. A 0.1 M ammonium acetate buffer (pH 3.0)-acetonitrile-tetrahydrofuran (72.5:12.5:15, v/v/v) mobile phase was found by means of the simplex method of solvent optimization to give excellent separation of the compounds. Solid-phase extraction (using RP C_{18} cartridges) of OTC from aqueous media and tissue gave overall recoveries

greater than 90 and 70 %, respectively. Degradation compounds had much lower recoveries, excluding their being monitored by the developed technique.

Previous research on the degradation of OTC during processing has focused on absolute changes in OTC. There have been no systematic studies on the rate of OTC degradation. The rate of OTC degradation was observed at 60, 70 and 80°C in several different aqueous and tissue media in order to ascertain the effects of the main factors commonly manipulated in meat processing practices (temperature, a_w, pH, additives). In contrast to observations for other compounds, OTC degradation (as measured by k_{obs}) was found to be independent of glycerol-adjusted water activities (a_w) in the range 0.6 to 1.0; entropy decreased as a_w was lowered. Monomeric (ortho) phosphate significantly increased k_{obs} whereas polymeric phosphates significantly decreased ($p < 0.05$) k_{obs}. The addition of calcium chloride to aqueous solutions significantly lowered k_{obs}, and decreased the entropy of the reaction, due to the formation of thermally stable OTC-Ca^{2+} complexes. Sodium chloride and sodium nitrite were found to have negligible effects upon k_{obs} in solution.

When studying OTC degradation in tissue matrices, several unusual anomalies were evident. Degradation of OTC in porcine tissue was significantly lower than that in aqueous buffer. Addition of metal-chelating agents (EDTA, polymeric phosphates) increased k_{obs} to values similar to that observed in aqueous solution. This was speculated to be due to decreasing the availability of polyvalent cations for OTC complexation, which decreased the formation of OTC-cation complexes. Additional evidence for the occurrence of metal complexation was provided by binding studies of OTC to BSA and

porcine tissue. The studies revealed that OTC binding to tissue was due in part to complexing protein-bound ions.

In contrast to observations in aqueous solution, sodium chloride and sodium nitrite in tissue increased k_{obs} at 60°C, whereas k_{obs} was lowered at 80°C. Sodium nitrite in tissue had a dramatically greater effect upon k_{obs} than aqueous sodium nitrite. Calcium chloride decreased k_{obs} in tissue, but to a much lesser extent than in aqueous solution.

The formation of several degradation compounds was noted. Application of the biexponential model to quantify the formation of one unknown compound was not successful, indicating that its formation kinetics did not obey a simple first-order model. Enthalpy/entropy compensation was exhibited by all treatments, indicating that common meat processing practices do not change the nature of the rate-limiting degradation mechanism, which according to previous literature and data obtained is an acid-catalyzed E2 elimination.

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1. INTRODUCTION

The world's population is increasing at an exponential rate. As a result, there is continuous pressure on available food resources to yield higher quantities with decreased inputs. This is accomplished by several means including mass production, selective breeding for favourable agronomic traits, utilization of more efficient farming practices, application of biotechnology, and increased utilization of by-products. Efficient farming practices includes the practice of intensive animal production, with animals confined to restricted areas for the purposes of feeding and management. This practice does allow for an increased output/input ratio, but at a cost. The close quarters are conducive to the spreading of disease, as well as increasing general stress levels. These factors, when combined, result in increased mortality rates, and ironically, decreased production. To offset this, xenobiotics (i.e. foreign compounds) are commonly used for the purposes of disease treatment, disease prophylaxis, and growth promotion. The range and number of xenobiotics allowed for use is vast, but the antibiotic class is the largest (MacNeil and Ellis, 1995). Antibiotics can treat or prevent disease by actively destroying microorganisms, or by severely limiting their ability to reproduce.

Oxytetracycline, a member of the tetracycline family of antibiotics, is one of the most commonly used antibiotics in animal husbandry. It is bacteriostatic in nature, and has applications in active disease treatment, prophylaxis, as well as growth promotant properties. Health Canada regulates OTC usage in terms of amount, species, withdrawal times, and the maximum residue level (MRL) allowable in edible tissues through

application of the Food and Drugs Acts and Regulations (Canada, 1985). Actual enforcement is the responsibility of veterinary meat inspectors at sanctioned meat processing facilities, as well as laboratory services provided by the Canadian Food Inspection Agency. Compliance with the regulations is generally good; however, the 1994-1995 Report on Chemical Residues in Meat and Poultry Products (Agriculture and Agri-Food Canada, 1995) indicates that violative occurrences with OTC are increasing in swine.

Because of the potential for OTC as well as other xenobiotic residues in food products, there is some concern with adverse health effects as a result of exposure to them. OTC is generally not known for its ability to cause acute reactions upon exposure, though it has been reported to occur. Better known are the adverse effects caused by exposure to degradation compounds of the tetracyclines. Degraded tetracycline preparations have been associated with reversible Fanconi syndrome, a form of kidney disease (Frimpter et al., 1963). This has been attributed specifically to the presence of anhydrotetracycline in old formulations. Heat-degraded OTC has also been shown to exhibit toxic effects, though identification of the compounds responsible for this has not been done (Tropilo et al., 1988).

Meat processing is the physical, chemical or enzymatic treatment of meat for the purpose of altering its original form. Processing serves the purposes of enhancing shelf-life, palatability, utility, and consumer preferences of a product. Thermal treatment of meat is a near ubiquitous process. Chemical treatments, such as the application of sodium chloride, phosphates, and sodium nitrite are also commonly used in meat

processing. Each of these additives serve a different purpose during the conversion of raw muscle tissue to edible meat products, due to their unique chemistries.

To date, there exists only limited information concerning the behaviour of OTC residues in tissue as a result of its exposure to common meat processing practices. Research to date has been limited to measuring the absolute changes in OTC amount as a function of a specific process, which makes extrapolation to other processing situations difficult. A better approach would be to determine the rate at which the amount of OTC changes as a function of processing parameters. This would allow for extrapolation of experimentally-derived results to other processes.

The primary goals of this project were to determine and quantify the effects of common processing parameters (heat; water activity; the presence of sodium chloride, calcium chloride or sodium nitrite) upon the degradation of OTC. The first objective was to develop a method that was capable of detecting and quantifying OTC as well as some of its common degradation compounds. Secondly, monitoring of OTC degradation as a function of time was required to quantify the rate of OTC degradation, as well as the rate of formation of degradation compounds. Thirdly, quantification of secondary kinetic parameters (energy of activation, changes in enthalpy and entropy) was performed to provide evidence for the interaction between processing parameters and OTC with respect to its rate of degradation. Fourthly, partitioning the effects of the processing parameter and the medium in which the degradation occurred (i.e. aqueous vs. tissue) was carried out to discriminate between processing and medium effects. As violative occurrences of OTC residues in pork tissue appear to be increasing (Agriculture and Agri-Food Canada, 1995), it was selected as the tissue for study.

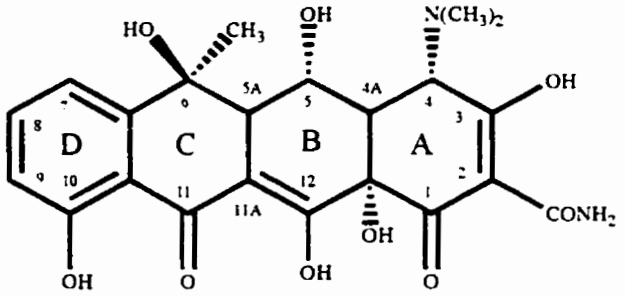
2. LITERATURE REVIEW

2.1 Oxytetracycline

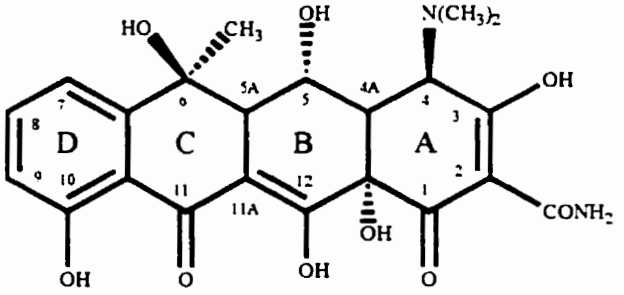
2.1.1 History

Antibiotics are a diverse array of chemical compounds that exhibit bacteriostatic and bacteriocidic properties towards susceptible microorganisms. Formal studies of the properties of antibiotics have been performed since the late nineteenth century (Rolinson, 1995). In 1935, sulphonamides, a family of related chemical agents, were the first antibiotics utilized successfully for the *in vivo* treatment of bacterial induced infections which also exhibited minimal toxicity towards a eucaryotic host (Fleming, 1995). To date, several hundred different antibiotics are characterized with regards to their chemical and therapeutic properties.

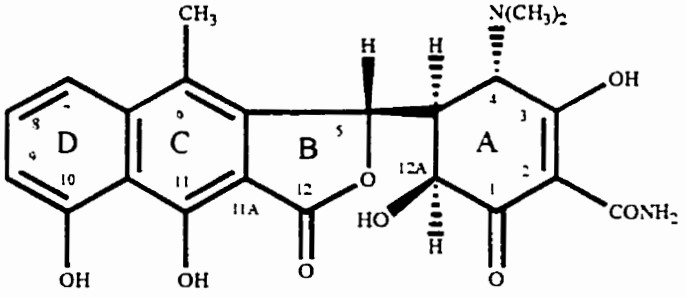
In 1949, chlortetracycline (CTC) was the first antibiotic of the tetracycline family isolated (Mitscher, 1978). Oxytetracycline (OTC) was isolated from the fermentation liquors of *Streptomyces rimosus* in 1949 at Pfizer Laboratories (Mitscher, 1978). The formal chemical abstracts indexing name for OTC (Figure 2.1) is 2-naphthacenecarboxamide, 4-(dimethylamino)-1, 4, 4a, 5, 5a, 6, 11, 12a-octahydro-3, 5, 6, 10, 12, 12a-hexahydroxy-6-methyl-1, 11-dioxo, 4s-4α, 4aα, 5α, 5aα, 6β, 12aα. The chemical abstracts service (CAS) registry number is 79-57-2. Tetracycline, a 5-dehydroxy form of OTC, is the parent substance of the tetracycline family of antibiotics for nomenclatural purposes.



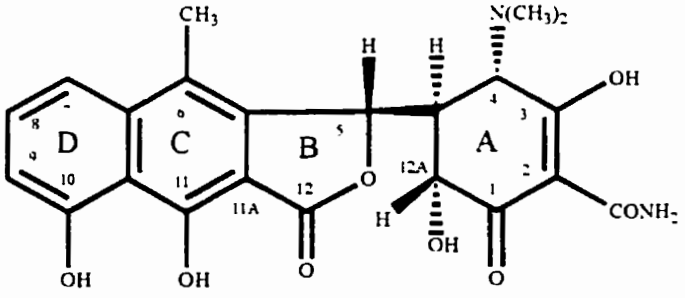
Oxytetracycline



4-epioxytetracycline



α-apooxytetracycline



β-apooxytetracycline

Figure 2.1. Structure of oxytetracycline and related degradation compounds.

2.1.2. Biosynthesis

Tetracyclines are secondary, polyketide-derived metabolites of actinomycetes, primarily *Streptomyces aureofaciens* and *S. rimosus* microorganisms. Polyketide and fatty acid biosynthesis involve head-to-tail condensation of acyl compounds, and thus both biosynthetic routes share some common reactions. The common reactions for the synthesis of both compounds involve acetyl, acyl and malonyl transferases, and β-ketoacyl synthase (Vining and Hopwood, 1995). The biosynthetic pathways diverge after creation of a β-ketoacyl intermediate; fatty acid biosynthesis involves subsequent reduction and dehydration reactions to generate fully reduced acyl groups, whereas additional acetyl groups are attached to the β-ketoacyl intermediate in polyketide synthesis.

The skeleton of all tetracyclines are synthesized from one malonic acid semiamide and eight malonyl-CoA units by monofunctional proteins that associate to form a multi-enzyme complex. The first intermediate in the biosynthesis of OTC is 6-methylpretetramid (Figure 2.2). Subsequent reactions include hydroxylation at C-4, hydration at C-12a, transamination and *N*-methylation at C-4, hydroxylation at C-6 and C-5, and reduction of the C-5a, C-11a double bond (Behal and Hunter, 1995).

2.1.3 Chemical Reactivity

Oxytetracycline is chemically stable in dry form or in aqueous solution if kept at refrigeration or subrefrigeration temperatures (Honikel et al., 1978; Iversen et al., 1989). However, at higher temperatures it becomes increasingly labile. Under mildly acidic or basic conditions, the degradation kinetics are dictated primarily by the C-5 and C-6

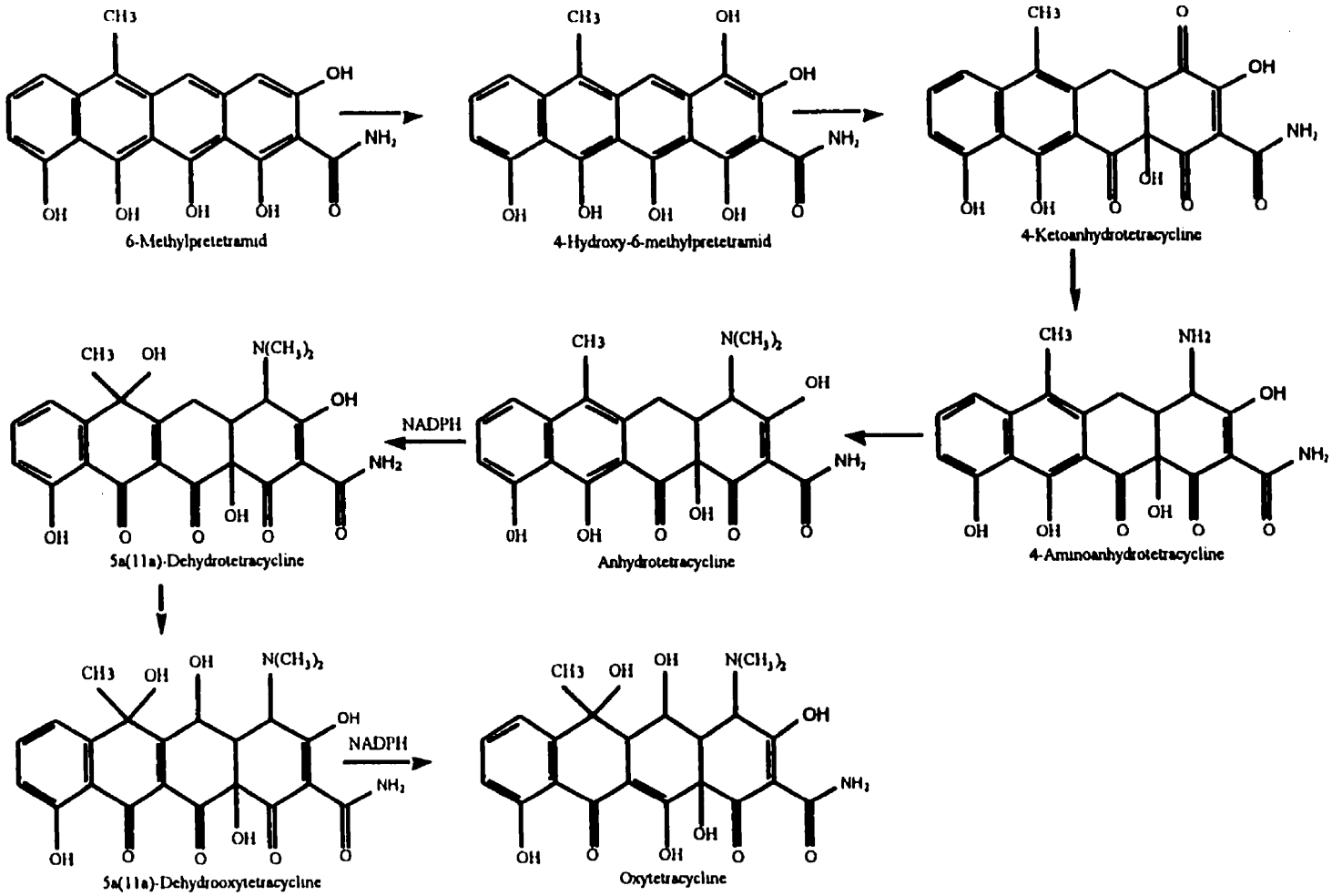


Figure 2.2. Partial biosynthetic route of oxytetracycline. Source: Adapted from Behal and Hunter (1995).

hydroxyl moieties, and the C-4 dimethylamino group (Mitscher, 1978). The C-6 hydroxyl is axial and trans to the C5a-H, making it sterically ideal for an E2 elimination reaction (Figure 2.3). The resultant anhydrooxytetracycline formed can be isolated in alkali media, though it is otherwise unstable, and undergoes scission of the B ring (mediated by the C-5 OH group) to produce the isomeric phthalides, α- and β-apooxytetracycline (α- and β-apoOTC) (Yasin and Jeffries, 1988). OTC undergoes first-order reversible epimerization of the C-4 dimethylamino group in a variety of aqueous solvents within the pH range of approximately 2-6 (McCormick et al., 1957); the rate of epimerization is dependent upon the nature of the ions present in the reaction media.

The toxicological significance of OTC thermal degradation products has been reported (Tropilo et al., 1988) though the chemical nature and quantity of the degradation compounds were not determined. It has been shown that epianhydrotetracycline, a degradation product of tetracycline, can cause nephropathy in susceptible individuals (Frimpter et al., 1963; Wegienka et al., 1964; Cleveland et al., 1965; Fulop and Drapkin, 1965).

Tetracyclines are strong metal chelating agents, with the site of chelation due to the 11, 12-β-dicarbonyl system and the A ring. At low pH, chelation occurs at the BCD rings and as the pH is increased, the A ring becomes involved with the carboxamide group and the 1 or 3-enol. Equilibrium constants (K_{eq}) for the chelates of multivalent positive ions with OTC range from log values of 4 to 9 (Albert, 1953).

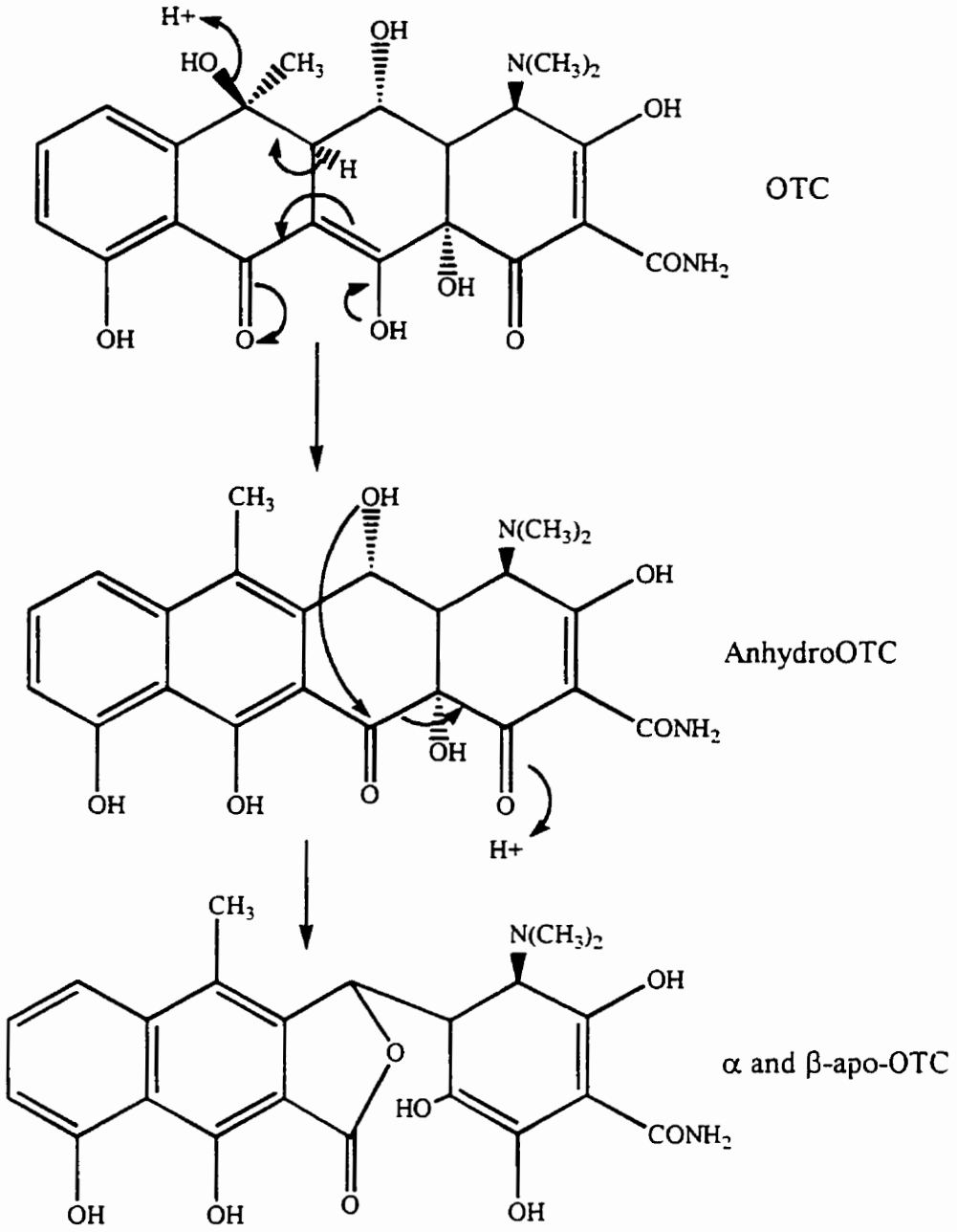


Figure 2.3. Degradation of oxytetracycline by C-5a:C-6 E2 elimination.

OTC also undergoes complexation with several unrelated classes of biochemicals (Higuchi and Bolton, 1959), as well as with ions. Equilibrium constants ranging from 2 to 30 have been reported for several biochemicals (Higuchi and Bolton, 1959). The interaction of tryptophan with OTC has a reported K_{eq} of 4. Human and bovine serum albumin (BSA) have been reported to complex with OTC with K_{eq} of log value 4 (Ma et al., 1973; Zia and Price, 1976). However, equilibrium constants are a function of both the method of determination, as well as protein concentration. It is known, however, that the predominant mechanism for OTC binding to protein is hydrophobic in nature, with bonding occurring at or near tryptophan residues. Other mechanisms, such as charge-transfer, hydrogen bonding and ionic interactions also influence the extent of interaction.

2.1.4 Analysis

Analysis for antibiotic residues in animal tissues has traditionally been performed using bioassay techniques. These techniques include diffusion systems (cylinder-plate, well-plate, pad-plate), turbidimetric systems, and immunological systems (Nelis and Leenheer, 1980; Hasselberger, 1993). Diffusion assays are capable of detecting 80-100 ppb OTC in animal tissue. However, tetracyclines have a high affinity for the tissue matrix (this has been attributed to hydrophobic binding and charge transfer mechanisms), thereby making diffusion assays inaccurate; lipophilic derivatives of tetracycline are particularly prone to this problem (Nelis and Leenheer, 1980). The official AOAC method 968.50 (Association of Official Analytical Chemists, 1990) is a non-specific microbiological plate assay (Hasselberger, 1993). Other assays such as the swab test on

premises (STOP) lacks sensitivity and specificity for detection of OTC residues (MacNeil et al., 1989).

HPLC methods have gained prominence for the analysis of OTC. The limit of detection for OTC residues using ultraviolet detectors is reported to be approximately 10 ppb (White et al., 1993). Reversed-phase chromatography is the preferred method for OTC analysis (Oka and Suzuki, 1984), though the exact nature of the separation mechanism is still unknown. Adjustment of the mobile phase pH alters the ionic form of the tetracyclines; at low pH, the cationic form of tetracyclines are predominant, so that interactions with negative counterions are then favoured (Knox and Jurand, 1975). However, increasing the ionic strength decreases the chromatographic capacity of tetracyclines, which conflicts with retention behaviour based solely on hydrophobic interactions. It is assumed that solutes in the mobile phase adsorb onto the packing, displacing the tetracyclines from the active sites. Mobile phases containing phosphate buffers give chromatographic efficiencies and capacities similar to those containing organic acids. These findings indicate that interaction of tetracyclines with free silanol groups of the silica support are in part responsible for the separation mechanism.

Polymeric columns are also used for separation work on tetracyclines (Naidong et al., 1990; Agasoster and Rasmussen, 1992; White et al., 1993). Polymeric columns avoid problems of interaction of tetracyclines with the free hydroxyl groups of the silica support of alkyl bonded columns and of reduced column lifetime due to the use of acidic mobile phases that are sometimes used for tetracycline analysis (Reeuwijk and Tjaden, 1986; Naidong et al., 1992). It is believed that the separation mechanism of tetracyclines on bare polystyrenedivinylbenzene (PSDVB) supports is due to π–π interactions

between the D ring of the tetracycline and the aromatic rings of the stationary phase (Naidong et al., 1990). It has been stated that the column efficiencies of polymeric and alkyl-bonded columns of similar particle sizes are similar, though some researchers have stated that resolution of PSDVB columns is poor in comparison to alkyl-bonded columns (Salisbury, 1993). PSDVB columns have the advantage of separating epimers of the tetracyclines; alkyl-bonded columns apparently lack this ability (Khan et al, 1987; Yasin and Jeffries, 1988; Naidong et al., 1990). However, other reports (Hoogmartens et al,, 1989) indicate the resolution of doxycycline and its epimers on reversed-phase columns; Barnes et al. (1985) successfully separated a mixture of OTC, α-apoOTC, β-apoOTC and anhydroOTC on an octyl-bonded column, with 4-epiOTC being partially resolved from OTC.

A method that has been used at the Health of Animals Laboratory (Canadian Food Inspection Agency, Saskatoon, SK) is based on the method of Oka et al. (1985). Extraction of the cationic species of tetracyclines from tissue is accomplished using an aqueous pH 4.0 EDTA buffer. Solid-phase extraction (SPE) C_{18} cartridges (Bond Elut C_{18}) are used to clean the extract by non-specifically binding drugs from the aqueous extract. Bond Elut C_{18} cartridges are preferred over Sep-Pak C_{18} and Baker 10 C_{18} cartridges due to superior adsorption capacities for tetracyclines (Oka et al., 1985). SPE cartridge consistency is vital for reproducibility. Carignan et al (1993) utilized a sample preparation procedure omitting the use of SPE to decrease variability in results; when tissue was spiked at 1 ppm, a day to day coefficient of variation (CV) of 13 % was obtained. The method of Oka et al. (1985) reported a CV less than 20 %. Oka et al. (1987) used tandem reversed-phase and ion-exchange cartridges for cleaning honey

samples for residue analysis; this was not found to be necessary for tissue samples. Mulders and Van de Lagemaat (1989) found that treating Sep-Pak C_{18} cartridges with a solution of 4 % dimethylchlorosilane prior to activation eliminated residual silanol groups; the resulting recoveries of OTC increased from 80 to 100 %. Drug elution from the columns is accomplished using a methanolic oxalic acid solution. This precludes the ability to concentrate the solution due to the decomposition of the tetracyclines by oxalic acid during evaporation (Ikai et al., 1987; Mulders and Van de Lageemaat, 1989). The tetracyclines are chromatographed on an Inertsil® reversed-phase C_8 column. Inertsil® columns have been end-capped to reduce the number of free silanol groups present, thereby decreasing polar interaction of tetracyclines with the support, subsequently reducing tailing and increasing resolution. A C_8 column has been used for the separation of tetracycline from its degradation products (4-epitetracycline, anhydrotetracycline and 4-epianhydrotetracycline) (Oka and Suzuki, 1984) and for the partial separation of OTC from 4 epi-OTC, α-apoOTC, β-apoOTC and anhydroOTC (Barnes et al., 1985). Iversen and coworkers (1989) reported that reversed-phase columns could not be used for the analysis of OTC residues in plasma of rainbow trout due to endogenous compounds coeluting with OTC. Instead, a cyano column was used to give good results. This is in contrast to the results of Bjorklund (1988) who reported using C_8 and C_{18} columns successfully for analyzing OTC from rainbow trout. Bjorklund (1988), however, used an SPE cartridge for isolation of the tetracyclines, whereas Iversen and coworkers (1989) did not. Ueno et al. (1992) describe a direct injection technique of rainbow trout serum spiked with OTC on a HISEP shielded hydrophobic phase column (polymer hydrophilic/hydrophobic phase bonded on silica gel).

The use of electrically driven separation methods (i.e. capillary electrophoresis, (CE)) for the separation of tetracyclines has been explored only recently. Impediments to the incorporation of such methods for the analysis of drug residues are primarily due to the difficulty in detecting low concentrations of material because of the relatively small sample sizes (nl vs µl in HPLC) used. This can be circumvented by sample concentration techniques, and research has shown that CE can be used successfully to separate different tetracycline mixtures (Chen and Gu, 1995; Pesek and Matyska, 1996). However due to the use of concentration steps, reproducibility does decrease. Additionally, no CE technique to date has been reported that is capable of separating the common OTC degradation products.

2.2. Biological Properties of Oxytetracycline

2.2.1 Medical/Agricultural Uses

Chopra (1985) reported that the major mode of the tetracyclines' inhibitory action upon bacterial growth is their ability to bind with a relative high affinity ($\log K_a \cong 6$) to the ribosomal A-site, thereby interfering with the binding of aminoacyl tRNA's to ribosomes which interferes with protein synthesis. At higher concentrations, binding to the ribosome P-site as well as inhibition of polypeptide chain termination also occur. Therefore, transport of tetracycline into the cell is a prerequisite for its bioactivity. Transport into the cell is dependent upon the type of bacteria. Gram-negative bacteria have an outer membrane in addition to the cytoplasmic membrane found in gram-positive bacteria. Transport of tetracyclines across this outer membrane is by means of passive diffusion through transmembrane protein pores and apolar

membrane regions. OTC, due to its relatively polar nature (water solubility, 5-31 mg/ml), diffuses primarily through the hydrophilic protein pores (Chopra, 1985). Transport of tetracyclines across the cytoplasmic membrane is by both passive diffusion as well as energy dependent systems. The primary energy dependent system involved is the carrier mediated transport system coupled to the proton motive force. Tetracycline resistance is due primarily to the ability of the organism to actively remove tetracyclines from the cytosol of the organism via other carrier mediated transport systems.

Tetracycline antibiotics are broad-spectrum antibiotics, effective against a wide range of microorganisms. Microorganisms of the genera *Staphylococcus*, *Streptococcus*, *Legionella*, *Chlamydia*, *Pasteurella* and *Mycoplasma* are generally sensitive to all members of the tetracycline family (minimum inhibitory concentrations 0.04-3.1 μg/ml) (Cunha, 1985). In addition to medical uses, however, tetracyclines are popular for use in veterinary formulas that are both active and prophylactic in nature (Gustafson and Kiser, 1985). As a result, the use of tetracyclines and other antibiotics for veterinary uses accounts for approximately half of all antibiotics produced, with a majority of this due primarily to use as feed additives (Shahani and Whalen, 1986).

The major prophylactic use of the tetracyclines is for their inter-related properties of growth promotion and disease prevention. It has been shown that antibiotic use at subtherapeutic levels results in reduced mortality, increased weight gains, lower feed to gain ratios, which results in lower production costs and hence increased returns on profit (Hays, 1986).

2.2.2 Regulatory Overview of Veterinary Drugs

In Canada, the registration and approval of veterinary drug products is carried out by the Bureau of Veterinary Drugs, Health Protection Branch by way of the Food and Drugs Acts and Regulations (MacNeil and Ellis, 1995), a process which approximately parallels that in the United States. To get approval, a sponsor must supply data with regards to the drug's clinical uses as well as its target species, human safety assessment, and procedures for analysis of the active ingredient in formulations as well as for analysis of residues in edible tissues at the established MRL. Human safety data includes information regarding metabolic studies of the drug in the target species, the form (metabolite) in which potential residues are found, and their distribution in the animal. Toxicity/carcinogenicity data, another component of the human safety data, is collected to establish an acceptable exposure level to the drug/metabolites. In Canada, there is not an established MRL for OTC, though for tetracycline the level is 0.25 mg/kg (0.25 ppm) for most edible tissues and fluids (MacNeil and Ellis, 1995).

Residue monitoring is the responsibility of the Canadian Food Inspection Agency (Canadian Food Inspection Agency, 1997). Both random monitoring, based on a national survey plan, and suspect monitoring is conducted by veterinary meat inspectors at slaughter plants. Suspect monitoring refers to analysis of animals with obvious lesions or that are of questionable health at the time of slaughter. Though compliance with residue levels is generally good, pork violative rates have been increasing. Levels of OTC that have been found in pork muscle tissue have been as high as 1.74 ppm, whereas hog kidneys have had levels up to 21.1 ppm, well in excess of acceptable residue levels (Agriculture and Agri-Food Canada, 1995).

2.2.3 Pharmacokinetics

2.2.3.1 Animal Species

The 1993 Canadian Compendium of Veterinary Products (Canadian Animal Health Institute, 1993) lists several injectable formulations meant for use in cattle, swine, and sheep. Additionally, several species of scientific and economic importance have also been tested for their internal distribution and disposition of OTC, including mice (Snell, et al., 1958), rats (Curl et al., 1988), horses (Horspool and Mckellar, 1990), rabbits (McElroy et al., 1987), buffalo (Varma and Paul, 1983), camels (Oukessou et al., 1992), turkeys (Dyer, 1989), and various farmed fish species (Bjorklund and Bylund, 1991). Pharmacokinetic parameters including half-lives ($t_{1/2}$) and volumes of distribution (V_d) have been calculated for these animals. Half-life indicates the time needed for the concentration of drug to reduce to one-half of its initial measured concentration; V_d is a measure of the distribution of the drug throughout the body, defined as the ratio of dose administered to the initial plasma concentration. Hence, a low V_d indicates that the drug is confined primarily to the circulatory system, whereas a high V_d indicates that the drug is distributed to other tissues of the body (Shargel and Yu, 1993). Table 2.1 summarizes these parameters for adult animals of economic importance. Kirkwood and Widdowson (1990) report that the elimination half-life of OTC can be modelled as a function of bodyweight by an allometric equation. Utilizing literature data, they were able to fit half-life data according to the allometric equation:

$$t_{1/2}(\text{final elimination phase}) = 160w^{0.20}$$

where w is the weight of the animal. This equation was found to be not significantly

Table 2.1. Half-lives ($t_{1/2}$) and volumes of distribution (V_d) of intravenously administered oxytetracycline formulations in different animal species.

Species/Animal	$t_{1/2}$ (min)	$V_d$area(L/kg)	Source:
Cow	540-618	0.80-0.87	Nouws et al., 1983
			Toutain and Raynaud, 1983
Pig	225-306	1.38-1.84	Xia et al., 1983
			Pijpers et al., 1990
Sheep	221	---	Kirkwood and Widdowson, 1990
Camels	460	0.80	Oukessou et al., 1992
Rabbit	54-120	0.55-1.37	McElroy et al., 1987
Turkey	43	3.60	Dyer, 1989
Rainbow Trout	4800	1.33	Grondel et al., 1989

different from the allometric equation describing metabolic turnover time. This relationship was determined using a database of terrestrial homeotherms including the cow, pig, camel, buffalo, sheep, goat, rabbit, pheasant, chicken and turkey. From Table 2.1, it is evident that this equation does not apply to aquatic life, as OTC elimination is also dependent on environmental temperature (Grondel et al., 1989; Bjorklund and Bylund, 1991). Similar correlations have not been determined for V_d, though differences have been noted for other pharmacokinetic parameters. Banting et al. (1985) reported that the area under the curve (AUC- a measure of drug availability) for the same dosage levels applied to calves and pigs were 2.6 times lower in pigs than calves. This in turn indicates that dosage levels would have to be substantially higher in swine than in calves to maintain equivalent therapeutic levels.

2.2.3.2 Tissue Type

Early reports on the pharmacokinetics of intravenously (iv) administered OTC indicated that the elimination of OTC was primarily by excretion via glomerular filtration (Kunin, 1967). Mevius et al. (1986a) had reported that in swine only 42-60 % of iv doses are eliminated renally, and that this clearance was urine flow dependent, and correlated well with creatinine clearance, indicating that OTC was excreted by glomerular filtration and tubular secretion. A similar mechanism was reported in dairy cows (Mevius et al., 1986b). The remainder of the dose is speculated to be eliminated by fecal excretion via losses incurred during enterohepatic recycling, and sequestration by bones. The effect of sequestration was speculated to be of significance since OTC

sequestration would be greater in young, growing animals, thereby resulting in potentially longer persistence of the drug in the animal.

The kinetic model of elimination of OTC has been controversial, due to different investigators using different assay procedures, dosage levels, and type of pharmacokinetic analyses (Banting et al., 1985). Both two and three compartment open models have been applied to pigs and cows (Xia et al., 1983); differences in conclusions have been speculated to be due mainly to the frequency in which initial blood sampling is performed. A large number of samples in the initial phases supports the three compartment model (Figure 2.4). The ratio of back diffusion to the central compartment for compartments 2 and 3 for cows and pigs is approximately 10, indicating that the elimination of OTC from the deep tissue compartment is significantly slower than from the secondary tissue storage compartment. The deep tissue compartment has been speculated to be the result of enterohepatic recycling or bone storage (Meijer et al., 1993). A graph of a typical plasma concentration-time curve for an iv administered dose of OTC is illustrated in Figure 2.5.

Intramuscular (im) administration of OTC has the advantage of maintaining therapeutic levels of OTC in an animal for extended time periods with only minimal animal handling. Rate of absorption of the drug from the depot site (k_a) has been speculated to be due to two factors; diffusion of the drug into the bloodstream and blood flow to the depot site (Bederka et al., 1971). If diffusion was the rate-limiting step for drug absorption from the depot site, it would be proportional to the concentration and would proceed by means of first-order absorption (Greenblatt and Koch-Weser, 1976). The primary determinant for k_a would be the drug partition coefficient. Lipophilic drugs

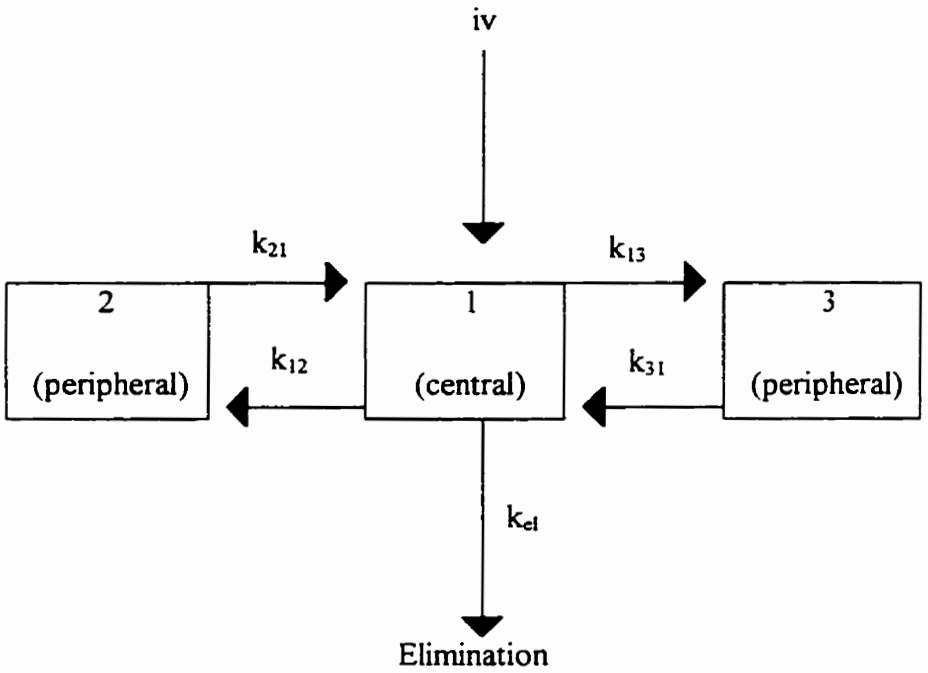


Figure 2.4. Schematic diagram of the three compartment open model.
Peripheral compartment 2 = tissue storage;
Peripheral compartment 3 = deep tissue storage;
Source: Mevius et al. (1986b).

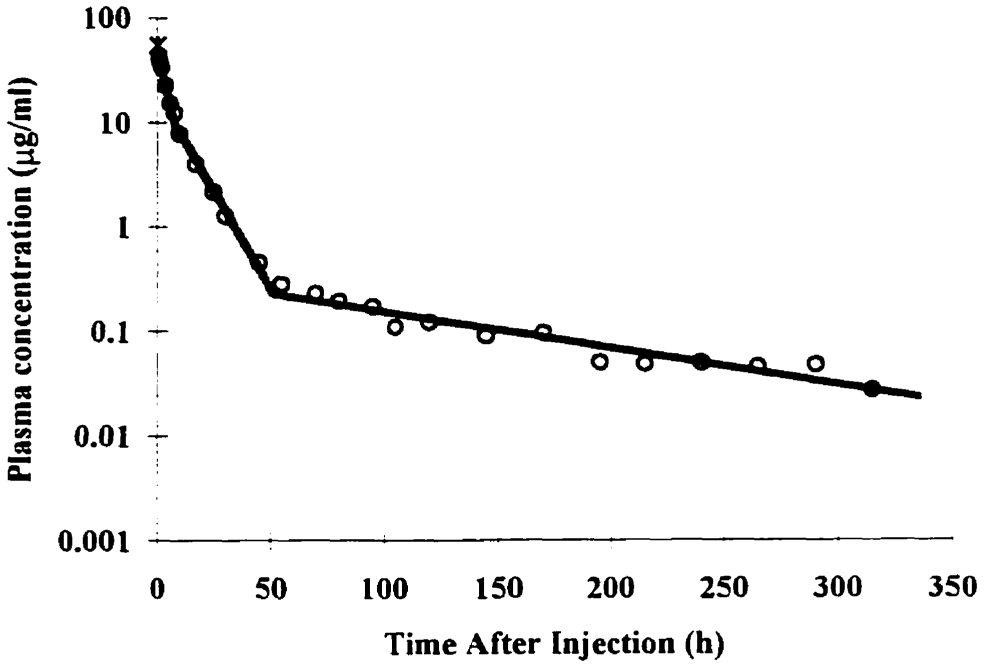


Figure 2.5. Measured concentrations (mean) and mean fitted plasma concentration time curve for oxytetracycline after a single iv administration of 40 mg/kg body weight to veal calves (n=5).
Source: Adapted from Meijer et al. (1993).

would diffuse directly through the membranes of capillary endothelial cells into circulation, whereas hydrophilic drugs can enter circulation only via the pores in the capillary membrane (Chopra, 1985). Lymph flow would only account for a small fraction of the drug removed from the injection site. However, for OTC, diffusion from the injection site is normally not the rate-limiting step. Nouws and Vree (1983) compared the absorption of im administered OTC preparations as a function of injection site; neck, shoulders, buttocks or subcutaneous injection (neck). Injection in the buttock region resulted in the lowest absorption and degree of bioavailability (83 %) for the formulation; the shoulder injection had a significantly higher bioavailability (99 %) as compared to an iv injection.

Absorption from im injection sites normally does not follow first-order kinetics, but is instead referred to as "non-linear absorption" (Greenblatt and Koch-Weser, 1976). This is due to several factors, including spreading of the injection volume to a larger area (thereby increasing k_a), increased osmotic pressure at the site of the injection (thereby drawing water into the site of injection, decreasing k_a), and drug precipitation at the injection site (decreasing k_a). The most significant factor affecting k_a is the amount of tissue damage at the injection site caused by the formulation (Mevius et al., 1986a and b; Nouws et al., 1990). The tissue damage itself has been attributed to both OTC and the delivery solvent (Rasmussen and Svendsen, 1976; Rasmussen, 1978). Tissue damage usually constitutes extensive oedema, fibro-angioblastic tissue, haemorrhages and muscle degeneration. Injection of OTC in aqueous solutions at concentrations less than 5 mg/ml has been reported to cause no visible tissue damage (Wright et al., 1954); however, the degree of reaction increases with increasing concentration. Polyvinyl pyrrolidone has

been reported as causing only minimal tissue damage (Mevius et al., 1986b); other vehicles such as aqueous 2-pyrrolidone and glycerol formaldehyde have been reported to cause extensive damage at injection sites (Nouws et al., 1990). The greater the degree of irritation, the greater the amount of residues found at the injection site. Mevius et al. (1986a) had proposed that tissue irritation effects cause variable residual OTC amounts due to two sequential events; an initial decrease in k_a due to a decrease in local blood circulation which in turn is due to initial tissue irritation, followed by an increase in blood flow and increasing k_a due to tissue inflammation. Intramuscular administration of OTC, therefore, provides prolonged release of OTC though it can be unpredictable. A schematic model (Figure 2.6) illustrates this unpredictability. Two or more different k_as are believed to exist, with the potential for a plasma concentration-time curve to exhibit both traditional and "flip-flop" kinetics during elimination. In traditional kinetics, k_a is higher than k_{el}, whereas "flip-flop" refers to the phenomena whereby k_a is lower than k_{el}, which affects the calculations required to estimate these parameters. The only way in which it can be verified that an im injection is exhibiting flip-flop kinetics is by comparison to an iv injected formulation.

Drug disposition in the body has been shown to be affected by disease status (Ames et al., 1983; Shargel and Yu, 1993). The two main mechanisms by which this occurs are the alteration of the elimination constant or alteration of storage compartments. These effects cannot be quantified due to inherent uncertainties in the disease process.

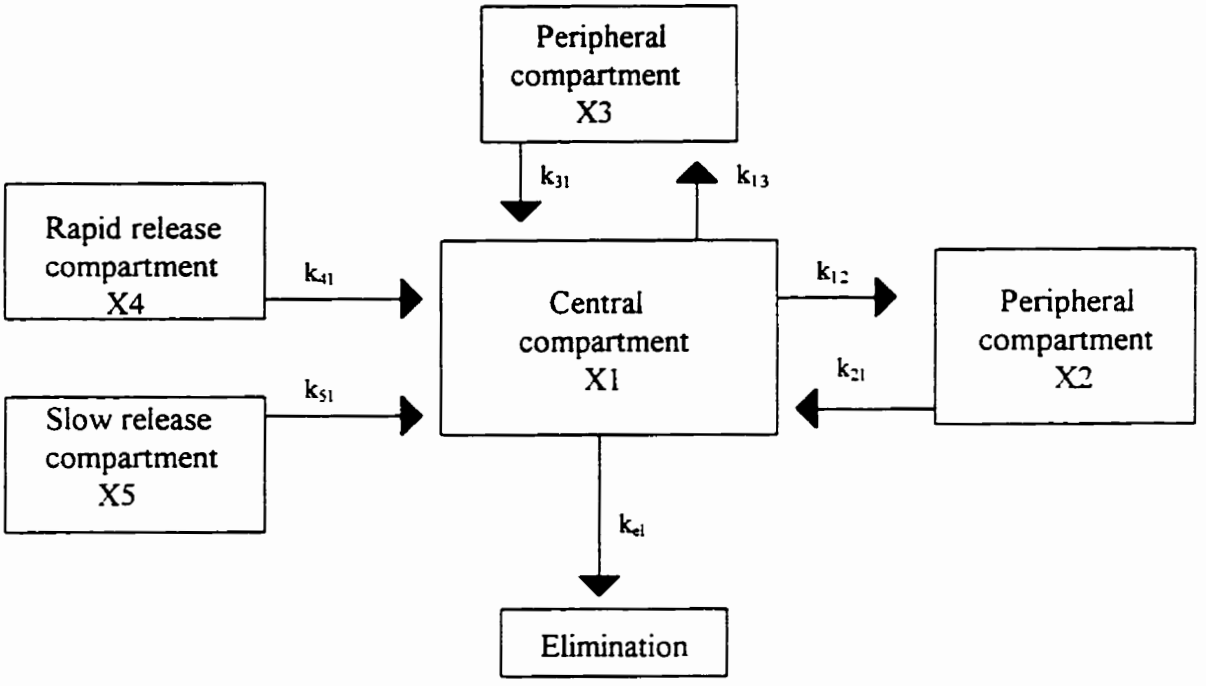


Figure 2.6 Open 3-compartment model with 2 compartments of absorption. Source: Adapted from Toutain and Raynaud (1983).

2.3 Effect of Processing on Oxytetracycline Stability

2.3.1 Meat Processing

Meat processing is defined as any physical, chemical or enzymatic treatment of meat that alters its original form (Romans et al., 1994). Processing serves the purposes of enhancing shelf-life, palatability, utility, and consumer preferences for a product. Processed meats include comminuted (e.g. sausages, meat patties), non-comminuted (e.g. ham, bacon), restructured and canned products. The common non-meat ingredients used for meat processing are water, nitrite, cure colour accelerators, salt, phosphates, plant proteins and other water-binding agents, and sweeteners, which may or may not be used in conjunction with heating (Claus et al., 1994). Nitrite is used primarily to prevent the outgrowth of *Clostridium botulinum* spores and subsequent toxin production as well as for colour and flavour formation; its presence in cured products is limited to levels below 200 ppm (Canada, 1994). Cure accelerators increase the rate of cure as well as having antioxidant properties to maintain the overall quality of a product. Salt is used for flavour, extracting proteins (subsequent increase in functionality), and extending shelf-life by lowering the water activity (a_w) of a product. Normal usage is approximately 2-3 % by weight of product. Phosphates are utilized for their ability to increase the water-holding capacity of meat as well as their antioxidant properties. Their use is limited to 0.5 % (dibasic orthophosphate basis) of the final weight of the product (Canada, 1994).

Heating is a near ubiquitous mode of meat processing. In addition to inactivation of microbes and enzymes, it induces several other changes in meat products. At 40-50°C, sarcoplasmic proteins begin to denature; at 50-65°C, myofibrillar proteins

denature with a subsequent decrease in water binding capacity and increased pH. At 65-80°C, collagen also begins to undergo changes similar to the myofibrillar proteins (Van Laack, 1994). Also during heating, 60-70 % of added nitrite will be decomposed (Okayama et al., 1991), though it was reported that cooking time, and not temperature is more important in determining the rate of nitrite destruction. The reported energy of activation (E_a) for nitrite decomposition is 13-14 kcal/mol (Olsman, 1973), which supports Okayama's and coworkers' findings. Polyphosphates are rapidly hydrolysed by *in situ* phosphatases present in meat (Molins, 1991), but these same enzymes are inactivated at temperatures greater than 60°C. Wazer and Callis (1958) reported that at pH 5-6, the rate of temperature induced hydrolysis of phosphates is slow.

The alteration of a_w in foodstuffs is one of the main methods used for increasing the shelflife of a food product. Several definitions for a_w exist. The first such expression was based on the escaping tendency of a solvent (also known as fugacity) from solution relative to that of pure solution. Later definitions used the approximation of the partial pressure of water above a solution/matrix relative to that of pure water (the two previous definitions tended to be within 1 % of each other, and are therefore regarded as being equal) (Fennema, 1985). By lowering a_w, you are decreasing the availability of water for physical, chemical, biochemical and microbiological reactions. As most reactions require the presence of a solvent media in which to occur, decreasing the availability of the solvent tends to decrease the rate of detiorative reactions. The a_w of most processed meat products ranges from 1.0 to 0.6 (Leistner et al., 1981). An a_w of 0.86 or lower prevents toxin production by *Staphylococcus aureus*, one of the main pathogens of concern in meat products.

Nitrite is a strong oxidizing agent ($HNO_2 + H^+ + e \rightarrow NO(g) + H_2O$, $E^\circ = 1.0$ V). It is by this reaction that the true curing agent, nitric oxide (NO), is formed which combines with myoglobin to form nitrosyl-hemochrome. Cure accelerators act as electron donors for the reaction, increasing the rate of the reaction. Nitrite also undergoes nitrosation reactions with proteins, and nitrosated products of tyrosine, tryptophan and phenylalanine have been isolated (Woolford et al., 1976).

Phosphates commonly used in meat processing are monomeric, dimeric, trimeric or formulations containing a mixture of varying degrees of polymerization (i.e. hexametaphosphate). They are used as antioxidants as well as to increase the water binding properties of cooked meats, the mechanism by which this occurs believed to be primarily a pH increasing effect, though evidence for actomyosin dissociation and myofibrillar protein dispersion exists (Lewis et al., 1986).

2.3.2 Stability Testing of Pharmaceutical Formulations

2.3.2.1 Methodology

Pharmaceutical stability has traditionally referred to the chemical stability of a drug substance in its dosage form (Carstensen, 1995). This is studied through the use of chemical kinetics, whereby the drug in a pure system or one including as few adjuvants as possible is evaluated to determine the manner and rate of degradative reaction mechanisms. This in turn allows for the determination of the stability of the formulation, which is of importance when establishing expiration dates. Stability tests on formulations are performed throughout the entire drug dosage development sequence.

In the USA, as well as in Canada, the general overall sequence as reported by Carstensen (1995) is as follows:

a) Synthesis/extraction of a series of promising bioactive compounds, based on theory or previously studied compounds of similar chemical structure.

b) Pharmacological evaluation of the series.

c) Selection of promising members of the series for further studies.

d) Small scale synthesis of promising compounds.

e) Toxicological investigations (chronic, acute) of formulations.

f) Large batch synthesis.

g) Phase I clinical testing (pharmacology in humans).

h) Phase II clinical testing (dose-level response investigations, side effects, bioavailability).

i) Phase III clinical testing (large scale).

j) Full production.

1) first production lots.

2) routine production.

3) formula change.

Stability studies of the drugs serve different purposes throughout the new drug development procedure. Stability studies during toxicological evaluations ascertain the purity of the preparations being studied, as well as evaluate the toxicological potential of degradation compounds. The primary purpose of drug formulation is to create a suitable delivery vehicle which is also stable. Full production can introduce unknown variables that affect drug stability, and stability testing during this phase is used to ascertain what

effect these unknown variables have on the formulation stability, and what the likely expiration date should be for the formulas. Quality assurance programs are carried out on a routine basis to assure stability of the drug production process.

Chemical kinetics is defined as the study of the rate of changing proportions of reactants and products (Carstensen, 1995). The rate of change can generally be classified by the order of reaction. Reaction order refers to the dependence of the reaction upon reactant/product concentration. A zero-order reaction proceeds at a rate independent of reactant concentration; a first-order reaction is dependent on the concentration of one reactant; and a second-order reaction is dependent on the concentration of two reactants or on the squared concentration of one reactant. These reaction orders and their differential and integrated forms are summarized in Table 2.2. OTC degradation is a second-order reaction, dependent upon the concentration of OTC and water (Connors et al., 1986). However, in solution, the concentration of water is extremely large (~ 55.5 M) relative to that of OTC concentrations normally encountered in pharmaceutical preparations or as meat residues; therefore, the degradation rate of OTC is effectively dependent upon the OTC concentration only (Connors et al., 1986); this reaction is referred to as a pseudo-first-order reaction.

Determination of the order of reaction may be accomplished via the van't Hoff method (Swinbourne, 1971). Briefly, both sides of the differential equation are transformed by a ln function. Solving for the unknown in the resulting equation gives the order of the reaction:

$$\ln(-\frac{d[\mathrm{A}]}{dt}) = \ln k + n\ln[\mathrm{A}] \qquad [2.1]$$

Table 2.2. Differential and integrated form expressions for different orders of rate reactions.

Order	Differential Form	Integrated Form	Units of the rate Constant
0	$-\frac{d[A]}{dt} = k$	$[A]_o - [A] = kt$	$M\ t^{-1}$
1	$-\frac{d[A]}{dt} = k[A]$	$[A] = [A]_o e^{-kt}$	t^{-1}
2	$-\frac{d[A]}{dt} = k[A]^2$	$\frac{1}{[A]} - \frac{1}{[A]_o} = kt$	$M^{-1}\ t^{-1}$
1+1	$-\frac{d[A]}{dt} = k[A][B]$	$\frac{1}{[B]_o - [A]_o} \ln \frac{[B][A]_o}{[A][B]_o} = kt$	$M^{-1}\ t^{-1}$

whereby n is the order of the reaction. These orders can be determined with respect to time (i.e. changes in concentration of the reactant during the course of the reaction) or with respect to concentration (i.e. changing initial concentrations of the reactant for several different experiments, and determine the immediate change in concentration). Determination of the order of reaction with respect to concentration is considered to give a true indication of the order of the reaction, as only initial changes in reactant concentration are measured, and subsequent product formation is only minimal. This prevents the formation of potential inhibitory or catalytic products which may alter the rate of further parent compound degradation. Linear regression of the appropriate integrated form of the equation can give estimates of the desired parameters.

Calculation of degradation rate constants can be accomplished by fitting the natural log transform of the concentration data to a linear model with respect to time. This is a standard integral method for the determination of such constants (Carstensen, 1995).

Chemical stability testing of pharmaceutical formulations is determined under several temperature regimes; these generally include the recommended storage temperature for the drug product, a lower than recommended temperature if the formulation is temperature sensitive, and a higher than recommended temperature (usually 15°C higher than recommended storage temperature) for accelerated testing purposes (Carstensen, 1995). Accelerated testing data can be utilized to determine the impact that excursions beyond the recommended storage conditions may have upon drug stability. This is accomplished by utilizing the rate constants obtained at the different temperatures to determine the energy of activation (E_a) values. The E_a gives an

indication of the "ease" with which a reaction can occur, and is determined by plotting the ln k_{obs} of the reaction rates as a function of 1/T(K). Two common theories of reaction rates exist; the collision theory and the absolute rate (transition state) theory (Chang, 1981). The collision theory is based on the kinetic theory of gases, in that the rate of a reaction can essentially be calculated if the physical size and mass of the reacting molecule, as well as the temperature, are known. The theory then allows for the calculation of the number of collisions occurring between reactants which then leads to the formation of products. However, two additional factors have to be considered. First, the steric factor must be accounted for, which refers to the requirement for the reactants to be properly oriented with respect to each other for a productive reaction to occur (i.e. entropy). Secondly, the frequency factor is required, which refers to the number of collisions occurring that are productive (i.e. E_a). The bases for calculating these factors is rooted in quantum mechanics, making the collision theory impractical to use. The transition state theory is favoured as it allows for the analysis of the energetic and entropic components of a reaction process (Carey and Sundberg, 1990). Briefly, the theory assumes that a reactant must reach a high energy, activated state before forming the product. Attainment of the activated (transition) state is the slowest, and therefore rate determining step of the reaction. This can then be treated as an equilibrium reaction:

$$A \rightarrow A^* \text{ (activated complex)} \qquad [2.2]$$

$$K^* = \frac{A^*}{A} \qquad [2.3]$$

The rate of decomposition of an activated complex is given as:

$$\text{rate } (k_r) = \frac{\mathrm{k}kT}{h} \qquad [2.4]$$

where k = the transmission coefficient (generally taken as 1), k is Boltzmann's constant (1.3805×10^{-16} erg K^{-1}) and h is Planck's constant (6.6256×10^{-27} erg s). The unit for the expression then becomes s^{-1} (a first order rate constant). However, from the following thermodynamic relations

$$\Delta G^* = \Delta H^* - T\Delta S^* \qquad [2.5]$$

$$\Delta G^* = -RT \ln K^* \qquad [2.6]$$

The following relationship between rate of reaction and temperature can be derived:

$$\text{rate } (k_r) = \frac{\mathrm{k}kT}{h} (e^{-\Delta H^*/RT})(e^{\Delta S^*/R}) \qquad [2.7]$$

whereby H and S are the enthalpy and entropy of activation, respectively. A linear plot of ln rate/T as a function of 1/T gives a slope of $-\Delta H^*/R$. The change in entropy (ΔS) can be determined by the relationship:

$$\Delta S = \frac{\Delta H^*}{T} + R \ln \frac{hk_r}{kT} \qquad [2.8]$$

In solution, enthalpy and entropy of activation reflect both the reacting species molecular changes and the reaction medium's effect upon the formation of the activated complex (Carey and Sundberg, 1990). Negative values of ΔH indicate a decrease in the energy content of activated complex due to breaking of bonds. Negative values of ΔS indicate a decrease in the entropy of the activated state, which corresponds to increased restrictions on movement. These increased restrictions can be due to formation of a more structured activated complex or due to increased ordering of the medium surrounding the complex.

Positive values of ΔS indicate an increase in the entropy of the activated state, which corresponds to decreased restrictions on movement. This can be due to formation of a less structured activated complex, or decreased ordering of the medium surrounding the complex.

2.3.2.2 Statistical Evaluation

Statistical evaluation of the parameters governing chemical stability kinetics is essential for determining the amount of confidence in the parameters' estimates. The essential parameters requiring these estimates are the rate constant and energy of activation. Confidence in the rate constant can be evaluated using linear and non-linear models. Techniques used to solve non-linear models give only approximations of the errors associated with the estimates, whereas linear models allow for more robust error calculations. In a linear model, reaction data are fitted to a linear equation. In first-order reactions, this takes the form of:

$$\ln [A] = \ln [A_o] - kt \qquad [2.9]$$

where A_o and A are the concentrations of the drug at time = 0 and t, respectively, and k is the rate constant. Confidence intervals for the rate constant can be calculated by treating the k values as individual observations (i.e. one k value from one set of time-concentration data) or as a function of several values (i.e. linear regression analysis). With linear regression analysis, all data points are used for the calculation of the k value, resulting in a large degrees of freedom which will give smaller estimates of the confidence interval for rate constants.

The precision of E_a can be determined by several different methods. Hill and Grieger-Block (1980) determined the standard error of E_a by utilizing the errors calculated when determining temperatures and rate constants utilizing the following formula:

$$(\Delta E_a/E_a)^2 = (T_2/T_2-T_1)^2 (\Delta T_1/T_1)^2 + (T_1/T_2-T_1)^2 (\Delta T_2/T_2)^2 + [1/\ln(k_2/k_1)]^2 [(\Delta k_1/k_1)^2 + (\Delta k_2/k_2)^2] \quad [2.10]$$

where:

$\Delta E_a/E_a$ = the standard error of E_a

$\Delta k_{1(2)}/k_{1(2)}$ = the standard errors of k_1 or k_2

$\Delta T_{1(2)}/T_{1(2)}$ = the standard errors of T_1 and T_2

$k_{1(2)}$, $T_{1(2)}$ = the reaction rate constant and temperature (K) at which it was determined

Another valid method for the determination of the precision of E_a is the method reported by Carstensen (1995). This method utilizes all data points for calculating E_a by performing a transformation on the observed data points as follows:

$$\ln [(1/t)*\ln(A_o/A)] = E_a/RT + \ln P \quad [2.11]$$

where:

t = time (min) at which the data point was collected

A_o = concentration of reactant at time t = 0 minutes

A = concentration of reactant at time t

T = temperature (K) at which the concentration was determined

R = gas constant (1.987 cal/K mol)

P = pre-exponential function

The advantage of this method is that because all data points are used in the calculation, the degrees of freedom is large and standard errors should be decreased. However, because of the exponential nature of the function, variance heterogeneity may occur which decreases the validity of the determined confidence interval.

2.3.3 History of Research on Processing and OTC Residues

Studies on the effects of processing upon incurred OTC residues in tissues has traditionally been limited to observing absolute changes in the amount of OTC as a function of the procedure (Rutczynska-Skonieczna, 1967; Scheibner, 1969, 1972; Honikel et al., 1978; Shakaryan et al., 1976; O'Brien et al., 1980, 1981; Ibrahim and Moats, 1994). A review by Moats (1988) summarized studies of the thermal inactivation of several classes of antibiotics. The review indicated that standard methodology has generally not been utilized; various food products of different sizes were subjected to different processes (e.g. boiling, frying, roasting) of varying time lengths. Studies such as this provide a limited amount of information, as the amount of OTC loss can only be duplicated under identical conditions. Recently, studies have been done on the rate of OTC loss as a function of processing parameters (Kitts et al., 1992; Rose et al., 1996). Kitts et al. (1992) performed a systematic study of the depletion of incurred OTC residues in farmed Chinook salmon, providing values for kinetic parameters and some statistical evaluation. Rose et al. (1996) studied the rate of OTC degradation in water and cooking oil. With a kinetic approach, it is possible to model losses of OTC in a system if parameters such as initial OTC concentration, size and shape of the tissue sample, and temperature are known. Such models could be used to

predict how much OTC may be left in a system after being subjected to a given process which allows for better estimates of dietary OTC intakes. Since the incidence of finding OTC residues in pork tissue, particularly at injection sites, appears to be increasing (Agriculture and Agri-Food Canada, 1995), the probability of these same products being consumed also increases. A study of the rate of breakdown of OTC as a function of different meat processing practices would allow the appropriate agencies to better estimate the potential dietary intake of this substance.

3. MATERIALS AND METHODS

3.1 Chemicals and Materials

All organic solvents were of Omnisolv grade (VWR-Canlab, Edmonton, AB); water was purified by means of a Milli-Q purification system (Millipore, Mississauga, ON). Oxytetracycline was obtained from Sigma (St. Louis, MO); 4-epiOTC, α-apoOTC and β-apoOTC were obtained from Spectrum Chemical (Gardenia, CA). Tetra-sodium pyrophosphate (SAPP), penta-sodium tripolyphosphate (STP) and sodium hexametaphosphate (SH) were obtained from Caledon Laboratories (Edmonton, AB). Orthophosphate (SP) and all other reagents were of analytical reagent grade. Bond-Elut C_{18} cartridges (500 mg) were purchased from Varian Canada (Mississauga, ON). Frozen porcine tissue (*semimembranosus* and *semitendinosus*) was obtained from the Food Product Innovation Program (University of Saskatchewan, Saskatoon, SK) and analyzed to verify the absence of OTC residues.

3.2 High-Performance Liquid Chromatography

3.2.1 Extraction and Analysis of OTC and Related Compounds from Aqueous and Tissue matrices

The liquid chromatograph consisted of a Beckman Model 342 HPLC system (Beckman Instruments, Berkeley, CA) containing a 20 µl injection loop, a Valco 2 µm precolumn screen filter (Supelco Canada, Mississauga, ON) a C_8 Inertsil (50 x 4.6 mm I.D.) guard-column and C_8 Inertsil (250 x 4.6 mm I.D.) analytical column (Lab Link, Rockford, IL) and a Waters 484 variable-wavelength detector (Millipore, Milford, MA).

A Hewlett Packard 3390A integrator (Hewlett Packard, Avondale, PA) set at an attenuation of 2 mV was used for peak-area quantification.

A statistical simplex design (Snee, 1979) was used to optimize mobile phase selectivity for the compounds and the design is as shown in Table 3.1. The solvents selected for the study were methanol (MeOH)(x1), tetrahydrofuran (THF)(x2) and acetonitrile (ACN)(x3). The selectivity of these solvents is due to their relative differences in solvent polarity, proton acceptor contribution, proton donor contribution and dipole contribution (Snyder, 1978) (Table 3.2). A solvent strength equivalent to 75:25 (v/v) 0.1M ammonium acetate (pH 3.0) buffer-acetonitrile was used as the starting point, as this is the recommended mobile phase in Method TTC-SP05 (Agriculture and Agri-Food Canada, 1990). The chromatographic optimization function (COF) was calculated according to the formula of Glajch et al. (1980);

$$COF = \sum_{i=1}^{k} \ln(R_i / R_d) \qquad [3.1]$$

where R_i is the resolution of the ith peak pair in the chromatogram, R_d is the desired peak-pair resolution in the chromatogram, and k is the number of peak pairs. R_d was set to a value of 1.5; peak pair(s) with a resolution (R_i) greater than or equal to 1.5 were given equal value. All possible peak-pair combinations were taken into account during the calculation of the COF values. Data were fitted to quadratic and special cubic models and analysed using the PROC REG procedure of the SAS Institute Inc. (1989) to determine the optimal mobile phase composition. Standards containing 1 mg/ml of the compounds were utilized for analysis.

Table 3.1. Ten-run simplex design for a triphasic organic solvent mixture.

Run	Coded Values for solvents			COF[a]
	Methanol	Tetrahydrofuran	Acetonitrile	
1	1.00	0.00	0.00	y1
2	0.00	1.00	0.00	y2
3	0.00	0.00	1.00	y3
4	0.50	0.50	0.50	y12
5	0.50	0.00	0.50	y13
6	0.00	0.50	0.50	y23
7	0.33	0.33	0.33	y123
8	0.66	0.17	0.17	y1123
9	0.17	0.66	0.17	y1223
10	0.17	0.17	0.66	y1233

Adapted from Snee (1979).
[a] COF, chromatographic optimization function.

Table 3.2. Selectivity parameter values of chosen organic solvents.

Solvent	P[a]	Xe[b]	Xd[c]	Xn[d]
Methanol	5.1	0.48	0.22	0.31
Tetrahydrofuran	4.0	0.38	0.20	0.42
Acetonitrile	5.8	0.31	0.27	0.42

Adapted from Snyder (1978)
[a]P, solvent polarity.
[b]Xe, proton acceptor contribution.
[c]Xd, proton donor contribution.
[d]Xn, dipole contribution.

The calibration curves for OTC (calculated on its free base) and 4-epiOTC were obtained at 350 nm. Calibration curves for α- and β-apoOTC were obtained at 250 nm. Purity determination of the 4-epiOTC, α- and β-apoOTC preparations were not carried out; however, Khan et al. (1992) reported the respective purities of preparations obtained from the same source to be 84.5 %, 94.3 % and 95.2 %. Current purities are claimed to be greater than 95% (Acros Organics, 1998). These purities were not taken into account when determining the regression equations and were assumed to be 100 % pure for calculation purposes.

Preparation methods for samples and standards are given in detail in Method TTC-SP05 of the Agriculture and Agri-Food Canada's Methods Manual (Agriculture and Agri-Food Canada, 1990). The efficacy of the procedure outlined in Method TTC-SP05 for the extraction of OTC and degradation products from fortified distilled water and pork muscle was investigated at concentration levels of 2000, 1000, 200 and 100 ng/ml (g). Distilled water (5 ml) or homogenized meat samples (5 g) were fortified with appropriate amounts of 10 or 1 mg/ml methanol stock solutions of the compounds to give the final desired concentrations. The samples were blended with 20 ml of 0.1 M Na_2EDTA McIlvaine buffer (pH 4.0), and centrifuged at 1000 x g for 10 min. The tissue plug was re-extracted twice more using 20 and 10 ml of buffer, respectively. The combined filtrates were applied to a 500 mg Bond-Elut C_{18} cartridge previously activated with methanol and water, and then washed with 20 ml of distilled water. The compounds were eluted with 7 ml of 0.01 M methanolic acid, and diluted to 10 ml with distilled water. Triplicate analyses for each concentration were obtained; the average of

each analysis was determined from duplicate injections. Peak area quantification was used to determine the recovery (%) from the two matrices.

3.2.2 Determination of OTC Degradation Kinetics

The HPLC system consisted of a Waters 600E pump, a Waters 715 ULTRA WISP autosampler set at an injection volume of 100 µl, and a Waters 996 photodiode array detector acquiring spectra in the wavelength range of 240-450 nm with a resolution of 1.2 nm and a data acquisition rate of 1 spectra/4 sec. Peak heterogeneity was assessed by the photodiode array software option of Millennium Chromatography Manager V 2.10 software. Peak area counts for OTC were obtained at 350 nm; peak area counts for other peaks were obtained at 250 nm. All other parameters are as indicated in section 3.2.1.

3.3 Determination of OTC Degradation Kinetics

3.3.1 Test Matrices/Media

Table 3.3 summarizes the composition of all aqueous media utilized during the determination of OTC degradation kinetics. Table 3.4 summarizes the composition of tissue media utilized during the determination of OTC degradation kinetics.

Table 3.3. Aqueous matrices utilized during study.

Number	Media Composition/Description
1	Distilled Water
2	Glycerol/distilled water 0.9 a_w @ 20°C
3	Glycerol/distilled water 0.8 a_w @ 20°C
4	Glycerol/distilled water 0.6 a_w @ 20°C
5	SP buffer (pH 5.5, 0.01 M)
6	SP buffer (pH 5.5, 0.05 M)
7	SP buffer (pH 5.5, 0.1 M)
8	SP buffer (pH 4.0, 0.1 M)
9	SP buffer (pH 7.0, 0.1 M)
10	SP buffer + 0.1 M NaCl (pH 5.5, 0.05 M)
11	SP buffer + 0.4 M NaCl (pH 5.5, 0.05 M)
12	Acetate buffer (pH 5.5, 0.01 M)
13	Acetate buffer (pH 5.5, 0.05 M)
14	Acetate buffer (pH 5.5, 0.1 M)
15	Acetate buffer + 0.01 M SP (pH 5.5, 0.05 M)
16	Acetate buffer + 0.03 M SP (pH 5.5, 0.05 M)
17	Acetate buffer + 0.05 M SP (pH 5.5, 0.05 M)
18	Acetate buffer + 0.01 M SAPP (pH 5.5, 0.05 M)
19	Acetate buffer + 0.03 M SAPP (pH 5.5, 0.05 M)
20	Acetate buffer + 0.05 M SAPP (pH 5.5, 0.05 M)
21	Acetate buffer + 0.01 M STP (pH 5.5, 0.05 M)
22	Acetate buffer + 0.03 M STP (pH 5.5, 0.05 M)
23	Acetate buffer + 0.05 M STP (pH 5.5, 0.05 M)
24	Acetate buffer + 0.01 M SH (pH 5.5, 0.05 M)
25	Acetate buffer + 0.03 M SH (pH 5.5, 0.05 M)
26	Acetate buffer + 0.05 M SH (pH 5.5, 0.05 M)
27	Acetate buffer + 0.01 M $CaCl_2$ (pH 5.5, 0.05 M)
28	Acetate buffer + 0.1 M $CaCl_2$ (pH 5.5, 0.05 M)
29	Acetate buffer + 0.003 M $NaNO_2$ (pH 5.5, 0.05 M)

Table 3.3. Tissue matrices utilized during study.

Number	Media Composition/Description
1	Porcine tissue diluted 9:1 with acetate buffer (pH 5.5, 0.1 M final conc.)
2	Porcine tissue diluted 3:2 with acetate buffer (pH 5.5, 0.1 M final conc.)
3	Porcine tissue diluted 9:1 with acetate buffer/NaCl solution (0.4 M NaCl, pH 5.5, 0.1 M final conc.)
4	Porcine tissue diluted 9:1 with acetate buffer/$CaCl_2$ solution (0.4 M $CaCl_2$, pH 5.5, 0.1 M final conc.)
5	Porcine tissue diluted 9:1 with acetate buffer/SP solution (0.5 % SP, pH 5.5, 0.1 M final conc.)
6	Porcine tissue diluted 9:1 with acetate buffer/SAPP solution (equivalent to 0.5 % SP, pH 5.5, 0.1 M final conc.)
7	Porcine tissue diluted 9:1 with acetate buffer/STP solution (equivalent to 0.5 % SP, pH 5.5, 0.1 M final conc.)
8	Porcine tissue diluted 9:1 with acetate buffer/SH solution (equivalent to 0.5 % SP, pH 5.5, 0.1 M final conc.)
9	Porcine tissue diluted 9:1 with acetate buffer/EDTA solution (0.2 M EDTA, pH 5.5, 0.1 M final conc.)
10	Porcine tissue diluted 9:1 with acetate buffer/$NaNO_2$ solution (200 ppm $NaNO_2$, pH 5.5, 0.1 M final conc.)

3.3.2. Protocol

The thermal stability of OTC was investigated at 60, 70, and 80°C (± 1°C) in the aqueous and tissue media listed in section 3.3.1. Distilled water solutions were adjusted to water activities (a_w's) of 0.9, 0.8 and 0.6 (± 0.01) at ambient temperature using glycerol, and the a_w's were verified by a Model CX-1 Decagon Water Activity Meter (Decagon Devices, Inc., Pullman, WA). All other media were prepared immediately before use. Three ml of the media were dispensed into 50 ml round-bottom glass centrifuge tubes (VWR-Canlab, Edmonton, AB) and 100 µl of 1 mg/ml methanolic OTC was added. The tubes were sealed with Teflon lined caps, placed in constant temperature water baths (Büchi, Switzerland) and removed at intervals ranging from 0 to 60 minutes, and cooled immediately in ice water. OTC was extracted from the aqueous

matrices as described in section 3.2.1. Come-up temperature profiles were monitored using an OMEGA microprocessor thermometer with a T-type thermocouple (Omega Engineering, Inc., Laval, QC). Steady-state temperatures (within 1°C of the desired temperature) were obtained within 10 minutes.

OTC degradation in porcine tissue was monitored in a similar manner. Porcine tissue was preground in a commercial blender, frozen, and stored at -20°C for a period no longer than one month prior to use. Ground tissue (5.0 ± 0.1 g) was added to 50 ml round bottom glass centrifuge tubes, and 100 µl of 1 mg/ml methanolic OTC was added. Immediately after preparation, 500 µl of the treatment was added to the tubes (section 3.3.1) and mixed. The tissue was packed to the bottom using a rubber-tipped syringe plunger, sealed, and thermally treated for 0 to 60 minutes. The come-up temperature profile of the geometric center of the tissue mass was monitored as indicated for the aqueous media.). Steady-state temperatures (within 1°C of the desired temperature) were obtained within 10 minutes.

3.3.3 Data Analysis

Thermal destruction constants (k_{obs}, min^{-1}) of OTC and their associated 95 % confidence intervals were obtained from linear regression equations, n=18, of the ln peak area counts as a function of time using Excel V 5.0 (Microsoft Corporation, Redmond, WA). Energy of activation (E_a, kcal/mol) values and the associated standard errors were calculated according to Hill and Grieger-Block (1980) using k_{obs} and standard errors obtained at 60 and 80°C. Changes in enthalpy (ΔH) and entropy (ΔS) were calculated

according to Carey and Sundberg (1990) using k_{obs} obtained at 60, 70 and 80°C. The isokinetic temperature was determined by the method of Leffler (1955) using all ΔH and ΔS values obtained. Power analysis for the k_{obs} obtained were calculated using obtained estimates of standard errors (60°C, -0.0008; 70°C, -0.002; 80°C, -0.005)

Where possible, estimation of the rates of formation and degradation of OTC degradation compounds was accomplished by fitting data to a four parameter biexponential equation:

$$A = P1e^{-k_d t} - P2e^{-k_f t} \qquad [3.2]$$

where P1 and P2 are pre-exponential constants, k_d is the rate of destruction of the unknown compound and k_f is the rate of formation of the unknown compound. The PROC NLIN using the DUD iterative procedure of SAS (SAS Institute Inc., 1989) was used to obtain estimates and corresponding standard errors of the parameters. Initial estimates and boundaries for PROC NLIN were provided by the manual technique of "feathering" the data (Shargel and Yu, 1993).

3.4 Determination of Meat/BSA Binding of OTC.

The determination of OTC binding was a modification of the method by Yuan et al. (1995). Using a commercial grinder, 2 g of porcine tissue was ground with 98 ml of 0.1 M sodium acetate buffer for 30 seconds. Three ml of the homogenate was blended with 2 ml of 0.065 % (w/v) dextran (MW 70,000), 0.6 % (w/v) activated charcoal solution in 0.1 M sodium acetate buffer, pH 5.5. A 100 µl portion of 100 mg/ml methanolic OTC was added to the mixture. The solution was mixed for times ranging

from 30 to 540 seconds, filtered though a glass microfibre filter to separate the charcoal from the supernatant, and analyzed for OTC content as outlined previously. The data was fitted to a four parameter biexponential equation:

$$B(t) = P1e^{-\alpha t} + P2e^{-\beta t} \qquad [3.3]$$

where B(t) is the amount (area counts) obtained for OTC at time t

α = the composite association constant of drug to charcoal (s^{-1})

β= the composite dissociation constant of drug from tissue/BSA (s^{-1})

The PROC NLIN procedure using the DUD iterative option of SAS (SAS Institute Inc., 1989) was used to obtain estimates and corresponding standard errors of the parameters. Initial estimates and boundaries for the parameters were provided by the manual technique of "feathering" the data (Shargel and Yu, 1993).

4. RESULTS

4.1 Analysis and Quantification of OTC and Degradation Compounds from Aqueous and Tissue Matrices

4.1.1 Optimization of Chromatographic Separation of OTC and Degradation Compounds

According to the solvent classification scheme of Snyder (1978) as cited by Glagch et al. (1980) the calculated polarity strength of a 75:25 (v/v) buffer-ACN mobile phase (assuming the aqueous buffer has the same eluting strength as water) would be 9.2. At a flow-rate of 0.6 ml/min, elution times for OTC and degradation products were: OTC, 10.5 min; 4-epiOTC, 11.2 min; α-apoOTC, 10.4 min; β-apoOTC, 31.2 min. Calculated compositions for equal polarity (equal to 75:25 (v/v) buffer-ACN) buffer-methanol and buffer-tetrahydrofuran mobile phases were 78:22 (v/v) and 82:18 (v/v), respectively. With the calculated buffers as the mobile phase, elution of the primary compound (OTC) occurred at 40 min with buffer-methanol and 23 min with buffer-tetrahydrofuran, respectively. Trial and error formulation of the buffer phases resulted in a final binary-phase composition of buffer-methanol 55:45 (v/v) (OTC, 10.8 min) and buffer-tetrahydrofuran 70:30 (v/v) (OTC, 12.0 min), corresponding to calculated polarity strengths of 7.9 and 8.3 respectively, lower than was calculated. The actual mobile phase compositions tested according to the ten-run simplex design were as indicated in Table 4.1. The COF values and adjusted retention times (unretained solvent time, 3.9 min) for the four components were obtained (Table 4.2). Relative standard deviations for the adjusted retention times and COF values were less than 5 and 10 % of the means,

Table 4.1. Mixture formulations for optimization experiment.

Run	MeOH	THF	ACN	Buffer
1	45.00	0.00	0.00	55.00
2	0.00	30.00	0.00	70.00
3	0.00	0.00	25.00	75.00
4	22.5	15.00	0.00	65.00
5	22.5	0.00	15.00	62.50
6	0.00	15.00	12.5	72.50
7	15.00	8.33	10.00	66.77
8	30.00	4.17	5.00	60.83
9	7.5	16.7	5.00	70.80
10	7.5	4.17	20.00	68.33

Values in % (v/v)

Table 4.2. Adjusted retention times and COF values for oxytetracycline and degradation compounds, and subsequent liquid chromatograph operating pressure (n=3).

Run	Adjusted retention time (min)					Pressure
	OTC	4-epiOTC	α-apoOTC	β-apoOTC	COF	p.s.i. (x 10^{-3})
1	6.9	6.7	6.7	49.6	-9.35	2.36
2	8.1	9.2	4.9	35.1	-0.09	2.47
3	6.6	7.3	6.6	27.4	-3.30	1.49
4	8.6	9.3	5.2	38.8	-0.79	1.89
5	9.3	9.6	9.6	60.2	-7.11	2.50
6	7.2	8.1	4.8	31.0	-0.32	1.92
7	8.3	8.5	6.2	39.0	-0.51	2.09
8	8.3	8.5	6.2	45.4	-1.43	2.20
9	7.9	8.7	4.8	31.6	-0.38	1.75
10	7.9	8.6	6.2	35.8	-0.70	2.30

respectively. In general, it was observed that mobile phases incorporating tetrahydrofuran (strong dipole interaction, weak proton donor) effected a better peak separation than those without tetrahydrofuran.

The COF values obtained from the experiments (runs 1-10) were fit to both quadratic and cubic equations. The response y as described by the quadratic model was:

$$E(y) = -8.86(x1) - 0.78(x2) - 3.21(x3) + 19.72(x1*x2) + 2.43\ (x1*x3) + 8.72(x2*x3) \quad [4.1]$$

where x1, x2 and x3 are the coded values of methanol, tetrahydrofuran and acetonitrile in the simplex design used for optimization. The response y as described by the cubic equation was:

$$E(y) = -8.74(x1) - 0.65(x2) - 3.08(x3) + 15.80(x1*x2) - 1.47(x1*x3) + 4.82(x2*x3) + 63.41\ (x1*x2*x3) \quad [4.2]$$

Tabulated F-ratios (α=0.05) for the quadratic and cubic models were 2.51 and 2.45, respectively. Calculated F-ratios for the lack of fit were 85 and 81, respectively, indicating that a significant lack-of-fit is present for both proposed models and that the models do not adequately describe the COF response as a function of the volumes of the three organic solvents. The mobile phase with the highest COF value (i.e. greatest resolution) was run 2 (Buffer-THF, 70:30). However, the operating pressure was high (2470 p.s.i.). Run 6 (Figure 4.1) has the second highest COF (-0.32) and also had a reasonably low operating back pressure (1.92 p.s.i.). This mobile phase was subsequently utilized for further experiments.

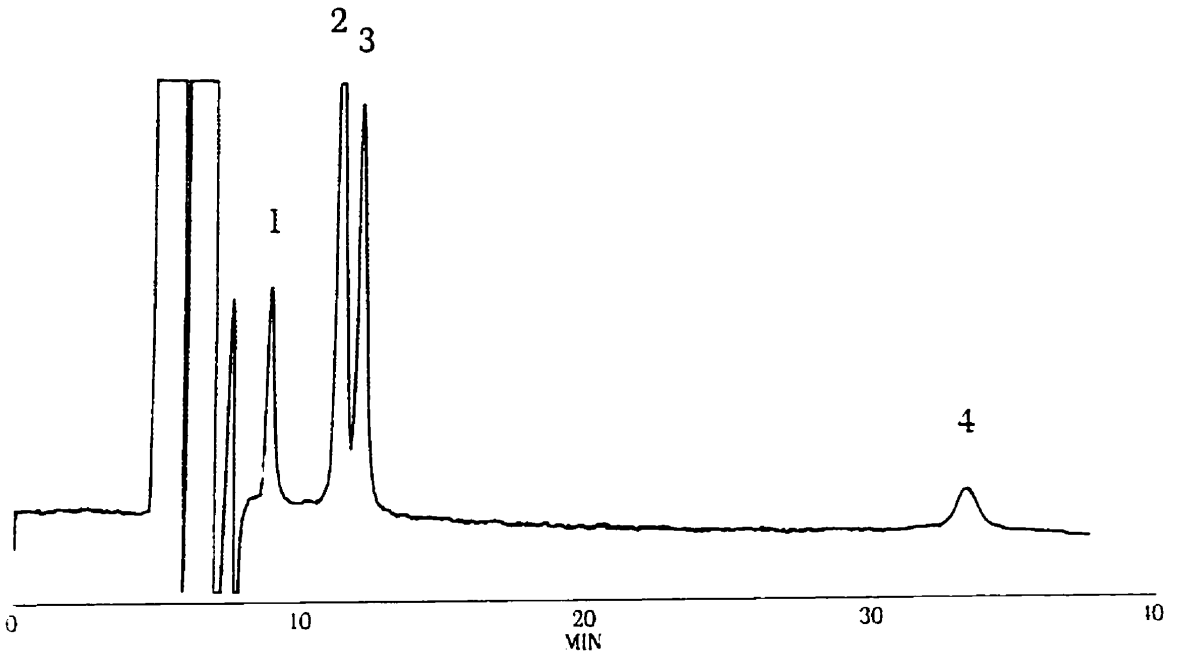


Figure 4.1 HPLC chromatogram of OTC and related degradation compounds from run 6. (1) α-apoOTC, (2) OTC, (3) 4-epiOTC, (4) β-apoOTC.

Table 4.3 Regression statistics for OTC, 4-epioOTC, α- and β-apoOTC for regression curves of peak-area counts as a function of concentration (ng/ml) (n=12).

Regression Statistics	OTC	4-epiOTC	α-apoOTC	β-apoOTC
Intercept	3173.7	1951.2	4917.6	-20189.7
S.E.[a]	1523.0	698.6	5325.8	13482.6
x-variable	255.1	246.0	764.2	658.0
S.E.	2.7	1.2	9.5	24.0
R^2	0.99	0.99	0.99	0.98

[a]S.E., standard error.

Regression statistics for the relationship between compound concentration and peak-area counts are shown in Table 4.3. The correlation coefficient exceeded 0.98 for all regression equations. The limits of detection for OTC, 4-epiOTC, α- and β-apoOTC were 40, 20, 50, and 140 ng/ml, respectively under the given experimental conditions.

4.1.2 Recovery of OTC and Degradation Compounds from Aqueous and Tissue Matrices

Recovery data of OTC and degradation compounds from distilled water and pork tissue are reported in Tables 4.4 and 4.5. Recovery of OTC was greater than 90 % from distilled water for all concentrations investigated. Recovery of 4-epiOTC, α- and β-apoOTC were lower than OTC, and the percentage recovery appeared to be dependent upon the initial concentration. Recovery of OTC and 4-epiOTC from tissue were significantly lower ($p < 0.05$) than from distilled water, whereas recoveries of α- and β-apoOTC were sharply decreased, with β-apoOTC recovery being reduced to less than 10 % at concentrations of 2000 and 1000 ng/ml, and no detectable levels being found at

concentrations of 200 and 100 ng/ml. Elution using acetonitrile, tetrahydrofuran and ethanol containing 0.01 M oxalic acid did not improve recoveries, and were highly irreproducible (data not shown).

Table 4.4. Recovery of OTC and degradation products from 5 ml of distilled water fortified at four different levels (n=3).

Spike level (ng/ml)	Recovery (%) (CV, %)			
	OTC	4-epiOTC	α-apoOTC	β-apoOTC
2000	99.0 (0.7)	83.4 (1.3)	88.9 (5.6)	84.3 (16.9)
1000	90.5 (4.7)	79.4 (2.1)	52.6 (9.3)	58.1 (12.7)
200	93.4 (6.0)	83.9 (6.7)	17.7 (33.0)	62.9 (25.0)
100	90.1 (16.7)	66.1 (12.3)	ND[a]	ND

[a] ND, not detected.

Table 4.5. Recovery of OTC and degradation products from 5 g of homogenized porcine tissue fortified at four different levels (n=3).

Spike level (ng/ml)	Recovery (%) (CV, %)			
	OTC	4-epiOTC	α-apoOTC	β-apoOTC
2000	83.7 (2.4)	57.5 (4.6)	40.0 (3.7)	8.9 (11.4)
1000	69.9 (3.2)	52.9 (4.8)	29.8 (6.3)	6.5 (6.2)
200	74.1 (5.3)	61.8 (10.5)	26.0 (11.4)	ND[a]
100	74.6 (12.5)	49.3 (3.8)	29.9 (10.0)	ND

[a] ND, not detected.

4.2 Effects of Treatments and Thermal Processing on OTC Degradation

4.2.1 Water Activity

The influence of glycerol adjusted water activities ranging from 0.6 to 1.0 upon the rate of OTC degradation at 60, 70, and 80°C, with the appropriate kinetic parameters, are shown in Tables 4.6 and 4.7. Rate constants ranged from -0.0043 to -0.0073 min^{-1} at 60°C, whereas at 80°C they ranged from -0.0510 to -0.0575 min^{-1}. No statistically significant difference was seen between rate constants at a given temperature, though trends ($0.05 < p < 0.20$) were noted. The calculated E_a for distilled water was higher than that obtained for the glycerol./distilled water solutions; additionally, ΔS values were positive for degradation in distilled water, whereas reactions in glycerol/distilled water solutions were negative. Change in enthalpy values ranged from 22 kcal/mol to 29 kcal/mol. The degradation of OTC at 80°C in distilled water is shown in Figure 4.2.

Table 4.6. OTC degradation parameters k_{obs} (min^{-1}) and E_a (kcal/mol) in aqueous media as affected by water activity.

		$k_{obs}(min^{-1})$[a]		E_a (kcal/mol)	
Medium	Temp (°C)	mean	95 % c.i.	mean	S.E.[b]
Distilled Water	60	-0.0043	(-0.0051, -0.0036)	30.14	2.81
	70	-0.0172	(-0.0185, -0.0159)		
	80	-0.0566	(-0.0599, -0.0532)		
Glycerol/distilled water	60	-0.0066	(-0.0089, -0.0043)	23.99	2.93
(0.9 a_w @ 20°C)	70	-0.0210	(-0.0240, -0.0179)		
	80	-0.0510	(-0.0556, -0.0463)		
Glycerol/distilled water	60	-0.0061	(-0.0089, -0.0032)	26.26	3.45
(0.8 a_w @ 20°C)	70	-0.0225	(-0.0262, -0.0188)		
	80	-0.0575	(-0.0633, -0.0517)		
Glycerol/distilled water	60	-0.0073	(-0.0100, -0.0046)	23.59	2.81
(0.6 a_w @ 20°C)	70	-0.0208	(-0.0231, -0.0184)		
	80	-0.0549	(-0.0595, -0.0502)		

[a] n=18, linear regression of $\ln[OTC_{time=t}] = \ln[OTC_{time=0}] - k_{obs}t$.

[b] standard error.

Table 4.7. Changes in enthalpy (ΔH, kcal/mol) and entropy (ΔS, cal/ mol K) for OTC degradation at different water activities.

Medium	ΔH (kcal/mol)	ΔS (cal/mol K)
Distilled Water	29.43	10.67
Glycerol/distilled water (0.9 a_w @ 20°C)	23.28	-6.97
Glycerol/distilled water (0.8 a_w @ 20°C)	25.55	-0.29
Glycerol/distilled water (0.6 a_w @ 20°C)	22.84	-8.06

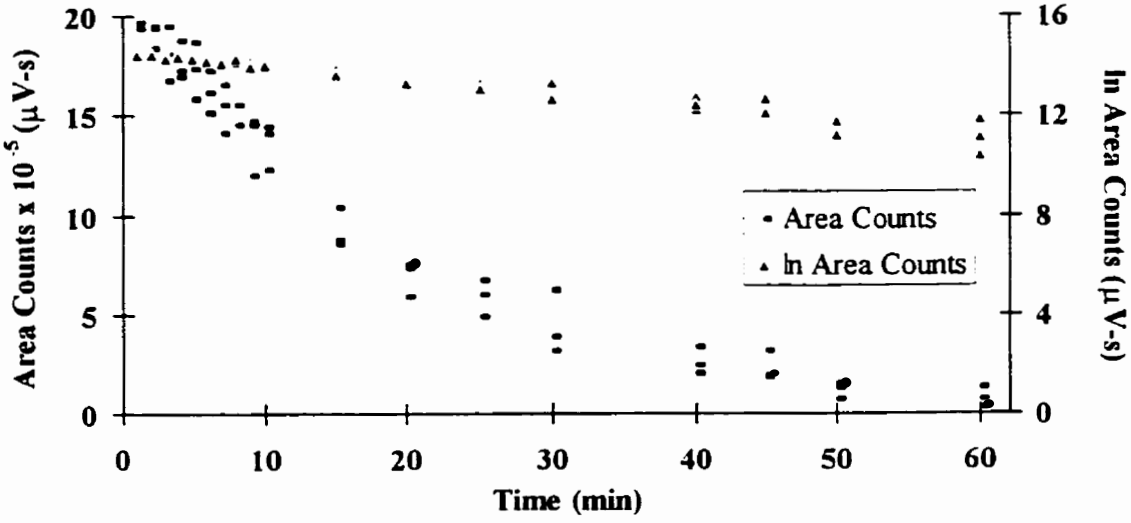


Figure 4.2. Concentration-time profile of OTC in 80°C distilled water.

4.2.2 Orthophosphate Buffer

Increasing phosphate buffer concentration from 0.01 to 0.1 M significantly increased most rate constants at temperatures from 60-80°C (Table 4.8). The buffer free degradation rate constant (k_o) was calculated using the equation (Carstensen, 1995):

$$k_{obs} = k_o + k_{HQ}[HQ] \qquad [4.3]$$

where:

k_o = rate constant at a buffer concentration of 0 M

k_{HQ} = rate constant as a function of phosphate buffer concentration

[HQ] = phosphate buffer concentration (M)

Plots of k_{obs} for OTC degradation at different phosphate concentrations and temperatures are shown in Figure 4.3. The k_o values at 60, 70 and 80°C were -0.0049, -0.0151 and -0.0586 min^{-1}, respectively, which compared favourably with the rate constants obtained in distilled water. The k_{HQ} values at 60, 70 and 80°C were -0.1360, -0.3442 and -0.3715 min^{-1}, respectively. The calculated E_a, ΔH and ΔS values (Table 4.9) for k_o (29.0, 28.3 and 7.6 kcal/mole respectively) also compared favourably with those obtained in distilled water. The qualitatively higher values for k_{HQ}, as well as the lower E_a, ΔH and ΔS values obtained for those solutions containing phosphate buffer, indicated that phosphate buffer had a strong catalytic effect upon the degradation of OTC.

Degradation of OTC in pH 4 and 7 phosphate buffers (0.1 M) was significantly ($p < 0.05$) lower at all temperatures than the corresponding degradation rates in pH 5.5 buffer. E_a, ΔH and ΔS values also differed substantially, indicating that the different pH and/or different amounts of buffer species affected the rate of degradation of OTC.

Table 4.8. OTC degradation parameters k_{obs} (min^{-1}) and E_a (kcal/mol) in aqueous media as affected by phosphate buffer and sodium chloride concentration, and pH.

		$k_{obs}(min^{-1})$[a]		E_a (kcal/mol)	
Medium	Temp (°C)	mean	95 % c.i.	mean	S.E.[b]
Phosphate Buffer	60	-0.0080	(-0.0101, -0.0059)	24.23	2.39
(pH 5.5, 0.01 M)	70	-0.0203	(-0.0215, -0.0192)		
	80	-0.0637	(-0.0669, -0.0604)		
Phosphate Buffer	60	-0.0089	(-0.0100, -0.0082)	24.88	4.65
(pH 5.5, 0.05 M)	70	-0.0280	(-0.0305, -0.0255)		
	80	-0.0747	(-0.0800, -0.0699)		
Phosphate Buffer	60	-0.0198	(-0.0210, -0.0185)	18.28	1.48
(pH 5.5, 0.1 M)	70	-0.0518	(-0.0558, -0.0479)		
	80	-0.0943	(-0.1006, -0.0880)		
Phosphate Buffer	60	-0.0053	(-0.0058, -0.0047)	28.38	2.35
(pH 4.0, 0.1 M)	70	-0.0195	(-0.0209, -0.0181)		
	80	-0.0600	(-0.0641, -0.0559)		
Phosphate Buffer	60	-0.0064	(-0.0070, -0.0057)	22.36	1.80
(pH 7.0, 0.1 M)	70	-0.0186	(-0.0201, -0.0172)		
	80	-0.0433	(-0.0453, -0.0412)		
Phosphate Buffer	60	-0.0110	(-0.0125, -0.0095)	22.12	2.12
+ 0.1 M NaCl	70	-0.0282	(-0.0318, -0.0245)		
(pH 5.5, 0.05 M)	80	-0.0730	(-0.0819, -0.0641)		
Phosphate Buffer	60	-0.0099	(-0.0115, -0.0084)	25.52	3.04
+ 0.4 M NaCl	70	-0.0238	(-0.0266, -0.0210)		
(pH 5.5, 0.05 M)	80	-0.0705	(-0.0816, -0.0593)		

[a] n=18, linear regression of $\ln[OTC_{time=t}] = \ln[OTC_{time=0}] - k_{obs}t$.

[b] standard error.

Table 4.9. Changes in enthalpy (ΔH, kcal/mol) and entropy (ΔS, cal/mol K) for OTC degradation at different phosphate buffer and sodium chloride concentrations, and pHs.

Medium	ΔH (kcal/mol)	ΔS (cal/mol K)
Phosphate Buffer (pH 5.5, 0.01 M)	23.52	-5.86
Phosphate Buffer (pH 5.5, 0.05 M)	24.18	-3.66
Phosphate Buffer (pH 5.5, 0.1 M)	17.58	-21.88
Phosphate Buffer (pH 4.0, 0.1 M)	27.68	5.83
Phosphate Buffer (pH 7.0, 0.1 M)	21.67	-11.86
Phosphate Buffer + 0.1 M NaCl (pH 5.5, 0.05 M)	21.41	-11.54
Phosphate Buffer + 0.4 M NaCl (pH 5.5, 0.05 M)	22.97	-9.10

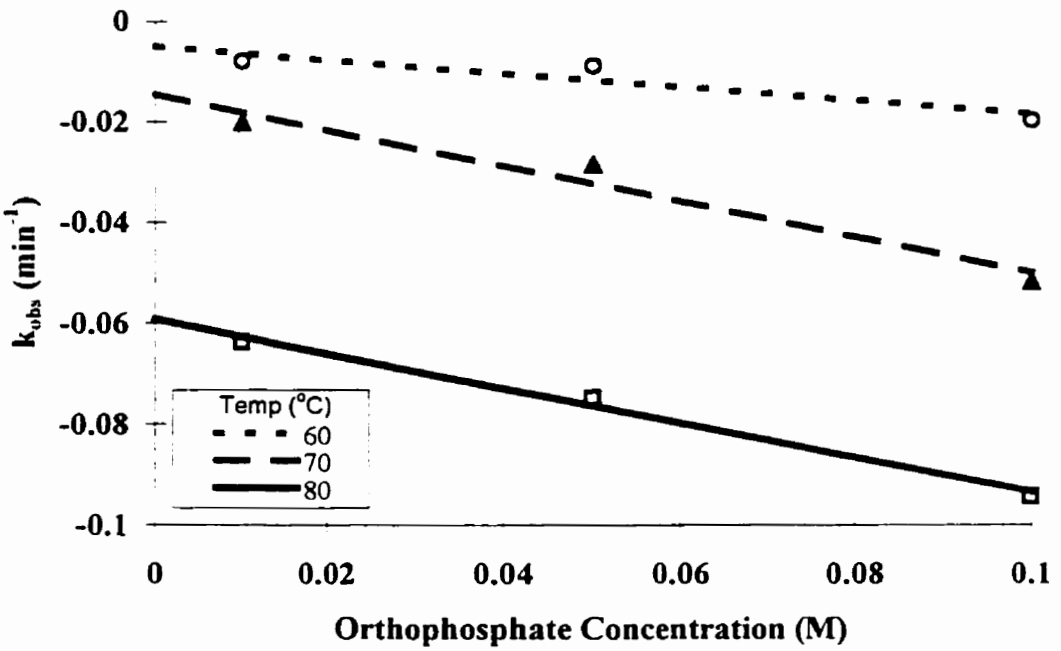


Figure 4.3. Rate of OTC degradation (k_{obs}, min^{-1}) at 60-80°C in orthophosphate buffers of different concentrations.

4.2.3 Sodium Chloride

The effect of ionic strength upon OTC degradation was investigated using two different concentrations of NaCl in 0.05 M phosphate buffer, pH 5.5. There were no significant differences ($p < 0.05$) in k_{obs} noted at all temperature-concentration combinations (Table 4.8). A substantial drop in entropy (-3.66 for control to -11.54 and -9.10 cal/mol K for 0.1 M and 0.4 M solutions of NaCl, respectively) was also noted (Table 4.9). All other kinetic parameters exhibited enthalpy/entropy compensation (section 4.2.8) which indicated that no change in the mechanism of OTC degradation occurred.

4.2.4 Acetate Buffer/Polymeric Phosphates

The rates of OTC degradation with associated kinetic parameters in acetate buffers are shown in Table 4.10. At 60°C, the rate constants, which ranged from -0.0063 to -0.0071 min^{-1}, were not significantly different between the varying acetate buffer concentrations at pH 5.5. At 70°C, the rate constant in 0.1 M acetate buffer (-0.0212 min^{-1}) was significantly greater than in 0.01 M acetate buffer (-0.0172 min^{-1}). At 80°C, the rate constant in 0.01 M acetate buffer (-0.0443 min^{-1}) was significantly lower than in 0.05 M or 0.1 M acetate buffer. The E_a values for the three buffers ranged from 22.86 to 26.36 kcal/mol. Analysis of covariance of k_{obs} as a function of temperature with acetate buffer concentration as the covariate indicated that buffer concentration did not have an identifiable influence upon the rate constants ($p > 0.05$). E_a values ranged from 18 kcal/mol to 25 kcal/mol.

Table 4.10. OTC degradation parameters k_{obs} (min^{-1}) and E_a (kcal/mol) in aqueous media as affected by acetate buffer concentration and inclusion of monomeric and polymeric phosphates.

Medium	Temp (°C)	$k_{obs}(min^{-1})$[a] mean	95 % c.i.	E_a (kcal/mol) mean	S.E.[b]
Acetate Buffer	60	-0.0063	(-0.0079, -0.0046)	22.86	2.35
(pH 5.5, 0.01 M)	70	-0.0172	(-0.0192, -0.0152)		
	80	-0.0443	(-0.0477, -0.0409)		
Acetate Buffer	60	-0.0064	(-0.0071, -0.0057)	26.36	2.30
(pH 5.5, 0.05 M)	70	-0.0203	(-0.0219, -0.0189)		
	80	-0.0612	(-0.0667, -0.0556)		
Acetate Buffer	60	-0.0071	(-0.0079, -0.0063)	23.80	2.14
(pH 5.5, 0.1 M)	70	-0.0212	(-0.0227, -0.0198)		
	80	-0.0542	(-0.0595, -0.0489)		
Acetate Buffer	60	-0.0075	(-0.0089, -0.0061)	24.36	2.22
+ 0.01 M SP	70	-0.0233	(-0.0259, -0.0208)		
(pH 5.5, 0.1 M)	80	-0.0602	(-0.0645, -0.0559)		
Acetate Buffer	60	-0.0067	(-0.0080, -0.0055)	27.50	4.20
+ 0.03 M SP	70	-0.0268	(-0.0310, -0.0227)		
(pH 5.5, 0.1 M)	80	-0.0703	(-0.0748, -0.0659)		
Acetate Buffer	60	-0.0081	(-0.0091, -0.0070)	23.80	2.51
+ 0.05 M SP	70	-0.0251	(-0.0281, -0.0221)		
(pH 5.5, 0.1 M)	80	-0.0617	(-0.0708, -0.0526)		

Table 4.10. Continued.

Medium	Temp (°C)	$k_{obs}(min^{-1})$[a] mean	95 % c.i.	E_a (kcal/mol) mean	S.E.[b]
Acetate Buffer	60	-0.0054	(-0.0064, -0.0043)	26.88	2.61
+ 0.01 M SAPP	70	-0.0160	(-0.0172, -0.0148)		
(pH 5.5, 0.1 M)	80	-0.0535	(-0.0592, -0.0478)		
Acetate Buffer	60	-0.0056	(-0.0064, -0.0048)	25.28	2.20
+ 0.03 M SAPP	70	-0.0182	(-0.0196, -0.0167)		
(pH 5.5, 0.1 M)	80	-0.0486	(-0.0523, -0.0449)		
Acetate Buffer	60	-0.0056	(-0.0063, -0.0048)	25.80	2.13
+ 0.05 M SAPP	70	-0.0173	(-0.0189, -0.0156)		
(pH 5.5, 0.1 M)	80	-0.0505	(-0.0535, -0.0475)		
Acetate Buffer	60	-0.0060	(-0.0070, -0.0051)	24.05	2.19
+ 0.01 M STP	70	-0.0174	(-0.0193, -0.0156)		
(pH 5.5, 0.1 M)	80	-0.0473	(-0.0516, -0.0430)		
Acetate Buffer	60	-0.0058	(-0.0063, -0.0053)	25.29	2.03
+ 0.03 M STP	70	-0.0188	(-0.0201, -0.0175)		
(pH 5.5, 0.1 M)	80	-0.0448	(-0.0474, -0.0422)		
Acetate Buffer	60	-0.0051	(-0.0065, -0.0037)	25.37	2.95
+ 0.05 M STP	70	-0.0155	(-0.0180, -0.0131)		
(pH 5.5, 0.1 M)	80	-0.0446	(-0.0512, -0.0381)		

Table 4.10. Continued.

Medium	Temp (°C)	$k_{obs}(min^{-1})$[a] mean	95 % c.i.	E_a (kcal/mol) mean	S.E.[b]
Acetate Buffer	60	-0.0050	(-0.0064, -0.0036)	24.52	2.97
+ 0.01 M SH	70	-0.0143	(-0.0156, -0.0130)		
(pH 5.5, 0.1 M)	80	-0.0408	(-0.0430, -0.0387)		
Acetate Buffer	60	-0.0052	(-0.0071, -0.0033)	23.72	2.70
+ 0.03 M SH	70	-0.0150	(-0.0171, -0.0130)		
(pH 5.5, 0.1 M)	80	-0.0396	(-0.0413, -0.0379)		
Acetate Buffer	60	-0.0052	(-0.0060, -0.0043)	23.88	2.06
+ 0.05 M SH	70	-0.0146	(-0.0153, -0.0138)		
(pH 5.5, 0.1 M)	80	-0.0400	(-0.0423, -0.0377)		

[a] n = 18, linear regression of $\ln[OTC_{time=t}] = \ln[OTC_{time=0}] - k_{obs}t$.

[b] standard error.

Table 4.11. Changes in enthalpy (ΔH, kcal/mol) and entropy (ΔS, cal/mol K) for OTC degradation at different acetate buffer and phosphate concentrations.

Medium	ΔH (kcal/mol)	ΔS (cal/mol K)
Acetate Buffer (pH 5.5, 0.01 M)	22.10	-10.60
Acetate Buffer (pH 5.5, 0.05 M)	25.69	0.21
Acetate Buffer (pH 5.5, 0.1 M)	23.07	-7.45
Acetate Buffer + 0.01 M SP (pH 5.5, 0.1 M)	23.66	-5.57
Acetate Buffer + 0.03 M SP (pH 5.5, 0.1 M)	26.81	3.68
Acetate Buffer + 0.05 M SP (pH 5.5, 0.1 M)	23.05	-7.23
Acetate Buffer + 0.01 M SAPP (pH 5.5, 0.1 M)	26.08	1.05
Acetate Buffer + 0.03 M SAPP (pH 5.5, 0.1 M)	24.57	-3.41
Acetate Buffer + 0.05 M SAPP (pH 5.5, 0.1 M)	25.00	-2.11
Acetate Buffer + 0.01 M STP (pH 5.5, 0.1 M)	23.43	-6.69
Acetate Buffer + 0.03 M STP (pH 5.5, 0.1 M)	23.22	-6.75
Acetate Buffer + 0.05 M STP (pH 5.5, 0.1 M)	24.64	-3.37
Acetate Buffer + 0.01 M SH (pH 5.5, 0.1 M)	23.83	-5.86
Acetate Buffer + 0.03 M SH (pH 5.5, 0.1 M)	23.03	-8.18
Acetate Buffer + 0.05 M SH (pH 5.5, 0.1 M)	23.14	-7.84

Varying phosphate concentration in 0.1 M acetate buffer did not have as significant an effect upon the rate of degradation as phosphate buffer alone. Increasing the concentration of phosphate in 0.1 M acetate buffer did not have any effect at 60 and 70°C; a significant increase occurred in 0.03 M SP at 80°C as compared to 0.1 M acetate buffer control. E_a, ΔH and ΔS values were qualitatively similar (Table 4.11).

SAPP appeared to have a weak inhibitory effect upon OTC degradation. Varying the concentration from 0.01 M to 0.05 M significantly decreased the rate of degradation at 60 and 70°C, at 80°C, this was not apparent. There was no difference observed between SAPP buffers. E_a, ΔH and ΔS values were qualitatively similar.

STP was inconsistent in its ability to decrease the rate of OTC degradation. No significant differences were noted at 60°C, whereas at 70°C all concentrations tested significantly ($p < 0.05$) decreased the rate of degradation. At 80°C, only 0.03 M STP significantly decreased k_{obs}. All other kinetic parameters were similar.

SH consistently decreased the rate of OTC degradation at most temperature-concentration combinations as compared to control. No significant differences were observed for k_{obs} within the different concentrations. All other kinetic parameters were similar to each other.

4.2.5 Calcium Chloride

The inclusion of $CaCl_2$ into acetate buffer at 0.01 M significantly decreased k_{obs} at 70 and 80°C over that of control (Table 4.12). At 0.1 M, $CaCl_2$ significantly decreased k_{obs} at 60, 70 and 80°C over that of the control; at 70 and 80°C k_{obs} was significantly lower than that for 0.01 M $CaCl_2$ solution at the same temperatures. Qualitatively, E_a, ΔH and ΔS were less than the corresponding parameters in the control and 0.01 M $CaCl_2$ (Table 4.13); however, the associated errors with these parameters were much greater.

4.2.6 Sodium Nitrite

The use of $NaNO_2$ at 0.003 M $NaNO_2$ (ca. 200 ppm sodium nitrite) did not have any significant effects upon OTC degradation. However, at 70°C, nitrite increased the mean k_{obs}, whereas at 80°C it decreased the mean k_{obs} over control ($0.05 < p < 0.10$). Qualitatively, all other kinetic parameters were lower in the nitrite containing solution than in the control solution.

Table 4.12. OTC degradation parameters k_{obs} (min^{-1}) and E_a (kcal/mol) in aqueous media as affected by calcium chloride and sodium nitrite.

		$k_{obs}(min^{-1})$[a]		E_a (kcal/mol)	
Medium	Temp (°C)	mean	95 % c.i.	mean	S.E.[b]
Acetate Buffer	60	-0.0071	(-0.0079, -0.0063)	23.80	2.14
(pH 5.5, 0.1 M)	70	-0.0212	(-0.0227, -0.0198)		
	80	-0.0542	(-0.0595, -0.0489)		
Acetate Buffer	60	-0.0044	(-0.0072, -0.0016)	24.39	4.23
+ 0.01 M $CaCl_2$	70	-0.0112	(-0.0148, -0.0076)		
(pH 5.5, 0.1 M)	80	-0.0357	(-0.0404, -0.0310)		
Acetate Buffer	60	-0.0028	(-0.0053, -0.0003)	16.85	6.09
+ 0.1 M $CaCl_2$	70	-0.0046	(-0.0076, -0.0015)		
(pH 5.5, 0.1 M)	80	-0.0119	(-0.0172, -0.0066)		
Acetate Buffer	60	-0.0070	(-0.0082, -0.0058)	21.48	2.24
+ 0.003 M $NaNO_2$	70	-0.0245	(-0.0265, -0.0226)		
(pH 5.5, 0.1 M)	80	-0.0436	(-0.0490, -0.0382)		

[a] n=18, linear regression of $\ln[OTC_{time=t}] = \ln[OTC_{time=0}] - k_{obs}t$.

[b] standard error.

Table 4.13. Changes in enthalpy (ΔH, kcal/mol) and entropy (ΔS, cal/mol K) for OTC degradation in the presence of calcium chloride and sodium nitrite.

Medium	ΔH (kcal/mol)	ΔS (cal/mol K)
Acetate Buffer + 0.01 M $CaCl_2$ (pH 5.5, 0.1 M)	23.74	-6.39
Acetate Buffer + 0.1 M $CaCl_2$ (pH 5.5, 0.1 M)	16.16	-30.04
Acetate Buffer + 0.003 M $NaNO_2$ (pH 5.5, 0.1 M)	20.75	-14.43

4.2.7. Porcine Tissue

4.2.7.1 Porcine Tissue and 0.1 M Acetate Buffer

Incorporation of OTC into porcine tissue with 10 % (w/w) of 1.0 M acetate buffer (final acetate concentration ≈ 0.1 M) significantly decreased the rate of OTC degradation at all tested temperatures as compared to 0.1 M acetate buffer (Table 4.14). The kinetic parameters were qualitatively similar between the two systems (Tables 4.15 and 4.11, respectively). Increasing the amount of acetate buffer to 60 % (w/w) of tissue (acetate concentration adjusted to give a final acetate concentration of 0.1 M), significantly decreased k_{obs} only at 80°C. However, the k_{obs} was significantly greater than the corresponding k_{obs} in the 10 % (w/w) mixture at 60 and 70°C. All secondary kinetic parameters were dramatically lower; the associated errors with E_a remained relatively constant. The E_a, ΔH and ΔS values for the 10 % (w/w) and 60 % (w/w) mixtures were 25.5 and 15.2 kcal/mol, 24.8 and 14.5 kcal/mol, and -3.26 and -32.6 kcal/mol, respectively.

4.2.7.2 Porcine Tissue and Phosphates

SP, SAPP, STP and SH were added to porcine tissue to give final concentrations equivalent to 0.5 % dibasic sodium phosphate. No significant differences in k_{obs} were observed between porcine tissue containing SP and porcine tissue containing acetate buffer alone (control). All kinetic parameters for tissue containing SP, however, were qualitatively lower than the control.

Table 4.14. OTC degradation parameters k_{obs} ($min.^{-1}$) and E_a (kcal/mol) in tissue media as affected by different treatments.

Medium	Temp (°C)	k_{obs}[a] mean	k_{obs}[a] 95 % c.i.	E_a (kcal/mol) mean	E_a (kcal/mol) S.E.[b]
Porcine Tissue	60	-0.0042	(-0.0055, -0.0029)	25.52	3.04
	70	-0.0138	(-0.0157, -0.0119)		
	80	-0.0372	(-0.0415, -0.0329)		
Porcine Tissue	60	-0.0089	(-0.0100, -0.0079)	15.24	1.52
3:2 dil. With buffer	70	-0.0195	(-0.0231, -0.0159)		
(pH 5.5)	80	-0.0327	(-0.0403, -0.0251)		
Porcine Tissue	60	-0.0082	(-0.0110, -0.0054)	14.64	1.46
+ 0.4 M NaCl	70	-0.0154	(-0.0192, -0.0116)		
(pH 5.5)	80	-0.0287	(-0.0321, -0.0253)		
Porcine Tissue	60	-0.0036	(-0.0047, -0.0024)	23.21	2.62
+ 0.4 M $CaCl_2$	70	-0.0098	(-0.0107, -0.0088)		
(pH 5.5)	80	-0.0260	(-0.0283, -0.0238)		
Porcine Tissue	60	-0.0065	(-0.0088, -0.0043)	19.41	2.66
+ SP (0.5 % final conc.)	70	-0.0164	(-0.0193, -0.0136)		
	80	-0.0344	(-0.0390, -0.0297)		

Table 4.14. Continued.

Medium	Temp (°C)	k_{obs}[a] mean	k_{obs}[a] 95 % c.i.	E_a (kcal/mol) mean	E_a (kcal/mol) S.E.[b]
Porcine Tissue	60	-0.0060	(-0.0068, -0.0052)	23.42	1.96
+ SAPP	70	-0.0167	(-0.0182, -0.0152)		
(equivalent to 0.5 % SP)	80	-0.0447	(-0.0469, -0.0424)		
Porcine Tissue	60	-0.0039	(-0.0047, -0.0031)	29.43	2.99
+ STP	70	-0.0167	(-0.0186, -0.0149)		
(equivalent to 0.5 % SP)	80	-0.0485	(-0.0546, -0.0424)		
Porcine Tissue	60	-0.0081	(-0.0101, -0.0061)	21.24	2.43
+ SH	70	-0.0251	(-0.0273, -0.0229)		
(equivalent to 0.5 % SP)	80	-0.0496	(-0.0560, -0.0433)		
Porcine Tissue	60	-0.0059	(-0.0089, -0.0028)	25.77	2.58
+ 0.2 M EDTA	70	-0.0183	(-0.0234, -0.0132)		
(pH 5.5)	80	-0.0535	(-0.0603, -0.0467)		
Porcine Tissue	60	-0.0076	(-0.0091, -0.0061)	11.38	1.74
+ $NaNO_2$	70	-0.0121	(-0.0141, -0.0100)		
(200 ppm final conc.)	80	-0.0200	(-0.0240, -0.0160)		

[a] n=18, linear regression of $\ln[OTC_{time=t}] = \ln[OTC_{time=0}] - k_{obs}t$.

[b] standard error.

Table 4.15. Changes in enthalpy (ΔH, kcal/mol) and entropy (ΔS, cal/mol K) for OTC degradation in treated porcine tissue.

Medium	ΔH (kcal/mol)	ΔS (cal/mol K)
Porcine Tissue (pH 5.5)	24.81	-3.26
Porcine Tissue 3:2 dil. with buffer (pH 5.5)	14.54	-32.60
Porcine Tissue + 0.4 M NaCl (pH 5.5)	13.95	-34.55
Porcine Tissue + 0.4 M $CaCl_2$ (pH 5.5)	22.41	-3.57
Porcine Tissue + SP (pH 5.5)	18.79	-20.46
Porcine Tissue + SAPP (pH 5.5)	22.77	-8.68
Porcine Tissue + STP (pH 5.5)	28.79	8.55
Porcine Tissue + SH (pH 5.5)	20.53	-14.81
Porcine Tissue + 0.2 M EDTA (pH 5.5)	25.07	-1.81
Porcine Tissue + $NaNO_2$ (pH 5.5)	10.61	-44.72

SAPP significantly increased k_{obs} at 80°C over control, with no substantial differences in the kinetic parameters from the control. STP significantly increased k_{obs} at 80°C, as well it substantially increased the remaining kinetic parameters over that of control. SH significantly increased k_{obs} at all temperatures at 60, 70 and 80°C. Kinetic parameters appeared to be slightly lower than that for control.

4.2.7.3 Porcine Tissue and EDTA, NaCl, $CaCl_2$ and $NaNO_2$

The addition of EDTA to a final concentration of 0.2 M in porcine tissue significantly ($p < 0.05$) increased the rate of OTC degradation at 80°C over control tissue. There were no significant differences in k_{obs} between tissue with EDTA and 0.1 M acetate buffer (Table 4.14). Kinetic parameters were qualitatively similar.

Addition of 0.4 M NaCl to porcine tissue significantly increased k_{obs} at 60°C and significantly decreased k_{obs} at 80°C compared to control (Table 4.14). Kinetic parameters were dramatically lower than those obtained for porcine tissue alone. Values for E_a, ΔH and ΔS for porcine control and NaCl treatment were 25.5 and 14.6 kcal/mol, 24.8 and 13.9 kcal/mol, and -3.2 and -34.5 kcal/mol, respectively.

The addition of $NaNO_2$ to tissue significantly increased k_{obs} at 60°C, whereas at 80°C it significantly decreased k_{obs} as compared to control. Changes in the remaining kinetic parameters were the most dramatic observed among all treatments vs. controls. Values for E_a, ΔH and ΔS of the $NaNO_2$ treatment were 11.4, 10.6 and -44.7 kcal/mol, respectively.

The presence of 0.4 M $CaCl_2$ in meat significantly lowered k_{obs} at 70 and 80°C. Kinetic parameters were not substantially different from that of the control.

4.2.8 Enthalpy/Entropy Compensation

A plot of the ΔH as a function of ΔS for all reactions studies is shown in Figure 4.4. Regression analysis of the data indicated that the isokinetic temperature of OTC degradation was 337 K. The 95 % confidence interval was 326 to 348 K, with an R^2 of 0.99.

4.2.9 Power Analysis of Degradation Data

Power curves for degradation of OTC using the assumed standard errors (Sec. 3.3.3) are shown in Figure 4.5. A power of 80 % was obtainable when the unknown true difference in k_{obs} was approximately 0.0035, 0.008, and 0.02 min^{-1} at 60, 70 and 80°C, respectively. A cursory evaluation of the kinetic data obtained revealed that few estimated differences were this great, indicating that, in general, the power of these tests was less than 80 %.

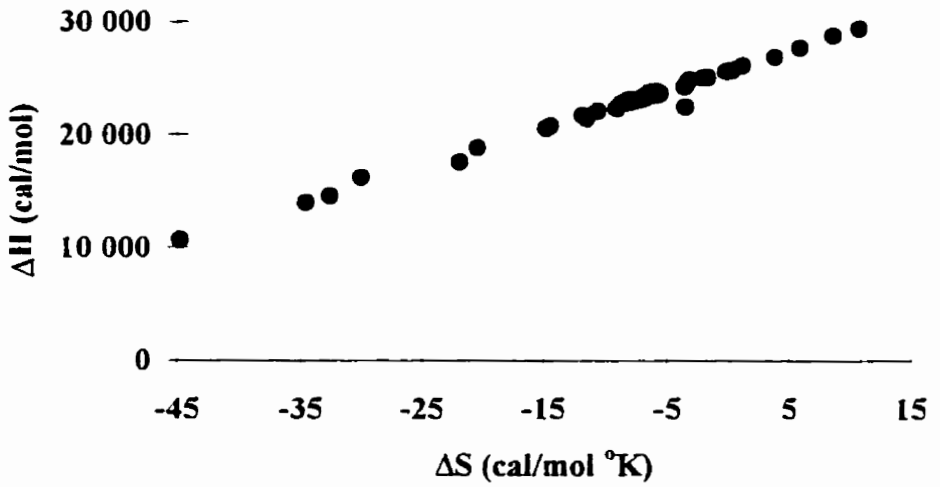


Figure 4.4. Enthalpy/Entropy compensation curve for OTC degradation under all experimental conditions.

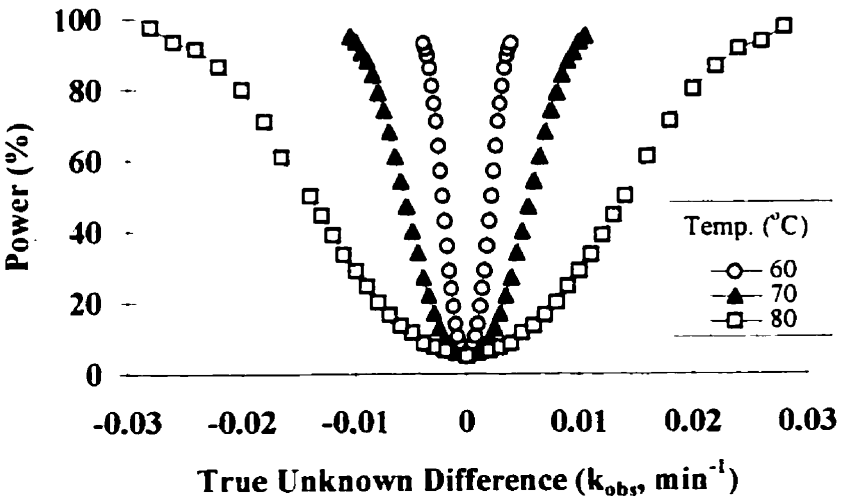


Figure 4.5. Power curves for OTC degradation at 60, 70 and 80°C.

4.2.10 Formation of Degradation Compounds

Quantification of degradation compounds could not be performed with accuracy for most treatments due to the presence of several overlapping peaks. Degradation of OTC in distilled water did allow for quantification of a chromatographic peak that eluted at 12 to 14 minutes. The photodiode array software indicated the peak was homogeneous in composition. Plots of average counts for the peaks at 60, 70 and 80°C are shown in Figure 4.6. A plot of the "feathering technique" used to obtain initial parameter estimates for SAS's PROC NLIN procedure is shown in Figure 4.7. As this is a crude technique, the actual grid search estimates and parameter boundaries used for PROC NLIN (Table 4.18) were set reasonably wide. Parameter estimates, standard errors, and 95 % confidence intervals obtained by PROC NLIN are given in Table 4.19. The calculated F-values for lack-of-fit analysis were 16, 3.07 and 2.86 for equations obtained at 60, 70 and 80°C, respectively. All F-values were significant at $p = 0.01$.

4.3 BSA/Tissue Binding of OTC

A typical plot of ln area counts as a function of time for OTC in the presence and absence of BSA is shown in Figure 4.8. Initial grid search estimates used for PROC NLIN procedure are shown in Table 4.18. Parameter estimates, standard errors and 95 % confidence intervals are given in Table 4.19. F-values for lack-of-fit analysis of the estimated models were all less than 1. None of the obtained results were significantly different from each other, though differences in mean β values were observed. Addition of phosphate to tissue numerically increased the β constant from 0.0014 to 0.0040 s^{-1}.

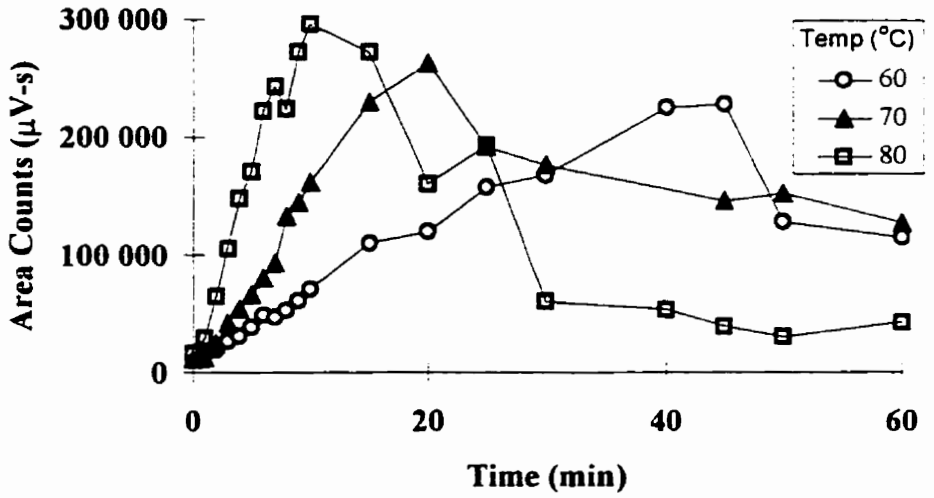


Figure 4.6. Formation of unknown OTC degradation compound at 60, 70 and 80°C in distilled water.

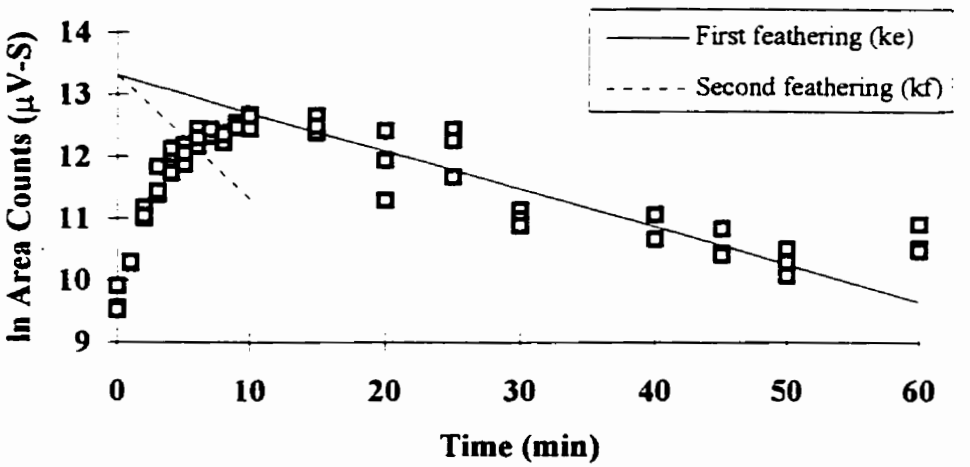


Figure 4.7. Illustrative example of the "feathering technique" used to obtained preliminary grid-search estimates for PROC NLIN.

Table 4.16. Preliminary grid search estimates used for PROC NLIN estimation of parameters for the biexponential model of unknown peak formation.

	Grid Search Estimates Used Parameters			
Temp (°C)	P1 (x 10^{-3})	P2 (x 10^{-3})	kf	kd
60	900 to 2000 by 200	900 to 2000 by 200	0.08 to 0.12 by 0.01	0.02 to 0.05 by 0.01
70	1000 to 2000 by 200	1000 to 2000 by 200	0.045 to 0.065 by 0.005	0.025 to 0.035 by 0.002
80	600 to 14 000 by 200	600 to 1400 by 200	0.20 to 0.45 by 0.05	0.045 to 0.070 by 0.005

Table 4.17. Parameter estimates obtained for biexponential model of unknown peak formation.

		Parameter Estimates			
Temp (°C)		P1 (x 10^{-3})	P2 (x 10^{-3})	kf	kd
60	Estimate	11	11	0.031	0.021
	Std. Error	216	216	0.106	0.084
	95 % c.i.	-423 , 445	-423 , 445	-0.181, 0.243	-0.147, 0.188
70	Estimate	18	18	0.044	0.032
	Std. Error	n.e.	n.e.	0.074	0.060
	95 % c.i.	n.e.	n.e.	-0.105, 0.194	-0.088, 0.152
80	Estimate	10	10	.137	0.066
	Std. Error	n.e.	n.e.	.009	0.003
	95 % c.i.	n.e.	n.e.	0.119, 0.154	0.059, 0.072

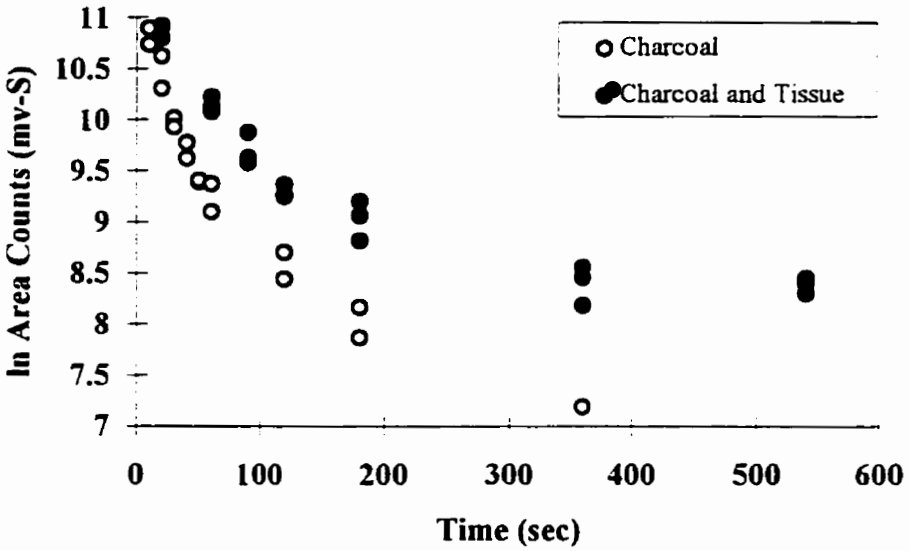


Figure 4.8. Adsorption of OTC from charcoal/dextran solution in the presence and absence of porcine tissue.

Table 4.18. Preliminary grid search estimates used for PROC NLIN estimation of parameters for the biexponential model of OTC -BSA/Tissue binding.

	Grid Search Estimates Used Parameters			
Solution	P1 ($\times 10^{-3}$)	P2 ($\times 10^{-3}$)	α	β
ALL	10 to 90 by 20	10 to 50 by 20	.016 to 0.024 by 0.002	0.001 to 0.005 by 0.002

Table 4.19. Parameter estimates obtained for biexponential model of OTC-BSA/Tissue binding.

		Parameter Estimates			
Solution		P1 ($\times 10^{-3}$)	P2 ($\times 10^{-3}$)	α	β
Charcoal	Estimate	71	7	0.048	0.0032
	Std. Error	5	5	0.009	0.0040
	95 % c.i.	59 , 83	0, 17	0.029, 0.067	-0.0054, 0.0118
BSA	Estimate	9	18	0.027	0.0026
	Std. Error	10	9	0.008	0.0020
	95 % c.i.	66, 107	0, 37	0.012, 0.043	-0.0014, 0.0066
BSA + NaCl	Estimate	79	17	0.021	0.0022
	Std. Error	9	11	0.006	0.0019
	95 % c.i.	61, 97	-6, 40	0.009, 0.033	-0.0017, 0.0061
BSA + SP	Estimate	76	37	0.027	0.0025
	Std. Error	9	8	0.0678	0.0008
	95 % c.i.	58, 97	19, 54	0.011, 0.043	0.0008, 0.0042
BSA + STP	Estimate	87	23	0.02782	0.0032
	Std. Error	6	6	0.00455	0.0010
	95 % c.i.	75, 99	11, 35	0.018, 0.037	0.0010, 0.0052
Tissue	Estimate	71	8	0.023	0.0014
	Std. Error	3	3	0.003	0.0009
	95 % c.i.	65, 77	3, 14	0.018, 0.028	-0.0006, 0.0033
Tissue + SP	Estimate	64	22	0.034	0.0040
	Std. Error	29	12	0.023	0.0024
	95 % c.i.	3, 126	-4, 47	-0.015, 0.083	-0.0011, 0.0091

5. DISCUSSION

5.1 Chromatographic Selectivity of Organic Solvents for OTC and Degradation Compounds

Application of the solvent classification scheme of Snyder (1978) for determining the elution strength of binary solvent systems was unable to accurately predict the chromatographic retention time of OTC. Trial and error buffer formulation was required to develop binary solvent systems of equivalent eluting strength for OTC. This apparent discrepancy was reproducible, and may be attributed to the alteration of the normal bulk properties of the base solvent or ion-pairing (Glajch et al., 1980). Differences in conformation have been show to have dramatic effects on polarity of tetracyclines, and are a function of their environment (Stezowski, 1976). Casey and Yasin (1985) as cited by Khan et al. (1987) reported that in deuterated DMSO, the enone ring of β-apoOTC assumes a half-chair conformation with the 4S, 4aS, and 12aR substituents in the axial position, whereas α-apoOTC occurs as a mixture of conformers with at least one of the 4S, 4aS, or 12aS substituents in the axial position. This may result in different intramolecular interactions between proximal functional groups.

Fitting the obtained COF data to quadratic and cubic models was unable to provide equations that accurately described the resolving power of the quaternary solvent systems. Quenouille (1959) had stated that polynomial models do not account for components in the mobile phase that are inert or have additive effects, and that a more complicated model would be needed to fit the data. Snee (1979) had proposed several models that can overcome these effects; these alternative models were not

investigated as some of the tested mobile phases resulted in good chromatographic separation of OTC from the degradation compounds. Naidong et al. (1990) achieved good separation of the four compounds using a poly(styrene-divinylbenzene) column (250 x 4.6 mm I.D.) maintained at 60°C using a one step gradient elution with a five-component pH 7.5 mobile phase. The mobile phase used in the present method offers the advantage of being simpler (triphasic mixture), and isocratic elution at ambient temperature is utilized.

5.2 Recovery of OTC and Degradation Compounds from Aqueous and Tissue Matrices

The extraction of OTC degradation compounds from aqueous and tissue matrices resulted in poor and irreproducible recoveries. Spurious peaks were not evident in the chromatograms, providing evidence that the breakdown and subsequent formation of other compounds did not occur. The utilization of different eluting solvents (i.e. acetonitrile, tetrahydrofuran) did not improve these recoveries. The present findings agree with Law et al. (1992) who had reported that elution of some hydrophobic material from RP C_{18} cartridges was more facile with methanol than acetonitrile. Hydrophobic interaction is therefore not the only retention mechanism operating in this recovery system, and other secondary interactions are also present. Mulders and Van de Lagemaat (1989) reported that the presence of residual silanols on C_{18} cartridges resulted in significant adsorption of tetracycline drugs onto the cartridges, resulting in reduced recoveries. The mechanism of interaction can be ionic (Law et al., 1992) or hydrogen bonding (Law and Weir, 1992). Drug recovery may also be reduced due to

specific drug-matrix or cartridge-matrix interactions (Law and Weir, 1992), whereby materials present in the matrix may interfere with the hydrophobic interaction between the compound and the C_{18} packing. Solid-phase extraction of low concentrations of OTC degradation products, particularly α- and β-apoOTC, was not possible with the present method.

5.3 Mechanism of Water Activity Effects upon OTC Degradation

The kinetics of chemical reactions tend to be at a maximum at water activities of 0.6-0.8 (Labuza, 1980). Below this range, the decrease in solute and water mobility will effectively hinder reactions (Yoshioka et al., 1992). Above this range, the effective solute concentration decreases due to increased free water and water of hydration, both being available for chemical reactions. Consequently, both of these phenomena decrease the rate of reaction. Thus, changing the water content of a matrix affects degradation by two general mechanisms; a concentration effect and a solute/water mobility effect.

Water mobility is commonly expressed in terms of τ_c, , which is referred to as correlation time or relaxation time; T_1, which is known as spin-lattice relaxation time and a_w, which is water activity (Yoshioka et al., 1995). Relaxation time refers to the time for a system initially at equilibrium to reach a new equilibrium when an external force is applied to the system. τ is a measure of the mobility of highly mobile water, whereas T1 represents the average mobility of highly mobile water and bound water. τ is inversely related to water content, whereas T1 is directly related to water content. τ is shown to be directly related to the inverse of T1 (Yoshioka et al, 1992). Water activity is also related to water content, though it does not appear to have a definable relationship with

τ or T1 (Warmbier et al., 1976). Values of τ will differ for free water, water of hydration, and bound water (Yoshioka et al., 1995). τ_c for bulk water at 25°C is given as 8 picoseconds (Franks, 1983). Bound water exhibits a τ value greater than hydration water, which in turn is greater than bulk water. The relationship of the rate of drug degradation to τ or T is dependent on the order of the reaction τ correlates with first-order (or pseudo-first order) reactions, whereas T1 correlates with second-order reactions (Yoshioka et al., 1992). Thus, the rate of OTC degradation is theoretically dependent upon τ, and not a_w. Rates of reactions in matrices of the same a_w but different components can significantly differ (Warmbier et al., 1976). Practically, however, a_w data are easier to obtain than other water mobility data.

Bell and Labuza (1994) had reported that as the a_w of a system decreased to approximately 0.6, the rate of most reactions increased with concurrent increase in E_a and ΔH, and decrease in ΔS. At 60 and 70°C, the mean rate of OTC degradation tended to be greater ($0.05 < p < 0.20$) in solutions of decreased a_w versus control. At 80°C, no trend was apparent. Contrary to Bell and Labuza (1994), E_a's and ΔH's decreased with increasing a_w, but there was an apparent decrease in entropy. As water mobility is lowered, the medium decreases entropy due to an increase in order of the solvent molecules (Duckworth , 1981). There was an apparent increase in k_{obs} at water activities below 1.0, which does agree with Bell and Labuza (1994). This apparent discrepancy is in agreement with Labuza (1980), illustrating that the phenomena of enthalpy/entropy compensation occurs in systems of similar properties. Bell and Labuza (1994) had stated that at a lower a_w, the potential for a change in system properties exist. This is due to

the decrease in the product formation constant (K*). The product formation constant was defined in equation 2.3 as:

$$K^* = [A^*] / [A]$$

where A* and A are concentrations of the drug in the activated state and non-activated state, respectively. A* will remain extremely small and will not change due to its unstable nature. A, however will increase, and thus K* should decrease. The free energy change as defined in equation 2.6:

$$\Delta G^* = - R T \ln(K^*)$$

will increase. Since entropy must decrease (due to decreased solvent mobility), enthalpy and subsequent E_a must increase. As a result, enthalpy/entropy compensation may not be seen; however, this was not the case for OTC degradation. It is known, however, that the particular solute used will alter the relationship between water activity and water mobility (Warmbier et al., 1976), and that glycerol solutions tend to have higher solvent mobilities than solutions having their a_w adjusted using different solutes. This may account for the data discrepancy of this study with that of Bell and Labuza (1994).

Enthalpy/entropy compensation is a phenomenon whereby in a series of related reactions involving minor changes in solvent, enthalpies and entropies will vary dependently with each other (Leffler, 1955). Thus, large ΔH values accompany large ΔS values. This relationship, if linear, is evidence that the mechanism of the reaction is the same for all tested series (Leffler, 1955). The high degree of correlation obtained between ΔH and ΔS for OTC degradation (Figure 4.4) indicated that the reaction mechanism did not differ under all tested conditions (Leffler, 1955). The isokinetic temperature (337 K = 64°C) revealed that below this temperature, degradation of OTC

by this mechanism was dominated by changes in enthalpy, whereas above this temperature, changes are dominated by changes in entropy (Leffler, 1955). The changes in the thermodynamic parameters indicated that the kinetics of the reaction were affected to a measurable extent while the reaction mechanism remained the same.

5.4 Buffer Effects Upon OTC Degradation

Vej-Hansen et al. (1978) were the first researchers to perform a limited systematic study of the effect of different buffers upon OTC degradation. They had concluded that acetate buffer over a range of 0.05 to 0.5 M had a significant though minor effect upon the rate of OTC degradation. At 60°C in pH 5.63, 0.05 M acetate buffer, they reported that k_{obs} was equivalent to 0.0055 $min.^{-1}$, which is just below the range of significance of the compiled statistics for pH 5.5, 0.05 M acetate buffer obtained in this study as shown in Table 4.12 .

OTC is subject to general acid-base catalysis (Connors et al., 1986). General acid-base catalysis is a reaction whereby a compound aids in proton transfer during the formation of the activated state in a reaction and is the rate limiting step (Jencks, 1969). The activity of a general acid/base catalyst is related to its pKa; plots of log $k_{[HQ]}$ as a function of pKa are positive for general base catalysts (i.e. the larger the pKa, the greater the activity) and negative for general acid catalyst (i.e. the larger the pKa, the less the activity). At pH 5.5, the predominant phosphate species is $H_2PO_3^-$, which has a pKa of ca. 7.2. Acetate has a pKa of 4.4. OTC degradation significantly ($p < 0.05$) increased in the presence of orthophosphate at pH 5.5, with an apparent linear relationship between phosphate concentration and k_{obs}. In contrast, analysis of covariance indicated that

acetate had a negligible effect upon k_{obs}. It thus can be concluded that OTC degradation is catalyzed by compounds that can act as general base catalysts. At pH 5.5, the acid degradation mechanism shown in Figure 2.3 would be the preferred one. Though the mechanism is acid catalyzed, the rate-limiting step requires removal of a proton (C5a - H), with subsequent dehydration at C-6.

Phosphate buffer was found to have different kinetic effects upon OTC degradation, depending upon the pH. Vej-Hansen et al. (1978) found that at pH's 1.6-3, no catalytic effect was apparent, whereas at pH 6.4-7.7, a catalytic effect over the range of 0.01 to 0.05 M is apparent. At pH 6.46, they obtained data for k_o and $k_{[HQ]}$ of 0.0054 and 0.29 $min.^{-1}$, respectively. At pH 5.5, k_o and $k_{[HQ]}$ of -0.0049 and -0.132 min^{-1} were obtained in the present study. The two k_o values were comparable. Due to different proportions of the active base at pH 5.5 and pH 6.46, comparisons of the $k_{[HQ]}$ would not be valid.

Increasing the phosphate buffer concentration decreased entropy. The decrease in entropy can be due to an increased ordering of the medium surrounding the reactants-products. As phosphate does act as a general base catalyst for OTC degradation, it would have to be aligned with OTC in order to be able to participate in the reaction, hence the loss in entropy. Enthalpy/entropy compensation was exhibited, which is evidence that the reaction mechanism was not altered, only enhanced. The extraction of the C-5 H appears to be the predominant rate limiting step in the degradation of OTC.

5.5 Polymeric Phosphate Effects Upon OTC Degradation

In general, polymeric phosphates decreased k_{obs}, with SH significantly ($p < 0.05$) decreasing k_{obs} at all tested temperatures. The secondary kinetic parameters did not qualitatively differ from that of the acetate buffer control. This, in combination with the fact that enthalpy/entropy compensation was exhibited, indicated that the reaction mechanism was not altered, and that the compounds did not appear to interact with OTC. Higuchi and Bolton (1959) reported that sodium hexametaphosphate exhibits negligible interaction with OTC. This indicates that the reaction mechanism was inhibited indirectly by the polymeric phosphates. Above 0.01 M, the effect was not concentration dependent as there was no difference in k_{obs} over the five-fold range of concentrations tested. The decrease in k_{obs} was more pronounced as the chain length of the polymeric phosphates increased. Polymeric phosphates are used as detergent aids; one of the mechanisms for their effectiveness is the ability to form ion spheres around dirt particles (Toy and Walsh, 1987). The size of ion spheres are independent of concentration. Though not a true complex, the presence of these spheres may interfere with the acid-catalyzed degradation of OTC. These ion spheres would affect molecular motion; techniques capable of measuring this motion (e.g. NMR) could be utilized to verify this speculation.

5.6 Ionic Strength Effects Upon OTC Degradation

Increasing phosphate buffer concentration from 0.05 to 0.1 M increased k_{obs}. This was shown to be due to the general base catalytic effect of phosphate buffer. The addition of NaCl to 0.05 M phosphate buffer did not have any effect upon k_{obs}. This is

evidence that ionic strength does not affect the transition state during OTC degradation (Connors, 1986), implying that the reaction does not undergo a change in ionization state. As shown in Figure 2.3, degradation of OTC by the proposed mechanism does not involve a change in ionic nature, thereby substantiating literature stating that this is a possible mechanism in OTC degradation.

There was a slight decrease in entropy upon addition of NaCl. The addition of NaCl would effectively increase the polarity of the medium (Connors, 1986). As OTC possesses polar functional groups, this would have resulted in some degree of increased ordering around the molecule. This indicates that minor changes in the matrix polarity (via addition of non-reactive ionic compounds) would not have an extensive effect upon k_{obs} given that water availability is still adequate.

5.7 Calcium Chloride Effects Upon Thermal Stability of OTC

Increasing the $CaCl_2$ concentration from 0 to 0.1 M decreased k_{obs} by approximately, 3, 4 and 5 fold at 60, 70 and 80°C respectively in 0.1 M acetate buffer. Additionally, the E_a decreased to 15 kcal/mol. Tetracycline forms stable complexes with most multivalent cations (Albert and Rees, 1956; Newman and Frank, 1976). The log value of the K_as for the formation of Fe^{3+}, Fe^{2+}, Mg^{2+} and Ca^{2+} complexes were reported to be 9.1, 5.6 (Albert, 1953), 3.8 (Albert and Rees, 1956) and approximately 2, respectively (Higuchi and Bolton, 1959). Calcium binds via the C-10 and C-11 oxygens forming primarily a 1:1 complex, a second cation can add to the C-12 and C-1 positions, though 2:1 complexes with OTC form much less readily (Newman and Frank, 1976). The binding of the C-10 and C-11 sites would interfere with the proposed degradation

mechanism by disrupting C-11 and C-12 enolization, which leads to aromatization of the C and D rings of the molecule and contributes to the driving force for the E2 elimination reaction at the C-5a:C-6 position.

The formation constant for the Ca^{2+}-OTC complex is not high relative to other ions, with log values of approximately 1-2 (Higuchi and Bolton, 1959), though it is thermally stable (Sina et al., 1974). There is no published information on the effects of temperature on formation of the complex itself. Increasing the Ca^{2+} concentration would increase the relative amount of Ca^{2+}-OTC complexes by way of an equilibrium shift, thereby decreasing k_{obs}, which is evident from the compiled data in Table 4.12.

The addition of $CaCl_2$ led to a dramatic decrease in entropy. As has been reported, OTC does form chelates with calcium and other cations. The formation of a chelate means a loss in the degree of ordering, hence the loss of entropy. As enthalpy/entropy compensation is still being exhibited, this implies that other mechanisms of OTC degradation are not occurring to an extent that measurably affect the kinetics of the reaction. This indicates that other degradative mechanisms are not favoured under the given reaction conditions.

Because of their ability to bind calcium, tetracyclines tend to accumulate in bone; a single therapeutic dose of tetracycline would last for at least 150 days in bone tissue (Van Brackle et al., 1994). In animals receiving prolonged treatment with OTC, it is a safe assumption that the amount of accumulation would be substantial. In products incorporating mechanically deboned meat (MDM), it is conceivable that bone particles are incorporated into the mixture, which may lead to potentially large amounts of thermally stable OTC in foodstuffs. Though pork products containing MDM are not

common (Campbell and Kenny, 1994), rendering is. Rendering is used to create pork byproducts, lard being the most common but also water soluble flavour concentrates. Bones are used in the rendering process. OTC is water soluble. Though current literature has not shown studies supporting this, it is reasonable to assume that carry over of OTC from bones to water-soluble byproducts is possible.

5.8 Sodium Nitrite Effects Upon Thermal Stability of OTC

Nitrite is a reactive molecule that can undergo oxidative, reductive and coupling reactions with phenols, amines, thiols and other organic functional groups (Walters and West, 1984). In acetate buffer, there were no significant differences in k_{obs}, though the mean k_{obs} increased at 70°C and decreased at 80°C (Table 4.12).

The addition of $NaNO_2$, however, did result in a decrease in entropy of the reaction. This, in addition to the slight changes in k_{obs}, implies that $NaNO_2$ and/or its degradation compounds are by some mechanism coordinating with OTC to react with it. The most probable mechanism would be an oxidative type of reaction, due to the relatively high oxidizing potential of nitrite. Since enthalpy/entropy compensation was exhibited, this implies that the reaction mechanism was not altered. Nitrite thus appears to be involved directly or indirectly at the site of the rate limiting step of the reaction.

5.9 Porcine Tissue Effects Upon Thermal Stability of OTC

OTC in porcine tissue matrix at pH 5.5 significantly decreased k_{obs} at all temperatures over that of pH 5.5, 0.1 M acetate buffer. E_a, ΔH and ΔS were not noticeably different from that of pH 5.5, 0.1 M acetate buffer. Additionally, the data

exhibited enthalpy/entropy compensation, and therefore it can be concluded that the mechanism of the reaction was not altered. The observed change in the rates of degradation can be attributed to two possible factors: 1) physical limitations on diffusion of the reactants due to inconsistencies in the tissue matrix. 2) chemical interference of the reaction. One major source of inconsistencies in the tissue matrix would be due to the presence of entrapped air pockets in the tissue, which can be as high as 13 % (v/v) in comminuted tissue products (Palombo et al., 1991). This may lead to local temperature differences within the meat matrix.

The addition of sodium chloride to OTC solution did not have any significant effect upon OTC degradation. However, addition of NaCl to tissue had dramatic effects upon k_{obs}. At 60°C, k_{obs} increased whereas at 80°C, k_{obs} decreased. Sodium chloride does increase the water holding capacity of meat, reduces cooking losses, and aids in the formation of a meat gel (Hamm, 1960; Clarke et al., 1987). Gels made from isolated meat proteins have bulk water of restricted mobility (Yasui et al., 1979), and water mobility does dramatically influence the rate of degradative reactions (Yoshioka et al, 1992, 1995). However, initial decreases in water mobility via changes in a_w will initially increase the rate of reactions (Table 4.6), which was not the case in the NaCl meat gel. NaCl does not have a direct effect upon OTC degradation (Table 4.8), nor should its property of slightly altering water mobility via gel formation have an effect upon OTC degradation, as was discussed in section 5.3. NaCl does, however, have the ability to displace divalent ions (calcium and magnesium) that are bound to muscle fibers (Bozler, 1955). Relatively large proportions (approximately 25%) of endogenous calcium and magnesium are tightly bound to muscle tissue to the extent that they cannot be removed

by strong chelating agents, such as EDTA (Bozler, 1955). At 60°C, myosin denaturation is complete, though myofibrillar protein (actin and actomyosin) denaturation is not complete until 75-80°C (Honikel, 1989), at which temperature a dramatic rearrangement of the tertiary structure of the protein occurs. It can be speculated that at 60°C, since myofibrillar proteins have not denatured, calcium is still bound with a relatively high affinity to the muscle proteins, though it can be selectively extracted by OTC. The presence of NaCl would act as an electrostatic shield for complexation to occur, reducing the amount of complexation. Less OTC would be in the thermal stable complex form, resulting in apparent increase in k_{obs}. At 80°C, the extensive protein denaturation and competitive displacement of calcium and magnesium ions from tissue by chloride ions would increase the effective concentration of both divalent ions for OTC complexation. This would increase the amount of OTC that exists in the thermally stable complex form, thereby decreasing the apparent k_{obs} at higher temperatures. The change in entropy for this medium was a relatively large negative number, which does imply that the OTC structure was restricted, similar to that exhibited by $CaCl_2$ solutions (Table 4.13).

In porcine tissue, increasing Ca^{2+} concentration to 0.4 M did not have as great an impact upon lowering k_{obs} as was observed in aqueous solutions; the presence of 0.1 M $CaCl_2$ in acetate buffer had a much greater k_{obs} lowering effect than 0.4 M $CaCl_2$ in porcine tissue. Addition of Ca^{2+} to tissue would result in substantial amounts of the ion being bound to the tissue (Bozler, 1955); this implies that much of the Ca^{2+} would, in effect, be unavailable for binding with OTC, thereby resulting in the formation of only a limited amount of the thermally stable OTC-Ca^{2+} complex. The relatively small change

in entropy is further evidence that the restricted OTC-Ca^{2+} complexes are not forming to a great extent. The use of metal chelating agents (EDTA, STP, SH) increased k_{obs}, which supported the conclusion that the meat mineral content was the predominant influence in OTC degradation. The lack of effect of $CaCl_2$ in tissue implies that the meat matrix is by some mechanism making the Ca^{2+} unavailable for complexing with OTC. It has been shown that OTC exhibits a relatively high degree of binding with BSA (log K_a = 3.85) though the mechanism of this interaction is predominantly hydrophobic, via primarily tyrosine and tryptophan residues (Higuchi and Bolton, 1959; Ma et al., 1973). Values of K_a for OTC binding to muscle tissue proteins has not been reported. Hydrophobic forces tend to be much weaker than polar attractive forces; intuitively this implies that OTC interaction with protein (i.e. tissue proteins) should not effect the water mediated degradation of OTC. Due to the relatively small change in entropy, it may be cautiously speculated that OTC still continues to form complexes with Ca^{2+}, albeit to a lesser extent due to protein selectively binding OTC as well as Ca^{2+}. With fewer complexes formed, the rate of degradation would be higher, as shown in Table 4.12.

The addition of polyphosphates to tissues generally increased k_{obs} at all temperatures. SH significantly ($p < 0.05$) increased k_{obs} at all temperatures versus tissue matrix alone, with the rates being similar to that of SH in buffer solutions. The different phosphates had widely varying effects upon the thermodynamic parameters of degradation. Sodium phosphate resulted in a decreased ΔS. Since it is known that orthophosphate has only negligible effects upon protein functionality in a buffered matrix (Lewis et al., 1986) the effect is due to interaction with OTC directly. It has been

shown that orthophosphate acts as a general base catalyst of OTC degradation, which is an entropy decreasing process.

SH and SAPP also decreased ΔS. Table 4.10 revealed that these compounds do not act as general base catalysts, and in fact tend to reduce degradation via indirect interaction with OTC. SAPP does have an effect on tissue matrices by breaking actomyosin complexes (Lewis et al. 1986). The increased protein in solution would result in a decreased water mobility in the gel (Hamm, 1960), but it has been shown that moderate reductions in water mobility do not affect OTC degradation dramatically (Table 4.6). Phosphates are strong chelators of metal ions, with polyphosphates generally being stronger chelators of metals than that of orthophosphates (Irani and Callis, 1962). Increasing temperature increases the amount of chelation (Molins, 1991), which is believed to be due primarily to a competitive effect between the thermostable phosphate-ion complex and the thermolabile metal ion-biological complexes (Irani and Callis, 1962). They are regarded as being effective chelators between pH's 5-12 (Wazer and Callis, 1958), particularly for iron, whereby lower pH increases the association constant (k_a) between phosphates and iron (Irani and Morgenthaler, 1963). Polyphosphates are less susceptible to the effects of pH upon their chelating ability than orthophosphates (Wazer and Callis, 1958). The logarithm of the association constants of ortho- and polyphosphates for monovalent cations ranges from 1-2 (Wazer and Callis, 1958). For divalent cations such as calcium and magnesium, orthophosphate forms complexes that have log K_a of 1-2; pyrophosphate forms complexes that have log K_a of 3-4; and tripolyphosphates form complexes that have log K_a of 6-7 (Wazer and Callis, 1958). Polyphosphates forms complexes with ferric and ferrous ions with log K_a of 22-

25 (Irani and Morgenthaler, 1963). The USDA has reported that the amount of calcium, magnesium and iron in meat is 16, 23 and 0.91 mg per 100g portion, respectively (USDA, 1997). Thus, the complexing of metal ions results in greatly reduced formation of thermostable OTC-cation complexes, thereby increasing k_{obs}. As the k_{obs} obtained were similar to that found in buffer alone, it can be concluded that physical inconsistencies in the tissue matrix did not affect OTC degradation.

The complexation of EDTA with metal ions is highly dependent upon pH; complexing is dependent upon the amount of the Y^{4-} species (Harris, 1991). At pH 5-6 (i.e. the approximate pH of postrigor meat) the log K_f for Mg^{2+}, Ca^{2+} and Fe^{2+} are approximately 3, 5 and 8 respectively. At pH 5.5, EDTA would be present predominantly in the diprotic form. The amount of the monoprotic species, with a pKa of 6.16 is approximately 0.2 of the total amount. It thus would have only negligible effect upon general base catalysis of OTC (Jencks, 1969). It has also been reported that EDTA does not form complexes with OTC (Higuchi and Bolton, 1959). It is assumed that upon addition of EDTA to a meat matrix, polyvalent cations would be made unavailable for binding by OTC, preventing the formation of thermally-stable complexes. Only the remaining matrix components would interact with OTC. Addition of 0.2 M EDTA to the tissue increased the rate of OTC degradation to levels that were not significantly different from that of 0.1 M acetate buffer. This indicated that the mineral content of tissue is primarily responsible for the increase in the thermal stability of OTC in tissue, which is in agreement with results obtained for the use of polyphosphates.

In tissue, the addition of $NaNO_2$ significantly increased k_{obs} at 60°C, whereas it significantly decreased it at 80°C ($p < 0.05$). The E_a decreased, though the fit of the kinetic parameters upon the isokinetic temperature line indicated that the mechanism for the degradation of OTC did not change. The drop in E_a, however, revealed that the rate limiting step for the degradation of OTC (elimination of the C-5a proton) has been affected. Nitrite is present in too small a quantity to effect the physical structure of tissue; as well nitrite does not appear to have a direct effect upon OTC as indicated by the k_{obs} obtained from solution experiments. This implies that nitrite affects and is affected by the tissues chemically, which subsequently affects the rate of OTC degradation. Nitrite tends to have minimal reactivity in a matrix unless reducing agents are present (Ando, 1973). The main component of tissue that has an influence upon k_{obs} is its mineral content. The presence of nitrite reduces the amount of non-heme iron in cooked meat as compared to no nitrite controls (Schricker and Miller, 1983). Several other unidentified nitrite reaction products also exist, some of which have been shown to have reducing properties (Sebranek et al., 1978), though there is no documentation that indicates the relative reactive strength of these chemical entities. The decrease in entropy was the largest among all treatments. The decrease indicates that the matrix (tissue and water) or OTC itself became much more structured during the course of the reaction. Physical changes in the matrix are unlikely, implying that the structure of OTC has become restricted by interaction with reaction products of nitrite and the tissue. The relatively low E_a of the reaction indicates that OTC degradation in this matrix is temperature insensitive relative to other tested matrices (e.g. in tissue only, k_{obs} increases ten-fold from 60 to 80°C, whereas in tissue with $NaNO_2$, k_{obs} increases three-fold).

Though nitrite does increase the rate of OTC degradation at lower temperatures, the large decrease in entropy for the reaction limits the temperature-induced increases in k_{obs}. Thus, OTC residues in cured meat products, in the absence of other influencing factors, would not be expected to change greatly during cookery practices.

5.10 Formation of OTC Degradation Compounds

The degradation of OTC under most experimental conditions resulted in the presence of several unknown chromatographic peaks during subsequent analysis. Previous evidence has indicated that the rate limiting step in OTC degradation is predominantly the E2 elimination mechanism at the C-5a:C-6 bond. However, the end product of this reaction is also labile, as evident from the several chromatographic peaks, suggesting the presence of several components in the reaction mixture. In distilled water, the amounts of these unknown compounds was less, and there was limited success in being able to monitor the formation of a compound responsible for a peak eluting at ca. 12 minutes under the given analytical conditions. At all temperatures, there was an initial increase in the unidentified compound, followed by a decrease. The logic for this trend is that assuming that the formation and degradation of this compound obeys first-order kinetics, initial formation of the compound would be favoured due to the large amount of the reactant (i.e. OTC). As the reaction proceeds, the amount of OTC decreases; less product forms, and the rate of degradation is greater than the rate of formation, resulting in a net decrease in the formation of the product. One common approach to quantify these rates is through application of the biexponential model (Shargel and Yu, 1993). SAS offers a procedure (PROC NLIN) that can enable the estimation of the parameters

for this model through an iterative procedure. In this procedure, initial estimates of the parameters are supplied by the user; the program subsequently performs a series of minor manipulations on the estimates in an attempt to minimize the differences between the given data and predicted values (i.e. minimize the residual sum of squares) using the parameter estimates. Utilizing the program algorithm, these manipulations continue until the differences no longer change; the reported values of the estimates are the last manipulated values. Variances of these estimates are not subject to the same protocols as for linear models, and are regarded as approximations only (SAS Institute Inc., 1989). The major problem associated with this method is that it is not known whether the estimates provided are the result of a global minima in residual deviations, or a local minima, in which case the parameter estimates are invalid. It was found that by altering the initial parameter estimates, the final obtained parameter estimates would differ substantially. Table 4.17 shows that the variances associated with the parameters are usually much larger than the estimate itself. In several instances, PROC NLIN could not provide approximations of the standard errors. Determination of the goodness of fit of the model through application of a lack-of-fit test revealed that the models at all temperatures exhibited significant lack-of-fit, indicating that the biexponential model is not appropriate for modelling the formation of unknown degradation compounds. The implications of this are that OTC degrades by several mechanisms and/or by different kinetics. Also, the biexponential model assumes that compound formation and degradation obey first-order kinetics, which may not be correct in this case. A mathematical model could be obtained only if sufficient data could be collected

accounting for the predominant degradation compounds and possible reaction mechanism.

5.11 OTC Binding to BSA/Tissue

Application of the biexponential model to the competitive binding data for OTC tissue/BSA data was valid. Standard errors of the estimates were generally less than the values of the estimates. Determination of the goodness-of-fit of the models by application of lack-of-fit tests indicated that lack-of-fit was insignificant (i.e. further improvements in the model would not be useful). However, the β values (coefficients of OTC dissociation from tissue/BSA) obtained from all treatments were not significantly different from each other.

Addition of NaCl or STP, a relatively strong chelating agent, did not affect the β term greatly in BSA solutions, which suggested that neither electrostatic shielding nor metal chelating action affected OTC binding to BSA; thus, the predominant attractive forces between OTC and BSA are not ionic in nature, nor are metal chelating effects involved. This implies that the binding of OTC to proteins is due to either hydrophobic or hydrogen bonding, which is in agreement with Ma et al. (1973).

It has been shown that when calcium is added to a tissue matrix, the increase in thermal stability of OTC is not as great if only buffer solution were used as a matrix.. Calcium and magnesium do have strong affinities for contractile proteins (Bozler, 1955). Kinetic data obtained from this project provided evidence that binding of OTC to calcium is inhibited due to the competition between protein and OTC for calcium. Further evidence for this is that the addition of phosphate, a weak metal chelator, to

porcine tissue homogenate, resulted in a dramatic increase in the β-term, showing that OTC was not held with as great an affinity to the tissue. Phosphate in pH buffered solution would not have a significant effect upon protein solubilities or functional properties of tissues (Lewis et al, 1986), thus the phosphate effect can be attributed to its other chemical properties (i.e. metal chelation). The metals chelated would include calcium and magnesium. As a result of chelation, the ions attached to the proteins would be unavailable for binding to OTC.

6. CONCLUSIONS

A method for the separation and quantification of oxytetracycline and related compounds through the application of isocratic reversed-phase liquid chromatography was developed. Extraction and quantification of common OTC degradation compounds from aqueous and tissue media was not as viable as it was for OTC. This was attributed to the potential degradation of the compounds in the matrices, or to the inability of the extraction methodology to separate the compounds from the matrices. Tissue matrices, due to their inherent complexity, decreased the extent and precision of OTC and degradation compound extraction. Monitoring the formation of degradation compounds would give further insight into the reactions occurring in food matrices. Further research to develop a viable extraction method for these compounds would be needed before successful monitoring of these compounds in tissues can occur.

The effects of heat, water activity, salts, phosphates, sodium nitrite and porcine tissue upon the rate of OTC degradation were determined. The rates of degradation were found to be significantly affected by the presence of phosphates (monomeric and polymeric), heat, nitrite, calcium and tissue. Water activity and salt levels of interest to meat processors did not appreciably effect the rate of OTC degradation. The secondary kinetic parameters obtained indicated that the rate limiting step in the degradation of OTC was the same for all processing condition combinations.

Water activities adjusted by means of glycerol did not significantly alter k_{obs}, though changes in the secondary kinetic parameters were noted. OTC degradation is affected by changes in a_w, though effects on k_{obs} would not be apparent until lower a_w's

or media of lower water mobility were utilized. Trends in k_{obs} changes were consistent with current theories on the effects of a_w upon reaction kinetics.

Orthophosphate and polymeric phosphates exhibited different effects upon OTC degradation. Monomeric phosphates catalysed the degradation of OTC, via general acid base catalysis. Polymeric phosphates decreased the rate of OTC degradation. The decrease in k_{obs} was dependent upon the type of polymeric phosphate used but not its concentration. The formation of ion spheres was a speculative explanation for this phenomenon.

In tissue, polymeric phosphates significantly increased k_{obs}. Polymeric phosphates are strong chelating agents, and induced similar effects as EDTA, another strong chelating agent. By making cations unavailable for OTC complexation, thermostable complexes could not be formed, resulting in increased k_{obs}. This indicated that the predominant mechanism by which OTC gains thermal stability in tissues is due to the mineral content.

The addition of calcium dramatically decreased k_{obs} in solution. Previous research has shown that OTC forms thermally stable chelates with calcium. The large decrease in entropy for thermal degradation of OTC in the presence of calcium data provided evidence of the formation of these complexes. The complexes interfere with OTC degradation by inhibiting aromatization of the C and D rings, which is the driving force for the rate limiting E2 elimination reaction. In tissue, the decrease in k_{obs} was not as dramatic, due to competitive binding of calcium by both OTC and tissue proteins. Competitive binding studies indicated that addition of chelating agents displaces OTC from protein binding mediated by metal complexation.

Nitrite in solution had a minimal effect upon the rate of OTC degradation, though secondary kinetic data indicated that nitrite is, in some manner, interacting with OTC. When added to porcine tissue, nitrite had a dramatic effect upon k_{obs}. Nitrite in the presence of reductants forms the active nitrosating agent in addition to other compounds. The low E_a indicated that the reaction was relatively independent of temperature. The low ΔS value revealed that a steric factor is involved in the reaction (i.e. proper alignment of OTC with the reactive nitrite-derived compounds).

The addition of sodium chloride to an aqueous solution of OTC did not significantly alter k_{obs}. Addition of sodium chloride to tissue, however, significantly increased OTC degradation at 60°C, and significantly decreased it at 80°C. Electrostatic shielding of divalent ions from OTC at low temperatures and displacement of divalent cations from tissues at high temperatures are believed to be the mechanisms occurring to explain this phenomena.

The presence of enthalpy/entropy compensation revealed that one reaction mechanism was the predominant rate limiting step. However, the number of degradation compounds that formed during thermal treatment increased as complexity of the reaction media increased. Analysis of the formation of one unknown compound revealed that its formation and degradation kinetics could not be modelled according to a biexponential equation. Subsequent degradation of OTC following the initial rate limiting step may proceed by way of several different mechanisms.

During meat processing, several of the studied parameters would occur simultaneously. To precisely model OTC degradation under such conditions would require quantifying interaction effects between the various parameters. This would

prove impractical, as the number of interactions would be immense. Instead, research efforts in this area should be directed towards identifying those interactions that are definable (i.e. first- or second-order). Higher-order interactions would be evaluated for their potential impact. If the impact is great, then application of several models, corresponding to different boundary conditions, may then prove more useful. These models, coupled with estimation of the amount of OTC residues present in raw tissues, could then be utilized to estimate the daily intake of OTC as well as the degradation products. The results obtained could be used for several purposes, the most notable when trying to correlate this data with atypical health patterns in affected populations.

Aspects of the protocol used in this study could be used for the study of other xenobiotics, though each would have its own unique chemistry, requiring modification of the procedures used. Much research on the degradation of other antibiotics is quantitative in nature, with few systematic studies performed on determining rates of reactions. Systematic studies would provide information more useful than that obtained from absolute quantitative studies for attempting to estimate the extent of breakdown of xenobiotics during a given cookery practice.

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IMAGE EVALUATION
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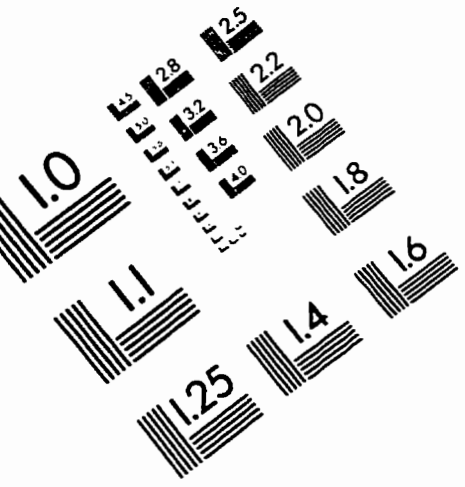

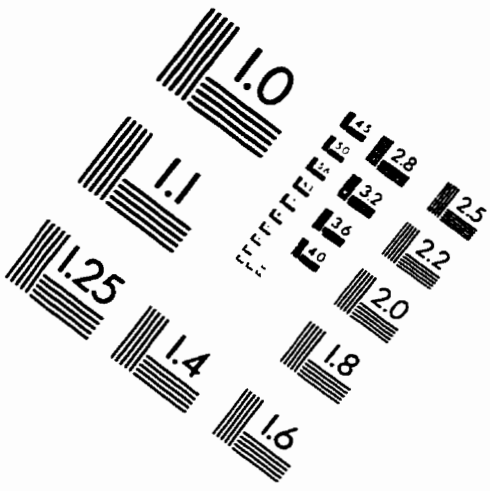

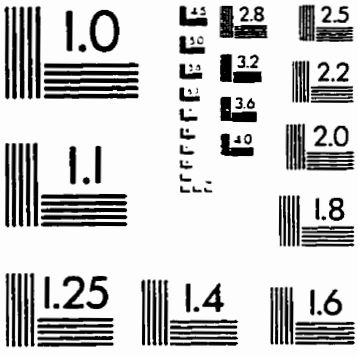

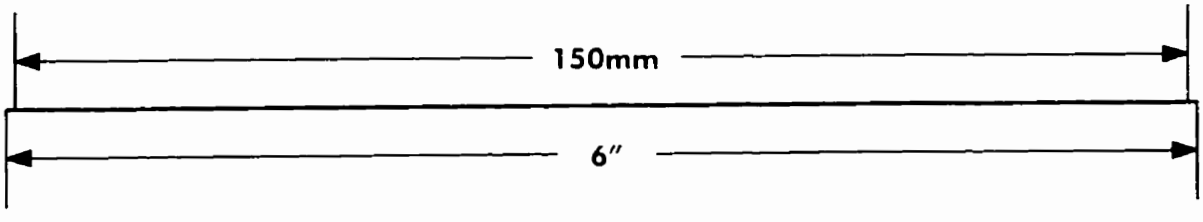

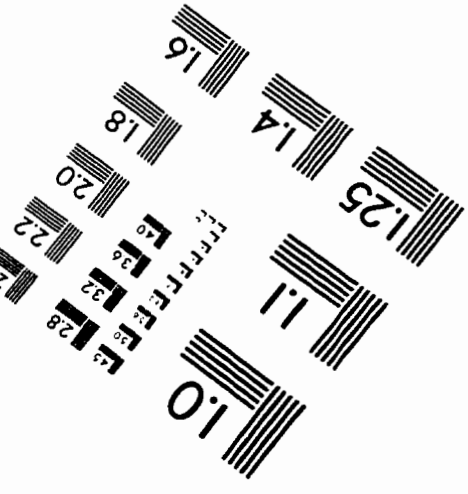

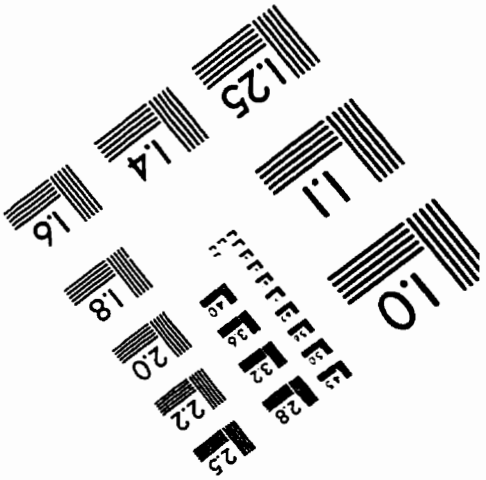

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Phone: 716/482-0300
Fax: 716/288-5989