DISSERTATION

Determination of Antibiotic, β-Agonist, and Non-Steroidal Anti-Inflammatory Drug Residues in Ground Beef from USDA Certified Organic, Natural, Conventional, and Market Cow and Bull Sources

Submitted by

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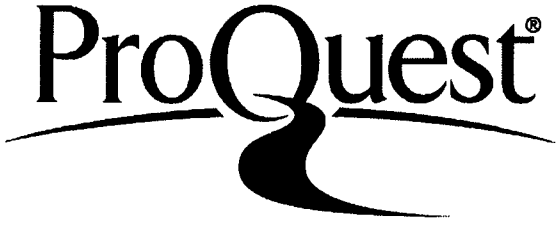

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ABSTRACT OF DISSERTATION

Determination of Antibiotic, β-Agonist, and Non-Steroidal Anti-Inflammatory Drug Residues in Ground Beef from USDA Certified Organic, Natural, Conventional, and Market Cow and Bull Sources

In recent years, consumer demand for organic and "natural" products has increased, partly due to a perception that such products are healthier and contain fewer additives, including veterinary drugs and growth promotants. The study presented herein compared occurrence of veterinary drug residues in ground beef samples reflecting different livestock production classifications. We collected ground beef samples (N = 400) consisting of 90.0 ± 4.0 % lean muscle tissue from a total of eight plants, two each reflecting production in the following categories: (1) USDA Certified Organic (n = 100); (2) USDA Process Verified Never Ever 3 (n =1 00); (3) conventionally raised fed beef (n = 100); and (4) ground beef derived from carcasses of market cows and bulls (n = 100). Liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC-MS) methods were developed for the following veterinary drugs: (1) Aminoglycosides (Gentamicin, Amikacin, and Neomycin); (2) β-lactams (Penicillin, Ampicillin, and Desfuroylceftiofur); (3) Fluoroquinolones (Danofloxacin and Ciprofloxacin); (4) Macrolides (Erythromycin, Tylosin, and Tilmicosin); (5) Phenicols (Florfenicol); (6) Sulfonamides (Sulfamethazine and

Sulfadimethoxine,); (7) Tetracyclines (Oxytetracycline, Chlortetracycline, and Tetracycline); (8) Streptogramins (Virginiamycin); (9) β-agonists (Ractopamine and Zilpaterol); and (10) non-steroidal anti-inflammatory drugs (Flunixin and Phenylbutazone). Residues exceeding their respective US tolerance limit were found in six ground beef samples. Two USDA Certified Organic samples contained Ampicillin residues exceeding US tolerance limits. One USDA Process Verified Never Ever 3 sample contained a residue of Ractopamine exceeding US tolerance limits. One sample from the market cow and bull category contained a residue of Sulfadimethoxine that exceeded US tolerance limits, one contained a residue of Ampicillin that exceeded US tolerance limits, and one contained a residue of Phenylbutazone that exceeded US tolerance limits. Residues of Phenylbutazone exceeding US tolerance limits were also found in one sample from the conventional production category. Additionally, residues (below the US tolerance limit) of several classes of veterinary drugs were found in samples from the USDA Certified Organic and USDA Process Verified Never Ever 3 production categories, a finding that clearly demonstrates violation of zero-tolerance statutes set forth by the National Organic Program and USDA Process Verified Never Ever 3 marketing descriptors. In the USDA Certified Organic production category, residues were detected in eight Ampicillin, seven Penicillin, three Sulfamethazine, one Sulfadimethoxine, and one Ractopamine sample. In the USDA Process Verified Never Ever 3 production category, residues were

detected in one Ampicillin, one Chlortetracycline, two Tetracycline, and six Ractopamine samples. These violations exceed the historical prevalence of veterinary drug residues reported by the National Residue Program and demonstrate the need for careful monitoring of animals administered veterinary drugs in order to prevent improper inclusion of unqualified animals in premium marketing programs, such as USDA Certified Organic and USDA Process Verified Never Ever 3 programs.

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So many things in life are fleeting, even the things we were once certain would never end. I am thankful to those who have stood by me throughout this journey. I have truly learned how much people care about me as they have stood by me during my tough times. Specifically, I want to thank my parents and family for their unconditional love and support. In addition, I want to thank Dr. Belk and Dr. Smith for their mentorship, understanding, continuous support, and faith in me. Finally, I want to acknowledge my fellow graduate students, who will remain my treasured friends forever. I will never forget those who never gave up on me.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

Objective of Dissertation

Determination of Antibiotic, β-Agonist, and Non-Steroidal Anti-Inflammatory Drug Residues in Ground Beef from USDA Certified Organic, Natural, Conventional, and Market Cow and Bull Sources

- The objective of the research presented herein was to determine differences in antibiotic, β-agonist, and non-steroidal anti-inflammatory drug residues in ground beef from USDA Certified Organic, USDA Process Verified Never Ever 3, conventional, and market cow and bull sources.

CHAPTER II

Literature Review

Beef Production Practices

In recent years, an increasing number of branded beef products have been made available to consumers. As a part of these branding programs, labeling claims pertaining to animal feed ingredients, animal handling, and post-slaughter processing of carcasses have been implemented as marketing tools. In an effort to establish specific guidelines for such labeling claims, the United States Department of Agriculture (USDA) has created and implemented regulations for the use of terms such as "organic" and "naturally raised." (1) In the research presented herein, samples of ground beef from USDA Certified Organic, USDA Process Verified Never Ever 3, conventional, and market cow and bull sources were collected and analyzed for the presence of antibiotics, β-agonists, and non-steroidal anti-inflammatory drugs (NSAIDs). The following is a brief description of USDA Certified Organic, USDA Process Verified Never Ever 3, and conventional beef, their legal definitions, and their labeling requirements. Beef labeled as "grass-fed" is another classification of beef, and grass-fed beef products can be organic, natural, or conventional, depending upon the production practices used,

documented, and validated according to the grass fed standards available through USDA-AMS. Because no grass-fed beef was specifically collected or analyzed in this study, it will not be described in further depth herein.

USDA Certified Organic Beef

In 1990, the Organic Food Production Act (OFPA) was passed by the US Congress. As a result of the OFPA, the National Organic Program (NOP) was approved by Congress in 2002, and it required USDA to develop national standards for organically produced agricultural products *(1)*. When the NOP standards were developed by the National Organic Standards Board, the Agricultural Marketing Service of the USDA (USDA-AMS) was charged with their administration.

Requirements for USDA Certified Organic products state that animals must be produced (a) without the use of antibiotics, growth promotants, pesticides, irradiation, sewage sludge, or genetic modification; (b) with access to outdoors; (c) by being fed only organic feed; and (d) by producers following a specific, accredited, Organic System Plan that outlines environmental stewardship protocols *(1)*. In addition to the on-farm and other animal production stipulations set forth by USDA-AMS, there are specific labeling standards for USDA Certified Organic products which state that (a) products labeled as "100 percent organic" must contain only organic ingredients; (b)

products bearing the word "organic" in the name must be comprised of at least 95% organically produced ingredients; (c) products that bear the wording "100 percent organic" or "organic" on the label may use the USDA organic seal; and (d) processed products containing at least 70% organic ingredients can use the phrase "made with organic ingredients" but may not use the USDA organic seal *(1)*. While some consumers believe USDA Certified Organic beef to be more healthy or nutritious, the National Organic Program was designed and implemented specifically as a marketing program *(1)*.

Natural Beef

Outside the category of USDA Certified Organic beef, assigning a specific definition to a classification of beef becomes very difficult. In recent years, however, the "natural" labeling of beef products has resulted in increased consumer confidence and, as a result, in price premiums for sectors of the industry participating in natural programs. By definition, most beef meets the USDA- Food Safety and Inspection Service's (USDA-FSIS) regulatory requirement to be labeled as natural. The USDA-FSIS defines natural as product that does not contain any flavoring agent, coloring ingredient, preservatives, or any other artificial or synthetic ingredient resulting in an end-product and its ingredients that are not more than minimally processed *(27)*. Because the USDA-FSIS statutory definition only pertains to processes conducted after slaughter, many beef packing companies petitioned

government agencies to generate production-specific marketing claims to facilitate further differentiation of their product, and, as a result, two standards were created by USDA.

The Never Ever 3 program is a USDA Process Verified Program that stipulates specific marketing claims that meat packers may make concerning the production practices implemented to generate their products. The Never Ever 3 program was created by the Audit Review and Compliance Branch of USDA-AMS and the specific tenets are: (1) no antibiotics may given, including through feed, water, or injection, from birth to slaughter; (2) the administration of growth promotants including natural hormones, synthetic hormones, estrus suppressants, β-agonists, or other synthetic growth promotants is prohibited from birth to slaughter; (3) animals may not be fed mammalian or avian by-products, including animal waste *(2)*. Animals may be fed fish by-products and vitamin and mineral supplements are also allowed *(2)*. All of the ground beef samples collected from the "natural" production category as a part of the research presented herein were derived from companies that voluntarily participated in the USDA Process Verified Never Ever 3 program.

In January of 2009, USDA-AMS released the "Naturally Raised" standard *(3)*. The marketing specifications (no antibiotics, no growth promotants, and no

mammalian or avian feed additives) are the same as the USDA Process Verified Never Ever 3 program requirements *(2, 3)*. The only notable difference is that the Naturally Raised standard is a Quality System Verified Program and not a Process Verified Program *(3)*. Quality System Verified Programs evaluate products on a predetermined standard (i.e., no antibiotics, no growth promotants, and no mammalian or avian feed additives) whereas process verified programs evaluate entire supply chain systems that deliver products that meet a set standard. As such, products bearing the "Naturally Raised" label are audited for compliance with the standard and production systems are audited for compliance with the USDA Process Verified Never Ever 3 standard.

Despite these standards, the understanding of the regulatory term "natural" is still mired by marketing claims that are not a part of the Naturally Raised or USDA Process Verified Never Ever 3 programs. According to Smith et al. *(52)*:

> Marketing and/or advertising claims made by specific "natural" beef producers include: (a) Cattle raised without antibiotics; no growth hormones added. (b) Beef without hormones, steroids or antibiotics; man hasn't messed with it. (c) No antibiotics, steroids, pesticides; laboratory tested and guaranteed. (d) Produced with sustainable agriculture, humane and proper animal care, and environmental

improvement; providing better nutritional health for you and your family. (e) Tested to be free of antibiotics, steroids and pesticides; fed all-vegetarian feed; humanely raised by small ranchers. (f) Minimally processed with no artificial ingredients. (g) Cattle do not receive antibiotics or growth hormone implants within 100 days pre-harvest. (h) No added hormones. (i) No antibiotics, no added hormones, as verified by 120-day affidavit. (j) Adhere to sustainable agriculture practices; follow highest standards of environmental stewardship; livestock never compete for food or water; livestock have shade or sprinklers; rotate livestock on pasture to enhance growth of desirable grasses and clover; pastureland is free of pesticides and herbicides; follow humane animal husbandry practices; can verify the source of all of our beef. (k) 100% vegetarian diet; no animal byproducts or animal fats—ever; no antibiotics—ever; no added hormones—ever; no artificial flavoring, coloring or chemical preservatives—ever. (l) Sausages made from cattle raised from birth without synthetic hormones or antibiotics; produced without the use of nitrates or nitrites. (m) Raised by farmers and ranchers who care about the animals and the environment in which they live; closely monitored from the farm to our stores to ensure compliance with our strict animal welfare and food safety quality standards. (n) Raised without supplemental hormones, antibiotics or animal byproducts. (o) Never,

ever—birth to box; no antibiotics or growth promotants. (p) Prohibited at any time during the lifetime of the animal are growth hormones (implant, injection or fed, of estradiol, progesterone, zeranoid, melengestrol acetate, steroids), antibiotics and animal byproducts. (q) Animal sourcing to birth with signed affidavits for each point of ownership *(52)*.

Almost all beef products meet the regulatory definition of natural. In addition, production supply chains that want to make verified claims about the production practices used to produce their products can participate in the USDA Process Verified Never Ever 3 program and producers of beef products that want to make marketing claims about their product pertaining to the production practices used may participate in the Naturally Raised program. In addition to these programs, companies may generate label claims that are approved y USDA-FSIS but are not a part of a USDA Process Verified or QSVP program.

Conventional Beef

Conventional beef is most easily defined as beef that is not a part of the marketing programs described above. Conventional beef, also known as "commodity beef," does not have a marketing-based definition but, rather, encompasses all beef production practices. As such, all cattle are "conventional" at birth and, when supply chain practices that meet the

requirements of USDA Certified Organic or Process Verified programs are implemented, accredited, and audited, end-products may be labeled as "Organic, "USDA Process Verified never Ever 3," or "Naturally Raised." It is important to note that food safety regulations do not differ among the different types of products and that all differences between types of products are based on regulatory statutes and marketing claims aimed toward consumer preference, and not toward safety. Additionally, a recent review of available scientific literature concerning the nutritional composition of meat found no significant differences when organic products were compared to conventional products (49).

Consumers believe that consuming beef products that adhere to specific production practices and, thus, qualify for specific marketing programs are of higher quality. In addition, from the descriptions of the differing production methods currently implemented to produce beef products, it is easy to perceive how consumers could be confused about differences between beef products bearing different labels. One of the main issues of concern for consumers is the use of exogenous chemicals, including growth-promoting hormones, antibiotics (both at therapeutic and sub-therapeutic concentrations), β-agonists, and NSAIDs. Consequently, in an effort to fully educate consumers about the products they choose, research has been conducted to determine amounts of certain exogenous chemical residues in meat products.

Furthermore, regulations are in place to ensure that, regardless of which meat product consumers choose, the product they purchase will not be harmful to their health. The following is a discussion of current regulations as they pertain to exogenous chemicals in meat products.

The Role of Specific US Government Agencies in Residue Monitoring

Regulating and monitoring pesticide, environmental contaminant, and veterinary drug residues is a shared responsibility between the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and USDA-FSIS *(28)*. Under the authority of the Federal Food, Drug, and Cosmetic Act, FDA establishes tolerances for veterinary drugs, food additives, and unavoidable environmental contaminants, while EPA, under the authority of the Federal Insecticide, Fungicide, and Rodenticide Act establishes tolerances for pesticides in foods *(28)*. Since 1967, USDA-FSIS has administered the National Residue Program (NRP), which functions to examine chemical residue concentrations in meat, poultry, and egg products *(28)*. Under the NRP, Scheduled Sampling Plans and USDA-FSIS Inspector Generated Sampling Plans are used to facilitate the monitoring of exogenous drug residues in meat. The goal of the Scheduled Sampling Plan is surveillance while the goal of inspector Generated Sampling is Investigation.

To accomplish the goal of detecting an agricultural chemical residue violation rate of $\geq 1.0\%$ in the animal population with a 95% probability, the Scheduled Sampling Plan operates in the following manner, "1) determine which compounds are of food safety concern; 2) use algorithms to rank the selected compounds; 3) pair these compounds with appropriate production classes; and 4) establish sample sizes" *(28)*. The Scheduled Sampling Plan serves not only to verify that public health standards are being met, but also to verify that Hazard Analysis and Critical Control Points (HACCP) plans that deem exogenous drug residues "a hazard not reasonably likely to occur" are in control.

According to the 2007 National Residue Program Data, scheduled sampling is conducted in the form of exposure assessments and exploratory assessments *(28)*. Exposure assessments are used "by FSIS, FDA, and EPA to determine the prevalence of residues in the Nation's meat, poultry, and egg products; by FSIS to condemn carcasses with violative concentrations of residue; by FDA to regulate producers when a sample contains violative concentrations of residues; by industry to retain product until the sample has been tested; and by industry to recall product that was not retained while the sample was tested, and found to contain violative concentrations of residue." Exploratory assessments are used "to reinvestigate animal populations from ongoing or previous exposure assessments if the violation rate is confirmed at one percent

or greater; to investigate animal populations when the compounds in question have no established tolerances; and to respond to intelligence reports from the field" *(28)*. The number of samples collected under Scheduled Sampling and the number of samples that exceeded the US tolerance limit for each veterinary drug included in the research presented herein for the most recent four years are shown in Table 1. Overall, the prevalence of veterinary drugs with residues exceeding US tolerances is < 1.0% for all of the drugs sampled in the current study with the exception of Flunixin, which was found at violative concentrations in almost 10.0% of samples in 2005 (Table 1).

Inspector Generated Sampling is also used as a part of the NRP to monitor meat products for violative concentrations of residues. Inspector Generated Sampling is not random but, rather, occurs when a Public Health Veterinarian suspects that an individual animal or population of animals may have violative concentrations of chemicals or contaminants *(28)*. Inspector Generated Sampling can be initiated due to animals' displaying signs of disease or abuse (fresh needle marks, etc.), producer history, or results from previous random sampling under the Scheduled Sampling Plan, and carcasses are retained until either on-site tests, such as the fast antimicrobial screening test (FAST) or the swab test on premises (STOP), or laboratory results can be obtained *(28)*.

The number of samples collected under Inspector Generated Sampling and the number of samples that exceeded the US tolerance limit for each veterinary drug included in the research presented herein for the most recent four years are shown in Table 2. Overall, the prevalence of veterinary drugs with residues exceeding US tolerances is < 1.0% for all of the drugs sampled in the current study (Table 2).

Determination of Tolerances / Maximum Residue Limits for Veterinary Drugs in Meat Products

A "tolerance" (the term used in the US) or "Maximum Residue Limit" (MRL) (the nomenclature used outside the US), for veterinary drugs is defined by the Codex Alimentarius Commission (CAC) as "the maximum concentration of residue resulting from the use of a veterinary drug that...is legally permitted or recognized as acceptable in or on a food...based on the type and amount of residue considered to be without any toxicological hazard for human health as expressed by the Acceptable Daily Intake (ADI), or on the basis of a temporary ADI that utilizes an additional safety factor" *(15)*. In general, countries or non government organizations (NGO) set a veterinary drug tolerance, or MRL, by evaluating toxicological research provided by the petitioning entity and setting an ADI which defines the level of intake of a specific compound over a lifetime below which there is considered to be no

appreciable risk to human health. An ADI is computed by dividing the no-observed-effect level (NOEL) of the drug found from toxicological studies by a safety factor which is usually 100 or 1000 (ADI = NOEL/Safety Factor). Once an ADI is set, residue data provided by the petitioning entity are analyzed using country-specific statistical models that take into account food consumption patterns, and an appropriate tolerance, or MRL, is set. However, because each country, or group of countries such as is the case in the European Union (EU), has a different process for evaluation of drugs and setting MRLs; they often vary, sometimes greatly, between countries, resulting in disruption of trade. As a result, it is important to fully understand the intricacies of each system in order to interpret each country's MRL regulations. Therefore, the following is a description of the specific parameters used by the US, the EU, and CAC to compute MRLs and a discussion of the implications of the different methods.

The United States' Methods for Determination of Veterinary Drug Residue Tolerances

In the United States, ADIs are set by expert members of a toxicological evaluation team and are calculated using the ADI formula previously given. In addition to animal toxicology data, companies must submit, where appropriate (such as in the case of antibiotics), data that elucidate the effects of the drug on human gut microflora. When both the animal toxicology and

human gut microflora have been evaluated and an ADI has been set for both, the lower ADI of the two is used as the official ADI for calculation of tolerances. It is important to note that the United States does not round ADI and, generally, uses the same number of significant figures in the ADI as was used in the toxicology trials *(26)*.

Once an ADI is set for a particular drug, the US-FDA evaluates residue data provided by the drug company, and any samples that are below the limit of quantitation (LOQ) or limit of detection (LOD) are "filled in" by assuming that the samples were produced from a lognormal distribution *(44)*. Subsequently, the dataset is checked by using a Shapiro-Francia test for lognormality. If the dataset is not lognormal, the "California rule" is applied, which sets the tolerance value three standard deviations above the sample mean (41). If the dataset passes the lognormal test, then up to three statistical tests may have to be applied, including (1) the upper 95% confidence limit on the 95^{th} percentile; (2) the 99^{th} percentile; and (3) the product of 3.9 times the upper limit of the median *(44)*. For datasets with fewer than 15 observations, the lowest value of the 3 methods is applied as the United States' residue level tolerance, whereas in datasets with $\geq$ 15 data points, the lowest value of the first 2 methods listed above is taken forward as the tolerance *(44)*.

In the United States, 100% of the ADI is accounted for by the cumulative total allocated to each item in the standard food basket (300 g muscle tissue, 100 g liver, 50 g kidney, 50 g fat, 100 g eggs, and 1.5 L of milk). When a new drug tolerance application is submitted, the applicant is allowed to denote what percentage of the ADI they would like allocated to each specific item in the food basket. Therefore, if the company has a specific use for the drug and knows that residues are likely to be present in higher concentrations in a specific tissue, based upon the recommended use, they can assign more of the ADI to that specific tissue *(26)*. By using the approach described above, the United States often has higher tolerance levels than other countries because: (1) the US does not round the ADI; (2) the US uses the same number of significant figures as was used in generating the toxicology data; (3) the US uses 100% of the ADI when creating a tolerance; and (4) the US partitions ADI throughout the standard food basket in a different manner than other countries. The main criticism of this approach and the usage of 100% of the ADI is that it does not allow for new product usages for the same drug without a lengthy and costly re-evaluation process *(26, 44)*.

The European Union's Methods for Determination of Veterinary Drug Maximum Residue Limits

In the EU, ADI are set in much the same way as in the US; however, once an ADI is set, it is rounded to 1 significant figure for the statistical calculations

involved in calculating an MRL, sometimes resulting in MRLs that are lower than US tolerances. The EU has two statistical methods for calculating MRLs of veterinary drugs. Method 1 for calculating MRLs dictates that data are to be treated as normally distributed, and the MRL is set at the upper 95% confidence interval of the 95th percentile *(44)*. In Method 2, no assumption is made about the distribution of the data; rather, the 75th percentile is computed using the Weibull procedure, which is known for consistently overestimating percentile values (thus creating an artificial worst-case scenario) (41). Subsequently, the 75th percentile value is doubled and set as the MRL *(44)*. Both EU Method 1 and Method 2 require that any values in residue datasets that are below the LOQ or LOD be substituted with a value that is ½ the LOQ, a requirement that artificially inflates the MRL values *(26, 44)*. Additionally, according to Salazar *(44)*, EU regulations do not stipulate when to use Method 1 or Method 2 or which value is set as an MRL when the results from the two methods differ. As a result of rounding of the ADI to one significant figure for statistical calculations, of poor guidance as to which method should be used for MRL calculation, of the assumption that the data are either normal or follow no distribution at all, and of substitution of values below the LOQ and LOD into residue datasets for statistical calculations, EU MRLs often differ from tolerances set by the US *(26, 44)*. In addition, the EU only uses the ADI of a particular drug as a reference value that may not be exceeded. In the EU, the cumulative total of the MRLs set for each tissue in the standard

food basket may not exceed the ADI, but will not necessarily equal 100% of it. Because the EU does not assign 100% of the ADI to the standard food basket, their values are often different than United States' tolerance limits for the same drug (Table 1) *(26)*. The CAC also sets MRLs based upon Recommendations from the Joint FAO / WHO Expert Committee on Food Additives (JECFA).

The Codex Alimentarius Commission's Methods for Determination of Veterinary Drug Maximum Residue Limits

The CAC is a third-party NGO that serves the function of creating non-binding international standards as guidance for government-affiliated bodies. In the case of setting MRLs for veterinary drugs, CAC does not independently evaluate drugs; rather, CAC relies on JECFA to conduct in-depth assessments of candidate drugs and recommend ADI and MRLs. JECFA computes ADI using the same (1 significant figure) method that the EU uses *(23)*. Subsequently, JECFA experts set MRLs based upon a statistical package that utilizes regression analysis for the estimation of upper limits of the 95% confidence interval on the 95th percentile of the population sampled *(23, 24, 44)*. Once JECFA has identified the MRL for each specific tissue in the standard food basket, they compute a theoretical maximum daily intake (TMDI) by summing the MRLs of all items in the standard food basket. JECFA will not recommend MRLs if the TMDI exceeds the ADI *(23)*. If the

TMDI exceeds the ADI, JECFA will adjust MRLs to lower concentrations and repeat the comparison. JECFA may also recommend that a MRL is "not necessary" or "unspecified" when there is a wide margin of safety between the TMDI and the ADI. Additionally, a "Temporary MRL" may be recommended by JECFA when a temporary ADI is assigned or when 100% of an ADI is accounted for in a current MRL designation but adequate residue data exists to re-evaluate the current ADI. Finally, JECFA can inform CAC that no MRLs can be established when there is no ADI set or when there are significant deficiencies in either the residue data provided or in the analytical methods *(23)*.

Because many countries defer to CAC MRLs as their sovereign MRLs, CAC is very careful to use highly conservative methods of MRL determination and, in some cases outlined above, will refrain from setting an MRL that does not meet CAC's strict standards. However, the CAC method is not above reproach. Similar to the EU method, the CAC method relies on rounding of ADI and, thus, can allow for less accurate comparison of TMDI and ADI. The CAC method of comparing TMDI to ADI has also come into question because the MRLs that are summed to compute the ADI are based upon the estimation of the upper 95% confidence interval of the 95^{th} percentile and, therefore, generate a near worst-case scenario estimates of amount of residue in a particular tissue. As a result, the JECFA has proposed to replace TMDI

with an estimated daily intake (EDI) model that uses the median value instead of the computed TMDI value *(23)*. This proposal has not yet been adopted by CAC because they do not believe that EDI is an acceptable estimator of acute toxicity hazards *(23)*. As a result, JECA is currently attempting to evaluate potential models that could effectively account for acute toxicity either separately or as a part of the EDI model.

As is evidenced from the description of US, EU, and CAC methods of MRL determination described herein, it is obvious how MRLs from different countries can differ, even drastically. When evaluating an MRL from a country it, therefore, becomes important to understand how the MRL was determined. In the cases of the US and the EU, both have a published, scientifically sound methodology of computing MRLs that, in some cases, generate MRLs that differ. Therefore, in order to harmonize trade, both nations could subscribe to the CAC MRL calculation method, which differs from both of the other methods. However, because nations have a right to maintain their sovereign governance and because the methods of both nations (and others not described herein) are sound, the US and EU are not likely to defer to CAC methods. The next section of this review of literature will discuss each family of drugs that were evaluated in the present study.

Overview of Specific Veterinary Drug Use in Cattle in the United States

The objective of the research presented herein was to determine differences in antibiotic, β-agonist, and NSAID residues in ground beef from USDA Certified Organic, natural, conventional, and market cow and bull sources. To that end, understanding specific parameters pertaining to each family of drugs is important. The following is a description of each family of veterinary drugs assessed in this study and methods of determination of those specific drugs in meat products. For each of the drugs described herein (excluding β-agonists), specific dosing and label information can be found in the *Compendium of Veterinary Products* and MRL/ tolerance concentrations can be found in Table 3.

Aminoglycosides

Aminoglycosides are bactericidal protein synthesis inhibitors that are effective against gram-negative aerobes *(6)*. Aminoglycosides consist of an aminocyclitol nucleus linked to at least 2 sugar groups, are highly polar and highly hydrophilic, and their mechanism of action is protein synthesis inhibition by one or more of the following processes: (1) RNA codon misreading by binding of the 30S ribosomal unit; (2) blocking ribosome / mRNA initiation process; (3) stabilizing tRNA-binding to the ribosome, preventing translocation; and (4) cell surface labializing *(6)*. Aminoglycosides have been used in the beef industry to treat bacterial infections by *Escherichia coli, Klebsiella, Proteus, Pseudomonas, Salmonella,*

Enterobacter, Shigella, Mannheimia hemolytica, Pasteurella, Haemophilus, Staphylococcus, and some *Streptococcus (6).* Because significant renal toxicity can be caused by consuming Aminoglycosides in excess of safe amounts, there is a zero tolerance for Amikacin and Gentamicin residues in meat products. The American Association of Bovine Practitioners "...being cognizant of food safety issues and concerns, encourages its members to refrain from the intramuscular, subcutaneous or intravenous extra-label use of aminoglycoside class antibiotics in bovines" *(6).* However, Aminoglycosides are sometimes administered in an extra-label manner under the care of a veterinarian. When such action is taken, significant withdrawal time (in excess of 240 days) is required, and due to change of ownership and poor record-keeping, some animals may accidentally enter the food chain *(6).* Further information on the Aminoglycoside-class antibiotics can be found in the *Compendium of Veterinary Products (11).*

In the present study, ultra-performance liquid chromatography triple-quadropole tandem mass spectrometry (UPLC-MS) methods were developed for the determination of the Aminoglycosides Gentamicin, Amikacin, and Neomycin in ground beef. Aminoglycoside antibiotics are poorly suited for determination by gas chromatography mass spectroscopy (GC-MS) because they are basic, hydrophilic, and thermally labile, and, therefore, derivitization is very difficult *(35).* However, due to these physiochemical properties,

research has employed the use of UPLC-MS and liquid chromatography mass spectrometry (LC-MS) methodologies to determine Aminoglycoside residues in meat. Babin and Fortier *(9)* developed a multi-drug residue analysis method that employed a reverse-phase column cleanup step, followed by a C18 extraction to separate analytes. The method developed by Babin and Fortier *(9)* was able to unambiguously identify residues of Dihydrostreptomycin, Gentamicin C1, and Neomycin in veal liver, kidney, and muscle tissue with a LOD range of 0.1 to 0.7 µg/kg.

Zhu et al. *(60)* developed a LC-MS method for the determination of 13 Aminoglycosides, including Gentamicin, Amikacin, and Neomycin. Samples of pork tissues (5.0 g) were mixed with 10.0 ml of trichloracetic acid, homogenized, and centrifuged at 1000 g for 5 min. This extraction procedure was repeated, the supernatants were combined, and 5.0 ml of 0.2 M heptafluorobutyric acid (HFBA) was added. Samples were subsequently centrifuged and subjected to solid phase extraction (SPE) on C18 cartridges. Samples were eluted with 6.0 ml acetonitrile:HFBA (80:20) and evaporated to a volume of 0.3 ml under a stream of nitrogen. Samples were reconstituted to 1.0 ml in 20 mM HFBA, filtered, and injected onto LC-MS for analysis *(60)*. The authors reported LOD of 558.2 µg/kg, 18.2 µg/kg, and 56.9 µg/kg for Neomycin, Amikacin, and Gentamicin, respectively *(60)*.

Kaufmann and Maden *(34)* developed a highly sensitive method for the determination of 11 Aminoglycoside antibiotics in muscle, liver, and kidney tissue that implemented trichloracetic acid extraction followed by SPE with a weak cation exchanger. The method developed by Kaufmann and Maden (31) was able to quantitatively detect all 11 of the Aminoglycoside antibiotics in all tissues in a range of 15.0 to 40.0 µg/kg, depending on the analyte and sample matrix *(34)*.

β-Lactams

The β-lactam antibiotics, which include the widely used penicillin antibiotics, are water soluble organic acids that, in their most basic form, contain a β-lactam ring, a thiazolidone ring, and an acyl side chain *(6)*. Penicillin antibiotics inhibit bacterial cell wall synthesis by preventing cross-linking of the peptidoglycan chain and are, thus, most effective against bacteria that are in the log phase of growth *(6)*. The penicillin group of antibiotics are effective against *Escherichia coli*, *Pasteurella, Staphylococcus*, *Actinomyces pyogenes, Listeria monocytogenes, Erysiplelothrix rhusiopathiae, Bacillus anthracis,* and *Streptococcus (6)*. Additionally, Ampicillin has been shown to be effective against all of the previously listed bacteria and *Campylobacter, Haemophilus, Moraxella, Salmonella,* and *Mannheimia hemolytica (6).* Desfuroylceftiofur is effective against *Streptococcus, Staphylococcus,* and gram negative bacteria *(6)*. Withdrawal times for β-lactams vary by dosage

and administration route and by specific drug, but times generally range between 10 and 20 days prior to slaughter *(11)*.

In the present study, UPLC-MS methods were developed for the β-lactam antibiotics Penicillin, Ampicillin, and Desfuroylceftiofur in ground beef. A LC-MS method for the determination of Desfuroylceftiofur residues in bovine and swine milk, muscle, and kidney was developed and tested across five laboratories for verification *(32)*. In the Hornish et al. *(32)* method, residues were extracted from samples with 0.4% dithioerythritol acid in 0.05 M, pH 9, borate buffer and were then subjected to SPE on a C18 column, followed by a strong anion exchange column and, subsequently, a strong cation exchange column. After sample cleanup via SPE, samples were subjected to LC-MS, and a LOD of 15.0 µg/kg was reported *(32)*.

Mastovska and Lightfield *(39)* reported the development and simplification of a method for the determination of 11 β-lactams in bovine kidney. Samples (1 g) were homogenized, extracted in 10.0 ml acetonitrile:water (80:20), centrifuged, subjected to SPE on a C18 cartridge, evaporated under nitrogen, reconstituted in water, and injected onto LC-MS for analysis. The authors did not report LOD or LOQ information *(39)*.

Goto et al. *(30)* developed a LC-MS method for the determination of Penicillin and Ampicillin (and other drugs) in bovine muscle, kidney, and liver. Goto et al. *(30)* homogenized 5 g of tissue in water and then pulled a 350 µl aliquot, which was subsequently centrifuged at 13,000 rpm for 30 min in a filtration centrifuge tube. Then, 50 µl aliquots were injected onto an LC-MS machine equipped with an electrospray ionization probe. The LOD reported by Goto et al. *(30)* was 2.0 µg/kg for all drugs.

Fagerquist et al. *(22)* developed an LC-MS method for determination of 10 β-lactam antibiotics in bovine kidney that included Penicillin, Ampicillin, and Desfuroylceftiofur. Fagerquist et al. *(22)* extracted kidney samples with acetonitrile and water (80:20) and centrifuged them at 4500 rpm for 5 min. Subsequently, the supernatant was decanted into a separate tube that contained C18 sorbent and inverted several times, allowing the sorbent material to thoroughly mix with the supernatant. Samples were then centrifuged, and the supernatant was evaporated under nitrogen until it reached a final volume of 1.5 ml, which was analyzed by LC-MS *(22)*. The dispersive SPE method of sample cleanup employed by Fagerquist et al. *(22)* drastically cuts down on sample preparation time and can allow for a larger number of samples to be run during each preparation. Fagerquist et al. *(22)* reported a limit of confirmation (LOC) of 10 µg/kg.

Fluoroquinolones

Fluoroquinolones are a highly similar group of antibiotics that share extensive activity against gram negative and gram positive aerobes but almost no activity against anaerobic bacteria *(6)*. Fluoroquinolones are bactericidal within 20 to 30 min of administration because they are topoisomerase inhibitors that (1) bind DNA gyrase and block movement of the replication fork; and (2) bind topoisomerase IV leading to DNA strand breaks *(6)*. Fluoroquinolones are used in the beef industry exclusively to treat Bovine Respiratory Disease, and extra-label use is strictly prohibited due to potential human health concerns *(6)*.

Carretero et al. *(16)* developed a LC-MS method able to detect 31 antimicrobials, including the Fluoroquinolones Enrofloxacin, Ciprofloxacin, Danofloxacin, Marbofloxacin, Ofloxacin, and Norfloxacin. Samples (1 g) were homogenized and extracted by pressurized liquid extraction (PLE), a process during which: (a) mixed with 11 g of ethylene diamine triacetic acid (EDTA)-washed sand and packed into the PLE machine; (b) heated for 5 min; (c) exposed to a solvent (not specified) for 10 min; (d) exposed to 1500 psi pressure; (e) exposed to nitrogen for 60 seconds; (f) flushed with water. The final elution volume of 40 ml was evaporated to 10 ml using a rotary evaporator, and 20 µl of sample was injected onto LC-MS for analysis. For

Ciprofloxacin and Danofloxacin (the analytes of interest in the research presented herein), the LOD was 3.0 µg/kg and the LOQ was 10.0 µg/kg *(16)*.

Yue et al. *(59)* developed a LC-MS method for the determination of 16 Quinolone and Fluoroquinolone residues in animal tissues, including Ciprofloxacin and Danofloxacin. Samples were extracted with acidified acetonitrile, defatted with hexane, concentrated in a rotary evaporator, and injected onto a LC-MS for analysis. The authors reported that the method was able to detect all 16 analytes at 10.0 µg/kg *(59)*.

Christodoulou et al. *(17)* developed a LC-MS method for the determination of ten Fluoroquinolone residues, including Ciprofloxacin and Danofloxacin in bovine liver. Samples (1.0 g) were extracted with 4.0 ml 0.1% trifluoroacetic acid (TFA), sonicated, and centrifuged. This extraction was repeated, and the supernatants were pooled and evaporated under a stream of nitrogen and reconstituted in 4.0 ml TFA. Samples were then subjected to SPE and were eluted with 1.5 ml TFA in acetonitrile. Samples were subsequently evaporated under a stream of nitrogen, reconstituted in TFA, and injected onto LC-MS for analysis *(17)*. The authors reported LOD for Ciprofloxacin and Danofloxacin of 3.0 µg/kg, and 5.0 µg/kg, respectively *(17)*.

Macrolides

In the present study, the presence of the Macrolide antibiotics Erythromycin, Tylosin, and Tilmicosin in ground beef was studied. The Macrolide group of antibiotics is composed of the 14-member ring class, which includes Erythromycin, the 15-member ring class, which includes Tulathromycin (not included in the research presented herein), and the 16-member ring class, which includes Tylosin and Tilmicosin *(6)*. The differences in structure among the Macrolides augment the treatment efficacy, administration route, and resistance profiles of each individual drug *(6)*. Most notably, Erythromycin can be administered orally, intravenously, or parenterally; Tilmicosin can only be administered subcutaneously; Tylosin can only be administered intramuscularly *(6, 11)*. The macrolide group of antibiotics uniformly inhibits protein synthesis by binding the 50S ribosomal subunit; however, the specific inhibition depends on the ring structure of the compound. Members of the 14-ring class, such as Erythromycin, interfere with translocation of peptidyl-tRNA; members of the 15-ring class interfere with the formation of the 50S ribosomal subunit; Tylosin, Tilmicosin, and other members of the 16-ring class inhibit the peptidyl transferase reaction *(6)*. Macrolide antibiotics are most commonly used to treat Bovine Respiratory Disease, but they are also effective against *Bacillus, Arcanobacterium, Erysiplelothrix rhusiopathiae, Listeria, Staphylococcus, Streptococcus, Rhodoccus equi, Enterococcus, Bordetella, Actinomyces, Clostridium,*

Bacteriodes, Fusobacterium, Actinobacillus, Brucella, Campylobacter, Haemophilus, Ehrlichia, Pasteurella, and *Mannheimia (6).*

Several methods of determination for macrolide antibiotics exist. Draisci et al. *(20)* developed a LC-MS method for the determination of Tilmicosin, Tylosin, and Erythromycin in beef samples. Samples (2 g) were extracted with 2 ml buffer and 10 ml chloroform and centrifuged for 10 min at 4000 g. The chloroform layer was removed from the resulting 3-layer solution and filtered through glass wool to remove any particulate matter. Subsequently, samples were subjected to SPE on a diol column, and the Macrolide antibiotics were eluted with 3 applications of 200 µl of 0.1 M ammonium acetate-methanol (50:50). Then, 1 ml of the resulting solution was injected onto LC-MS *(20)*. The LOQ reported by Draisci et al. *(20)* was 30, 20, and 50 µg/kg, for Tylosin, Tilmicosin, and Erythromycin, respectively.

Po-on Tang et al. *(43)* developed a LC-MS method for the determination of the Macrolides Tylosin, Erythromycin, the Fluoroquinolones Ciprofloxacin Danofloxacin, and the Streptogramin Virginiamycin (discussed below). Samples (1 g) were mixed with 5.0 ml acetonitrile and centrifuged, and 0.5 ml of acetonitrile in 0.1 M TRIS buffer was added. Subsequently, samples were evaporated under a stream of nitrogen and brought back up to a volume of 1.0 ml with the previously mentioned buffer *(43)*. After samples were

reconstituted, they were defatted by adding 1 ml n-hexane and centrifuged. Thereafter, the bottom layer was decanted and filtered through a 0.45 µm syringe filter. Samples were then subjected to SPE on Oasis HLB™ columns and injected directly onto the LC-MS for determination. Po-on Tang et al. *(43)* reported a LOD of 0.1 µg/kg for both Erythromycin and Tylosin.

Dubois et al. *(21)* developed a LC-MS method for the determination of Tylosin, Tilmicosin, and Erythromycin. Samples (5 g) were extracted with 25 ml of pH 10.5 TRIS buffer and subsequently centrifuged at 3000 g for 10 min. Thereafter, the supernatant was decanted and extracted a second time with 25 ml of TRIS buffer and centrifuged again. The supernatant was filtered through glass wool and subjected to SPE on Oasis HLB™ columns, where the samples were eluted with 95:5 methanol:30% ammonia. Samples were subsequently dried under nitrogen and reconstituted in 500 µl of 80:20 NH_4:acetonitrile and injected onto LC-MS for determination *(21)*. The authors reported the ability to detect all of the studied compounds over the entire range of half of the EU MRLs to twice EU MRLs, which is the requirement for a method to be accepted for use in testing of samples by EU standards *(21)*.

Phenicols

The Phenicol group, including Florfenicol, which was studied in the research presented herein, is a lipid-soluble group of antibiotics that has a broad spectrum of efficacy in cattle. Florfenicol, which is approved for intramuscular and subcutaneous administration in cattle, is bacteriostatic, and works by inhibiting protein synthesis at the 50S ribosomal subunit *(6).* Florpfenicol is an effective bacteriostatic agent against *Pasteurella hemolytica, Pasteurella multicida, Haemophilus sommus, Haemophilus influenzae, Escherichia coli, Moraxella, Klebsiella, Enterococcus, Shigella, Serpulina hyodysenteriae, Bacteroides, Fusobacterium, Bordetella bronchoseptica, Proteus, Neisseria, Cornyebacterium pyogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis, Streptococcus zooepidemicus, Staphylococcus aureus, Staphylococcus epidermidis,* and *Clostridium (6).*

There is limited implementation of LC-MS methodologies thus far for the determination of Florfenicol in bovine muscle tissues. However, Yamada et al. *(57)* developed a LC-MS method for the simultaneous determination of 130 veterinary drugs, including Florfenicol. Samples (5 g) were extracted with 95:5 acetonitrile:methanol, defatted with n-hexane, evaporated, and reconstituted in methanol prior to analysis by LC-MS. The authors were able to detect Florfenicol at 2.0 µg/kg and reported a LOQ of 8.0 µg/kg *(57).*

Sulfonamides

Sulfonamides are broad-spectrum bacteriostatic antibiotics that compete with Para Amino Benzoic Acid for incorporation into the scheme for folic acid synthesis, leading to decreased RNA, and, thus decreased protein synthesis *(6)*. Sulfonamide drugs are commonly paired with Diaminopyrimidines or Trimethoprim, resulting in synergistic effects and, commonly, bactericidal effects *(6)*. The general spectrum of bacteria susceptible to Sulfonamides includes *Bacillus, Erysipelothrix rhusiopathiae, Listeria monocytogenes, Streptococcus, Nocardia, Staphylococcus, Enterococccus, Brucella, Enterobacteriaceae, Actinobacillus, Haemophilus, Pasteurella, Clostridium perfringens, Bacteroides,* and *Fusobacterium (6)*. Sulfadimethoxine can be administered intravenously, via an oral bolus, and through drinking water in cattle; and Sulfamethazine can be administered as a granular feed additive or as an oral bolus*(6)*.

Van Eeckhout et al. *(55)* developed a LC-MS method for the determination of eight Sulfonamides, including Sulfamethazine and Sulfadimethoxine, in bovine kidney. Samples (5 g) were homogenized, mixed with 10 ml methane, placed in an ultrasonic bath for 10 min and, subsequently, centrifuged at 3500 g for 10 min. The supernatant was decanted, and the pellet was re-extracted following the same procedure. The supernatants were then combined and centrifuged at 3500 g for 10 min, and 100 µl was injected onto LC-MS for

analysis *(55)*. The authors reported detection of all eight Sulfonamide drugs at 50.0 µg/kg *(55)*.

Yamada et al. *(57)*, using the same method described above for Phenicol analysis, reported LOD for Sulfadimethoxine of 0.2 µg/kg, and LOQ of 0.6 µg/kg.

Tetracyclines

The Tetracycline group of antibiotic drugs is one of the oldest classes of drugs still in use today. In the present study, LC-MS methods were developed for the determination of Chlortetracycline, Tetracycline, and Oxytetracycline, which were discovered in 1948, 1953, and 1950, respectively *(6)*. The Tetracycline antibiotics are bacteriostatic and work by binding to the 30S ribosomal subunit, thus preventing access of tRNA to the ribosome-mRNA complex, consequently preventing addition of amino acids to a peptide chain *(6)*. Tetracyclines have a broad spectrum of efficacy; however, due to prolonged use over the past 60 years, many bacteria are resistant. The organisms that are expected to be susceptible to treatment with Tetracyclines are *Bacillus, Arcanobacterium pyogenes, Erysipelothrix rhusiopathiae, Listeria monocytogenes, Actinobacillus, Bordetella, Brucella, Francisella tularensis, Heamophilus, Pasteurella multicida, Mannheimia haemolytica, Yersinia, Campylobacter fetus, Rickettsia, Chlamydia, Actinomyces,* and

Fusobacterium (6). In addition, the Tetracycline antibiotics are reported to have variable effects on *Staphylococcus, Streptococcus, Enterococcus, Enterobacter, Escherichia coli, Klebsiella, Proteus, Salmonella, Clostridium, Bacteroides,* and *Dichelobacter nodosus (6). Pseudomonas aeruginosa, Serratia,* and *Mycoplasma* are reported to be completely resistant to Tetracycline antibiotics *(6).* Administration of Tetracyclines varies by the specific drug being applied and its intended uses. Tetracycline can be administered via an oral bolus in calves or in water through soluble powders for cattle *(6).* Chlortetracycline can be administered in water through a soluble powder or as a feed additive in a granular form *(6).* Oxytetracycline can be administered in an oral bolus in calves, in an injectable solution, as a feed additive, and through water via a soluble powder *(6).*

Oka et al. *(42)* developed a LC-MS method for the determination of four Tetracycline antibiotics, including Chlortetracycline, Tetracycline, and Oxytetracycline. Samples (5 g) were extracted with 50 ml 0.1 M EDTA-McIlvaine buffer (pH 4.0) and centrifuged at 850 g for 5 min. Subsequently, the samples were filtered and subjected to SPE on C18 cartridges, where they were eluted from columns with 10 ml ethyl-acetate followed by 20 ml of 5:95 methanol:ethyl-acetate. Samples were then dried under a stream of nitrogen and reconstituted in 0.1 ml water, and 50 µl was injected onto a LC-MS for analysis *(42).* The authors reported that their method could reliably detect 100

µg/kg of Chlortetracycline, Tetracycline, and Oxytetracycline in bovine muscle tissue *(42)*.

Blanchflower et al. *(13)* developed a LC-MS method for the determination of Chlortetracycline, Tetracycline, and Oxytetracycline in bovine muscle and kidney tissues. Frozen tissue samples (5 g) were extracted with glycine-HCL buffer (45 ml, 0.1 M glycine in 1.0 M HCL) and homogenized for 1 min. Subsequently, 5 g ammonium sulfate was added, shaken into the sample solution for 30 seconds, and allowed to stand for 10 min. Then the samples were centrifuged at 2000 g for 10 min, and the supernatant was filtered through a plug of glass wool *(13)*. The entire extraction procedure was performed a second time, and the supernatants were combined and subjected to SPE on an isolute cyclohexyl column. Samples were eluted in 7 ml of methanol, evaporated under nitrogen, and reconstituted in 0.5 ml of 20 mM oxalic acid:acetonitrile (80:20). Reconstituted extracts were centrifuged for 10 min at 5000 g, and 250 µl of each was injected onto LC-MS for analysis *(13)*. The authors reported results indicating that their method could accurately detect Chlortetracycline, Tetracycline, and Oxytetracycline in a range of 50 µg/kg to 200 µg/kg *(13)*.

Goto et al. *(30)*, using the method previously described to extract β-lactam antibiotics, reported a LOD of 2.0 µg/kg for Chlortetracycline, Tetracycline, and Oxytetracycline in muscle tissue.

Streptogramins

Streptogramin drugs, including Virginiamycin, which was studied in the research presented herein, are a mixture of a Macrolactone and a cyclic hexadepsipeptide *(6)*. Streptogramins are considered bactericidal due to the synergy of the Macrolactones, which block the substrate site of the peptidyl transferase center on the 50S ribosomal subunit and increase the affinity of the ribosome for cyclic hexadepsipeptides, thus inhibiting protein synthesis *(6)*. Virginiamycin is effective against gram-positive and anaerobic bacteria and is most effective against *Leptospira, Haemophilus,* and *Serpulina hyodysenteriae (6)*. Virginiamycin is administered to cattle as a feed additive to optimize efficiency by promoting good health, increase rate of weight gain, and reduce the incidence of liver abscesses *(11)*.

There is a very limited amount of literature available describing LC-MS determination of Virginiamycin or Streptogramins in muscle tissue. Yamada et al. *(57)*, using previously described methods for Phenicol extraction, established a LOD of 2.0 µg/kg and a LOQ of 8.0 µg/kg for Virginiamycin. Lauridsen et al. *(37)* developed a GC-MS method for the determination of

Virginiamycin in swine muscle tissue in concentrations as low as 1.0 µg/kg. The authors fed pigs Virginiamycin for 45 days and found no antibiotic residues in muscle samples collected immediately after slaughter, even in samples from pigs that were given no withdrawal time from the drug *(37).*

Non-Steroidal Anti-Inflammatory Drugs

In the present study, residues of the NSAIDs Phenylbutazone and Flunixin in ground beef were studied. In general, NSAIDs are used as anti-inflammatory, anticoagulant, analgesic agents to control pyrexia associated with Bovine Respiratory Disease or Mastitis, to control inflammation from Endotoxemia associated with toxic Metritis, Peritonitis, Endocarditis, or acute *Salmonellosis*, and as a long-term therapy for the management of osteoarthritis *(18, 49).* The entire group of NSAID drugs function by blocking the production of prostaglandin via binding and obstructing the action of cyclooxegenase, and the properties of NSAID action (therapeutic and anti-inflammatory) are directly proportional to the amount and type of prostaglandin that is impeded *(18).*

Flunixin is the only NSAID labeled for use in beef and dairy cattle in the US, and it is only approved for intravenous administration *(49).* Due to ease of administration, subcutaneous extra-label administration of Flunixin has been reported and has been banned by the Animal Medicinal Drug Use

Clarification Act *(49)*. The Food Animal Residue Avoidance Databank (FARAD) recommends a single dose withdrawal period of 30 days and a multiple dose withdrawal period of 60 days for cattle given intramuscular injections of Flunixin *(49)*.

Phenylbutazone is a commonly prescribed NSAID that is popular because of its long half-life (which allows for only once daily administration) *(49)*. Because Phenylbutazone has been found to cause Aplastic Anemia, Leukopenia, Agranulocytosis, and Thrombocytopenia in humans, there is a zero tolerance for residues of the drug in any product *(6)*. In 2003, the Food and Drug Administration-Center for Veterinary Medicine (FDA-CVM) banned the use of Phenylbutazone in female dairy cattle over 20 months of age due to an increasing rate of violative residues in meat samples collected from dairy cattle *(49)*. The withdrawal time recommended by FARAD for animals administered Phenylbutazone intramuscularly is 55 days *(49)*.

Yamada et al. *(57)*, using previously described methods for extraction of Phenicols, reported a LOD for Flunixin in animal tissues of 0.3 µg/kg and a LOQ of 1.0 µg/kg.

Asea et al. *(7)*, developed a LC-MS method for the determination of Phenylbutazone in animal tissues. Samples (2 g) were extracted using an

unidentified solution and centrifuged for 5 min at 2850 g, after which the supernatant was decanted and the extraction was repeated twice. Subsequently, the pooled supernatants were centrifuged at 2850 g for 10 min and evaporated to near-dryness under a stream of nitrogen *(7)*. The samples were reconstituted in an unidentified solution and subjected to SPE on a florisil SPE cartridge, where they were eluted in 12 ml of an unidentified solution. Samples were then evaporated to dryness and reconstituted in 400 µl mobile phase, and 50 µl was injected onto a LC-MS for analysis. The authors reported a LOD of 3.0 µg/kg and a LOQ of 10.0 µg/kg for Phenylbutazone in animal tissues *(7)*.

Igualada et al. *(33)*, developed a LC-MS method for the determination of four NSAID drugs including Flunixin. Samples (1 g) were homogenized, and 5.0 ml of 0.25 M HCL was added and vortexed into the sample mixture for 16 hr. Subsequently, 500 µl of 0.3 M sodium phosphate tri-basic 12-hydrate solution was added, and the mixture was brought to a pH of 7.0 by adding 2.0 N sodium hydroxide. Samples were then extracted three times with 4 ml ethyl-acetate, and the combined supernatants were evaporated to dryness under a stream of nitrogen. Samples were reconstituted in 250 µl of 50:50 formic acid:methanol, and 20 µl was injected onto LC-MS for analysis. The authors reported a LOD of 25.0 µg/kg and a LOQ of 31.0 µg/kg for Flunixin in bovine muscle *(33)*.

β-Agonists

β-adrenergic receptor agonists (β-agonists) are fed to cattle in the United States in order to increase protein accretion and decrease fat deposition *(8, 48, 53)*. For this reason, they are often called repartitioning agents. The use of β-agonists in cattle feed is prohibited in the EU by Council Directive 96/22/EC; however, Ractopamine and Zilpaterol were approved for use as growth promoting substances for cattle in the United States in 2003 and 2006, respectively *(8)*. In the research presented herein, methods were developed for the determination of the β-Agonists Zilpaterol and Ractopamine in ground bovine muscle tissue.

While the specific mechanism of action of β-agonists is not completely understood, it has been postulated that the effects could be (a) an increase in protein synthesis; (b) a decrease in muscle degradation; or (c) a combination of both *(8)*. In addition, β-agonists decrease fat deposition *(8, 25)*. The combined effect of increased protein accretion and decreased fat deposition results in carcasses that are heavier muscled and leaner *(8)*. Due to the controversy surrounding the use of β-agonists and differing regulations in countries throughout the world, there is significant interest in developing sensitive methods, including LC-MS methods, for their determination in animal tissues, urine, feces, and plasma.

Antignac et al. *(5)* developed a LC-MS method for the determination of Ractopamine in pork liver, kidney, retina, muscle, lung, and urine. Samples (15 g) were freeze-dried and ground, and 15 ml acetate buffer was added, stirred, and centrifuged (2000 g for 15 min). Subsequently, the supernatant was collected, the methanol was evaporated, 400 µl of *Helix Pomatia* buffer was added, and hydrolysis of conjugated metabolites was carried out for 15 hr at 60°C *(5)*. Thereafter, the supernatant was subjected to SPE on ChromP© and Screen DAU columns and was finally eluted in 6 ml of ethyl-acetate:32% ammonium hydroxide (97:3). Once the eluted samples were evaporated to dryness, they were reconstituted in 50 µl of acetic acid in water:methanol (95:5) and injected onto LC-MS for analysis. The authors reported a LOD of 0.028 µg/kg *(5)*.

Munoz et al. *(41)* developed a LC-MS method for the determination of β-agonists (including Ractopamine and Zilpaterol) and Penicillin drugs in meat tissues. The authors did not report extraction or cleanup procedures but reported extensive chromatographic parameters. The authors did not report LOD or LOQ for their assay but did state that it met the requirements for assay validation for use in the EU (0.5 – 2.0 X MRL) *(41)*.

Shelver and Smith *(47)* developed a LC-MS assay for the determination of Zilpaterol in sheep urine, liver, kidney, and muscle tissue. Samples (5 g) were added to 10 ml of pH 9 borate buffer, homogenized, and centrifuged at 10,000 g for 10 min. Thereafter, the extraction process was repeated two times, and the supernatants were pooled, filtered, and subjected to SPE, where they were eluted with methylene chloride:isopropyl alcohol:30% aqueous ammonium hydroxide (ratio not given), dried under a vacuum stream of nitrogen, and reconstituted in 1 ml methanol:10 mM ammonium acetate. The authors did not report a LOD or LOQ but did report Zilpaterol concentrations in muscle tissues in ng/g concentrations *(47)*.

Kootstra et al. *(36)* developed an LC-MS method for the determination of nine β-agonists, including Ractopamine, in bovine muscle tissue. Samples (5 g) were added to 5 ml TRIS buffer (pH 9.5) and 5 mg of protease and digested overnight at 60°C. Subsequently, the samples were hydrolyzed in *Helix Pomatia* buffer for 2 hr, and 10 mM sodium hydroxide was added drop-wise until the pH of the solution was > 12. Next, the solution was extracted with 15 ml ethyl-acetate, evaporated under a stream of nitrogen, reconstituted in 4 ml of methanol:water (80:20), and defatted twice with heptanes. Sample cleanup was performed on molecular imprinted polymer columns, and 100 µl was then injected onto a LC-MS for analysis *(36)*. The authors reported a LOD of 0.87 µg/kg *(36)*.

Conclusion

The beef industry is highly segmented, and, as a result, each individual segment uses essentially every possible means to increase the value of their product. Producers take advantage of marketing programs such as USDA Certified Organic to increase the value of their animals when they are sold. Feedlots can capitalize on the same marketing programs, but many routinely feed growth-promoting substances as a means to produce a high volume of product at a low input cost. Packers and retailers also capitalize on marketing programs and, in addition, create and advertise specific brands of beef that reflect specific traits that consumers purchase based upon their perception of advantages of what is being marketed. The result of this segmented marketing approach is that consumers are typically uninformed regarding the beef products they are buying because they do not fully understand the exact meaning of the marketing claims. In addition, consumers generally do not understand the use of antibiotics in animal husbandry, most especially as a regular feed ingredient used to promote weight gain, nor do they understand the rationale of extra-label administration of veterinary drugs. Instead, what consumers perceive is that beef products from animals never fed antibiotics or growth promotants are healthier for consumption, and, as a result, they may be willing to pay more for those products. Therefore, in an effort to more accurately describe to consumers the differences in agricultural chemical

residues in ground beef from different sources, the project presented herein was conducted.

Previous research has also aimed to elucidate differences in the presence of veterinary drugs and pesticides in beef products from specific production categories. *(51)* analyzed 20 samples each of muscle, adipose tissue, kidney, and liver for residues of anabolic steroids, environmental contaminants, Clenbuterol (a β-agonist), two tranquilizers, six Sulfonamides, including Sulfamethazine and Sulfadimethoxine from steers, heifers, and cows at eight packing plants from organic, natural, conventional, cull cow, and chronically ill sources. The authors reported a LOD of 0.025 μg/g for the Sulfonamides and found no detectable residues in any of the tissues assayed *(51)*. Usborne *(54)* compared natural and conventional beef purchased in supermarkets in Canada and reported no violative residues of Sulfonamides, antibiotics, heavy metals, growth promotants, parasiticides, polychlorinated biphenyls, pentachlorophenol, or pesticides.

Smith et al., *(50)* analyzed 64 muscle, 60 adipose, 36 liver, and 26 kidney samples from organic, natural, and conventional beef sources for the presence of anabolic steroids, xenobiotics, Penicillin, Tylosin, Erythromycin, Sulfonamides, including Sulfamethazine and Sulfadimethoxine, Tetracycline, Oxytetracycline, Chlortetracycline, and pesticides. The authors reported that

no detectable residues of any veterinary drugs were found in any production category, while pesticide residues were found in all production categories.

In order to revisit the previous research done on this subject, the current research was conducted with the objective of determining differences in antibiotic, β-agonist, and non-steroidal anti-inflammatory drug residues in ground beef from USDA Certified Organic, USDA Process Verified Never Ever 3, conventional, and market cow and bull sources.

CHAPTER III

Determination of Antibiotic, β-Agonist, and Non-Steroidal Anti-Inflammatory Drug Residues in Ground Beef from USDA Certified Organic, Natural, Conventional, and Market Cow and Bull Sources

ABSTRACT

In recent years, consumer demand for organic and "natural" products has increased, partly due to a perception that such products are healthier and contain fewer additives, including veterinary drugs and growth promotants. The study presented herein compared occurrence of veterinary drug residues in ground beef samples reflecting different livestock production classifications. We collected ground beef samples (N = 400) consisting of 90.0 ± 4.0 % lean muscle tissue from a total of eight plants, two each reflecting production in the following categories: (1) USDA Certified Organic (n=100); (2) USDA Process Verified Never Ever 3 (n=100); (3) conventionally raised fed beef (n=100); and (4) ground beef derived from carcasses of market cows and bulls (n=100). Liquid chromatography coupled with triple-quadrupole mass spectrometry (UPLC-MS) methods were developed for the following veterinary drugs: (1) Aminoglycosides (Gentamicin, Amikacin, and Neomycin); (2) β-lactams (Penicillin, Ampicillin, and Desfuroylceftiofur); (3) Fluoroquinolones (Danofloxacin and

Ciprofloxacin); (4) Macrolides (Erythromycin, Tylosin, and Tilmicosin); (5) Phenicols (Florfenicol); (6) Sulfonamides (Sulfamethazine and Sulfadimethoxine,); (7) Tetracyclines (Oxytetracycline, Chlortetracycline, and Tetracycline); (8) Streptogramins (Virginiamycin); (9) β-agonists (Ractopamine and Zilpaterol); and (10) non-steroidal anti-inflammatory drugs (Flunixin and Phenylbutazone). Residues exceeding their respective US tolerance limit were found in six samples. Two USDA Certified Organic samples contained Ampicillin residues exceeding US tolerance limits. One USDA Process Verified Never Ever 3 sample contained a residue of Ractopamine exceeding US tolerance limits. One sample from the market cow and bull category contained a residue of Sulfadimethoxine that exceeded US tolerance limits, one contained a residue of Ampicillin that exceeded US tolerance limits, and one contained a residue of Phenylbutazone that exceeded US tolerance limits. Residues of Phenylbutazone exceeding US tolerance limits were also found in one sample from the conventional production category. Additionally, residues (below the US tolerance limit) of several classes of veterinary drugs were found in samples from USDA Certified Organic and USDA Process Verified Never Ever 3 production categories, a finding that clearly demonstrates violation of zero-tolerance statutes set forth by the National Organic Program and USDA Process Verified Never Ever 3 marketing descriptors. In the USDA Certified Organic production category, residues were detected in eight Ampicillin, seven Penicillin, three

Sulfamethazine, one Sulfadimethoxine, and one Ractopamine sample. In the USDA Process Verified Never Ever 3 production category, residues were detected in one Ampicillin, one Chlortetracycline, two Tetracycline, and six Ractopamine samples. These violations exceed the historical prevalence of veterinary drug residues reported by the National Residue Program and demonstrate the need for careful monitoring of animals administered veterinary drugs in order to prevent improper inclusion of unqualified animals in premium marketing programs, such as USDA Certified Organic and USDA Process Verified Never Ever 3 programs.

INTRODUCTION

In recent years, consumers have become more conscientious about the food products they choose to purchase, and, as a result, the market for organic and natural beef products has increased dramatically. Despite research that indicates no difference in the nutritional composition of organic meat products when compared to conventional meat products *(19)*, some consumers still prefer organic products over conventional products due to perceived health and/or safety benefits *(58)*. Of specific issue in meat products are residues of veterinary drugs used during production to treat animal disease or to promote weight gain.

The use of veterinary drugs in animal production is closely regulated. The use of veterinary drugs is prohibited in cattle that are part of a USDA Certified Organic program. In addition, USDA has published the USDA Process Verified Never Ever 3 and Naturally Raised standards that allow meat product companies to make labeling claims that stipulate that no veterinary drugs, growth promotants, or mammalian or avian feed were used during the production of their products *(2, 3)*. The result of having many beef marketing programs that make numerous production-practice claims is that consumers are often not fully informed about the residue status of the products they are buying. This issue is further exacerbated in international beef marketing because foreign countries do not agree on policies concerning veterinary drug use and allowable residue tolerances of those veterinary drugs in meat products (Table 3). Each country establishes veterinary drug tolerances (as they are called in the US) or maximum residue limits (MRL) (as they are called outside the US) based upon their own methods and requirements. Research is needed to compare the prevalence of veterinary drug residues across beef products generated by use of differing production strategies so that consumers, regulators, foreign governments, and third-party nongovernment organizations, such as the Codex Alimentarius Commission, can make informed decisions concerning organic, natural, and conventional products.

Correspondingly, the objective of the research presented herein was to determine differences in antibiotic, β-agonist, and non-steroidal anti-inflammatory drug residues in ground beef from USDA Certified Organic, USDA Process Verified Never Ever 3, conventional, and market cow and bull sources. To accomplish this objective, the prevalence of veterinary drug residues exceeding US tolerance limits in ground beef samples was determined using ultra-performance liquid chromatography coupled with triple-quadrupole tandem mass spectrometry (UPLC-MS).

MATERIALS AND METHODS

Sample Collection

Ground beef samples (N=400) consisting of 90.0 ± 4.0 % lean muscle tissue were collected from two facilities in each of the following categories: (1) USDA Certified Organic (n=100); (2) USDA Process Verified Never Ever 3 (n=100); (3) conventional (n=100); and (4) ground beef from market cows and bulls (n=100). Ground beef samples weighing ~ 435 g were collected from the final grinder immediately prior to packaging and placed into Whirlpak™ bags over the course of an entire production shift (2-8 hr) from each facility. Subsequently, samples were shipped on ice to PharmCats Bioanalytical Services (2005 Research Park Circle, Manhattan, KS 66502) where they were frozen at -80°C when received and until analysis.

Tissue Preparation

Frozen (-80°C) tissue samples were thawed for 48 to 72 hr at 4°C, and approximately 5.0 g was homogenized thoroughly in a 50 ml centrifuge tube using a Polytron PT 10-35 BT (Kinematica, Inc., Bohemia, NY) Homogenizer with a Kinematica PT-DA 3012/2MEC B-154 Generator. Then, 0.5 g of homogenized sample was weighed into a 15 ml conical polypropylene centrifuge tube, and standard curves and blanks were prepared by spiking homogenates of blank ground beef (obtained from a privately owned steer that was never given any veterinary drugs) with standard solutions prepared according to the following procedures.

To detect all of the compounds of interest, five tissue extraction methods were developed and five accompanying UPLC-MS methods were developed for analysis on a Waters Acquity UPLC-TQD (Waters Corporation, Milford, MA) equipped with a Waters Acquity bridged ethyl hybrid (BEH) C18 column (1.7 µm, 2.1 x 100 mm, 35°) or a BEH C18 column (1.7 µm, 2.1 x 50 mm) for analyte separation. Specific groups (Pools) of compounds analyzed by each extraction and UPLC method are outlined in Table 4. Each extraction was carried out by running all eight sets (one set from each beef packing plant from which samples were collected) in one week's time, one extraction method per week. Standards were prepared fresh for each week, and stocks

were kept at -20°C. Working stocks for spiking were used only on the day of preparation and the next day (kept at -20°C), so that no diluted standards were more than 24 hrs old.

Because products in the USDA Certified Organic and USDA Process Verified Never Ever 3 categories have a zero tolerance for residues of veterinary drugs, the use of highly sensitive methodologies that were able to detect residues of veterinary drugs at concentrations below those of many government-approved methods were appropriate. However, the methods reported herein are not the approved FDA validated methods that would be used to make a regulatory determination of a violative residue. The methods were not validated according to Good Laboratory Practices (GLP) requirements nor were they conducted under GLP standards. Therefore, while the results were set against U.S. regulatory tolerances for reporting, the methods are not necessarily the same as those applied in a regulatory setting. Methods were determined for the following veterinary drugs: (1) Aminoglycosides (Gentamicin, Amikacin, and Neomycin); (2) β-lactams (Penicillin, Ampicillin, and Desfuroylceftiofur); (3) Fluoroquinolones (Danofloxacin and Ciprofloxacin); (4) Macrolides (Erythromycin, Tylosin, and Tilmicosin); (5) Phenicols (Florfenicol); (6) Sulfonamides (Sulfamethazine and Sulfadimethoxine); (7) Tetracyclines (Oxytetracycline, Chlortetracycline, and Tetracycline); (8) Streptogramins (Virginiamycin); (9) β-agonists (Ractopamine and Zilpaterol);

and (10) non-steroidal anti-inflammatory drugs (Flunixin and Phenylbutazone).

Standard Curve and Sample Spiking—β-Lactams, Sulfonamides, β-Agonists, and Fluoroquinolones

Separate 1 mg/ml composite solutions for the compounds listed in Pools A, B, and C in Table 4 were prepared by weighing the appropriate amount of each compound and dissolving in 50% acetonitrile, bringing the solution to 10 ml in a volumetric flask. Desfuroylceftiofur, Ractopamine, and Zilpaterol were prepared in 50% acetonitrile in separate 1 mg/ml solutions according to printed instructions accompanying their commercial packaging. The complete Pool ABC composite solution was prepared by adding 0.1 ml each of the 1 mg/ml solutions of the compounds in Pool A, Pool B, and Pool C to 0.4 ml acetonitrile to make a solution that was 100,000 ng/ml in each analyte. The Pool ABC composite solution was diluted serially with 100% acetonitrile to make calibration stocks ranging from 10 to 20,000 ng/ml. These served as the working stock solutions to be used for spiking blank ground beef samples for the standard curve. The Pool ABC composite solution was diluted 1:10 by adding 50 µl to 0.5 g tissue to give 5, 10, 20, 50, 100, 200, 500, 1,000, and 2,000 ng/g tissue concentrations (Table 5).

The internal standard (IS) solution for Extraction 1 (Pools A, B, and C; Table 4) was prepared by adding 100% acetonitrile to the commercially packaged internal standards (Penicillin G-d7, Sulfamethazine-d4, Ractopamine-d6, Zilpaterol-d7, and Ciprofloxacin-d8) (Table 4) to make 1 mg/ml solutions. Each standard solution was then diluted 1:10 in acetonitrile to make 100,000 ng/ml solutions. The combined IS solution was prepared by adding 0.2 ml of each 100,000 ng/ml solution to 3.0 ml acetonitrile, and the final IS spiking solution was thus 5,000 ng/ml for each internal standard. Samples and standards were spiked with 50 µl of this IS solution to give a 10-fold dilution to 500 ng/g. Quality control samples were prepared at the 20, 50, and 200 ng/g concentrations as spiked blanks.

Extraction of Standards and Samples—β-Lactams, Sulfonamides, β-Agonists, and Fluoroquinolones

The methods for extraction of the β-lactams, Sulfonamides, β-agonists, and Fluoroquinolones listed in Pools A, B, and C of Table 4 were adapted from several previously published methods *(12, 17, 38, 39)*. One ml of water was added to each 15 ml tube. Samples were then vortexed for 2 to 3 min to mix the spiking compounds thoroughly with the water and tissue. Then, 4 ml of 100% acetonitrile was added and vortexed 5 to 10 min, and samples were subsequently centrifuged at 4,000 rpm at 4°C for 10 min (Thermo IEC Centra-CL3R Refrigerated Centrifuge, Thermo-Fisher Scientific, Pittsburgh, PA).

The supernatant fraction was decanted into clean, labeled 15 ml polypropylene tubes containing 0.25 g of Bakerbond C18 40 µm Prep LC packing (Baker #7025-00) and vortexed for 5 min. Tubes were then centrifuged at 4,000 rpm at 4°C for 10 min, and the supernatant fractions were decanted into clean, labeled 15 ml tubes and evaporated at 40°C and 20 psi Nitrogen (Turbovap LV Concentration Workstation, Caliper Life Sciences, Hopkinton, MA) for approximately 2 hr. The dried extracts were reconstituted by adding 300 µl of 15% acetonitrile, vortexing briefly, and filtering through a nylon syringe filter, 13 mm, 0.2 µ (Fisher #09-720-5), and a 3 ml Luer slip-tip syringe. The filtered extract was deposited directly into a 300 µl insert of an amber glass vial for analysis by UPLC-MS, according to the conditions outlined in Table 4.

Standard Curve and Sample Spiking—Macrolides, Streptogramins, and NSAIDS

Separate 1 mg/ml solutions of each compound listed in Pool D in Table 4 were prepared and dissolved in 100% methanol, bringing the solution to 10 ml in a volumetric flask. Virginiamycin was prepared separately as a 1 mg/ml solution in methanol according to printed instructions accompanying their commercial packaging. The 1 mg/ml solutions were combined by adding 0.1 ml of each solution to 0.7 ml 100% acetonitrile for a 1:10 dilution to 100,000 ng/ml. This composite solution was diluted serially with 100% acetonitrile to

make calibration stocks ranging from 10 to 10,000 ng/ml. These working stock solutions were used for spiking blank ground beef samples for the standard curve. The dilution into the sample was 1:10 by adding 50 µl solution to 0.5 g tissue to give 1, 2, 5, 10, 20, 50, 100, 200, 500, 1,000 ng/g tissue concentrations (Table 5).

The IS solution was prepared by weighing the appropriate amount of Roxithromycin and dissolving in 100% methanol, bringing the solution volume to 10 ml in a volumetric flask. Flunixin-d3 and Phenylbutazone-d9 were prepared separately as 1 and 2 mg/ml solutions in methanol, respectively, according to printed instructions accompanying their commercial packaging. The combined IS solution was prepared by adding 80 µl of Roxithromycin solution diluted to 100,000 ng/ml in acetonitrile, 80 µl of Flunixin-d3 solution diluted to 100,000 ng/ml in acetonitrile, and 40 µl of Phenylbutazone-d9 solution at 2 mg/ml to 3.8 ml acetonitrile. The final IS spiking solution was, thus, 2,000 ng/ml Roxithromycin, 2,000 ng/ml Flunixin-d3, and 20,000 ng/ml Phenylbutazone-d9; samples and standards were spiked with 50 µl of this IS solution to give a 10-fold dilution to 200, 200, and 2,000 ng/g, respectively. Quality control samples were prepared at the 20, 50, and 200 ng/g concentrations as spiked blanks.

Extraction of Standards and Samples—Macrolides, Streptogramins, and NSAIDS

The methods for extraction of the Macrolides, Streptogramins, and NSAIDS listed Pool D in Table 4 were adapted from several previously published methods *(10, 14, 39, 43)*. One ml of water was added to each 15 ml tube. Samples were then vortexed for 2to 3 min to mix the spiking compounds thoroughly with the water and tissue. Four ml of 100% acetonitrile was added, vortexed 5 to10 min, and samples were then centrifuged at 4,000 rpm at 4°C for 10 min. The liquid supernatant fractions were transferred to clean, labeled 50 ml polypropylene centrifuge tubes, 15 ml of water was added, and the tubes were vortexed 1 min. The mixture was loaded onto Nexus C18 columns (3 ml, 60 mg, Varian Abs-Elut #12103101) that had been conditioned with 3 ml acetonitrile followed by 3 ml water. After loading, each column was washed with 3 ml of 5% acetonitrile and dried with vacuum for 5-10 min, and samples were eluted with 3 ml of 50% acetonitrile into clean, labeled 15 ml tubes. The samples were then evaporated at 40° C and 20 psi nitrogen for approximately 3 hr. The dried extracts were reconstituted by adding 200 µl 15% acetonitrile and vortexing briefly. The extract was transferred into a 300 µl insert of an amber glass vial for analysis by UPLC-MS according to the conditions outlined in Table 4.

Standard Curve and Sample Spiking—Aminoglycosides

Separate 1 mg/ml solutions of each compound listed in Pool E in Table 4 were prepared and dissolved in 10 ml of acetonitrile:water:acetic acid (20:78:2) in a 20 ml polypropylene vial. The 1 mg/ml composite solution was diluted to 100,000 ng/ml and then diluted serially to make calibration stocks ranging from 10 to 10,000 ng/ml. These calibration stocks served as the working stock solutions to be used for spiking blank ground beef samples for the standard curve. The dilution into the sample was 1:10 by adding 50 µl to 0.5 g tissue to generate 1, 2, 5, 10, 20, 50, 100, 200, 500, 1,000 ng/g tissue concentrations (Table 5).

The IS solution was prepared by weighing the appropriate amount of Tobramycin and dissolving in 10 ml of the acetonitrile:water:acetic acid solution in a polypropylene vial. The final IS solution was prepared by diluting the Tobramycin solution to a concentration of 2,000 ng/ml. Samples and standards were spiked with 50 µl of this IS solution to give a 10-fold dilution to 200 ng/g. Quality control samples were prepared at the 20 and 100 ng/g concentrations as spiked blanks.

Extraction of Standards and Samples—Aminoglycosides

The methods for extraction of the Aminoglycoside antibiotic compounds listed in Pool E of Table 4 were adapted from previously published methods *(60)*. This involved using polypropylene containers at every stage of the

extraction and analysis, as the Aminoglycosides adhere to glass. Two ml of 5% Trichloroacetic Acid was added to each standard or sample in a 15 ml tube. Samples were then vortexed for 5 to10 min to mix the spiking compounds thoroughly with the water and tissue. Subsequently, samples were centrifuged 4,000 rpm at 4°C for 10 min. The liquid supernatant fractions were transferred to clean, labeled 15 ml polypropylene centrifuge tubes, 0.5 ml of 0.2 M Heptafluorobutyric Acid (HFBA) was added, and the tubes were vortexed 1 min and centrifuged at 4,000 rpm at 4°C for 10 min. The supernatant was then loaded onto Nexus C18 columns (3 ml, 60 mg, Varian Abs-Elut #12103101) that had been conditioned with 3 ml acetonitrile, 3 ml water, and 3 ml 0.2 M HFBA. After loading, each column was washed with 3 ml of water, dried under vacuum for 5 to10 min, and eluted with 3 ml of acetonitrile with 0.2 M HFBA:water (80:20) into clean, labeled 15 ml tubes. The samples were then dried at 40° C and 20 psi nitrogen for approximately 2 hr. The dried extracts were reconstituted by adding 200 µl mobile phase A:mobile phase B (80:20) (Table 4) and vortexing briefly. The extracts were transferred into 250 µl polypropylene vials for analysis by UPLC-MS according to the conditions outlined in Table 4.

Standard Curve and Sample Spiking—Tetracyclines

A composite 1 mg/ml solution of the compounds listed in Pool F in Table 4 was prepared and dissolved in 100% methanol, bringing the solution to 10 ml

in a volumetric flask. The 1 mg/ml composite solution was diluted to 100,000 ng/ml in methanol. The solution was then diluted serially with 100% methanol to make calibration stocks ranging from 20 to 20,000 ng/ml. These calibration stocks served as the working stock solutions to be used for spiking blank ground beef samples for the standard curve. The dilution into the sample was 1:10 by adding 50 µl to 0.5 g tissue to generate 2, 5, 10, 20, 50, 100, 200, 500, 1,000, and 2,000 ng/g tissue concentrations (Table 5).

The IS solution was prepared by weighing the appropriate amount of Demeclocycline and dissolving in 100% methanol, bringing the solution to 10 ml in a volumetric flask. This solution was diluted 1:10 to 100,000 ng/ml in methanol. The IS spiking solution was then prepared by adding 80 µl of the 100,000 ng/ml Demeclocycline solution to 3.92 ml methanol. The final IS spiking solution was, thus, 2,000 ng/ml. Samples and standards were spiked with 50 µl of this IS solution to give a 10-fold dilution to 200 ng/g. Quality control samples were prepared at the 20 and 100 ng/ml concentrations as spiked blanks.

Extraction of Standards and Samples—Tetracyclines

The methods of extraction for the Tetracycline antibiotics listed in Pool F of Table 4 were adapted from previously published methods *(45)*. Two ml of McIlvaine extraction buffer was added to each 15 ml tube and vortexed 5 to10

min. Subsequently, the samples were centrifuged at 4,000 rpm at 4°C for 10 min, and the supernatant fractions were transferred to 3 ml syringes with a nylon syringe filter, 25 mm, 0.45 µ (Fisher #09-719F), using a transfer pipet to avoid the top layer of fat. Samples were then loaded onto Nexus C18 columns (3 ml, 60 mg, Varian Abs-Elut #12103101) that were previously conditioned with 3 ml methanol followed by 3 ml water. After loading, columns were washed with 3 ml of 5% methanol and dried under vacuum for 5-10 min. Samples were eluted with 3 ml of 100% methanol into clean, labeled 15 ml tubes and evaporated at 40° C and 20 psi nitrogen for approximately 1 hr. Dried extracts were reconstituted by adding 200 µl of 15% acetonitrile and vortexing briefly, and the extract was transferred into a 300 µl insert of an amber glass vial for analysis by UPLC-MS according to the conditions outlined in Table 4

Standard Curve and Sample Spiking—Phenicols

A 1 mg/ml solution of Florfenicol (Pool G; Table 4) was prepared and dissolved in 100% methanol, and brought to a volume of 10 ml in a volumetric flask. The solution was then diluted 1:10 to 100,000 ng/ml in methanol. The 100,000 ng/ml solution was then diluted serially with 100% methanol to make calibration stocks ranging from 10 to 5,000 ng/ml. These calibration stocks served as the working stock solutions used to spike blank ground beef samples for the standard curve. The dilution into the sample was

1:10 by adding 50 µl to 0.5 g tissue to generate 1, 2, 5, 10, 20, 50, 100, 200, and 500 ng/g tissue concentrations (Table 5).

The IS solution was prepared by diluting the 1 mg/ml solution to 2,000 ng/ml in 100% methanol and bringing the solution to 10 ml in a volumetric flask. Samples and standards were spiked with 50 µl of this IS solution to give a 10-fold dilution to 200 ng/g. Quality control samples were prepared at the 20 and 100 ng/ml concentrations as spiked blanks.

Extraction of Standards and Samples—Phenicols

The method of extraction of the Phenicol antibiotics listed in Pool G of Table 4 were adapted from previously published methods *(31)*. Two ml of ethyl acetate was added to each 15 ml tube, vortexed 5 to10 min, and centrifuged at 4,000 rpm at 4°C for 10 min. The supernatant fractions were then transferred to clean, labeled 15 ml tubes and evaporated at 40° C and 20 psi nitrogen until approximately 0.2 ml water remained. Then, 0.5 ml methanol was added, vortexed briefly, and 10 ml water was then added and vortexed. Samples were subsequently loaded onto Nexus C18 columns (3 ml, 60 mg, Varian Abs-Elut #12103101) that were previously conditioned with 3 ml methanol followed by 3 ml water. After loading, columns were washed with 3 ml of water and dried under vacuum for 5 to10 min. Samples were eluted with 3 ml of 100% methanol into clean, labeled 15 ml tubes and evaporated at 40° C and

20 psi nitrogen for approximately 1 hr. The dried extracts were reconstituted by adding 200 µl of 5% methanol and vortexing briefly. The extracts were transferred into a 300 µl insert of an amber glass vial for analysis by UPLC-MS according to the conditions outlined in Table 4.

Analysis of Data

Differences in the prevalence of samples exceeding the US tolerance limit in each production class were determined using a Fishers Exact Test in the PROC FREQ procedures of SAS *(46)* (Table 6). Additionally, Differences in the prevalence of detectable residues in the USDA Certified Organic production class and the USDA Process Verified Never Ever 3 production class were determined using a Fishers Exact Test in the PROC FREQ procedures of SAS *(46)* (Table 7).

In order to determine the limit of detection (LOD) for each compound, the standard deviation of the baseline noise for each compound using blank samples included with each extraction run was multiplied by three and the average blank concentration value was added. To compute the limit of quantitation (LOQ), the LOD was multiplied by 10 and adjusted based upon chromatography of samples *(4)*. Limits of detection and LOQ for each compound assayed are presented in Table 5.

RESULTS AND DISCUSSION

The incidence of samples within each production classification (USDA Certified Organic, natural, conventional, and ground beef from market cows and bulls) that exceeded the US tolerance limit is presented in Table 6. There were no statistically significant differences between the production classes. Two samples from the USDA Certified Organic production class contained Ampicillin residues exceeding the US tolerance limit of 10 ng/g. In the USDA Process Verified Never ever 3 production category, one sample contained a residue of Ractopamine exceeding the US tolerance limit of 30 ng/g (Table 6). In the conventional production category, one residue of Phenylbutazone, a drug which has a zero tolerance limit in beef muscle tissue, was found (Table 6). In the market cows and bulls production class, 1 sample contained Ampicillin residues exceeding the US tolerance limit of 10 ng/g. Additionally, one sample from the market cows and bulls production class contained Sulfadimethoxine residues exceeding the US tolerance limit of 100 ng/g, and one sample contained a residue of Phenylbutazone (Table 6).

Due to standards set forth by the National Organic Program, any residue of a veterinary drug (except those allowed) found in a USDA Certified Organic product is considered violative. Similarly, there is a zero tolerance for the presence of unapproved veterinary drugs in beef derived from USDA Process

Verified Never Ever 3 sources. The incidence of samples in the USDA Certified Organic and USDA Process Verified Never Ever 3 production categories that contained detectable amounts of veterinary drugs are presented in Table 7. A significantly ($P < 0.05$) higher number of detectable Ampicillin and Penicillin residues were found in samples from the USDA Certified Organic ground beef samples when compared to samples from the USDA Process Verified Never Ever 3 production category (Table 7). Conversely, a significantly ($P < 0.05$) higher number of detectable Ractopamine residues was found in samples from the USDA Process Verified Never Ever 3 production category when compared to the USDA Certified Organic production category (Table 7). It is important to note that these samples contained residues of veterinary drugs that are significantly below the US tolerance limit. Detectable residues not exceeding the US tolerance limit in samples from the conventional and market cows and bulls production categories were not presented in tabular form.

In the USDA Certified Organic production category, β-lactam, Sulfamethazine, Sulfadimethoxine, and Ractopamine residues were detected in ground beef samples (Table 7). β-lactam residues were found at both facilities tested, 13 over the course of 4 hr in one facility, and two over a span of 4 hr at the other facility (followed by a 4 hr span without any detectable residues found). Of the three samples with detectable concentrations of

Sulfamethazine, all were from the same plant and were collected within a period of two hr (Table 7). Additionally, 3 samples in the USDA Certified Organic production category contained detectable residues of both Ampicillin and Penicillin.

In the USDA Process Verified Never Ever 3 production category, samples were found to contain detectable residues of Ractopamine, Tetracyclines, and Ampicillin (Table 7). Of the six samples that had detectable concentrations of Ractopamine, all were collected from the same facility within a two hr time window (Table 7). In addition, one sample contained detectable residues of both Tetracycline and Chlortetracycline.

Due to the finding of positive samples within short succession of one another, the authors evaluated the potential for laboratory carryover of samples and concluded that the patterns of the repeated positive samples (which included multiple negatives in-between) were not suggestive of laboratory carryover. In addition, there were multiple samples where a single detected concentration was not followed by subsequent positive samples. Separate analyses were carried out by the same personnel, so carryover would have been the result of protocol violations and not protocol design. Furthermore, in the reported sample sets where repeated low concentrations were found in the samples over several hours, the concentrations were often similar, suggesting that trace

contamination from previous samples of similar concentration was not a possible explanation.

In the research presented herein, samples were collected from finished ground beef production lines. Consequently, determination of single animal origin for any given sample was impossible because every sample almost certainly contained meat from numerous animals. As a result, it is impossible to discern if the residues found were the cumulative sum of the specific veterinary drug residues from all of the animals represented in a composite 435 g ground beef sample, or if the trimmings from only one animal contained residues. It is possible that trimmings from a single animal could account for detectable residues in several ground beef samples due to carcass fabrication techniques (such as commingling primals and trimmings from different carcasses in the grinding operation). Furthermore, it is possible that beef trimmings from one animal left residual veterinary drug traces in the grinding machinery that tainted subsequent product. While this sampling technique did not allow for unambiguous determination of individual animals, the samples clearly represent what a consumer would purchase and consume. Sampling ground beef, therefore, brings to light the effects that even a single violative animal can have on a final product and highlights the importance of producers and feeders removing any animals that were treated with antibiotics or other prohibited substances from the USDA Certified Organic and USDA Process

Verified Never Ever 3 and Naturally Raised programs. Animals in these programs that are treated with antibiotics at any time should be sold as "conventional" product once the proper withdrawal periods for the drugs administered have been met.

Previous research has also aimed to elucidate differences in the presence of veterinary drugs and pesticides in beef products from specific production categories. Smith et al., *(51)* analyzed 20 samples each of muscle, adipose tissue, kidney, and liver for residues of anabolic steroids, environmental contaminants, Clenbuterol (a β-agonist), two tranquilizers, six Sulfonamides, including Sulfamethazine and Sulfadimethoxine from steers, heifers, and cows at eight packing plants from organic, natural, conventional, cull cow, and chronically ill sources. The authors reported a LOD of 0.025 μg/g for the Sulfonamides and found no detectable residues in any of the tissues assayed*(51)*. Usborne *(54)* compared natural and conventional beef purchased in supermarkets in Canada and reported no violative residues of Sulfonamides, antibiotics, heavy metals, growth promotants, parasiticides, polychlorinated biphenyls, pentachlorophenol, or pesticides. Smith et al, *(50)* analyzed 64 muscle, 60 adipose, 36 liver, and 26 kidney samples from organic, natural, and conventional beef sources for the presence of anabolic steroids, xenobiotics, Penicillin, Tylosin, Erythromycin, Sulfonamides, including Sulfamethazine and Sulfadimethoxine, Tetracycline,

Oxytetracycline, Chlortetracycline, and pesticides. The authors reported that no detectable residues of any veterinary drugs were found in any production category, while pesticide residues were found in all production categories *(29)*. Additionally, the US conducts the National Residue Program that monitors animals from all production classes for the presence of residues exceeding US tolerance limits. Historically (2004-2007), < 1.0% of all of the drugs sampled in the current study have been found to contain residues exceeding their respective US tolerance limit when sampled as a part of the National residue Program *(29)*. One notable exception is Flunixin, which was found at violative concentrations in almost 10.0% of samples collected across all bovine production classes in 2005. These findings are in stark contrast to the data present herein which demonstrate a 2.5% incidence of of samples exceeding US tolerance limits. One possible reason could be the enhanced sensitivity of the methods used in the present study. The LOD for the Sulfonamide detection methods presented herein are 50 to 100 X lower than the LOD reported by Smith et al *(51)*. Additionally, the sample size was very small both in the Smith et al., (1994, 1997) research and the present research. Future research should focus on collecting more samples from more facilities within each category over an extended period of time to fully discern the prevalence of detectable veterinary drug residues in USDA Certified Organic and USDA Process Verified Never Ever 3, Naturally Raised, conventional and market cow and bull sources.

In the research presented herein, UPLC-MS methods for the determination of 19 antimicrobials, two β-agonists, and two NSAIDS were developed and applied to ground beef samples from USDA Certified Organic, USDA process Verified Never Ever 3, conventional, and market cow and bull sources. At least one residue of a veterinary drug exceeding US tolerance limits was found in all production class categories. Additionally, residues (below the US tolerance limit) of several classes of veterinary drugs were found in samples from the USDA Certified Organic category, a finding that clearly demonstrates violation of zero-tolerance statutes set forth by the National Organic Program. USDA Process Verified Never Ever 3 product also contained detectable residues of certain veterinary drugs. These findings demonstrate the need for careful monitoring of animals administered veterinary drugs in order to prevent incorrect inclusion of unqualified animals in premium programs, such as USDA Certified Organic and USDA Process Verified Never Ever 3 programs, and to ensure that withdrawal times are honored. Due to the financial disincentives associated with the loss of an animal destined for sale in one of these premium programs (to conventional marketing strategies), and based upon the prevalence of detectable veterinary drug residues in the current study, government oversight, including testing of samples for veterinary drug residues, and mandating compliance with label claims, is the only method of protecting consumers and value-added brands of meat products. Consumer confidence in premium beef programs could be

severely compromised by the accidental or egregious violations of veterinary drug statutes and/or marketing claims.

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Table 1. Number of samples collected and exceededing the US tolerance limit under the Scheduled Sampling Plan for 2004-2007. Number of samples tested and the number of violative samples is the cumulative total of the samples collected in the Beef Cows, Bovine, Bulls, Dairy Cows, Heavy Calves, Heifers, and Steers categories.

Veterinary drug class	Veterinary drug compound	Number of samples tested 2004	Number of violative samples 2004	Number of samples tested 2005	Number of violative samples 2005	Number of samples tested 2006	Number of violative samples 2006	Number of samples tested 2007	Number of violative samples 2007
Aminoglycosides	Gentamicin	1372	2	1294	1	1451	4	1173	0
Aminoglycosides	Amikacin	1372	0	1294	0	1451	0	1173	0
Aminoglycosides	Neomycin	1372	0	1294	0	1451	2	1173	0
β-Lactams	Penicillin	1372	3	1294	0	1451	0	1173	0
β-Lactams	Ampicillin	NI	N/A	1294	0	1451	0	1173	0
β-Lactams	Desfuroylceftiofur	NI	N/A	1294	0	1451	0	1173	0
Fluoroquinolones	Danofloxacin	NI	N/A	NI	N/A	NI	N/A	1173	0
Fluoroquinolones	Ciprofloxacin	NI	N/A	NI	N/A	NI	N/A	1173	0
Macrolides	Erythromycin	1372	0	1294	0	1451	0	1173	0
Macrolides	Tylosin	1372	0	1294	0	1451	0	1173	0
Macrolides	Tilmicosin	1372	1	NI	N/A	1451	0	1173	0
Phenicols	Florfenicol	50	0	157	1	270	0	373	0
Sulfonamides	Sulfamethazine	1495	0	1632	0	1451	4	1590	3
Sulfonamides	Sulfadimethoxine	1495	0	1632	0	1451	1	1590	2
Tetracyclines	Oxytetracycline	1372	0	1294	0	1451	0	1173	1
Tetracyclines	Chlortetracycline	1372	0	1294	0	1451	0	1173	0
Tetracyclines	Tetracycline	1372	0	1294	0	1451	0	1173	0
Streptogramins	Virginiamycin	NI	N/A	NI	N/A	NI	N/A	NI	N/A
NSAIDs[1]	Flunixin	213	3	159	15	1451	5	NI	N/A
NSAIDs[1]	Phenylbutazone	2001	0	3213	2	1742	0	NI	N/A
β-Agonists	Zilpaterol	NI	N/A	NI	N/A	NI	N/A	NI	N/A
β-Agonists	Ractopamine	NI	N/A	240	0	4	0	306	0

Table 2. Number of samples collected and the number of samples that exceeded the US tolerance limit under the Inspector Generated Sampling Plan for 2004-2007.

Veterinary drug class	Veterinary drug compound	Number of samples tested 2004[1]	Number of violative samples 2004[1]	Number of samples tested 2005[2]	Number of violative samples 2005[2]	Number of samples tested 2006[3]	Number of violative samples 2006[3]	Number of samples tested 2007[4]	Number of violative samples 2007[4]
Aminoglycosides	Gentamicin	94146	108	104611	100	68511	148	108711	74
Aminoglycosides	Amikacin	94146	1	104611	5	68511	2	108711	0
Aminoglycosides	Neomycin	94146	26	104611	26	68511	46	108711	28
β-Lactams	Penicillin	94146	389	104611	342	68511	420	108711	435
β-Lactams	Ampicillin	94146	0	104611	6	68511	12	108711	12
β-Lactams	Desfuroylceftiofur	94146	0	104611	0	68511	0	108711	0
Fluoroquinolones	Danofloxacin	94146	0	104611	0	68511	0	108711	0
Fluoroquinolones	Ciprofloxacin	94146	0	104611	0	68511	0	108711	0
Macrolides	Erythromycin	94146	0	104611	0	68511	0	108711	0
Macrolides	Tylosin	94146	2	104611	1	68511	0	108711	1
Macrolides	Tilmicosin	94146	27	104611	35	68511	41	108711	18
Phenicols	Florfenicol	94146	0	104611	0	68511	0	108711	0
Sulfonamides	Sulfamethazine	94146	26	104611	36	68511	0	108711	39
Sulfonamides	Sulfadimethoxine	94146	114	104611	114	68511	173	108711	159
Tetracyclines	Oxytetracycline	94146	35	104611	44	68511	45	108711	38
Tetracyclines	Chlortetracycline	94146	0	104611	0	68511	0	108711	0
Tetracyclines	Tetracycline	94146	7	104611	19	68511	18	108711	7
Streptogramins	Virginiamycin	94146	0	104611	0	68511	0	108711	0
NSAIDs[1]	Flunixin	94146	38	104611	115	68511	139	108711	271
NSAIDs[1]	Phenylbutazone	94146	0	104611	0	68511	0	108711	6
β-Agonists	Zilpaterol	NI	N/A	NI	N/A	68511	N/A	N/A	NI
β-Agonists	Ractopamine	NI	N/A	NI	N/A	68511	N/A	N/A	NI

[1]Number of samples and number of violations found are from Table 116b, 2004 NRP red book. Samples were totaled from the Bulls, Steers, Beef Cows, Dairy Cows, and Heavy Calves categories
[2]Number of samples and number of violations found are from Table 136b, 2005 NRP red book. Samples were totaled from the beef Cows, Bulls, Dairy Cows, Heavy Calves, Heifers, and Steers categories.
[3]Number of samples and number of violations found are from Table 40b, 2006 NRP red book. Samples were totaled from the Beef Cows, Bovine, Bulls, Dairy Cows, Heavy Calves, Heifers, and Steers categories.
[4]Number of samples and number of violations found are from Table 40b, 2006 NRP red book. Samples were totaled from the Beef Cows, Bovine, Bulls, Dairy Cows, Heavy Calves, Heifers, and Steers categories.

Table 3. Tolerances and maximum residue limits (MRL) allowed in cattle muscle tissue for specific veterinary drug compounds reported by the United States (US), European Union (EU), and by the Codex Alimentarius Commission (CAC) in ng chemical allowed/g ground beef muscle tissue.

Veterinary drug class	Veterinary drug compound	US tolerance limit	EU MRL	CAC MRL
Aminoglycosides	Gentamicin	ZT	50	NR
Aminoglycosides	Amikacin	ZT	NR	NR
Aminoglycosides	Neomycin	1200	500	500
β-Lactams	Penicillin	50	NR	NR
β-Lactams	Ampicillin	10	50	NR
β-Lactams	Desfuroylceftiofur	1000	1000	1000
Fluoroquinolones	Danofloxacin	200	200	200
Fluoroquinolones	Ciprofloxacin[2]	NR	NR	NR
Macrolides	Erythromycin	100	200	NR
Macrolides	Tylosin	200	100	NR
Macrolides	Tilmicosin	100	50	100
Phenicols	Florfenicol	300	NR	NR
Sulfonamides	Sulfamethazine	100	NR	100000
Sulfonamides	Sulfadimethoxine	100	NR	NR
Tetracyclines	Oxytetracycline	2000	100	200
Tetracyclines	Chlortetracycline	2000	100	200
Tetracyclines	Tetracycline	2000	100	200
Streptogramins	Virginiamycin	NR	NR	NR
NSAIDs[1]	Flunixin	25	20	NR
NSAIDs[1]	Phenylbutazone	ZT	NR	NR
β-Agonists	Zilpaterol	NR	NR	NR
β-Agonists	Ractopamine	30	NR	NR

[1] Non-Steroidal Anti-Inflammatory Drug

[2] Ciprofloxacin is the target residue for Enrofloxacin

NR indicates that there are tolerance concentrations set for the target tissues but that no regulations are established for beef muscle tissue

ZT indicates that there is no tolerance set in any cattle tissue and, therefore, there is a zero tolerance for this drug in any cattle tissue

SOURCE: *(40, 56)*

Table 4. Ultra-performance liquid chromatography (UPLC) mass spectrometer and gradient parameters for veterinary drugs. Transition parameters include the ion transition (m/z), ionization mode (electrospray positive [ES+] or electrospray negative [ES-]), cone voltage (V), and collision voltage (eV) for each compound analyzed. Gradient parameters include run time, the composition and percentage of mobile phases, and flow rate for entire Pools (A-G) of compounds.

	Mass spectrometer parameters					Gradient parameters			
	Transition (m/z)	Ion mode	Dwell time (sec)	Cone voltage (V)	Collision voltage (eV)	Run time (min)	Mobile phase A	Mobile phase B	Flow (ml/min)
							0.1% formic acid in water	0.1% formic acid in ACN: MeOH 90:10	
Pool A—β-Lactams									
Penicillin G	335>160	ES+	0.1	25	11	0.0	85.0%	15.0%	0.2
Ampicillin	350>106	ES+	0.1	30	15	1.0	75.0%	25.0%	0.2
Desfuroylceftiofur	430>125	ES+	0.1	40	41	4.0	72.0%	28.0%	0.2
Penicillin G-d7 (IS)	342>183	ES+	0.1	25	13	5.0	40.0%	60.0%	0.2
						6.5	30.0%	70.0%	0.2
						7.0	5.0%	95.0%	0.2
						8.0	85.0%	15.0%	0.2
						8.5	85.0%	15.0%	0.2
Pool B—Sulfonamides, β-Agonists							0.1% formic acid in water	0.1% formic acid in ACN: MeOH 90:10	
Sulfamethazine	279>186	ES+	0.1	35	17	0.0	85.0%	15.0%	0.2
Sulfadimethoxine	311>156	ES+	0.1	35	17	6.0	78.0%	22.0%	0.2
Sulfamethazine-d4 (IS)	283>186	ES+	0.1	40	19	8.0	45.0%	55.0%	0.2
Zilpaterol	262>244	ES+	0.1	30	13	9.0	45.0%	55.0%	0.2
Ractopamine	302>164	ES+	0.1	30	17	9.5	5.0%	95.0%	0.2
Zilpaterol-d7 (IS)	269>25	ES+	0.1	30	15	10.5	85.0%	15.0%	0.2
Ractopamine-d6 (IS)	308>168	ES+	0.1	30	15	11.0	85.0%	15.0%	0.2
Pool C—Fluoroquinolones							0.1% formic acid in water	0.1% formic acid in ACN: MeOH 90:10	
Ciprofloxacin	332>288	ES+	0.1	40	17	0.0	85.0%	15.0%	0.2
Danofloxacin	358>340	ES+	0.1	45	21	4.0	80.0%%	20.0%	0.2
Ciprofloxacin-d8 (IS)	340>296	ES+	0.1	40	19	5.5	70.0%	30.0%	0.2
						6.0	5.0%	95.0%	0.2
						7.0	85.0%	15.0%	0.2
						7.5	85.0%	15.0%	0.2
Pool D—Macrolides, Streptogramins, NSAIDs							0.1% formic acid in water	0.1% formic acid in ACN: MeOH 90:10	
Erythromycin	734>158	ES+	0.1	40	31	0.0	85.0%	15.0%	0.2
Tilmicosin	869>174	ES+	0.1	90	45	1.0	60.0%	40.0%	0.2
Tylosin	916>174	ES+	0.1	65	39	6.0	15.0%	85.0%	0.2
Roxithromycin (IS)	838>158	ES+	0.1	40	33	6.5	15.0%	85.0%	0.2
Virginiamycin	526>508	ES+	0.1	30	13	7.0	5.0%	95.0%	0.2
Roxithromycin (IS)	838>158	ES+	0.1	40	33	8.0	85.0%	15.0%	0.2
Flunixin	297>279	ES+	0.1	45	23	8.5	85.0%	15.0%	0.2
Phenylbutazone	309>92	ES+	0.1	35	27				
Flunixin-d3 (IS)	300>282	ES+	0.1	45	25				
Phenylbutazone-d9(IS)	318>93	ES+	0.1	40	27				
Pool E—Aminoglycosides							20 mM HFBA in ACN: water 5:95	20 mM HFBA in ACN: water 50:50	
Amikacin	586>163	ES+	0.1	40	33	0.0	80.0	20.0%	0.2
Gentamicin	478>322	ES+	0.1	90	15	4.0	5.0	95.0%	0.2
Neomycin	615>161	ES+	0.1	70	29	6.5	5.0	95.0%	0.2
Tobramycin (IS)	468>163	ES+	0.1	45	21	7.0	80.0	20.0%	0.2
Pool F—Tetracyclines							0.1% formic acid in water	0.1% formic acid in ACN: MeOH 90:10	
Tetracycline	445>410	ES+	0.1	30	21	0.0	85.0%	15.0%	0.2
Oxytetracycline	462>444	ES+	0.1	35	21	4.5	50.0%	50.0%	0.2
Chlorotetracycline	479>462	ES+	0.1	35	19	5.0	5.0%	95.0%	0.2
Demeclocycline (IS)	465>448	ES+	0.1	35	17	7.0	85.0%	15.0%	0.2
						7.5	85.0%	15.0%	0.2
Pool G—Phenicols							100% water	100% MeOH	
Florfenicol	356>336	ES-	0.1	30	9	0.0	95.0%	5.0%	0.5

Chloramphenicol (IS)	321>152	ES-	0.1	35	15	0.4	95.0%	5.0%	0.5
						1.0	0.0%	100.0%	0.5
						2.0	0.0%	100.0%	0.5
						2.2	95.0%	5.0%	0.5
						3.0	95.0%	5.0%	0.5

ACN=Acetonitrile
HFBA=Heptafluorobutyric Acid
IS=Internal Standard
MeOH=Methanol

Table 5. Limit of detection, limit of quantitation, and standard curve range for ultra-performance liquid chromatography coupled with triple-quadropole tandem mass spectrometry of specific veterinary drug compounds.

Drug Class	Veterinary drug compound	Limit of detection (ng/g)	Limit of quantitation (ng/g)	Standard curve range (ng/g)
Aminoglycosides	Gentamicin	4.6	46.0	10.0-1000.0
Aminoglycosides	Amikacin	8.5	85.0	10.0-1000.0
Aminoglycosides	Neomycin	3.6	36.0	10.0-1000.0
β-Lactams	Penicillin	1.0	9.9	1.0-1000.0
β-Lactams	Ampicillin	0.8	8.3	1.0-1000.0
β-Lactams	Desfuroylceftiofur	25.0	253.0	20.0-1000.0
Fluoroquinolones	Danofloxacin	1.3	13.4	1.0-500.0
Fluoroquinolones	Ciprofloxacin[2]	1.2	11.9	1.0-500.0
Macrolides	Erythromycin	7.1	71.0	1.0-1000.0
Macrolides	Tylosin	0.8	8.5	1.0-1000.0
Macrolides	Tilmicosin	2.1	21.0	1.0-1000.0
Phenicols	Florfenicol	12.3	123.0	10.0-1000.0
Sulfonamides	Sulfamethazine	0.3	3.2	1.0-500.0
Sulfonamides	Sulfadimethoxine	0.5	4.5	1.0-500.0
Tetracyclines	Oxytetracycline	3.1	31.0	2.0-2000.0
Tetracyclines	Chlortetracycline	5.7	57.0	2.0-2000.0
Tetracyclines	Tetracycline	2.8	28.0	2.0-2000.0
Streptogramins	Virginiamycin	11.5	115.0	1.0-1000.0
NSAIDs[1]	Flunixin	1.3	13.0	1.0-1000.0
NSAIDs[1]	Phenylbutazone	47.0	467.0	50.0-1000.0
β-Agonists	Zilpaterol	5.7	56.6	5.0-500.0
β-Agonists	Ractopamine	0.3	2.7	1.0-500.0

[1] Non-Steroidal Anti-Inflammatory Drug
[2] Ciprofloxacin is the target residue for Enrofloxacin

Table 6. Number of ground beef samples from USDA Certified Organic, USDA Process Verified Never Ever 3, conventional, and market cow and bull sources exceeding the United States tolerance limit for specific veterinary drug residues.

Veterinary Drug Class	Veterinary drug compound	US tolerance limit (ng/g)[2]	USDA Certified Organic (n=100)	USDA Process Verified Never Ever 3 (n=100)	Conventional (n=100)	Market cows and bulls (n=100)
Aminoglycosides	Gentamicin	ZT	0	0	0	0
Aminoglycosides	Amikacin	ZT	0	0	0	0
Aminoglycosides	Neomycin	1200	0	0	0	0
β-Lactams	Penicillin	50	0	0	0	0
β-Lactams	Ampicillin	10	2	0	0	1
β-Lactams	Desfuroylceftiofur	1000	0	0	0	0
Fluoroquinolones	Danofloxacin	200	0	0	0	0
Fluoroquinolones	Ciprofloxacin[3]	NR	0	0	0	0
Macrolides	Erythromycin	100	0	0	0	0
Macrolides	Tylosin	200	0	0	0	0
Macrolides	Tilmicosin	100	0	0	0	0
Phenicols	Florfenicol	300	0	0	0	0
Sulfonamides	Sulfamethazine	100	0	0	0	0
Sulfonamides	Sulfadimethoxine	100	0	0	0	1
Tetracyclines	Oxytetracycline	2000	0	0	0	0
Tetracyclines	Chlortetracycline	2000	0	0	0	0
Tetracyclines	Tetracycline	2000	0	0	0	0
Streptogramins	Virginiamycin	NR	0	0	0	0
NSAIDs[1]	Flunixin	25	0	0	0	0
NSAIDs[1]	Phenylbutazone	ZT	0	0	1	1
β-Agonists	Zilpaterol	NR	0	0	0	0
β-Agonists	Ractopamine	30	0	1	0	0

[1] Non-Steroidal Anti-Inflammatory Drug

[2]SOURCE: *(40, 56)*

[3] Ciprofloxacin is the target residue for Enrofloxacin

NR indicates that there are tolerance concentrations set for the target tissue but that no regulations are established for beef muscle tissue

ZT indicates that there is no tolerance set in any cattle tissue and , therefore, there is a a zero tolerance for this drug in any cattle tissue

Table 7. Incidence of detectable veterinary drug residues in ground beef samples from USDA Certified Organic and USDA Process Verified Never Ever 3 sources.

Veterinary drug class	Veterinary drug compound	Limit of detection (ng/g)	USDA Certified Organic (n=100)	USDA Process Verified Never Ever 3 (n=100)
Aminoglycosides	Gentamicin	4.6	0	0
Aminoglycosides	Amikacin	8.5	0	0
Aminoglycosides	Neomycin	3.6	0	0
β-Lactams	Penicillin	1.0	7^{z}	0^{y}
β-Lactams	Ampicillin	0.8	8^{z}	1^{y}
β-Lactams	Desfuroylceftiofur	25.0	0	0
Fluoroquinolones	Danofloxacin	1.3	0	0
Fluoroquinolones	Ciprofloxacin[2]	1.2	0	0
Macrolides	Erythromycin	7.1	0	0
Macrolides	Tylosin	0.8	0	0
Macrolides	Tilmicosin	2.1	0	0
Phenicols	Florfenicol	12.3	0	0
Sulfonamides	Sulfamethazine	0.3	3	0
Sulfonamides	Sulfadimethoxine	0.5	1	0
Tetracyclines	Oxytetracycline	3.1	0	0
Tetracyclines	Chlortetracycline	5.7	0	1
Tetracyclines	Tetracycline	2.8	0	2
Streptogramins	Virginiamycin	11.5	0	0
NSAIDs[1]	Flunixin	1.3	0	0
NSAIDs[1]	Phenylbutazone	47.0	0	0
β-Agonists	Zilpaterol	5.7	0	0
β-Agonists	Ractopamine	0.3	1^{y}	6^{z}

[1] Non-Steroidal Anti-Inflammatory Drug

[2] Ciprofloxacin is the target residue for Enrofloxacin

[z,y] number of positive samples within a row lacking common superscript letters differ ($P < 0.05$).

SOURCE: *(40, 56)*