DISSERTATION

RACTOPAMINE WITHDRAWAL, DEPLETION, AND RESIDUE TESTING IN BEEF CATTLE

Submitted by

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ABSTRACT

RACTOPAMINE WITHDRAWAL, DEPLETION, AND RESIDUE TESTING IN BEEF CATTLE

Studies were conducted to evaluate use of ractopamine hydrochloride (RH) in beef cattle production and the effect of various withdrawal times and depletion periods on residues in tissues and fluids collected from live and harvested animals. Primary objectives of these studies were: i) to develop and validate a LC-MS/MS assay to determine if detectable and quantifiable levels of RH can be detected in digestive tract-derived edible offal items of cattle resulted from tissue residues or residual ingesta contamination; ii) to determine presence of ractopamine in tissues after 12 h, 2, 4, and 7 days of withdrawal (in comparison to negative control cattle which did not receive RH); iii) to develop U.S. beef industry best practices for RH use for export to the Chinese market; and iv) to test the impact of withdrawal from ractopamine hydrochloride in the diets of feedlot cattle for 2, 4, or 7 days on residues for parent and total ractopamine in muscle, fat, rendered tallow, and large intestines in contrast to a true negative control group as well as validate and test feed samples to verify ractopamine presence using LC-MS/MS protocols.

In the first study, tissue samples and corresponding rinsates from 10 animals were analyzed for parent and total ractopamine (tissue samples only). The lower limit of quantitation was between 0.03 - 0.66 ppb depending on tissue type, and all tissue and rinsate samples tested had quantifiable concentrations of ractopamine. The greatest concentration of tissue specific ractopamine metabolism (represented by higher total vs. parent ractopamine levels) were observed in liver and small intestine. Contamination from residual ingesta (represented by

detectable ractopamine in rinsate samples) only was detected in small intestine, with a measured mean concentration of 19.7 ppb (+/- 12.2 ppb). Taken together, these results underscored the importance of the production process and suggested that improvements may be needed to reduce likelihood of contamination from residual ractopamine in digestive tract-derived edible offal tissues for market.

In the second study, liver and muscle samples were collected after 2, 4, and 7 days of withdrawal from RH due to regulatory issues surrounding 12-h samples. Parent and total ractopamine residues in individual liver samples ranged from a minimum of 3.40 and 3.46 ppb, respectively, for the control treatment group, to a maximum of 3.54 and 14.19 ppb, respectively, for the 2-day withdrawal treatment group. For the individual muscle samples, parent and total ractopamine concentrations ranged from below the limit of quantification (0.12 ppb) in the control samples, to 1.13 (parent ractopamine) and 1.72 ppb (total ractopamine) in 2-day withdrawal samples. Therefore, overall, parent and total ractopamine concentrations detected in the liver and muscle samples fell far below the MRL set by Codex and FDA. The greatest parent and total ractopamine levels (282.40 and 289.85 ppb, respectively) were detected after 12 h withdrawal in individual large intestine samples, followed by small intestine (142.26 and 181.91 ppb, respectively) and omasum (109.70 and 116.90 ppb, respectively) samples. Because detectable levels of ractopamine were identified in tissues collected from control animals (i.e., animals not receiving RH in their ration), further research was conducted to determine potential sources of ractopamine contamination, and frequency and accuracy of testing in global markets. For example, eight feed-grade tallow samples were analyzed for parent and total ractopamine presence as a potential source of contamination, especially in cattle not receiving ractopamine in their rations. Ractopamine concentrations of 0.40 to 50.80 ppb were obtained for these tallow

samples. While this could potentially explain the detectable levels of ractopamine residues found in control samples and the fact that 7-day withdrawal did not result in non-detectable levels, further research looking at tallow recycling and residual proteins in tallow is necessary to understand the implications of contaminated tallow on residue levels across tissues. Data from the current study may be useful in developing new recommendations for RH use and withdrawal to beef cattle producers in the U.S. who intend to export to global markets.

Results from the third study revealed several items of interest, for example; RH declines rapidly in the lower GI of beef cattle, with levels below detection by day four. Additionally, there is a very small likelihood of RH cross-contamination via tallow inclusion in diets.

Finally, the fourth study indicated that RH residues can, in fact, be quite low; however, because of limits of detection which are above zero, it is nearly impossible to quantify a level as 0.00 ppb, making zero tolerance requirements insurmountable. Overall, results of these studies were promising in that they showed that RH levels were lower than once thought, but there is a long way to go before zero-tolerance requirements can be met.

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CHAPTER 1*

Literature Review

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1.1 Introduction

Animal production generating meat, milk, leather, and wool accounts for more than 50% of the value of agricultural products in the United States (USDA-NIFA, 2018). Additionally, livestock products on a global level provide an estimated 13% of total energy and 28% of protein in diets consumed (USDA-NIFA, 2018). Latest population projections by the United Nations indicate that a current global population of 7.6 billion will increase by nearly one billion people in the next 12 years (UN-ESA, 2017). By 2050, estimates suggest that we will reach a population of 9.8 billion people, and by the new millennia of 2100, we could reach up to an estimated 9.6 to 12.3 billion people (Figures 1.1 and 1.2; Gerland et al., 2014; UN-ESA, 2017). As the global population continues to increase at an alarming rate, so does the necessity to feed more people with fewer resources. Livestock and meat production will be tasked with providing a substantial amount of nutrients and high-quality protein in the future to avoid a calorie deficit globally because livestock can be used to help convert grasses to food on non-cultivable lands. For this reason, the livestock industry has relied heavily on technologies, such as anabolic implants and supplementation with beta-adrenergic receptor agonists, to aid in increased production efficiencies (Dilger, 2015; Stewart, 2013).

Urbanization and development also impact animal production as arable land mass continually decreases at a time when there is an ever-increasing need for food. Therefore,

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production efficiency and sustainability have become major focuses for livestock producers. Overall, the goal of sustainable agriculture is to meet society's current food and textile needs without compromising the ability of future generations to meet these needs (Kuhlman and Farrington, 2010; Spiertz, 2010). While the challenge of feeding a growing population is clear, the main concern is whether it can be done sustainably, equitably, and quickly enough to keep up with the growing demand for other resources, such as biofuels (Spiertz, 2010). Agriculturalists have been faced with the dilemma of keeping up with the burgeoning demand for bio-based commodities (food, feed, fiber, and fuel) while also complying with, and satisfying stricter constraints in regard to, product safety and environmental impact (Spiertz, 2010). So, what can be done to face these challenges head on?

Two primary technologies used to more sustainably and efficiently produce livestock and meat are anabolic implants and dietary supplementation with beta-adrenergic receptor agonists, otherwise known as beta-agonists (Johnson, Ribeiro, and Beckett, 2013). Beef cattle producers have used growth promotants for more than 50 years, although the technologies have improved over that period of time. These compounds make animals more efficient by increasing average daily gain of beef cattle with less feed, known as feed efficiency, and thus enhancing the amount of lean muscle produced per unit of inputs (Johnson, Ribeiro, and Beckett, 2013). Anabolic implants and beta-agonists used in the United States have been embraced by livestock producers due to growth-promoting characteristics which create economic benefits while also allowing for more sustainable animal production (Centner, Alvey, and Stelzleni, 2014). It is estimated that 90% of agricultural growth, to feed an ever-growing population over the next several decades, must come from more intense production on land currently used for agricultural purposes (Neumeier and Mitloehner, 2013).

Utilization of production technologies, or biotechnologies, has the potential to help in this regard due to improved animal growth, lean yield, and feed efficiency using the same land mass (Neumeier and Mitloehner, 2013). In fact, Stackhouse et al. (2012) demonstrated that use of growth-promoting technologies in feedlot systems in California decreased the carbon footprint, ammonia emissions, and cost of beef production. Beta-agonist use during cattle feeding decreased ammonia emissions, resulting in a 7% decrease in ammonia loss from the full production system (Stackhouse et al., 2012). When a combination of ionophores, hormonal growth implants, and beta-agonists were used, ammonia emissions were further decreased and the carbon footprint was decreased by 2.2 kg carbon dioxide equivalent (Stackhouse et al., 2012). Decreased emissions and improved efficiency improve the overall sustainability of livestock production through generation of more lean protein production (meat and by-products) with fewer inputs, such as grain, water, and land mass (Anderson et al., 2004; Dilger, 2015).

Unfortunately, the shift of consumer preference and political policy positions to natural and organic food production, both in the United States and abroad, has generated trade barriers for products from animals receiving anabolic implants, beta-agonists, and other biotechnologies. For this reason, it is advantageous for politicians, consumers and producers to better understand these technologies and the challenges associated with them in trade.

1.2 Production Technologies

Use of anabolic steroids, in the form of time-releasing ear implants, have been approved by the United States Food and Drug Administration (FDA); they are characterized as safe and effective growth-promoting agents. Hence, producers implant more than 90% of all feedlot cattle in the United States (Johnson, Ribeiro, and Beckett, 2013; USDA-APHIS, 2013). Since 1954, the FDA has subjected all anabolic implants to strict scrutiny before approval (Stewart,

2013) via the New Animal Drug Application (NADA) process. This process requires demonstration and validation that new drugs do not harm people who consume the animal, the animal itself, nor the environment, and that they work as intended (FDA, 2017). Additionally, the FDA uses scientific data to establish acceptable limits for the compounds in meat so that human consumption does not lead to harmful effects (FDA, 2017; Stewart, 2013). The anabolic agents used in beef cattle implants consist mainly of three naturally occurring hormones (estradiol, progesterone, and testosterone) and two synthetic hormones (zeranol and trenbolone acetate; Stewart, 2013).

These compounds are necessary for normal development, growth, and reproduction of humans and food animals, meaning that people are generally not at risk from consuming food from animals treated with the small quantities that are used to improve efficiency of production with these implants because the additional hormone concentrations present following treatment are miniscule compared to the hormones already generated endogenously in animals and that are normally found in meat products (FDA, 2017). Synthetic hormones must also go through a tedious approval process involving information and/or toxicological testing of laboratory animals to determine safe levels in edible animal products (FDA, 2017).

Implants are small pellets which contain growth promotants that are released gradually over time, thus increasing the circulating levels of somatotropin and insulin-like growth-factor 1 (Stewart, 2013). Growth hormone is then secreted at an accelerated rate, leading to augmented muscle development (Stewart, 2013). Three different hormonal implant strategies exist: androgenic implants, such as trenbolone acetate (TBA) and testosterone; estrogenic implants, such as estradiol 17-β (E_2), estradiol benzoate, and zeranol; and combination implants (androgen plus estrogen), such as TBA plus E_2 (Johnson, Ribeiro, Beckett, 2013). In general, use of

hormonal implants has been shown to increase growth rates by up to 28% while improving feed efficiency and lean muscle mass by up to 20% and 10%, respectively (Johnson, Ribeiro, and Beckett, 2013; Stewart, 2013). Furthermore, there is an additive effect when utilizing combination estrogenic/androgenic implants (Stewart, 2013). In fact, feed efficiency is improved an additional 6 to 14% with combination implants versus estrogen-only implants (Stewart, 2013). It is also estimated that combination TBA/E_2 implants increase carcass protein by 10% compared to non-implanted steers, assisting in sustainability of production (Johnson, Ribeiro, and Beckett, 2013).

Yet another production technology that improves sustainability in livestock production is dietary supplementation with beta-agonists, which are used in feed during the last three to six weeks of finishing (generally for 28-42 days). Beta-agonists are feed additives which are used to improve feed efficiency and promote growth in livestock (Kootstra et al., 2005). Beta-agonists are synthetic compounds which bind to G protein-coupled beta-receptors on cell surfaces in muscle, fat, and other tissues of animals, including humans and livestock (Johnson, 2014; Mersmann, 1998). When beta-agonists bind to adrenergic receptors on cells, they increase muscle mass via hypertrophy, while also decreasing fat accretion/lipid synthesis (Neumeier and Mitloehner, 2013). In other words, beta-agonists lead to increased protein synthesis and decreased muscle protein degradation (Mersmann, 1998) and fat production—hence, some refer to them as "repartitioning agents" because they repartition how nutrients are utilized in metabolism. Beta-agonists are used in human medicine for a number of reasons, such as asthma treatment, but are strictly used as growth promotants in livestock production as they enhance growth and alter body composition (Anderson, Moody, and Hancock, 2004; Mersmann, 1998). Beta-agonists in livestock production stimulate skeletal muscle growth without an increase in

natural hormone levels (Centner, Alvey, and Stelzleni, 2014). Livestock producers have utilized this technology to increase body weight of swine and cattle, eventually leading to heavier carcasses and thus more meat production.

Ractopamine hydrochloride and zilpaterol hydrochloride are the two beta-agonists approved by the FDA for use in food animal species in the United States (Dilger, 2015). Ractopamine is approved for use in swine, turkeys, and cattle and binds to beta-1 receptors, whereas zilpaterol is only approved for use in cattle and binds to beta-2 receptors (Arp et al., 2014; Centner, Alvey, and Stelzleni, 2014; Dilger, 2015). These beta-agonists are also approved for use in other countries, such as Brazil, Canada, South Korea, and Mexico; however, they have been banned in several places, as well, such as China and the European Union (Dilger, 2015). In the United States, zilpaterol currently is not used in any feeding systems. In a meta-analysis of research data that included more than 50 comparisons for both ractopamine and zilpaterol, dietary supplementation in cattle presented notably increased weight gain, hot carcass weight, *Longissimus* muscle area, and efficiency of gain to feed (Lean, Thompson, and Dunshea, 2014).

Ractopamine and zilpaterol were both subjected to approval processes similar to the FDA's approval process for anabolic implants. A NADA begins with the United States FDA to ensure safety and effectiveness, and then it is extended to the Center for Veterinary Medicine. From there, the Office of New Animal Drug Evaluation investigates the drug and surveillance and compliance data are accumulated. This process is overseen by FDA veterinarians, animal scientists, biologists, and toxicologists, and takes several years before a decision is made based on scientific evidence. It is a robust system that leaves no credible reason to believe that the drugs that are used are unsafe. According to the makers of livestock growth promoting

technologies, today, the NADA process can take up to 20 years to result in a newly approved technology, and can cost in excess of $25-$100 million.

Ractopamine, specifically, underwent an extensive approval process through the FDA in order to calculate the no-observed-adverse-effect-level (NOEL or NOAEL; 0.125 mg/kg/day) and the acceptable daily intake (ADI; 1.25 μg/kg/day), which was completed in December of 1999 (FDA, 1999). Its use as a growth promotant was approved in 2000; since then, however, ractopamine use has remained contentious (Centner, Alvey, and Stelzleni, 2014). After years of scientific review, the Codex Alimentarius Commission, an intergovernmental food standards-setting body with over 180 members, voted to adopt a maximum residue limit (MRL) by a narrow vote of 69 to 67 in 2012 (Bottemiller, 2012). The Codex Alimentarius Commission considers recommendations from the Joint FAO/WHO Expert Committee on Food Additives and scientific evidence when voting to adopt maximum residue limits. This vote made it significantly less difficult for countries with higher tolerances, such as the United States and Canada, to challenge those with zero tolerance residue policies associated with trade for ractopamine residues in meat products because these policies are more restrictive than the global standard (Bottemiller, 2012). Countries with zero tolerance policies include China, the European Union, and Taiwan (Bottemiller, 2012). In addition, as procedures for importing beef tissues (and particularly beef liver) in Egypt evolve, restrictions in that country are increasing. Even with an international Codex Alimentarius standard, there have been instances in which exports from the United States (and other countries) into countries with zero tolerance policies were denied due to ractopamine levels that were under the global MRL. And, too, the sample handling and testing methods in such countries can be contentious as they impact results of testing. This controversy

remains relevant as ractopamine hydrochloride is still commonly fed to livestock; issues with zilpaterol are less relevant as the compound is not currently used in North America.

Notwithstanding the lack of use, zilpaterol also has undergone the NADA approval process; however, maximum residue limits/tolerances have not yet been adopted for this beta-agonist (Arcella et al., 2016) because, when this compound is used, there generally is a 2-day withdrawal from the compound before shipment to packing plants. The Joint FAO/WHO Expert Committee on Food Additives has recommended maximum residue limits for zilpaterol in cattle based on several different assessments of the scientific literature, but the Codex Alimentarius Commission has not voted on the issue (Arcella et al., 2016). The recommended limits for cattle are 3.3 μg/kg (or ppm) in kidney, 3.5 μg/kg in liver and 0.5 μg/kg in muscle (Arcella et al., 2016).

Although zilpaterol was approved as a feed additive for beef cattle in the United States in 2006, several reports of animal welfare concerns arose in the summer of 2013 (Boyd et al., 2015). Consequently, the manufacturer removed zilpaterol from the market as it did not want to contribute to animal welfare problems. Zilpaterol has been linked to increased respiration rates and panting scores in cattle, and also lameness (Boyd et al., 2015; Grandin, 2014). It is important to note that correlation does not equal causation, and research on this topic clearly states that these issues are likely multifactorial (Boyd et al., 2015; Grandin, 2014). Nevertheless, any drug that does, in fact, contribute negatively to animal welfare cannot be administered.

Before animal welfare related to beta-agonist feeding became a concern, multiple studies were conducted investigating the additive effects of feeding ractopamine or zilpaterol in conjunction with administration of an anabolic implant. Baxa et al. (2010) investigated effects of zilpaterol hydrochloride and the steroidal implant Revalor-S (a combination implant of

trenbolone acetate and estradiol 17-β) on feedlot performance, carcass characteristics, and skeletal muscle composition in finishing steers. When compared to control cattle, those receiving only zilpaterol and those receiving only the Revalor-S steroidal implant exhibited improvements in growth performance and carcass characteristics (Baxa et al., 2010). As expected, cattle that received the combination treatment presented the greatest increase in average daily gain and feed efficiency, as well as an additive increase in hot carcass weight, longissimus multifidus area, and dressing percentage (Baxa et al., 2010). A similar study investigating performance of finishing beef steers in response to anabolic implant and zilpaterol hydrochloride was conducted with differing concentrations of trenbolone acetate and estradiol (Parr et al., 2011), with similar results of additive responses.

In the Parr et al. (2011) study, the higher dose of trenbolone acetate plus 17-β estradiol with a more gradual release period ameliorated steer performance and hot carcass weight (Parr et al., 2011). Bryant et al. (2010) conducted yet another study comparing effects of ractopamine and steroidal implants with differing trenbolone acetate and 17-β estradiol concentrations in finishing steers. Adapted results from this study are shown in Figure 1.3. Holding days on feed constant and compared to control cattle, average daily gain increased by 21% with one anabolic implant; over time this effect was amplified with a second anabolic implant to nearly 27%, and another 2% with two anabolic implants and dietary administration of ractopamine for the last 28 days of feeding (Bryant et al., 2010). This cumulative and additive effect on growth performance and carcass traits also was observed for gain to feed ratios (feed efficiency) and carcass weight, with carcass weight increased by more than 100 pounds compared to control cattle (Bryant et al., 2010). These increases alone could lead to an economic benefit of hundreds of dollars, albeit which is dependent on market values.

Despite the international trade-related controversy surrounding use of beta-agonists as growth-promotants, the benefits to sustainability and animal production are obvious. The looming trade issues associated with evolving and ever-better abilities to detect extremely low concentrations of residues in tissues, coupled with unscientific import policies, could impact future ractopamine use.

1.3 Trade Barriers to Exports

Unfortunately for livestock producers in the United States, various barriers to trade, both tariff and non-tariff, adversely affect export markets and create caustic disputes with other countries. One of the barriers results from banning the use of certain growth compounds; China alone lists 146 controlled compounds. These banned products include, but are not limited to, anabolic growth-promoting implants and beta-agonists (along with melengestrol acetate and many more compounds), often with a lack of scientific evidence to support the decision-making process.

One of the most hotly-contested trade bans in the world resulted from the 1988 European Third-Country Directive that essentially restricted use of natural hormones to strictly therapeutic treatments, while banning utilization of synthetic anabolic agents and the importation of both implant-treated animals and meat from animals to which implants were administered (Johnson, 2015). This ban was implemented despite conclusions published in several reports by a Scientific Working Group of 22 notable European scientists that was formed by the Commission of the European Communities (the forerunner to the European Union; EU) and led by Professor G. E. (Eric) Lamming of the U.K., that clearly refuted any human health consequences of using anabolic growth technologies in livestock production. By 1989, the European Union fully implemented this ban on meat and meat products from animals which were administered

anabolic growth promotants (Johnson, 2015). This created a major disturbance in meat trade between the United States (along with other countries) and the European Union. While there have been many attempts to resolve the issue through World Trade Organization dispute consultations, settlement panels, formal appeals, and arbitration proceedings, it remains problematic to this day as the EU, although losing all attempts to maintain the ban in the WTO, has retained policies to prevent the use of such growth technologies.

In addition to the ban on use of anabolic compounds, use of beta-agonists as growth promotants in farm animals also was banned in the European Union in 1996 (Centner, Alvey, and Stelzleni, 2014). This ban occurred before the European Union conducted any research regarding ractopamine or zilpaterol and prior to beta-agonists entering the market as growth promotants in livestock (Centner, Alvey, and Stelzleni, 2014). Nearly 10 years later, after the JECFA reconfirmed the acceptable daily intake and maximum residue limits of ractopamine, the European Food Safety Authority began an investigation to evaluate the compound's safety because it had not done so before adding it to the list of banned veterinary drugs (Bories et al., 2009). Their investigation considered available information about ractopamine from previous research, including studies examining effects on laboratory animals, dogs, monkeys, pigs, cattle, and humans (Bories et al., 2009). Although no new research was conducted, panelists concluded that the detailed investigation did not provide enough evidence to overturn the ban because it was not clearly stated that the consumption of ractopamine residues by humans was safe (Bories et al., 2009), despite approval by FDA in the United States. Hence, the ban was political, as there was no reason to believe that there was a risk to humans, and therefore the decisions resulted in a non-tariff barrier to trade. Nonetheless, the issue will not dissipate any time soon,

and especially as more countries push for zero tolerance, or other non-scientific protocols, relative to beta-agonist residues.

Trade with China also remains contentious because of banned products, which include zearanol, trenbolone acetate, and beta-agonists. The Chinese banned products are of significant concern because most North American cattle feeders administer melengestrol acetate as a feed additive to heifers for the purpose of suppressing estrus while increasing body weight; this is a banned compound for beef production in China, which completely closes this market to any cattle North American producers that feed heifers.

Ractopamine residues have been in the news frequently since maximum residue limits were approved by Codex. Directly after Codex voted to approve the international standards for ractopamine limits, Michael Hansen of Consumers International asserted, "We are concerned that with this vote, Codex is becoming another politicized global body, rather than the science-based consensus body it has managed to be so far" (Bottemiller, 2012). However, the chief veterinarian for the National Cattlemen's Beef Association stated, "It is paramount that science is the foundation for all decisions made in the international community. Today, the Codex commission proved they are willing to trust science and make decisions based on facts rather than politics" (Bottemiller, 2012). Regardless of what side of the issue one is on, it is clear that ractopamine residues have been, and will continue to be, a challenge to the meat industry.

Ractopamine is especially problematic in exported products because tissue concentrations of the compound can be affected by enzymatic reactions that occur during tissue handling. "Parent ractopamine" is the amount of the compound detected in tissues that result directly from the feed additive itself (Churchwell et al., 2002; Elanco, 2003). But, some countries choose to test for "total ractopamine," which reflects the combination of "parent" ractopamine plus its

metabolites (Churchwell et al., 2002). In some postmortem tissues, ractopamine metabolites can undergo enzyme hydrolysis in the presence of beta-glucuronidase to artificially increase the amount of parent ractopamine detected; the consequence can be additional and misleadingly-high parent ractopamine concentrations in tissue—perhaps leading to rejection by the importing country (Smith and Shelver, 2002). Beta-glucuronidase is an enzyme that can transform endogenous and exogenous compounds into toxic compounds and otherwise troublesome compounds (Looijer-van Langen and Madsen, 2010). The amount by which tissue concentrations of parent ractopamine are increased is dependent on the tissue, the amount of metabolites present, and the amount of beta-glucuronidase that is in the tissue, along with the temperature at which tissue samples are maintained (higher temperatures result in more rapid conversion of metabolites to parent compound); liver has especially high concentrations of beta-glucuronidase. This is also true in humans, and it has been shown that variability among individuals is quite striking, making a case for the large biological variability in animal studies as well (Sperker et al., 1997). As an example of how this may result in export problems, consider a customs port in Egypt (which is extremely warm) where over 80% of livers from the United States are shipped; sample handling in Egypt is critical to export success if inspectors test for parent ractopamine concentrations.

In addition, maximum residue limits (MRLs) set by the FDA are based on concentrations of parent ractopamine levels rather than total levels in the target tissues on which the NADA was based (mainly, liver and muscle) (Elanco, 2003). Where Codex Alimentarius is concerned, it is much more difficult to determine whether MRLs are based on parent or total as these data are not included in the standard (Codex, 2015). The main concern for producers in the United States who are exporting to countries with limits lower than the regulatory requirements is that the tests

used are often specifically for total ractopamine, which results in an escalated quantifiable ractopamine residue that is misleading, and they may be testing off-target tissues to which the MRLs should actually not apply.

Moreover, cross-contamination potential for compounds that are provided to animals in feed are not uncommon in feedlots or at processing plants. In other scenarios, unexpected results of testing frequently occur. Zeranol, a naturally occurring estrogen-like mycotoxin, can be detected in cattle (and particularly in their livers) that have never received a zeranol anabolic implant treatment (i.e., Ralgro) because zearalenone can be generated by certain *Fusarium* species in grains that are fed to the cattle (Kennedy, 1998). At processing plants, further contamination of hormones and beta-agonists can occur when residual compounds are transmitted from gastrointestinal tissues onto processing equipment, leading to positive tests on subsequently cross-contaminated tissues that are not necessarily representative of the actual residual amount.

1.4 Conclusions

Use of growth technologies has helped the livestock and meat industries to make great strides towards sustainability and efficiency of production. It is necessary that such technologies continue to be used if we are to provide high-quality, nutrient-dense proteins to a rapidly growing global human population. Regrettably, not all policies (and, particularly, import policies) around the globe agree about the appropriateness of use for growth technologies. Research on this topic is ongoing, but it may be necessary to establish acceptable MRLs (even as marketing conditions) for off-target tissues if such tissues are to be continually tested for ractopamine concentrations, and particularly if they are tested for total ractopamine concentrations, in destination markets. In the meantime, livestock producers around the world,

and particularly in the United States, are going to have to look for different ways to recover the losses incurred through the lost opportunity of exporting to countries with rigorous zero-tolerance ractopamine requirements for imports. They should also be cautious about cross-contamination and false positives that may occurs as a consequence of other compound sources. Because the United States relies heavily on beef and pork exports, these marketing obstacles create tremendous barriers to trade. Implementation of current production technologies in the livestock industry leads to an additional $250 per head advantage over the animals not receiving growth promotants. If cattle were to average 1400 pounds at a value of $1.50/hundredweight, this would be the equivalent of a 17% increase in value due to the use of growth promotants (a combination, not just ractopamine). This advantage is meaningless, however, if export markets are lost because of banned or otherwise controlled compounds. The countries involved in the current debate surrounding growth promotants must find common ground so that proper nutrition can be delivered in a safe, economical, and sustainable manner.

1.5 Justification

In general, these studies were conducted to better understand the implications of ractopamine usage for U.S. beef production and future exports as zero tolerance standards are further explored and implemented. It was imperative that withdrawal times, differing sources of contamination, and the odds of various tissues testing above or below the MRL were investigated. In past research, ractopamine has been scrutinized from a health concern standpoint, although this has primarily been a trade barrier and not food safety issue (Centner, Alvey, and Stelzleni, 2014). Therefore, in these studies, it was critical to examine the true

residual contamination in various tissues and fluids, often after withdrawal, to determine the risk of ractopamine presence above the MRL.

Subjecting animals in these studies to withdrawal brings up another important point, however. The on-label use of ractopamine hydrochloride according to the NADA supports a zero-day withdrawal time, making any deviations from that off-label or extra-label (Elanco, 2003). According to the Veterinary Feed Directive, off-label or extra-label use of medicated feed is prohibited (FDA, 2015a). Because of the issues surrounding export and residues above the MRL for tissues such as the abomasum, many processors have moved toward a 48 h ractopamine withdrawal requirement for harvested cattle. For this reason, 2-day, 4-day, and 7-day withdrawal periods were investigated in these studies; however, this is actually off-label and could cause regulatory challenges. Moreover, although the results from these studies suggest that increased withdrawal times may be beneficial in achieving ractopamine levels below the MRL, it would be precarious to make such a recommendation with the current on-label use in place.

Lastly, it is unclear at what point the economic benefit of utilizing ractopamine hydrochloride no longer has an impact. Nearly all easily accessible data regarding the economics of ractopamine are relative to a zero-day practical withdrawal time because, as previously mentioned, applying or requiring a longer withdrawal time is considered off-label use (Elanco, 2003). According to the U.S. FDA Guidance for Industry 207, a practical zero withdrawal represents the shortest amount of time between the administration of the last dose of a drug and slaughter (FDA, 2015b). Once again, because most data are associated with the practical zero withdrawal and commonly consumed tissues, it is difficult to determine the point in time at which there is no longer a benefit to producers if an extended withdrawal were the only way to achieve ractopamine residue concentrations below the MRL for muscle.

The studies presented in this dissertation were thus extraordinarily important in realizing what withdrawal times were most effective, even though a withdrawal time cannot necessarily be recommended currently. Further, these studies helped bring about an awareness of potential sources of contamination in beef cattle production, such as tallow and meat and bone meal. Ultimately, the work displayed odds associated with exporting a large number of offal tissues that would otherwise be worth much less for consumption in the U.S. Consequently, this research cannot be ignored.

Table 1.1. United States Food and Drug Administration and Codex maximum residue limits (MRLs) for ractopamine hydrochloride in regulatory tissues for beef.

Tissue	**U.S. FDA (ppb)**	**CODEX (ppb)**
Kidney	N/A	90
Liver	90	40
Fat	N/A	10
Muscle	30	10

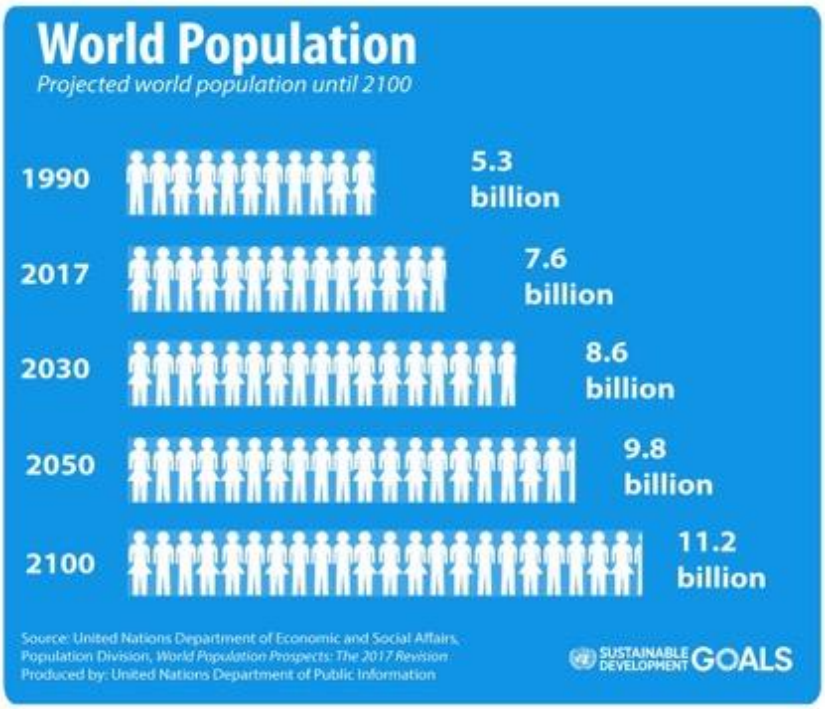

Figure 1.1. Projected world population from 1990 to 2100.

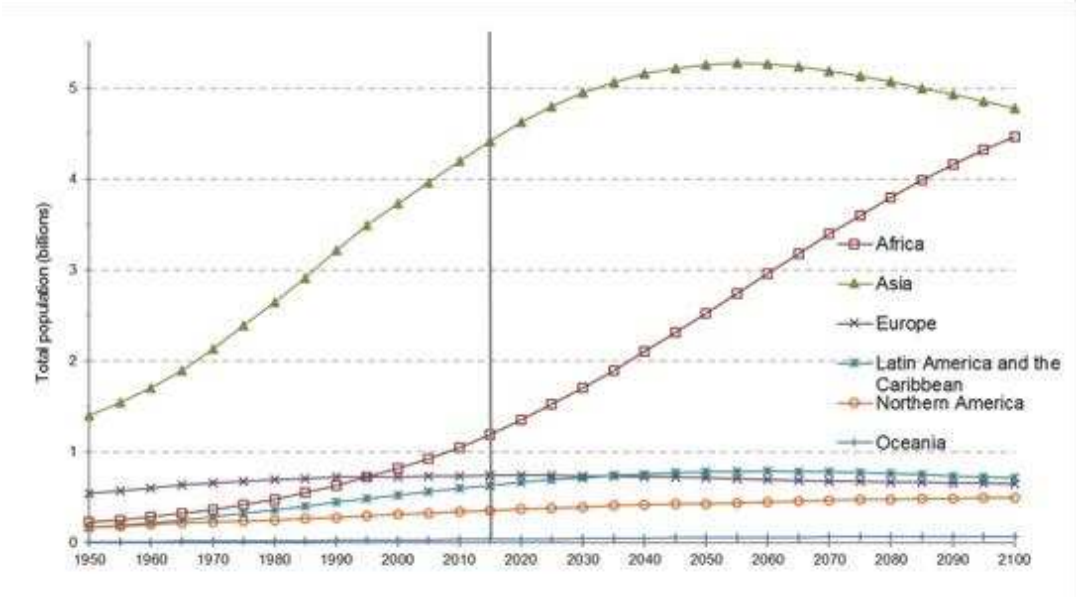

Figure 1.2. Levels and trends of the world's population by region. Source: UN-ESA World Population Prospects (2017).

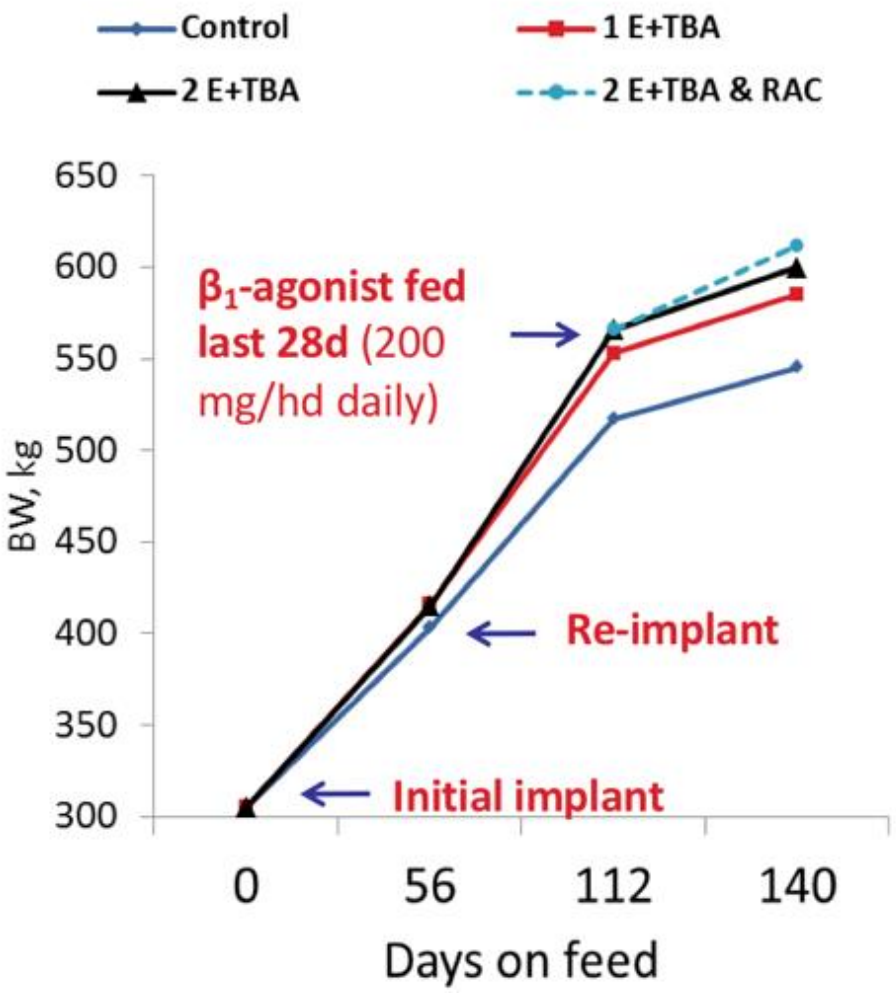

Figure 1.3. Additive effect of growth enhancement during finishing via steroidal implants and beta-agonists (E + TBA: 17-β estradiol plus trenbolone acetate; RAC = ractopamine hydrochloride). Adapted from Bryant et al., 2010.

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CHAPTER 2**

Quantification of ractopamine residues on and in beef digestive tract tissues

Summary

Ractopamine hydrochloride is a commercial beta-adrenergic agonist commonly used as a dietary supplement in cattle production for improved feed efficiency and growth promotion. Currently, regulatory target tissues (as approved in the New Animal Drug Application with FDA) for ractopamine residue testing are muscle and liver. However, other tissues have recently been subjected to testing in some export markets for U.S. beef, a clear disregard for scientific maximum residue limits associated with specific tissues. The overall goal of this study was to develop and validate a LC-MS/MS assay to determine whether detectable and quantifiable levels of ractopamine in digestive tract-derived edible offal items (i.e., abomasum, omasum, small intestine, and reticulum) of cattle resulted from tissue residues or residual ingesta contamination of exposed surfaces of tissues (rinsates). Tissue samples and corresponding rinsates from 10 animals were analyzed for parent and total ractopamine (tissue samples only). The lower limit of quantitation was between 0.03 - 0.66 ppb depending on the tissue type, and all tissue and rinsate samples tested had quantifiable concentrations of ractopamine. The highest concentration of tissue specific ractopamine metabolism (represented by higher total vs. parent ractopamine levels) were observed in liver and small intestine. Contamination from residual ingesta (represented by detectable ractopamine in rinsate samples) only was detected in small intestine,

** A large portion of Chapter 2 has already been published at: Davis, H.E., C. Badger, P. Brophy, I. Georgnaras, T.J. Burnett, J. Scanga, K.E. Belk, and J. Prenni. 2019. Quantification of ractopamine residues on and in beef digestive tract tissues. *J of Anim Sci*. https://doi.org/10.1093/jas/skz263.

with a measured mean concentration of 19.72 ppb (+/- 12.24 ppb). Taken together, these results underscore the importance of the production process and suggest that improvements may be needed to reduce the likelihood of contamination from residual ractopamine in digestive tract-derived edible offal tissues for market.

Introduction

Beta-adrenergic agonists, otherwise known as beta-agonists, are commonly used in the livestock industry for growth promotion (Anderson, Moody, and Hancock, 2004; Kootstra et al., 2005). One type of beta-agonist, ractopamine hydrochloride, is a phenethanolamine compound, similar to endogenous catecholamines, and has been approved for use as a growth promotant in food-animal production in several countries (Johnson, Ribeiro, and Beckett, 2013). These synthetic compounds bind to G protein-coupled beta-receptors on differing cell surfaces (e.g., muscle and fat) in livestock (Mersmann, 1998; Johnson, 2014), increasing muscle mass via hypertrophy while also decreasing fat accretion/lipid synthesis. Ractopamine hydrochloride (RH; Elanco Animal Health, Greenfield, IN) is a commercial beta-adrenergic agonist commonly used as a dietary supplement in cattle production for improved feed efficiency and growth promotion.

When beta-agonists such as ractopamine are used in livestock production, they are known to increase protein synthesis while decreasing degradation of protein and production of fat (Mersmann, 1998). For this reason, beta-agonists are sometimes referred to as repartitioning agents because they are capable of altering utilization of nutrients during animal metabolism, for example from adipose tissue toward muscle (Anderson et al., 2004). While beta-agonists are used for disease treatment in human medicine (e.g., for respiratory challenges such as asthma), they are used strictly for growth promotion in food-animal production (Mersmann, 1998; Anderson et al., 2004).

As the world population continues to increase, it is becoming increasingly important to produce more nutritionally-rich food with fewer resources (K.C. et al., 2018). In fact, sustainably feeding the next generation is one of the most crucial challenges of the 21st century (K.C. et al., 2018). Because beta-agonists have the ability to stimulate skeletal muscle growth without increasing hormone levels, cattle and swine producers have widely adopted this technology to improve meat yield (Centner, Alvey, and Stelzleni, 2014). In 2000, use of ractopamine for the purpose of increasing muscle, reducing fat, and promoting better feed efficiency in animals was approved by the U.S. Food and Drug Administration (FDA). Since then, it has been used in livestock production in over 20 countries, but concerns remain regarding potential human health risks (Centner, Alvey, and Stelzleni, 2014). Furthermore, because of multiple geo-political issues, the European Union, China, and Russia have restricted and even banned use of ractopamine, as well as the importation of meat with detectable levels of ractopamine (Bories et al., 2009; Centner, Alvey, and Stelzleni, 2014). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) is a scientific authority that assesses safety of residues of veterinary drugs in food (Centner, Alvey, Stelzleni, 2014). The Codex Alimentarius Commission then utilizes these assessments and recommendations to adopt maximum residue limits (MRLs) as international standards (Centner, Alvey, and Stelzleni, 2014). The JECFA has reviewed residue safety data several times (JECFA, 1992; JECFA, 2004; JECFA, 2006; JECFA, 2010) and recommended an Acceptable Daily Intake (ADI) for consumers and MRLs for edible tissues. Maximum residue limits for ractopamine were thus developed through this lengthy and scientifically rigorous process for muscle, liver, kidney, and fat tissues and were adopted in 2012 (Codex, 2015). No such limits exist for digestive tract-derived edible offal tissues or other edible tissues such as

tendons and bones, creating concerns in export markets where these tissues are more frequently imported and consumed (Centner, Alvey, and Stelzleni, 2014).

Currently, global standards for ractopamine detection are based on analysis of intact ractopamine ("parent") related to the feed additive itself. In 2012, a candidate method recognized by the Expert Review Panel of AOAC International (AOAC, 2015) was developed and validated for quantitation of ractopamine in bovine, swine, and turkey tissues using liquid chromatography coupled with tandem mass spectrometry (Burnett et al., 2012; Ulrey et al., 2013). Nine separate laboratory groups were then tasked with analyzing bovine muscle, bovine liver, swine muscle, swine liver, and turkey muscle to quantify ractopamine utilizing this method (Ulrey et al., 2013). Based upon AOAC benchmarks, this method was deemed acceptable due to reproducibility among these laboratories and tissues (Ulrey et al., 2013). The method received Final Action status at the end of 2012 and is a major testing practice utilized globally for ractopamine quantitation (Ulrey et al., 2013). Alternative testing methods approved by the U.S. Department of Agriculture for detection of parent ractopamine include the commercially available enzyme-linked immunosorbent assay (ELISA) kit developed by Randox Food Diagnostics (Randox Laboratories Ltd.; Crumlin, United Kingdom) (Randox, 2018). However, cattle have been shown to metabolize ractopamine hydrochloride into four major metabolites – two ring A monoglucuronides, one ring B monoglucuronide, and one diglucuronide (Elanco, 2003; Tang et al., 2016). These glucuronide metabolites can be disrupted/deconjugated by beta-glucuronidase (Tang et al., 2016), releasing the free ractopamine for detection. Thus, enzymatic treatment of the tissue enables detection of the total ractopamine pool which represents the free ractopamine plus the glucuronide metabolites. It is important to note that while the enzymatic hydrolysis increases the sensitivity to detect ractopamine in tissues, the total ractopamine pool is not

appropriate for direct comparison to MRLs as these limits were established based on detection of the marker residue (free ractopamine; Burnett et al., 2012).

This study fills an important knowledge gap in our understanding of total ractopamine residue accumulation in offal tissues, and the potential for cross contamination with the drug to occur during processing. Here, we present a validated LC-MS/MS assay for quantification of parent and total ractopamine in multiple tissue types, including liver, muscle, abomasum, omasum, small intestine, and reticulum, and evaluate the hypothesis that residual ingestate could contribute to the detection of ractopamine in digestive tissues. Results of this work improve our understanding of the levels of ractopamine residues in beef tissues that are reflective of global eating habits.

Materials and Methods

Sample collection and storage. Individual heifers (N = 10), originating from a commercial feedlot producer which fed ractopamine HCl (24.6 g/ton for 32 days) according to U.S. label direction (fed right up to loading; < 4 h between loading to stun), were identified during the slaughter process. Samples from the digestive tract (abomasum, omasum, small intestine, and reticulum) as well as muscle and liver were collected following complete commercial processing in a large-scale harvest facility. Samples (minimum of 100 g tissue) were placed into sterile sample bags, placed on ice and transported to Colorado State University (Fort Collins, CO) for processing and analysis. Additionally, five known ractopamine HCl-free tissue samples (one of each for muscle, liver, omasum, small intestine, and reticulum) were collected and then pooled to be used as a matrix background for each tissue type.

Materials. Ractopamine HCl certified reference standard (1.0 mg/ml) was purchased from Sigma-Aldrich (St. Louis, MO). Ractopamine-d6 HCl internal standard (1 mg with exact

weight packaging) was purchased from Toronto Research Chemicals (North York, ON, Canada). β-Glucuronidase (from Helix pomatia, type HP-d, aqueous solution, ≥ 100,000 units/ml) and sodium acetate (NaOAc) was purchased from Sigma-Aldrich (St. Louis, MO). Ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO), water (LCMS grade), methanol (LCMS grade), formic acid (Pierce LCMS grade) and acetonitrile (LCMS grade) were purchased from Thermo Fisher Scientific (Waltham, MA).

Sample preparation. One hundred to 150 g of digestive tract tissue (abomasum, omasum, small intestine, and reticulum) was weighed into a sterile plastic zip bag and 1 ml LCMS-methanol per g of tissue was added to generate the rinsate samples. The tissue was submerged, shaken, and massaged for 1 min to release any remaining ingesta from the external tissue surface. Tissue was then carefully transferred to a clean cutting surface and liquid (rinsate) was decanted into a clean pre-weighed 250 ml conical tube. The rinsate was frozen for at least 3 h at -80°C and then lyophilized for 24 - 48 h. The rinsed tissue was chopped into small (~ 3 × 3 cm) pieces, flash frozen in liquid N_2, and homogenized using a Robot Coupe Blixer V4 (Robot Coupe USA; Jackson, MS). Muscle and liver tissue were homogenized in the same way without rinsing. Two subsamples (5 +/- 0.5 g) of each tissue type homogenate were obtained and placed into separate 50 m conical tubes. Tissue homogenate and lyophilized rinsate were stored at -80°C until extraction. Rinsate solids were resuspended at 1 ml per 10 mg and tissue homogenate at 4 mL per 1 g with methanol containing 25 ng/mL of the internal standard (IS). Samples were sonicated for 30 min, vortexed at 4°C for 10 min, and incubated for 30 min at -80°C. The samples were centrifuged at 3,000 × *g* for 10 min to remove remaining solids. The supernatant was transferred and two aliquots of 1 mL were collected in microcentrifuge tubes for analysis (the remaining supernatant was stored at -80°C). The 1 ml aliquots were centrifuged again at 4°C

for 10 min at 12,000 × *g* and the supernatant was transferred to glass vials. One aliquot was analyzed directly for parent ractopamine and the other was processed as described below for total ractopamine analysis.

The 1 ml supernatant aliquot from the extraction above was evaporated to dryness under nitrogen. The sample was resuspended in 200 µl of 25 mM NaOAc buffer (pH 5.2). Four µl β-glucuronidase was added and the sample was mixed thoroughly by gentle vortex. The sample was incubated at 65°C for 2 h in a sand bath. Four hundred µl of methanol were added and the sample was mixed thoroughly by gentle vortex followed by centrifugation at approximately 2025 × *g* for 5 min. Supernatant was carefully removed and placed into a clean glass vial for LC-MS analysis.

Recovery samples (control tissues) were fortified prior to extraction with ractopamine standard equal to 5, 10, and 20 ppb and allowed to sit undisturbed for 15 – 20 min on ice before proceeding with extraction as described above.

UPLC-MS analysis. Samples were analyzed on a Synapt G2-Si Q-TOF mass spectrometer (Waters Corporation) with a standard flow ESI source coupled to a Waters Acquity I-Class UPLC equipped with a reverse phase 1.0 mm × 50 mm Waters Acquity UPLC HSS T3 column (1.8 µm particles). The UPLC was operated at 0.400 ml min^{-1} and the column temperature was thermostatically controlled at 50°C using the column heater onboard the UPLC stack. Buffer A was water (Fisher Scientific, LCMS Grade) with 2 mM ammonium formate, buffer B was acetonitrile (Fisher Scientific, LCMS Grade) with 0.1% formic acid (Pierce, LCMS Grade). One µl of sample was directly injected onto the column. The UPLC gradient is described in Table 2.1.

Data were acquired at 10 Hz across the mass range of 50-1000 m/z. Collisional energy of each identified transition was optimized in the trap cell of the TriWave (302→164.106: CE=11, 302→284.1627: CE=9). Transfer cell collisional energy was turned off and no mobility separation was conducted to maintain ion transmission efficiency. Targeted enhancement was turned on and configured to target the product ion of interest. The final method consisted of two MS/MS functions: the first targeted ractopamine (302 m/z →164.106, CE=11, Targeted Enhancement=164.106), and the second targeted the internal standard, ractopamine-d_6 (308 m/z →168.17 m/z, CE=11, Targeted Enhancement=168.17 m/z). For identification confirmation, 284.1627 m/z was utilized. These transitions agree with established method s in the literature for detection of ractopamine by LC-MS/MS (Burnett et al., 2012; Tang et al., 2016). The spectra were LockMass corrected using leu-enkephalin (Sigma Aldrich) to provide accurate mass data.

Peak picking and integration were performed using Quanlynx software (V 4.2 SCN983, Waters Corporation). Peak areas for each sample (included standard curve) were normalized to the peak area of the internal standard in that sample. Quantification of samples was performed using linear regression (no weighting) against a matrix matched external standard curve. Separate standard curves were generated for each matrix type (muscle, liver, abomasum, omasum, reticulum, small intestine and rinsate) from control tissue fortified with ractopamine standard post extraction (ranging from 0.1-50 ng/ml). Accuracy and precision were determined for each tissue type and the pooled rinsates based on triplicate injections of the fortified control matrix at ractopamine concentrations of 1, 5, and 25 ng/ml. The limit of detection was calculated as 3 x standard deviation of the blank signal/slope of the linear regression curve and the limit of quantitation was calculated as 10 x standard deviation of the blank signal/slope of the linear regression curve (Table 2.2).

Survey of ractopamine hydrochloride presence in select beef cuts

Background. In addition, a random survey of select tissues during a single production shift harvesting conventionally raised cattle at a commercial beef harvest facility was conducted. Currently, variety meats of heart, kidney, liver, omasum, tendons, outside skirt, inside skirt, hanging tender, oxtails, beef bones, feet, cheek meat, head meat, oxlips, backstrap, tunic tissue, and tongues derived from cattle less than 30 months of age are approved for export to China. A survey was conducted by sampling 10 of these tissues (outside skirt, inside skirt, oxtails, beef bones, feet, cheek meat, head meat, oxlips, backstrap, and tunic tissue) in the production environment of a commercial beef harvest facility during a conventional production shift [10 tissues x 5 samples of each x 1 commercial beef harvest facility x 2 ractopamine assays (parent and total) = 100 samples in total] to ascertain the likelihood of ractopamine contamination occurring due merely to processing oversights. Where necessary, validation of tests to cover the beef variety meat export possible tissues was completed. The remaining tissues included in the approved export list have previously been collected, validated, and analyzed so they were excluded from the survey portion of this study.

Sample collection. Samples were collected at random during one production shift at a commercial beef harvest facility from conventionally raised cattle following normal production processes. All samples were collected aseptically, using a new pair of gloves to prevent cross-contamination between samples and were placed in individual sterile Whirl-Pak bags (Nasco; Fort Atkinson, WI). Upon collection, samples were placed in direct contact with ice and transported to Colorado State University (Fort Collins, CO) where they were stored at -20°C until processing for ractopamine quantitation.

Beef bones proved challenging for this task as the harvest facility did not process them. That said, whole femurs were collected, frozen, and then cut into ~ ½ slices at the Colorado State University Veterinary School using a band saw. Similarly, feet are not typically processed at the plant that samples were collected from, thus a laboratory method of boiling on a hot plate and slicing open with a scalpel to de-hide the feet was used to process the feet prior to tissue homogenization for ractopamine quantitation. Once all tissues were collected and ready for processing, the homogenization, extraction, and UPLC-MS.

Results for both studies

Assay demonstrates effective and reproducible quantification of ractopamine across multiple tissue types.

Extraction efficiency was evaluated by fortification of control tissue samples with 5, 10, or 20 ppb of ractopamine before extraction. Measured recoveries ranged from 36% to 131%, with the majority of test samples > 75% (% recovery = measured/fortified x 100). These results indicate that the simple extraction protocol utilized in this study was effective and reproducible across the range of concentrations detected in the samples. All extractions were performed using a stable isotope labeled internal standard.

Accuracy and precision of the assay were determined by evaluation of control tissue samples fortified after extraction in triplicate at ractopamine concentrations of 1, 5, and 25 ng/ml. The average % CV of ractopamine detection for each tissue type and the rinsate (evaluated as a pool of rinsates from abomasum, omasum, reticulum, and small intestine) were all less than 2%. Average % accuracy for ractopamine quantitation for each tissue type were all better than 6% and was 10% for the pooled rinsates (% CV = standard deviation of measured

values/average of measure values) x 100; % accuracy = average of measured values – fortification level/fortification level) x 100.

Limits of detection and quantitation were determined for all tissue types (Table 2) and were comparable with published methods for muscle and liver, and well below the current Codex Alimentarius Commission Maximum Residue Limit (MRL) of 10 ppb for muscle (Codex, 2015). Importantly, this sensitivity was achieved using a very simple, high throughput extraction protocol that does not include a solid phase extraction step as is common in most published protocols for ractopamine detection (Burnett et al., 2012; Ulrey et al., 2013).

Parent and total ractopamine varies across tissue types.

Both parent and total ractopamine were measured in muscle, liver, abomasum, omasum, small intestine, and reticulum from 10 animals (Table 2.3; Figures 2.1 – 2.6). Total ractopamine concentrations represent parent ractopamine plus ractopamine metabolites. As expected, the highest concentration of ractopamine metabolites were measured in liver tissue (80.93 +/- 39.8 ppb; represented by the difference between the total and parent ractopamine concentration) (Figure 2.2). However, ractopamine metabolites were also detected in abomasum (23.52 +/- 47.6 ppb) and small intestine (16.97 +/- 20.2) (Table 2.3; Figures 2.5 and 2.6). All of the digestive tract offal tissues contained higher concentrations of ractopamine than muscle (Table 2.3; Figures 2.1 – 2.6). As an example, the total ractopamine concentration in muscle samples was 5.37 +/- 0.95 ppb while the total ractopamine values of other tissues ranged from 13.19 +/- 12.7 ppb (reticulum) to 105.04 +/- 38.4 ppb (liver) (Table 2.3; Figures 2.1 – 2.6).

Residual ingesta may contribute to ractopamine in some offal tissues.

To determine if residual ingesta in the digestive tract could be contributing to measured ractopamine levels in tissue homogenates, rinsate samples were generated by gently massaging

the tissue samples in methanol to release any residual ingesta into solution. This rinsate sample was then extracted and assayed for parent ractopamine. The rinsate from the small intestine contained an average parent ractopamine concentration of 19.71 +/- 12.2 ppb, whereas all other digestive tissue rinsates contained < 2.19 +/- 1 ppb of ractopamine (Table 2.4). This suggests that commercial processing procedures were insufficient to remove all residual ingesta from digestive tissues and in particular from the small intestine.

Ractopamine presence in select beef cuts collected at random from a commercial beef harvest facility was variable, but relatively low.

The samples from the survey were collected at random across a conventional production shift and were in no way related to one another. In general, both parent and total ractopamine residue concentrations were extremely low across all tissue types, with only one backstrap sample exceeding the Codex MRL for muscle at 18.19 ppb; however, the backstrap is on off-target tissue and should not be held to the Codex standard (Table 2.5). The odds of samples testing below the MRL for muscle is more important to producers, though, as these are the data that will truly help make purchasing, feeding, and processing decisions. For this random survey, there was a 100% chance of falling below the 10.0 ppb MRL for all 10 tissues (parent ractopamine) and 9 out of 10 tissues (total ractopamine) (Figure 2.7). However, the probability of falling below the 0.1 ppb MRL (near the limit of detection) to have more confidence in being able to reach a zero residue was much lower (Figure 2.7). The greatest likelihood of achieving the 0.1 ppb cutoff for both parent and total ractopamine was observed in head meat (100%), cheek meat (80%), oxlips (60%), backstrap (60%), and feet (60%) (Figure 2.7).

Discussion and Conclusions

To our knowledge, this study represented the first published report of ractopamine residues in/on digestive edible offal tissues from beef cattle. Results demonstrated the development and validation of a sensitive and accurate analytical method for quantitation of ractopamine in multiple tissues with importance relevant to global eating habits. Furthermore, results demonstrated potential for substantial contamination from residual ingesta on tissues (specifically digestive tissues) collected at commercial beef harvest facilities even after commercial processing procedures. This finding has important implications for harvest facilities to ensure that processing procedures are sufficient to reduce or eliminate contamination from residual ingesta.

These results are of even further significance when considering zero-tolerance trade policies—albeit for off-target tissues—for ractopamine residues that have been implemented in markets including China, the EU, and Russia. These countries will refuse or destroy imported product with any detectable concentration of ractopamine upon arrival (Centner, Alvey, Stelzleni, 2014). Importantly, all of the offal tissues analyzed in this study (abomasum, omasum, small intestine, and reticulum) contained detectable levels of ractopamine.

Currently, the U.S. meat industry relies heavily on export markets, exporting over 120,000 metric tons of beef to Hong Kong, alone, in 2018, valued at $966 million (USMEF, 2018). As trade negotiations continue, it will be of critical importance for beef producers to keep in mind that different countries/foreign trading blocs have different policies in regards to ractopamine residues. While the tissues and rinsates investigated in this study were off-target items, it will continue to be important for beef producers and commercial beef harvest facilities to develop methods for reducing ractopamine residues in edible offal products meant for export

to avoid refusal of products that have no regulatory MRL. Moreover, continued research must be conducted to evaluate how these offal tissues could be contaminated with ractopamine upon beef cattle harvest and fabrication and to explore practical measures to prevent such contamination. Although the probability of several of these tissues falling below the MRL was high, reaching 100% in many cases, there were circumstances in which higher residue levels were observed. For this reason, it is challenging to state what the true risk to producers of feeding ractopamine may be in different tissues without withdrawal data and a better understanding of contamination in general.

Table 2.1. UPLC gradient

Time (min)	% A	% B
0	99.0	1.0
0.2	99.0	1.0
2.2	70.0	30.0
3	1.0	99.0
4.25	1.0	99.0
4.5	99.0	1.0
6.5	99.0	1.0

Table 2.2. Limits of Detection (LOD) and Quantitation (LOQ)

Tissue Type	LOD (ppb)	LOQ (ppb)
Muscle	0.03	0.11
Abomasum	0.09	0.32
Liver	0.02	0.06
Omasum	0.01	0.05
Small Intestine	0.03	0.09
Reticulum	0.01	0.03
Rinsate	0.02	0.06

Table 2.3. Mean parent and total ractopamine residue concentrations (ppb) in six tissue types (muscle, liver, abomasum, omasum, small intestine, and reticulum) from cattle fed ractopamine hydrochloride after a practical 12-hour (0 day) withdrawal. Total ractopamine metabolites were calculated as the difference between the total ractopamine and parent ractopamine measurements with propagation of the standard deviations.

Tissue Type	Parent Ractopamine (ppb)	Total Ractopamine (ppb)	Total Ractopamine metabolites (ppb)
Muscle	4.42 +/- 0.6	5.37 +/- 0.9	0.95 +/- 1.1
Liver	24.11 +/- 10.7	105.04 +/- 38.4	80.93 +/- 39.8
Abomasum	15.77 +/- 6.1	39.29 +/- 47.2	23.5 +/- 47.6
Omasum	27.88 +/- 24.5	28.47 +/- 26.4	0.59 +/- 36.0
Small Intestine	27.68 +/- 12.1	44.65 +/- 16.2	16.97 +/- 20.0
Reticulum	11.39 +/- 12.5	13.19 +/- 12.7	1.8 +/- 17.9

Table 2.4. Mean parent concentrations (ppb) from rinsates from four different tissue types (abomasum, omasum, small intestine, and reticulum) from cattle fed ractopamine hydrochloride after a practical 12-hour (0 day) withdrawal.

Tissue Type	Parent Ractopamine (ppb)
Abomasum	2.19 +/- 1
Omasum	0.56 +/- 0.7
Small Intestine	19.71 +/- 12.2
Reticulum	0.48 +/- 0.2

Table 2.5. Parent and total ractopamine concentrations from a survey of tissues collected during one production shift at a commercial beef harvest facility from conventionally-raised cattle.

Tissue	Parent Ractopamine (ppb)	Total Ractopamine (ppb)	Tissue	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Outside Skirt	1.12	1.76	Cheek Meat	ND	ND
Outside Skirt	1.63	3.14	Cheek Meat	ND	ND
Outside Skirt	0.96	2.23	Cheek Meat	ND	ND
Outside Skirt	1.47	2.29	Cheek Meat	0.21	0.60
Outside Skirt	1.51	2.39	Cheek Meat	ND	ND
Inside Skirt	0.34	0.43	Head Meat	ND	ND
Inside Skirt	1.53	3.34	Head Meat	ND	ND
Inside Skirt	2.47	3.86	Head Meat	ND	ND
Inside Skirt	1.36	2.27	Head Meat	ND	ND
Inside Skirt	1.24	1.62	Head Meat	ND	ND
Oxtails	0.08	0.10	Oxlips	ND	ND
Oxtails	0.14	0.31	Oxlips	ND	ND
Oxtails	ND	0.12	Oxlips	ND	ND
Oxtails	ND	ND	Oxlips	0.56	1.17
Oxtails	ND	ND	Oxlips	0.29	0.54
Beef Bones	ND	0.37	Backstrap	0.32	1.13
Beef Bones	0.17	0.35	Backstrap	1.42	18.19
Beef Bones	ND	0.41	Backstrap	ND	ND
Beef Bones	0.13	0.92	Backstrap	ND	ND
Beef Bones	0.29	0.64	Backstrap	ND	ND
Feet	ND	ND	Tunic Tissue	1.13	1.45
Feet	ND	ND	Tunic Tissue	1.02	1.03
Feet	ND	ND	Tunic Tissue	0.90	1.21
Feet	0.60	1.18	Tunic Tissue	2.39	2.60
Feet	0.14	0.34	Tunic Tissue	0.80	1.20

ND = Non-detectable; less than limit of detection (0.08 ppb).

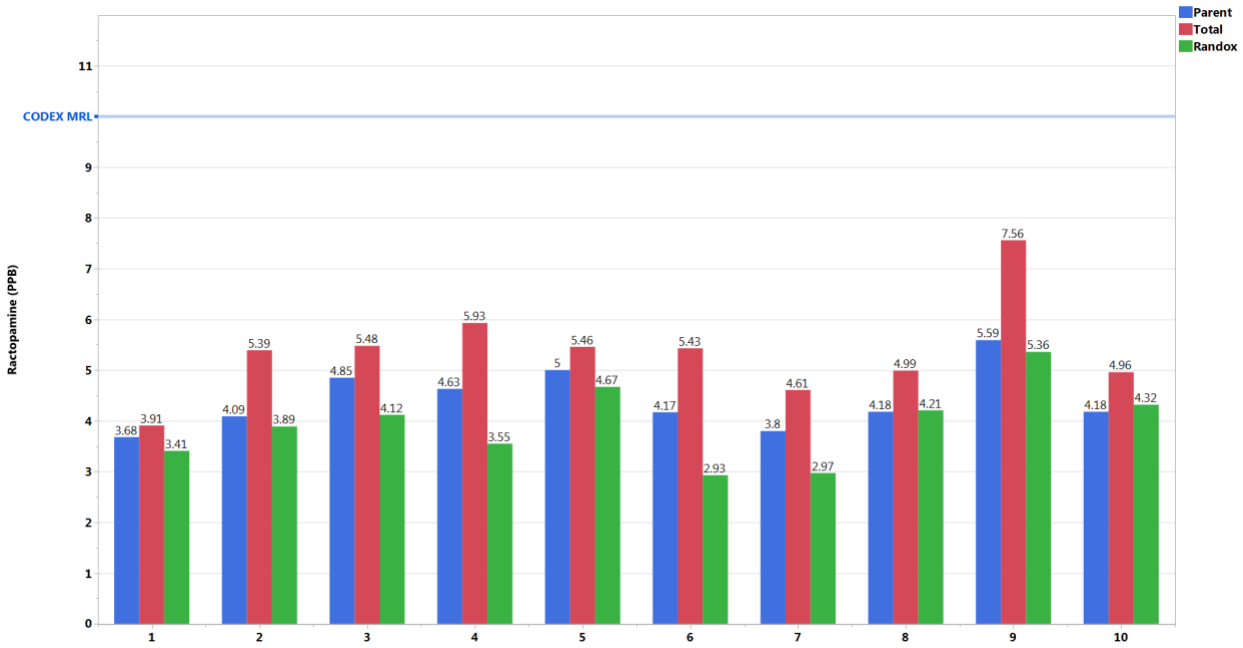

Figure 2.1. Parent and total ractopamine residue concentrations (ppb) in muscle samples collected from 10 heifers fed ractopamine hydrochloride (24.6 g/ton for 32 days) based on liquid chromatography mass spectrometry analysis and Randox. (Source: Elanco Animal Health, 2017).

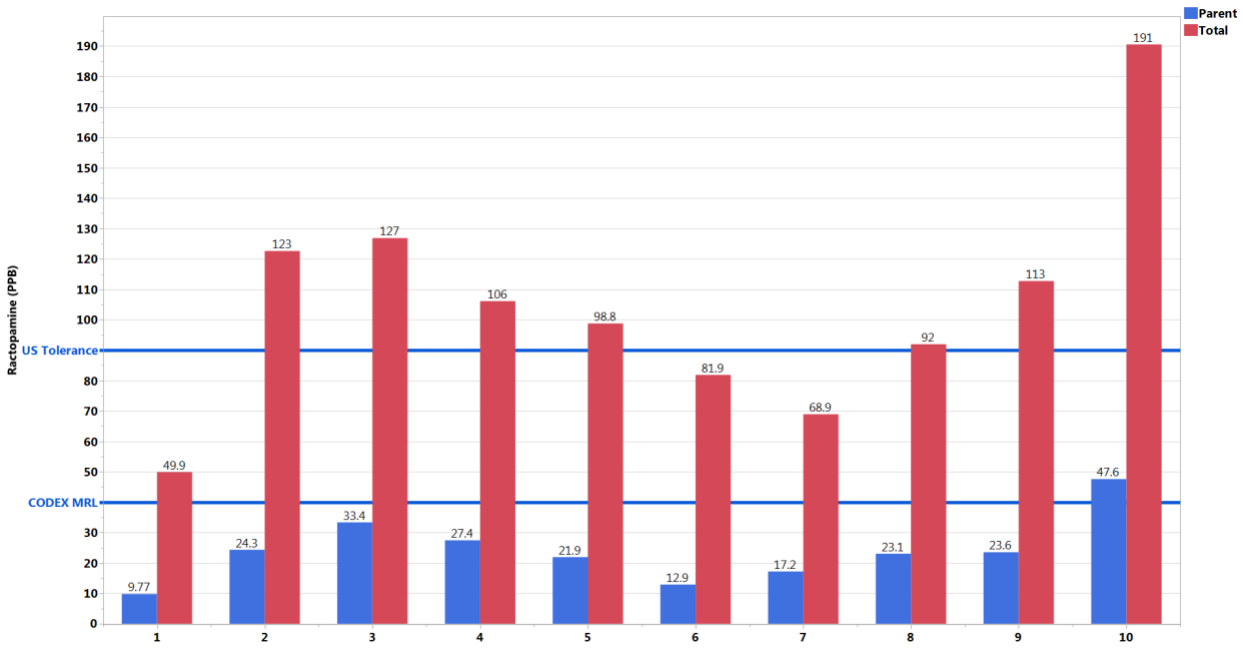

Figure 2.2. Parent and total ractopamine residue concentrations (ppb) in liver samples collected from 10 heifers fed ractopamine hydrochloride (24.6 g/ton for 32 days) based on liquid chromatography mass spectrometry analysis. (Source: Elanco Animal Health, 2017).

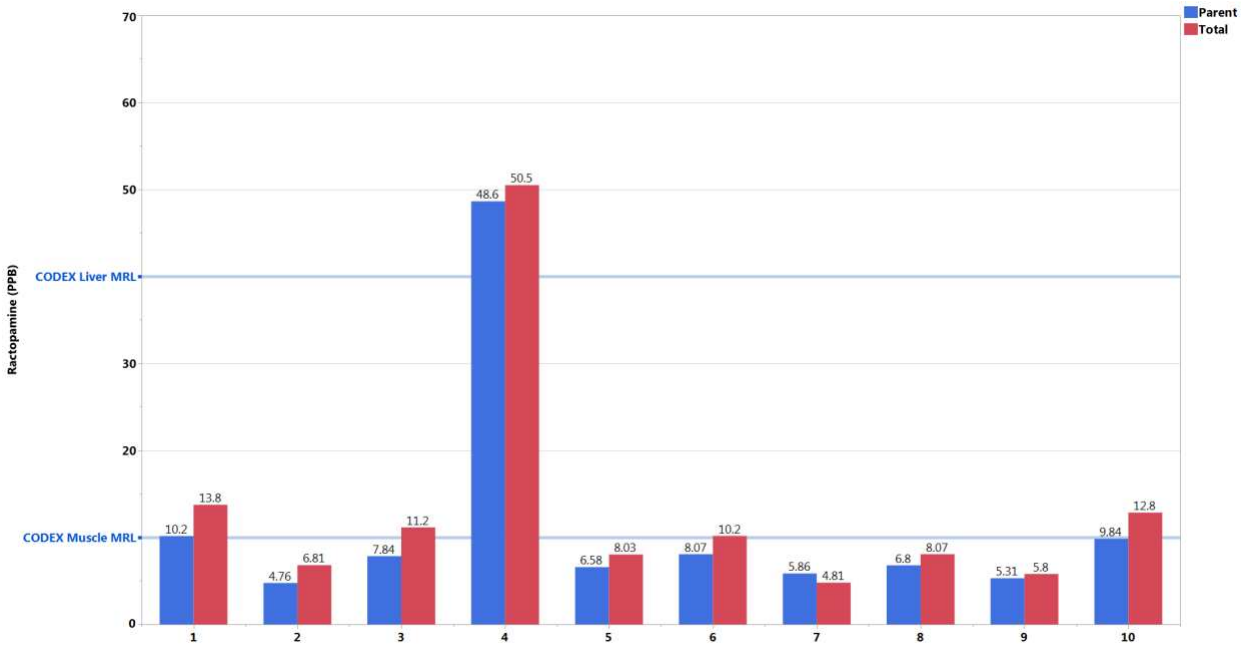

Figure 2.3. Parent and total ractopamine concentrations (ppb) in reticulum samples collected from 10 heifers fed ractopamine hydrochloride (24.6 g/ton for 32 days) based on liquid chromatography mass spectrometry analysis. (Source: Elanco Animal Health, 2017).

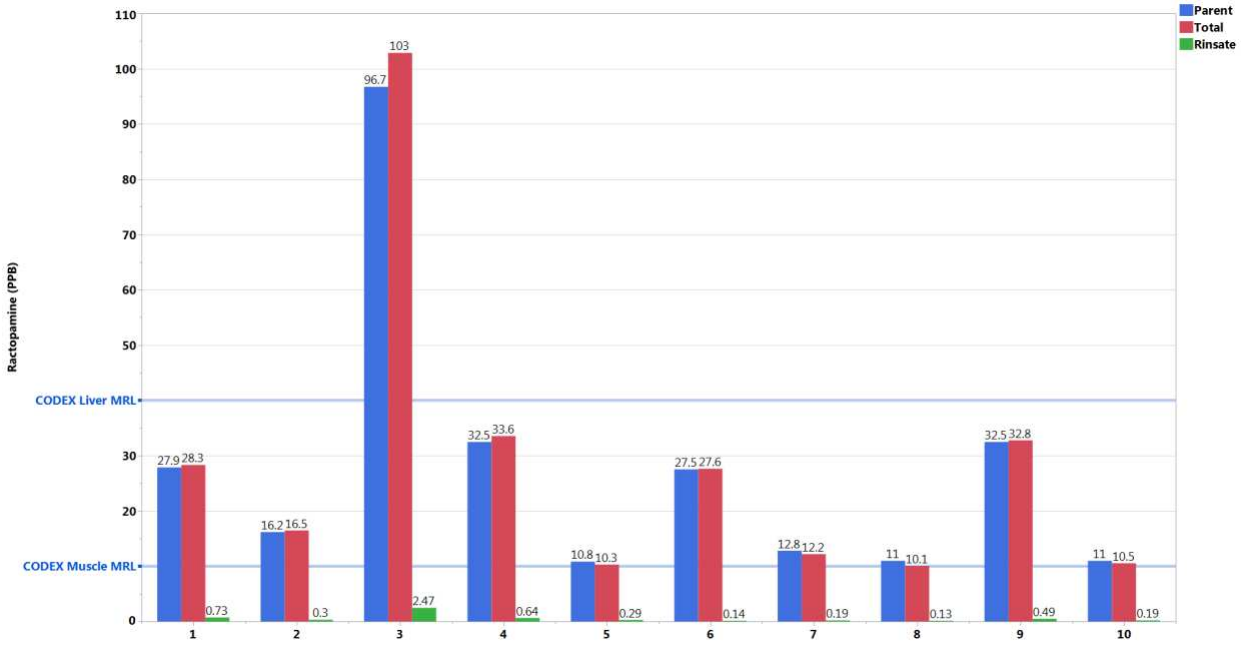

Figure 2.4. Parent and total ractopamine concentrations (ppb), as well as rinsates, in omasum samples collected from 10 heifers fed ractopamine hydrochloride (24.6 g/ton for 32 days) based on liquid chromatography mass spectrometry analysis. (Source: Elanco Animal Health, 2017).

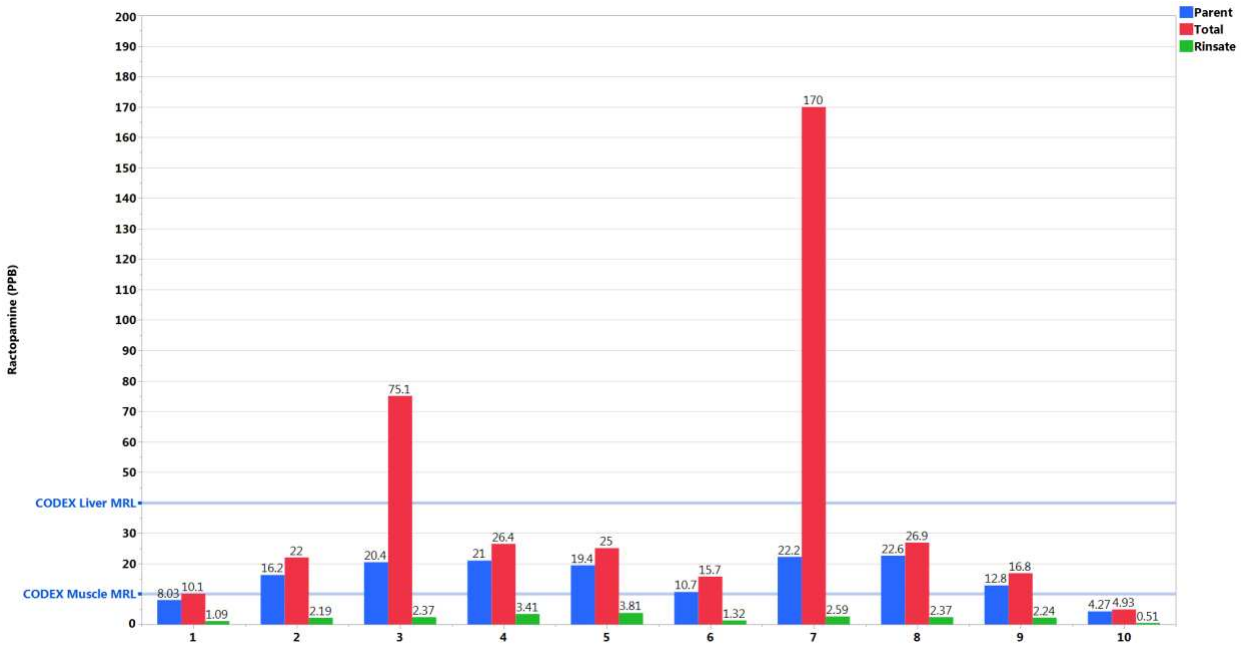

Figure 2.5. Parent and total ractopamine concentrations (ppb), as well as rinsates, in abomasum samples collected from 10 heifers fed ractopamine hydrochloride (24.6 g/ton for 32 days) based on liquid chromatography mass spectrometry analysis. (Source: Elanco Animal Health, 2017).

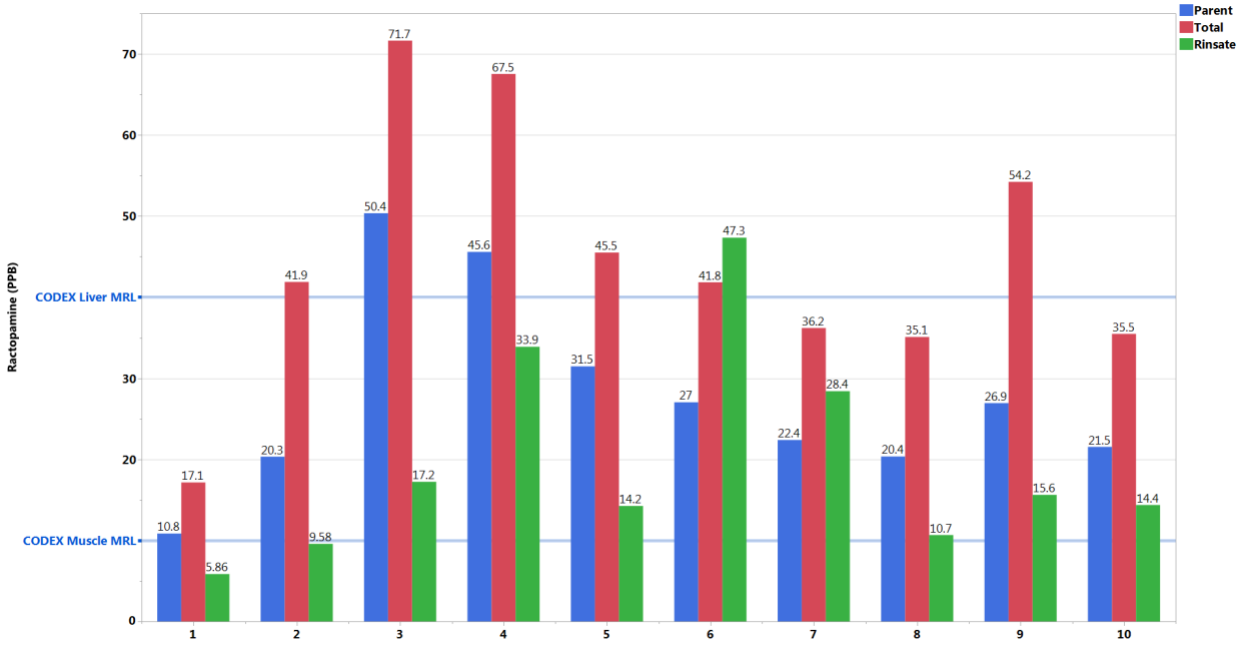

Figure 2.6. Parent and total ractopamine concentrations (ppb), as well as rinsates, in small intestine samples collected from 10 heifers fed ractopamine hydrochloride (24.6 g/ton for 32 days) based on liquid chromatography mass spectrometry analysis. (Source: Elanco Animal Health, 2017).

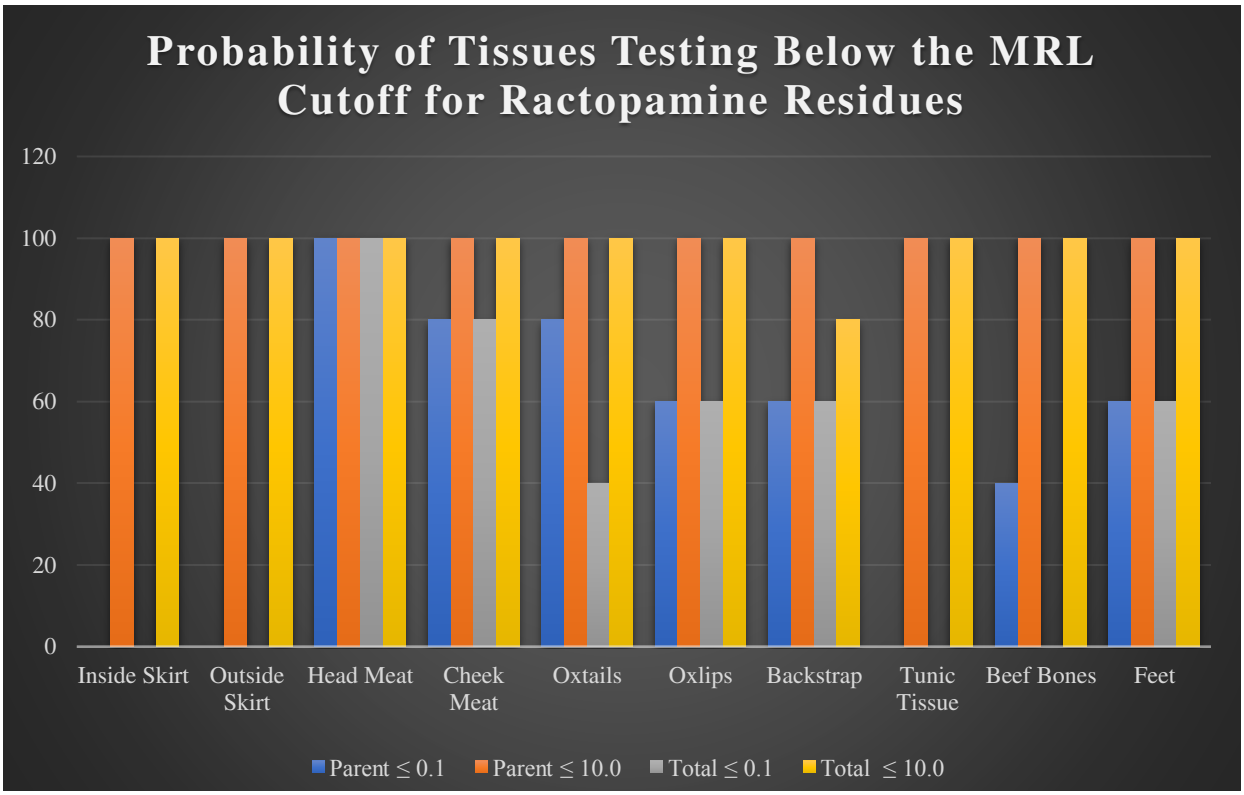

Figure 2.7. The probability of 10 tissues (collected at random during a single shift at a commercial beef harvest facility) testing below the maximum residue limit for ractopamine detection, given two cutoff levels [0.1 ppb (near the limit of detection for liquid chromatography mass spectrometry) and 10.0 ppb (the Codex MRL for muscle)].

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CHAPTER 3

Effects of ractopamine withdrawal in yearling steers

Summary

Due to developing meat trade issues associated with use of the beta-agonist, ractopamine hydrochloride (RH), in livestock production, the current study was conducted to assess the impact of improving withdrawal procedures from RH during cattle finishing. Current maximum residue limits (MRL) for ractopamine in liver, a target tissue, are 40 ppb (Codex) and 90 ppb [U.S. Food and Drug Administration (FDA)]. Muscle, another target tissue, has an MRL of 10 ppb (Codex) and 30 ppb (FDA). Maximum residue limits and tolerances are based upon parent rather than total ractopamine (i.e., ractopamine + ractopamine glucuronides); however, many countries have begun subjecting tissues to residue testing based upon total ractopamine assays and in off-target tissues, presenting a major challenge to the U.S. beef industry. Therefore, the objectives of these studies were to determine the concentration of residues of parent ractopamine and total ractopamine in various tissues and rendered tallow from animals fed RH and subjected to RH withdrawal times ranging from 12 h to 7 days, as well as animals that did not receive RH. Moreover, a dose and depletion study assessing ractopamine in the lower gastrointestinal (GI) tract of fistulated steers was conducted.

Ractopamine concentrations in processed tissue samples were determined by a multiple reaction monitoring assay performed using ultra performance liquid chromatography coupled to a tandem quadrupole mass spectrometry (UPLC-MS/MS). The method, developed and validated at Colorado State University for each matrix type, was adapted from the AOAC official method 2011.23 (Ulrey et al., 2013). Peak picking and integration were performed using QuanLynx software. Quantification of ractopamine residues in samples and quality control was performed

using linear regression against an external matrix-matched standard curve. The distribution of parent and total ractopamine concentrations in each tissue type was evaluated separately using a general linear mixed model approach. Analysis of variance included a fixed effect of withdrawal time with a random effect of block (day collected; study one only).

In the first study, parent and total ractopamine residues in individual liver samples ranged from a minimum of 3.40 and 3.46 ppb, respectively, for the control treatment group, to a maximum of 3.54 and 14.19 ppb, respectively, for the 2-day withdrawal treatment group. For the individual muscle samples, parent and total ractopamine concentrations ranged from below the limit of quantification (0.12 ppb) in the control samples, to 1.13 (parent ractopamine) and 1.72 ppb (total ractopamine) in 2-day withdrawal samples. Therefore, overall, parent and total ractopamine concentrations detected in the liver and muscle samples fell far below both the MRL and tolerance set by Codex and FDA. The highest parent and total ractopamine levels (282.40 and 289.85 ppb, respectively) were detected after 12 h withdrawal in individual large intestine samples, followed by small intestine (142.26 and 181.91 ppb, respectively) and omasum (109.70 and 116.90 ppb, respectively) samples.

Based on the finding that detectable levels of ractopamine were obtained in tissues collected from control animals (i.e., animals not receiving RH in their ration), a second study was conducted to determine potential sources of ractopamine contamination. For example, eight feed-grade tallow samples were analyzed for parent and total ractopamine presence as a potential source of contamination, especially in cattle not receiving ractopamine in their rations. Ractopamine concentrations of 0.40 to 50.80 ppb were obtained for these tallow samples. While this could potentially explain the detectable levels of ractopamine residues found in control samples and the fact that 7-day withdrawal did not result in non-detectable levels, further

research is necessary to understand the implications of contaminated tallow on residue levels across tissues. The data from the current study may be useful in the development of new recommendations for RH use and withdrawal to beef cattle producers in the U.S. who intend to export to global markets.

Introduction

Beef cattle producers have relied on growth promotants for over 50 years to increase production and improve feed efficiency (Johnson, Ribeiro, and Beckett, 2013). While steroidal implants have been historically used for growth promotion through increased muscle leanness, increased average daily gain, and feed intake stimulation, beta-adrenergic receptor agonists (beta-agonists) were approved in the early 2000s as a class of orally active growth promotants used in beef cattle finishing (Johnson, Ribeiro, and Beckett, 2013). Beta-agonists are used as dietary supplements in feed during the last three to six weeks of cattle finishing for improved feed efficiency and growth promotion (Johnson, Ribeiro, and Beckett, 2013; Kootstra et al., 2005). They bind to G protein-coupled beta receptors on cell surfaces, thus increasing muscle mass via hypertrophy and decreasing lipid synthesis and fat deposition (Johnson, 2014; Mersmann, 1998; Neumeier and Mitloehner, 2013). Beta-agonists in livestock production stimulate skeletal muscle growth without increasing hormone levels, which eventually leads to heavier carcasses with fewer inputs and, therefore, an economic benefit to producers (Centner, Alvey, and Stelzleni, 2014).

There are only two beta-agonists, ractopamine hydrochloride and zilpaterol hydrochloride, approved by the U.S. Food and Drug Administration (FDA) for use in food animal species for growth promotion (Dilger, 2015). Both ractopamine and zilpaterol were subjected to new animal drug approval (NADA) processes, which are quite robust systems

ensuring the safety and effectiveness of the compounds. Ractopamine binds to beta-1 receptors and is approved for use in swine, cattle, and turkeys while zilpaterol binds to beta-2 receptors and is only approved for use in cattle (Arp et al., 2014; Centner, Alvey, and Stelzleni, 2014; Dilger, 2015). Although these beta-agonists are approved for use in the United States and countries such as Brazil, Mexico, and Canada, they have been banned in other locations such as China and the European Union (Dilger, 2015). Zilpaterol is not currently used in beef cattle finishing in the United States for animal welfare reasons; however, ractopamine is still commonly used by livestock producers.

Despite the extensive approval process for beta-agonists and the adoption of maximum residue limits (MRL) by the Codex Alimentarius Commission, an intergovernmental food standard setting committee, ractopamine use remains contentious (Bottemiller, 2012). Certain countries have begun adopting zero tolerance policies, which are clearly more restrictive than the global standard, for ractopamine use and residues, creating marketing issues for export from countries with higher tolerances, such as Canada and the United States (Bottemiller, 2012). Sample handling and testing methods are crucial when holding exporters accountable to the global standard, and these are often just as contentious as ractopamine use, itself. Current MRLs for ractopamine in liver, a target tissue, are 40 ppb (Codex) and 90 ppb (FDA). Muscle, another target tissue, has an MRL of 10 ppb (Codex) and 30 ppb (FDA). Maximum residue limits and tolerances are based upon parent rather than total ractopamine (ractopamine + ractopamine glucuronides); however, many countries have begun subjecting tissues to residue testing based upon total ractopamine assays in both on- and off-target tissues, presenting a major challenge to the U.S. beef industry. For this reason, it is critical that livestock producers using ractopamine

have data available to them to make withdrawal decisions before harvesting and exporting products to any countries with stricter ractopamine residue policies.

For example, in May of 2017, an off-target U.S. beef variety meat (i.e., an abomasum) was selected for testing upon import into South Korea and found to contain residues that violated the Codex MRL established for parent ractopamine concentrations in muscle. Because of the finding, the plant that produced the tissue was delisted for further exports to South Korea. This outcome led to significant concern among the beef industry. A short time later, a consortium of industry trade associations representing cattlemen (NCBA), packers (NAMI) and exporters (USMEF) jointly suggested to cattle feeders that a withdrawal should be implemented for ractopamine hydrochloride in the diets of feedlot cattle to eliminate concerns about tissues testing positive for the compound in export markets.

In August of 2017 (as a consequence of the South Korean plant delisting), potential ramifications to the beef industry of foreign sampling and ractopamine testing programs directed towards off-target variety meat tissues were discussed. It was determined that very little was known about the depletion curves associated with off-target tissue concentrations of ractopamine upon withdrawal, particularly if countries choose to test for total (parent plus hydrolyzed metabolites) ractopamine concentrations. Therefore, these studies were meant to allow formulation of recommendations for ractopamine withdrawal times during cattle feeding to achieve appropriate depletion in a variety of meat tissues.

The objective of the first study was to determine the concentration of residues of parent ractopamine and total ractopamine in 10 tissue types from animals fed ractopamine hydrochloride (RH; 250-275 mg/hd/day for 32 days) and subjected to RH withdrawal times of 12 h, 2 days, 4 days, or 7 days. The primary objective of the second study was to test the impact of

withdrawal from ractopamine hydrochloride in the diets of feedlot cattle for 2, 4, or 7 days on residues for parent and total ractopamine in muscle, fat, rendered tallow, and large intestines in contrast to a true negative control group. Additionally, feed samples were collected, validated, and tested to verify ractopamine presence using liquid chromatography mass spectrometry (LC-MS/MS) protocols. Note that residue concentrations in tissues from cattle managed to achieve a practical 0-day withdrawal (12 hours) were not tested in this experiment.

The objective of the third study was to determine dose response and depletion curves of ractopamine hydrochloride (RH) in the lower gastrointestinal (GI) tract of fistulated (i.e., cannulated – both rumen and duodenal) steers either receiving or not receiving RH as part of the daily ration.

Materials and Methods

Design. Study one was performed in two identical phases, consisting of six pen blocks (30 pens) of yearling steers totaling 1,200 to 1,500 head in each phase. Tissues were collected and analyzed only from animals fed during phase two of this study, which began in October 2017. Cattle in each phase were fed RH at 250-275 mg/hd/day for 32 days and relative to different withdrawal times (12 h, 2 days, 4 days, and 7 days). One additional pen block of cattle, designated as the untreated (control) group, did not receive RH in their ration.

Study two was designed with a total of N = 75 experimental units (EU; small pens of cattle from which individual cattle were randomly sampled for testing) were assigned to 5 treatment groups across separate feedlot pens: 1) a negative control [fed no ractopamine and no feed-tallow; fed from verified clean feed trucks; Control (No Tallow)]; 2) cattle fed ractopamine hydrochloride (at approximately 250-300 mg/hd/d), but withdrawn from treatment at 2 days before harvest (2 day); 3) withdrawn from treatment at 4 days before harvest (4 day); 4)

withdrawn from treatment at 7 days before harvest (7 day); or 5) a control with feed-tallow [fed no ractopamine but received feed-tallow; Control (With Tallow)]. This led to a total of n = 15 x 5 treatment groupings for 75 samples for each tissue/matrix type (muscle, fat, rendered tallow, and large intestine).

During feeding, bunk samples provided to each treatment pen [four total pens – two for Control (No Tallow) cattle, one for ractopamine-fed cattle, and one for Control (With Tallow) cattle] were collected five times each for subsequent LC-MS/MS analysis to determine dietary ractopamine inclusion. Upon appropriate withdrawal times, cattle were removed from the larger ractopamine-fed pen into smaller withdrawal pens and a feed sample was collected from the 7-day, 4-day, and 2-day pens for three additional feed samples. Further, three composite tallow samples were collected over the feeding period (28 days) for a total of 26 feed samples. At harvest, all test cattle were traced through harvest processing under USDA-FSIS inspection at a commercial beef harvest facility. As disassembly proceeded, tissues were identified via tag transfer and traced such that all tissues were collected from the same animal carcasses. From each EU in each treatment group, four 'products' were collected/manufactured and tested for both parent and total ractopamine concentrations. 'Products' included muscle, fat, and large intestine tissue at harvest, as well as rendered tallow that was manufactured in-laboratory from fat collected during harvest. Total sample numbers included 75 EU x 4 'products' = 300 samples. Total tests for ractopamine residue quantification = 75 EU x 4 'products' x 2 tests (parent and total) = 600 tests plus 26 x 2 test for feed for a grand total of 652 tests.

In the third study, four steers (n = 2 not receiving RH and n = 2 receiving RH at the approved dosage) were assessed from -3 days (still receiving RH if on the RH treatment) to 13

days post-withdrawal to determine the amount of RH present and length of time required for RH to clear the GI tract should a contamination event of low levels occur.

Sample collection. Samples in study one were collected at a commercial beef harvest facility in Northern Colorado after the animals were slaughtered and the carcasses commercially processed. Steers were harvested in a balanced design, over two days, according to four withdrawal times (12 h, 2 days, 4 days, and 7 days), or no withdrawal for steers not fed RH (control). Samples collected included 10 different tissue types (i.e., liver, muscle, heart, tendon, tongue, abomasum, omasum, rumen/reticulum, small intestine, and large intestine) from two animals per pen of cattle which received RH (for a total of 12 animals per treatment group), and one animal per pen for cattle which did not receive RH (for a total of six control animals) (Table 3.1). Each tissue type was collected within animal, and randomization was achieved based on a random order of cattle entering the harvest facility from the holding pens. Pre-randomization was not plausible due to the unknown order of cattle advancement. Further randomization occurred based upon tissue collection from approximately every 20 head, except where there was a break in the lot. Tag transfer was instrumental in ensuring that samples were collected from the correct number of animals for each treatment.

Samples were collected from identified carcasses/removed organ sets as they were conveyed along the chain in the beef harvest facility. All samples were collected aseptically, using a new pair of gloves to prevent cross-contamination between samples. In order to keep track of selected carcasses, differing identification systems were developed. Extra numbered USDA Food Safety Inspection Service gang tags were printed for each selected carcass, and tags were used for identification at muscle, tendon, and tongue collection stations as carcasses moved through facility processes. Each viscera set was marked liberally with red carcass ink so all

viscera samples could be identified and collected, either on the evisceration table upstairs or in the basement of the harvest facility, after proper processing. Once marked with carcass ink and identified, abomasum, rumen/reticulum, and omasum samples were tagged with cattle ear tags with corresponding identification numbers. Small and large intestine samples were identified based upon presence of carcass ink. Upon proper identification, samples were collected at their respective locations.

Before tissue sample collection, livers were washed, and then a cross-section was trimmed on the viscera table before the remaining tissues were sent down the chute for further processing. Muscle samples were trimmed by plant personnel on the carcass rail as carcasses proceeded down the line. After USDA inspection, hearts were washed and collected. Flexor tendons from the foreshank were collected on the harvest floor after hide removal. Tongues were collected from the chain immediately following inspection and washing in the head cabinet, a device in which offal items such as tongues are washed with an antimicrobial solution. The remaining tissues were collected at downstream offal processing areas in the beef harvest facility.

Because abomasum tissues were not being processed at this facility at the time of collection, they were rinsed out with hot water and then placed in a cold-water bath before being bagged. Omasum samples were split and entered the normal processing vat of hot water (60-61°C) and were collected following spinning within the vat. Rumen/reticulum samples underwent the same washing and spinning process in a large vat of hot water (60°C) and a combination of commercial sanitizers. They then entered a refiner with a slightly lower temperature (52°C) and the same sanitizer mixture before collection. Both small and large intestines were identified, stripped of their internal linings, flushed with water, and collected.

Collected samples were placed in individual sterile Whirl-Pak bags (Nasco; Fort Atkinson, WI) (or bags from the plant in the case of small and large intestine samples which were too large for Whirl-Pak bags) and positioned in direct contact with ice in boxes or coolers. It is important to note that in some tissues, ractopamine glucuronides can be converted to ractopamine, making it crucial for each tissue to be placed in direct contact with ice to reduce enzymatic activity and to be subsequently frozen such that the tissues do not thaw at any point prior to sample processing for ractopamine analysis. Samples were transported to Colorado State University (Fort Collins, CO) where they were stored in a -20°C freezer until processing for ractopamine quantification.

For study two, samples were collected at a commercial beef harvest facility in California. Steers were harvested in a balanced design, in order of the previously mentioned five treatments: 1) a negative control [fed no ractopamine and no feed-tallow; fed from verified clean feed trucks; Control (No Tallow)]; 2) cattle fed ractopamine hydrochloride (at approximately 250-300 mg/hd/d), but withdrawn from treatment at 7 days before harvest (7 day); 3) withdrawn from treatment at 4 days before harvest (4 day); 4) withdrawn from treatment at 2 days before harvest (2 day); or 5) a control with feed-tallow [fed no ractopamine but received feed-tallow; Control (With Tallow)]. Samples collected included four different tissue/matrix types (i.e., muscle, fat, tallow, and large intestine) 75 animals (randomly assigned for shipment from each of the five-treatment group small pens (EU). Each tissue/matrix type was collected within animal. Pre-randomization was not plausible due to the unknown order of cattle advancement. Tag transfer was instrumental in ensuring that samples were collected from the correct animals for each treatment. All attempts were made to collect each tissue within animal, and no exceptions were made in this experiment.

Samples were collected from carcasses or their respective removed organ sets as they were conveyed along the chain in the beef harvest facility. All samples were collected aseptically, using a new pair of gloves to prevent cross-contamination between samples. Never-tear tags were printed for each carcass, and tags were used for muscle, fat, tallow, and large intestine collection stations for the sake of identification as carcasses moved throughout the facility. Because carcasses came in order of 1 – 75, it was not necessary to mark viscera sets with carcass ink as has been done in the past for identification purposes.

Muscle samples were trimmed by plant personnel on the carcass rail as carcasses proceeded down the line. Fat samples and tallow samples were also collected as carcasses were moving on the rail. Large intestine samples were identified and collected on the viscera table, separate from the other collection stations. Collected samples were placed in individual sterile Whirl-Pak bags (Nasco; Fort Atkinson, WI) and positioned in direct contact with ice in boxes or coolers. It is important to note that in some tissues, ractopamine glucuronides can be converted to ractopamine, making it crucial for each tissue to be placed in direct contact with ice to reduce enzymatic activity and to be subsequently frozen such that the tissues do not thaw at any point prior to sample processing for ractopamine analysis. Samples were transported/shipped to Colorado State University (Fort Collins, CO) where they were stored in a -20°C freezer until processing for ractopamine quantification.

In study three, four gastrointestinal samples were obtained: two samples were collected from the rumen, [rumen fluid (RF) and rumen solid (RS)], one sample from the duodenum [duodenal fluid (DF)], and one fecal sample. Samples were collected into 50 ml conical tubes (Thermo Fisher Scientific; Waltham, MA) and stored at -20°C until processing.

Tissue homogenization. For the first two studies, tissues were cryogenically homogenized prior to ractopamine extraction procedures. Treatment groups within each tissue type were identified based on tag transfer data so that control samples could be processed first to avoid cross-contamination. Approximately 100 g of each tissue were chopped into small (~ 3 cm x 3 cm) pieces, flash frozen in liquid nitrogen (N_2), and homogenized using a Robot Coupe Blixer V4 (tendons; Robot Coupe USA; Jackson, MS) or a Nutribullet food processor (all other tissues; Capital Brands, LLC; Los Angeles, CA). Two subsamples of tissue homogenate, each weighing 1 ± 0.5 g, were placed in separate 5 ml conical tubes and stored at -80$_o$C until extraction.

In-laboratory tallow rendering. In the case of tallow, in-laboratory rendering was necessary as the commercial beef harvest facility used did not have its own rendering facility. Thus, subcutaneous fat samples (> 100 g each) were collected from each of the treatment groups and rendered following the Essential Rendering Overview by Meeker and Hamilton. Each sample was cut into small (~ 3 cm x 3 cm) pieces, placed in a sterile 600 ml Pyrex beaker (Corning, Inc.; Corning, NY), and microwaved for 6 min in a Panasonic Countertop Microwave (The Genius Sensor 1250W; Panasonic Corp.; Kadoma, Osaka Prefecture, Japan). After cooking, the remaining fat solids were removed using sterile forceps, and the temperature was obtained using an infrared thermometer. The average temperature after cooking was 134.5°C ± 7.1 (standard deviation). The liquid portion was carefully poured into two 50 ml centrifuge tubes (VWR International, LLC; Radnor, PA). The first tube was filled to 30 ml and centrifuged for 20 min at 2000 RPM (20°C; Beckman Model TJ-6 Centrifuge; Beckman Coulter, Inc.; Indianapolis, IN). This portion was used for ractopamine analysis while the second tube was labeled “bulk” and stored in the -20°C freezer should any further testing be required.

Analytical method. An adaptation of the AOAC final action method was developed and used for the analytical method (Davis et al., 2019; Ulrey et al., 2013). Two aliquots of each sample were analyzed, one for parent ractopamine and one for total ractopamine (ractopamine + glucuronides). All aliquots were processed as described in the sample extraction procedure, described below, while samples intended for total ractopamine analysis were further processed as described in the enzyme hydrolysis for total ractopamine procedure. All fluid samples from study three were freeze-dried by removing the caps and replacing with tin foil and lyophilized for 4 days or until all water had evaporated. Once dried, the samples were homogenized using a lab spatula. From there, samples were treated the same as tissue samples for extraction.

Sample extraction. Tissue and fluid homogenate samples were extracted with 4 ml per gram of extraction solution [methanol (MeOH) containing 25 ng/ml of the internal standard ractopamine-d_6]. Samples were sonicated for 30 min, vortexed at 4°C for 10 min, and incubated for 30 min at -80°C. The samples were then centrifuged for 10 min at 12,000 x *g* and two 1 ml aliquots of the supernatant were transferred to glass vials. The remaining supernatant was stored at -80°C. One aliquot was analyzed directly for parent ractopamine by liquid chromatography mass spectrometry (LC-MS) and the other underwent further processing for total ractopamine analysis.

Enzyme hydrolysis for total ractopamine. The 1 ml supernatant aliquot from the extraction procedure for total ractopamine was evaporated to dryness using a nitrogen dryer. The sample was re-suspended in 200 µl of 25 mM sodium acetate (NaOAc) buffer (pH 5.2). An aliquot of 4 µl β-glucuronidase solution (100,000 units/ml) was added and the sample was mixed thoroughly by gentle vortex. The sample was incubated at 65°C for 2 h in a sand bath. An aliquot of 400 µl of MeOH was added and the sample was again mixed thoroughly by gentle vortex

followed by centrifugation at approximately 2,025 x *g* for 5 min. The supernatant was carefully removed and placed into a clean glass vial for LC-MS analysis.

Preparation of the calibration curve. Control tissue was obtained from carcasses of animals that were not fed RH. Control tissue was homogenized and extracted as detailed above and then spiked with RH and the internal standard. A serial dilution was performed to generate an 11-point standard curve ranging from 0.05-50 ng/ml. The standard curve range was optimized for each tissue to capture the appropriate concentration of the samples.

Sample analysis via LC-MS/MS. Samples were analyzed by UPLC-MS/MS using a system comprised of a Waters Synapt G2-Si with a standard flow electrospray ionization (ESI) source coupled to a Waters Acquity 1-Class UPLC equipped with a reverse phase 1.0 mm x 50 mm Waters Acquity UPLC HSS T3 column (1.8 µm particles). The UPLC was operated at 0.400 ml/min and the column temperature was thermostatically controlled at 50°C using a column heater. Mobile Phase A (MPA) was water (Fisher Scientific, LC-MS Grade) with 2 mM ammonium formate (Table 2). Mobile Phase B (MPB) was acetonitrile (Fisher Scientific, LC-MS Grade) with 0.1% formic acid (Pierce, LC-MS Grade) (Table 3.2). The sample injection volume was 1 µl.

Data were acquired at 10 Hz across the mass range of 50-1000 m/z. The transfer cell collisional energy (CE) was turned off and no mobility separation was conducted to maintain ion transmission efficiency. Targeted enhancement was turned on and configured to target the product ion m/z 164.106. The method consisted of two MS/MS functions: the first was selective for ractopamine (302 m/z → 164.106, CE = 11, Targeted Enhancement = 164.106) and the second was selective for the internal standard [ractopamine-d_6 (308 m/z → 168.17 m/z, CE = 11, Targeted Enhancement = 168.17 m/z). A second transition for ractopamine (302 m/z →

284.1627 m/z) was utilized for identification confirmation only. The spectra were LockMass corrected to provide accurate mass data.

Peak picking and integration were performed using QuanLynx software (Waters Corporation). Peak areas for each sample were normalized to the peak area of the internal standard in that sample. Quantification of samples and quality control was performed using linear regression against the matrix matched standard curve. Separate standard curves were generated for each matrix type. The concentrations of parent ractopamine and total ractopamine (ractopamine + ractopamine glucuronides) were individually reported for each sample.

Statistical analysis. Parent and total ractopamine concentrations were analyzed separately for each tissue/matrix type using a general linear mixed model in SAS (version 9.4; Cary, NC). Analysis of variance included a fixed effect of withdrawal time and random effect of block (only for study one as samples were collected over two days). Data are reported as least squares means using a significance level of $\alpha = 0.05$. For the statistical analysis of study one, two carcasses, one from the 2-day and one from the 7-day withdrawal groups, were removed due to condemnation of the carcasses on collection day, leading to the loss of all visceral tissues.

Results and Discussion

Limits of detection (LOD) and quantification (LOQ) of the UPLC-MS/MS analysis for residue levels (ppb) of parent and total ractopamine in the different tissue types evaluated in this study, are shown in Table 3.3. Least squares mean residues of parent and total ractopamine in each of the 10 tissues are shown in Table 3.4, while ractopamine concentrations in the individual samples of liver, muscle, heart, tendon, tongue, abomasum, omasum, rumen/reticulum, small intestine, and large intestine analyzed are presented in the Appendix in Supplemental Tables 3.1 through 3.10.

Control samples. An unexpected finding of ractopamine residues being detected in most of the tissues collected from carcasses of cattle that did not receive RH (control treatment) (Table 3.4, Supplemental Tables 3.1 – 3.10), suggested that contamination may have occurred at some point at the feedlot and/or during harvesting and collection procedures. Mean concentrations of both parent and total ractopamine were above the detection limit of the analysis (Table 3.3) in eight of the 10 tissues, with only parent ractopamine in muscle and parent and total ractopamine in rumen/reticulum concentrations averaging 0 ppb (Table 3.4). One example of said research was the collection and analysis of eight feed-grade tallow samples for parent and total ractopamine presence, especially in cattle that did not receive ractopamine in their rations. Ractopamine concentrations of 0.40 to 50.80 ppb were obtained for these tallow samples (data not shown in tables). While this could potentially explain the presence of ractopamine residues in the tissues of animals not fed RH, further research is necessary to understand the implications of contaminated tallow on residue levels across tissues.

Liver. Mean concentrations of ractopamine in liver samples, regardless of treatment group (i.e., withdrawal time), ranged from 3.40 to 3.48 ppb (parent ractopamine) and 3.53 to 8.84 ppb (total ractopamine) (Table 3.4). As can be seen, mean levels of parent ractopamine among the control and 2-, 4-, and 7-day withdrawal treatment groups were numerically similar; however, statistical differences ($P < 0.05$) were noted among each withdrawal time (Table 3.4). With regards to mean total ractopamine concentrations, residue levels in liver samples from the control and 4- and 7-day withdrawal treatments were lower (3.53 to 3.96 ppb; $P < 0.05$) than that of the 2-day withdrawal treatment group (8.84 ppb) (Table 3.4). Based on the results obtained for the individual liver samples, the highest residue concentration observed was 14.19 ppb (2-day withdrawal) from the total ractopamine analysis (Supplemental Table 3.1). Overall, parent and

total ractopamine concentrations (LS means and individual samples; Table 3.4 and Supplemental Table 3.1) were far below the MRL set by Codex (40 ppb) and the tolerance set by FDA (90 ppb). Twelve h withdrawal samples were not collected for liver samples.

Muscle. In study one, muscle samples also were only collected after 2-, 4-, and 7-days of cattle withdrawal. The current MRLs for muscle are 10 ppb (Codex) and 30 ppb (FDA). Residue levels in all muscle samples analyzed in this study (LS means and individual samples; Table 3.4 and Supplemental Table 3.2) were below this threshold. More specifically, regardless of RH withdrawal duration, mean parent ractopamine concentrations in muscle samples ranged from 0.00 (i.e., below the LOD; control) to 0.73 (2-day withdrawal) ppb, while mean total ractopamine levels ranged from 0.04 (control) to 1.15 (2-day withdrawal) ppb (Tables 3.3 and 3.4). Mean residue levels of ractopamine (parent and total) for the 7-day withdrawal and control treatment groups were lower ($P < 0.05$) than those of the 2- and 4-day withdrawal groups (Table 3.4).

In study two, muscle sample least squares mean estimates ranged from non-detectable [ND; limit of detection (LOD) = 0.12 ppb] to 0.76 ppb (parent) and ND to 1.22 ppb (total) ractopamine (Tables 3.5 and 3.6). Non-detectable residues were observed in both Control (With Tallow) and Control (No Tallow) treatment groups, and the highest residues were observed in the 2-day withdrawal treatment (Tables 3.5 and 3.6). That said, the residue levels in muscle samples analyzed in this study were below the current MRL and tolerance for muscle [10 ppb (Codex) and 30 ppb (FDA)]. Mean residue levels of ractopamine (parent and total) for all treatment groups were different ($P < 0.05$), with the exception of the two control groups (Tables 3.5 and 3.6).

Heart. All remaining tissues were collected for control animals and at every withdrawal time (12 h, 2 days, 4 days, and 7 days). Mean concentrations in heart samples, regardless of treatment group, ranged from 0.84 to 3.31 ppb (parent ractopamine) and 0.94 to 5.14 ppb (total ractopamine) (Table 3.4). With regards to mean parent ractopamine concentrations, the residue level in heart samples from the 4-day withdrawal treatment (0.84 ppb) was lower ($P < 0.05$) than that of the 12 h withdrawal group (3.31 ppb) (Table 3.4). Additionally, mean concentrations of total ractopamine in heart samples from the control, 2-, 4-, and 7-day withdrawal treatments were lower (0.94 to 1.52 ppb; $P < 0.05$) than that of the control treatment group (5.14 ppb) (Table 3.4). Based on the results obtained for the individual heart samples, the highest residue concentration observed was 14.01 ppb (12 h withdrawal) from the parent ractopamine analysis (Supplemental Table 3.3).

Tendon. Regardless of treatment, mean parent ractopamine concentrations in tendons from the foreshank ranged from 0.58 (control) to 4.85 (12 h withdrawal) ppb, while mean total ractopamine residues ranged from 1.07 (control) to 13.74 (12 h withdrawal) ppb (Table 3.4). Mean residue levels of ractopamine (parent and total) for the 2-, 4-, and 7-day withdrawal and control treatments groups were lower ($P < 0.05$) than those of the 12 h withdrawal groups (Table 3.4). The highest observed residue level (based upon individual samples) was 24.84 ppb (total ractopamine) (Supplemental Table 3.4). The tendon is an off-target tissue, meaning it cannot officially be held to the standards set by Codex and FDA.

Tongue. Mean concentrations of ractopamine in tongue samples, regardless of treatment, ranged from 0.33 to 3.94 ppb (parent ractopamine) and 0.36 to 8.01 ppb (total ractopamine) (Table 3.4). In regards to mean parent ractopamine concentrations, residue levels in tongue samples from the control, and 2-, 4-, and 7-day withdrawal treatment groups were lower (0.33 to

1.57 ppb; $P < 0.05$) than that of the 12 h withdrawal group (3.94 ppb) (Table 3.4). The same general trend was observed for the total ractopamine concentrations in which residue levels from the control, 2-, 4-, and 7-day withdrawal treatments were lower (0.36 to 2.41 ppb; $P < 0.05$) than that of the 12 h withdrawal group (8.01 ppb) (Table 3.4). Based on the results obtained for individual tongue samples, the lowest residue concentration observed was 0.18 ppb (control) and the highest residue concentration was 10.19 ppb (12 h withdrawal), both from the total ractopamine analysis (Supplemental Table 3.5).

Abomasum. Regardless of treatment, mean concentrations of ractopamine in abomasum samples ranged from 4.27 to 16.59 ppb (parent ractopamine) and 4.28 to 20.05 ppb (total ractopamine) (Table 3.4). With regards to mean parent ractopamine concentrations, residue levels in abomasum samples from the control, 2-, 4-, and 7-day treatment groups were lower (4.27 to 5.28 ppb; $P < 0.05$) than that of the 12 h withdrawal treatment group (16.59 ppb) (Table 3.4). Similarly, mean total ractopamine residue levels from the control, 2-, 4-, and 7-day withdrawal groups were lower (4.28 to 5.80 ppb; $P < 0.05$) than that of the 12 h withdrawal group (20.05 ppb) (Table 3.4). Based on the individual abomasum samples, the highest observed residue concentration was 71.36 ppb (12 h withdrawal) from the total ractopamine analysis (Supplemental Table 3.6).

Omasum. Regardless of RH withdrawal time or lack of RH in rations, mean parent ractopamine concentrations in omasum samples ranged from 2.57 (control) to 76.80 (12 h withdrawal) ppb, while mean total ractopamine levels ranged from 2.46 (control) to 81.33 (12 h withdrawal) ppb (Table 3.4). Mean residue levels of ractopamine (parent and total) for the control and 4-day withdrawal treatment groups were lower ($P < 0.05$) than those of the 12 h, 2- and 7-day withdrawal groups (Table 3.4). Additionally, the 2- and 7-day withdrawal groups were

lower ($P < 0.05$) than the 12 h withdrawal group for both the parent and total ractopamine analyses in omasum samples (Table 3.4). The highest residue concentration from the individual omasum samples was 116.90 ppb (total ractopamine) (Supplemental Table 3.7).

Rumen/reticulum. Mean parent ractopamine concentrations in rumen/reticulum samples, regardless of treatment group, ranged from 0.00 (i.e., below the LOD; control) to 5.81 (12 h withdrawal) ppb, while mean total ractopamine levels ranged from 0.00 (i.e., below the LOD; control) to 6.80 (12 h withdrawal) ppb) (Tables 3.3 and 3.4). For both parent and total ractopamine, mean residue levels for the control, 2-, 4-, and 7-day withdrawal groups were lower ($P < 0.05$) than those of the 12 h withdrawal groups (Table 3.4). More specifically, mean parent ractopamine concentrations were lower (0.00 to 0.65 ppb; $P < 0.05$) than that of the 12 h withdrawal group (5.81 ppb) (Table 3.4). The same trend was observed for the total ractopamine concentrations (Table 3.4). In individual rumen/reticulum samples, the highest observed value was 11.02 ppb, while the lowest observed value fell below the LOD of 0.15 ppb (Table 3.3); both of these values were from the total ractopamine analysis (Supplemental Table 3.8).

Small intestine. Mean concentrations of ractopamine in small intestine samples, regardless of treatment group, ranged from 2.10 to 53.67 ppb (parent ractopamine) and 2.57 to 77.85 ppb (total ractopamine) (Table 3.4). For both parent and total ractopamine concentrations, mean residues from the control, 2-, 4-, and 7-day withdrawal groups were lower ($P < 0.05$) than those of the 12 h withdrawal treatment (Table 3.4). Based on the results obtained for the individual small intestine samples, the highest residue concentration observed was 181.91 ppb (12 h withdrawal) from the total ractopamine analysis (Supplemental Table 3.9).

Large intestine. For study one, mean concentrations of ractopamine in large intestine samples, regardless of treatment group, ranged from 20.35 to 160.61 ppb (parent ractopamine)

and 17.31 to 183.69 ppb (total ractopamine) (Table 3.4). As was observed for the majority of tissue types analyzed, mean levels of parent and total ractopamine for the 2-, 4-, 7-day withdrawal, and control treatment groups were statistically similar ($P \geq 0.05$, Table 3.4); however, for both parent and total ractopamine concentrations, mean residues from these treatment groups were lower ($P < 0.05$) than those of the 12 h withdrawal treatment (Table 3.4). Based on the results obtained for the individual large intestine samples, the highest residue concentration observed was 289.85 ppb (12 h withdrawal) from the total ractopamine analysis (Supplemental Table 3.10).

For study two, the residue levels in large intestines were higher than the other products tested (muscle, fat, and tallow). It is important to remember, however, that the large intestine is not a target tissue for testing and the levels present in the current study are much lower than those in previous experiments. In terms of raw data, the minimum and maximum residue values quantified from large intestine samples were below the limit of detection (LOD = 0.32 ppb) and 20.74 ppb, respectively (total ractopamine). Regardless of treatment, mean parent ractopamine concentrations in large intestine samples ranged from 0.41 (control groups) to 7.42 (7-day withdrawal) ppb, while mean total ractopamine residues ranged from 0.52 [Control (No Tallow)] to 8.45 (7-day withdrawal) ppb (Tables 3.5 and 5.6). Mean levels of ractopamine (parent and total) for the 2-day and 4-day withdrawal groups and both control groups were lower ($P < 0.05$) than those of the 7-day withdrawal treatment (Tables 3.5 and 3.6). Again, because the large intestine is an off-target tissue, it cannot officially be held against any standards set by Codex and the FDA.

Fat. Least squares means estimates of fat samples followed the same trends as muscle, with the highest observable levels (0.22 and 0.49 ppb) detected in 2-day withdrawal samples and

the lowest below detection in both control groups (LOD = 0.12 ppb) (Tables 3.5 and 3.6). The 2-day and 4-day withdrawal groups (0.22 and 0.14 ppb, respectively) were not ($P \geq 0.05$) different for parent ractopamine, but they were different ($P < 0.05$) for total (0.10 and 0.29 ppb, respectively) (Tables 3.5 and 3.6). For parent ractopamine residues, the 4-day withdrawal group was not different ($P \geq 0.05$) from the 7-day withdrawal group; however, it was different ($P < 0.05$) from this treatment in the total ractopamine analysis (Tables 3.5 and 3.6). The 7-day withdrawal groups and both control treatments did not differ ($P \geq 0.05$) (Tables 3.5 and 3.6).

Tallow. Mean concentrations in tallow samples, regardless of treatment group, ranged from 0.02 to 0.07 ppb (parent ractopamine) and 0.03 to 0.06 ppb (total ractopamine) (Tables 3.5 and 3.6). In the parent ractopamine analysis, the mean residue level in tallow samples from the 7-day withdrawal group (0.07 ppb) was different ($P < 0.05$) from all other treatments (Table 3.5). The remaining treatments did not differ ($P \geq 0.05$) with residue levels of 0.04, 0.03, 0.02, and 0.02 ppb (Table 3.5). With regards to mean total ractopamine concentrations, the residue levels in tallow samples from the 2-, 4-, and 7-day withdrawal treatments (0.05, 0.05, and 0.06 ppb, respectively) were similar ($P \geq 0.05$) (Table 3.6). However, the 2- and 4-day treatments also did not differ ($P \geq 0.05$) from the control groups (both 0.03 ppb), while the 7-day withdrawal treatment residue did) (Table 3.6).

Feed. Feed samples also were tested for ractopamine presence, and the highest concentrations observed were in the ration used to feed all cattle that received ractopamine plus tallow, with a maximum value of 7,064.05 ppb. However, significantly lower residues were observed in control diets, both with and without tallow, with maximums of 139.81 and 25.39 ppb, respectively. While these numbers do not reflect true “negative” controls as far as feed values are concerned, this did not seem to affect tissue/matrix controls, which were relatively

low or below detection (Tables 3.5 and 3.6). After 2, 4, and 7 days of withdrawal, ractopamine was still present in feed samples collected, but there was a steep decline from the ractopamine plus tallow treatment group, with the maximum residue observed from these three treatments being 22.14 ppb (7 day). Once again, this did not appear to affect the tissue/matrix results. These data suggested that if ractopamine is present on a feedlot, even if great care is taken to prevent cross-contamination, there is potential for ractopamine to spread into ractopamine-free or "natural" rations.

Dose and depletion. All sample types (rumen fluids, rumen solids, duodenal fluids, and fecal fluids) showed drastic regression of ractopamine concentration after three full treatment days. The treatment steers' intestinal fluid total ractopamine levels continuously declined until day 13 in all regions, as represented in Figure 3.10. Treated steers received ractopamine for three days (days -3, -2, and -1) after which ractopamine was removed and samples were collected for an additional 13 days (days 0 – 13). Following removal of ractopamine from the diets in treated steers, a sharp decrease of parent and total ractopamine were observed, with levels reaching the control level (still above the limit of detection) and remaining constant by day 7 (Figures 3.4 and 3.5; Table 3.7).

Conclusions. In general, the results of these studies suggested that after extended withdrawal times of 2, 4, and 7 days, residues in certain tissues *can* fall below the liver and muscle MRL set by Codex. However, residue levels in omasum, small intestine, and large intestine samples were higher than the muscle MRL even after extended withdrawal, although they did meet the liver MRL. That said, after 7-day withdrawal, detectable levels of ractopamine were obtained in most of the tested tissues. Because zero tolerance requirements recently set by China, Taiwan, and the European Union relate to use and not necessarily residues, they will not

be met either way. This presents major marketing/export challenges to producers using beta-agonists. Upon analyzing for parent and total ractopamine, large intestine, small intestine, and omasum samples had the highest numerical residue levels. It is unclear why ractopamine residues were detected in control (animals not treated with RH) samples. Feed-grade tallow was collected in an attempt to determine potential sources of contamination. Results varied greatly, leading to further research on the subject. While these data suggested that extended withdrawal times are generally adequate to meet current MRL, it is important to recognize that visceral tissues sampled frequently test above MRL.

As all of the tissues analyzed in these studies were off-target tissues with the exception of liver and muscle, it is imperative to understand that they cannot officially be held to the MRL set by Codex and the FDA. In many cases, however, off-target tissues have been tested in export markets, making it is critically important to further understand presence of ractopamine residues in these off-target tissues. Part of this understanding comes from realizing the probability of sample residues falling below the MRL. For this reason, all samples were subjected to an additional analysis to determine the odds of meeting these standards (Figures 3.6 – 3.9). The MRL cutoffs used for this particular analysis were 0.1 ppb (near the limit of detection for liquid chromatography mass spectrometry ractopamine quantitation) and 10.0 ppb (the muscle MRL).

Results for study one suggested that at 0.1 ppb, it was nearly impossible to achieve more than a 60% probability of falling below the cutoff, and even in this case it was for only a very select set of samples (muscle, tongue, and rumen/reticulum specifically after extended withdrawal) (Figures 3.6 and 3.8). The risk, therefore, of testing positive for ractopamine in all other samples, and the aforementioned tissues at shorter withdrawal times, was high for both parent and total ractopamine (Figures 3.6 and 3.8). Contrastingly, the probability of falling below

the MRL of 10.0 ppb (for parent ractopamine) was much greater for liver, muscle, heart, tendon, tongue, and rumen/reticulum, with nearly all withdrawal times approaching 100% (Figures 3.7 and 3.9). Unsurprisingly, the probability of falling below the 10.0 ppb MRL was lower for total ractopamine in liver, likely due to metabolites (Figures 3.7 and 3.9). The tissues that were of the highest risk of not meeting the MRL in either case regardless of withdrawal time were abomasum, omasum, small intestine, and large intestine (Figures 3.6 – 3.9). Once again, while these are currently off-target tissues based on Codex and FDA MRL, zero tolerance standards are becoming more common and these tissues can and will be affected by such requirements.

- Extended withdrawal times of 2, 4, and 7 days *can* result in residues under the liver and muscle MRL set by Codex, but residue levels in **omasum, small intestine, and large intestine** were higher than the Codex muscle MRL, even after extended withdrawal.
- New zero tolerance requirements/recommendations by China, Taiwan, and the European Union present major marketing/export challenges because most of the tested tissues, even after 7-day withdrawal, are above the limit of detection.
- All tissues evaluated in the study (aside from liver and muscle) were off-target tissues and, therefore, should never be officially held to a Codex MRL.
- Tissues collected from negative control cattle (i.e., cattle that did not receive ractopamine hydrochloride in the diet) were generally positive at low levels for the compound. In follow-up, but not necessarily related to the initial study, feed tallow samples collected from the same cooperating feedyard two months later contained ractopamine levels of **0.4 to 50.8 ppb,** suggesting that negative control sample cattle during study one may have been compromised.

- The fact that tissues derived from negative control cattle tested positive for ractopamine made determination of conclusive withdrawal time recommendations to meet export requirements difficult to develop, leading to the development and implementation of study two.

Results of study two suggested that it is possible to collect true negative control samples from a commercial beef harvest facility, but this is relative to limit of detection (Tables 3.5 and 3.6). In this study, fat, tallow, and large intestine samples were all statistically similar to the control groups at the 7-day withdrawal period. Realizing that ractopamine levels in this study were much lower than those present in study one, the challenge still remains that U.S. beef export markets are now requiring or recommending zero tolerance standards for use and residues. For this reason, it was critically important to understand risk involved in these products testing above the MRL for ractopamine, so probabilities were tested to better understand what percentage of the samples from study two fell below the limit of detection and the Codex MRL for muscle (Figures 3.10 and 3.11). The probability that muscle, fat, and tallow would fall below the 10.0 ppb MRL was 100% across all treatments for both parent and total ractopamine (Figure 3.11). However, this was not true for large intestine samples, holding true to the fact that large intestine residues are generally among the highest collected (Figure 3.11). This means that there is a greater risk associated with exporting large intestines. Further, at the 0.1 ppb cutoff, probabilities are much more variable, creating more concern for risk of rejection of exported product based on a positive ractopamine value (Figure 3.10).

While the results from the second study indicated that it was possible to collect negative control samples in some cases, there was clearly still contamination from an unknown source creating ramifications for the results. This was observed in both feed samples collected as well as

tissue samples, which led to the dose and depletion study of the lower gastrointestinal tract of fistulated steers.

When the third study was designed, it was imperative that contamination between was considered to avoid any further challenges with depletion. Considerable care was taken to prevent cross-contamination, but untreated (control) steers still exhibited ractopamine presence, suggesting another potential issue. During the three-day treatment, ractopamine levels (both parent and total) in all sample types from ractopamine-treated steers continued to rise, while levels in samples from the control steers remained relatively steady (Figures 3.4 and 3.6). After the treatment period, ractopamine was removed and a rapid decline of ractopamine concentration was observed, suggesting a dose response of ractopamine in the lower GI of cattle. Because of the remaining issues with control samples presenting with positive ractopamine levels, another contamination study was conducted. Overall, these studies proved that ractopamine levels vary greatly, and extended withdrawal times are necessary to near the MRL for several of the visceral tissues, such as omasum, small intestine, and large intestine. This trend was found across all studies, and was deemed an extremely important conclusion in terms of export markets as these items are typically not consumed in the U.S.

Table 3.1. Treatment groups (ractopamine hydrochloride withdrawal times) and number of animals sampled per treatment.

Treatment (Withdrawal Time)	Number of Pens	Animals Sampled per Pen	Total Animals Sampled per Treatment Group	Tissues Collected
12 h	6	2	12	all tissues *except liver and muscle*
2 days	6	2	12	all tissues
4 days	6	2	12	all tissues
7 days	6	2	12	all tissues
Control	6	1	6	all tissues

Table 3.2. Ultra-performance liquid chromatography (UPLC) gradient for LC-MS/MS sample analysis of 10 tissue types for ractopamine quantification.

Time (min)	% MPA^	% MPB*
0	99.0	1.0
0.2	99.0	1.0
2.2	70.0	30.0
3	1.0	99.0
4.25	1.0	99.0
4.5	99.0	1.0
6.5	99.0	1.0

^ Mobile Phase A: water with 2 mM ammonium formate
* Mobile Phase B: acetonitrile with 0.1% formic acid

Table 3.3. Limit of detection (LOD) and limit of quantification (LOQ) for parent and total ractopamine concentration residues (ppb) for the 10 tissues collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Tissue Type	LOD (ppb)	LOQ (ppb)
Liver	0.002	0.006
Muscle	0.04	0.12
Heart	0.02	0.08
Tendon	0.02	0.07
Tongue	0.03	0.11
Abomasum	0.028	0.093
Omasum	0.50	1.67
Rumen/Reticulum	0.15	0.51
Small Intestine	0.22	0.73
Large Intestine	0.44	1.45

Table 3.4. Least-squares means of parent and total ractopamine concentrations (ppb) for 10 tissues collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times prior to slaughter (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Tissue	Treatment	Parent Ractopamine (ppb)			Total Ractopamine (ppb)		
		LSMean	95% CI Low	95% CI High	LSMean	95% CI Low	95% CI High
Liver	2 days	3.48$_{a}$	3.46	3.49	8.84$_{a}$	7.70	9.97
	4 days	3.44$_{b}$	3.42	3.45	3.96$_{b}$	2.88	5.04
	7 days	3.43$_{bc}$	3.41	3.44	3.60$_{b}$	2.47	4.73
	Control	3.40$_{c}$	3.38	3.42	3.53$_{b}$	2.00	5.06
Muscle	2 days	0.73$_{a}$	0.59	0.87	1.15$_{a}$	0.99	1.32
	4 days	0.56$_{a}$	0.42	0.69	0.71$_{b}$	0.55	0.87
	7 days	0.23$_{b}$	0.09	0.37	0.26$_{c}$	0.09	0.43
	Control	0.00$_{b}$	-0.20	0.19	0.04$_{c}$	-0.19	0.27
Heart	12 h	3.31$_{a}$	1.67	4.95	5.14$_{a}$	4.21	6.07
	2 days	2.63$_{ab}$	0.91	4.35	1.23$_{b}$	0.26	2.21
	4 days	0.84$_{b}$	-0.80	2.48	0.94$_{b}$	0.01	1.87
	7 days	1.92$_{ab}$	0.20	3.64	1.52$_{b}$	0.54	2.50
	Control	1.36$_{ab}$	-0.96	3.68	1.04$_{b}$	-0.28	2.35
Tendon	12 h	4.85$_{a}$	4.02	5.69	13.74$_{a}$	11.94	15.53
	2 days	2.81$_{b}$	1.93	3.68	4.89$_{b}$	3.00	6.78
	4 days	2.70$_{b}$	1.86	3.53	2.88$_{bc}$	1.08	4.67
	7 days	1.72$_{bc}$	0.84	2.59	1.95$_{c}$	0.07	3.83
	Control	0.58$_{c}$	-0.60	1.75	1.07$_{c}$	-1.47	3.61
Tongue	12 h	3.94$_{a}$	3.67	4.22	8.01$_{a}$	7.45	8.56
	2 days	1.57$_{b}$	1.28	1.86	2.41$_{b}$	1.83	2.99
	4 days	1.04$_{c}$	0.77	1.32	1.25$_{c}$	0.69	1.80
	7 days	0.57$_{d}$	0.28	0.86	0.62$_{c}$	0.04	1.20
	Control	0.33$_{d}$	-0.06	0.71	0.36$_{c}$	-0.43	1.14

Tissue	Treatment	Parent Ractopamine (ppb)			Total Ractopamine (ppb)		
		LSMean	95% CI Low	95% CI High	LSMean	95% CI Low	95% CI High
Abomasum	12 h	16.59$_{a}$	12.16	21.02	20.05$_{a}$	15.50	24.58
	2 days	5.28$_{b}$	0.64	9.92	5.80$_{b}$	1.05	10.55
	4 days	4.52$_{b}$	0.09	8.95	4.61$_{b}$	0.08	9.14
	7 days	4.33$_{b}$	-0.31	8.97	4.35$_{b}$	-0.40	9.10
	Control	4.27$_{b}$	-1.99	10.53	4.28$_{b}$	-2.13	10.68
Omasum	12 h	76.80$_{a}$	68.85	84.75	81.33$_{a}$	73.12	89.54
	2 days	22.29$_{b}$	13.95	30.63	22.73$_{b}$	14.13	31.34
	4 days	5.92$_{c}$	-2.03	13.88	6.13$_{c}$	-2.07	14.34
	7 days	19.47$_{b}$	11.13	27.81	20.19$_{b}$	11.58	28.79
	Control	2.57$_{c}$	-8.67	13.82	2.46$_{c}$	-9.15	14.06
Rumen/Reticulum	12 h	5.81$_{a}$	5.36	6.26	6.80$_{a}$	6.29	7.31
	2 days	0.65$_{b}$	0.18	1.12	0.89$_{b}$	0.35	1.43
	4 days	0.24$_{b}$	-0.21	0.69	0.25$_{b}$	-0.26	0.77
	7 days	0.07$_{b}$	-0.41	0.54	0.07$_{b}$	-0.47	0.60
	Control	0.00$_{b}$	-0.64	0.64	0.00$_{b}$	-0.72	0.72
Small Intestine	12 h	53.67$_{a}$	40.07	67.27	77.85$_{a}$	60.53	95.16
	2 days	20.42$_{b}$	6.16	34.68	28.74$_{b}$	10.58	46.90
	4 days	19.18$_{b}$	5.58	32.78	21.78$_{b}$	4.47	39.10
	7 days	15.08$_{b}$	0.82	29.34	16.03$_{b}$	-2.13	34.19
	Control	2.10$_{b}$	-17.13	21.33	2.57$_{b}$	-21.91	27.06
Large Intestine	12 h	160.61$_{a}$	138.94	182.28	183.69$_{a}$	161.86	205.52
	2 days	44.25$_{b}$	21.52	66.98	48.84$_{b}$	25.95	71.74
	4 days	32.73$_{b}$	11.06	54.40	35.83$_{b}$	14.00	57.66
	7 days	26.75$_{b}$	4.02	49.48	27.78$_{b}$	4.89	50.68
	Control	20.35$_{b}$	-10.30	50.99	17.32$_{b}$	-13.56	48.19

$_{abcd}$Means without a common superscript within a column, by tissue, differ ($P < 0.05$).

Table 3.5. Least squares means of parent ractopamine concentrations (ppb) in muscle, fat, rendered tallow, and large intestine from steers fed five different treatments: 1) a negative control [fed no ractopamine and no feed-tallow – fed from verified clean feed trucks; Control (No Tallow)]; 2) cattle fed ractopamine hydrochloride (at approximately 250-300 mg/hd/day), but withdrawn from treatment at 7 days before harvest (7 day); 3) withdrawn from treatment at 4 days before harvest (4 day); 4) withdrawn from treatment at 2 days before harvest (2 day); or 5) a control with feed-tallow [fed no ractopamine but fed feed-grade tallow; Control (With Tallow)].

Tissue/Matrix	Treatment	Parent			
		LSMean	95% CI Low	95% CI High	SE[1]
Muscle	2 day	0.76[a]	0.65	0.88	0.06
	4 day	0.59[b]	0.47	0.71	
	7 day	0.32[c]	0.20	0.44	
	Control (With Tallow)	<	0.00	0.12	
	Control (No Tallow)	<	0.00	0.12	
Fat	2 day	0.22[a]	0.13	0.31	0.04
	4 day	0.14[ab]	0.05	0.22	
	7 day	0.08[bc]	0.00	0.17	
	Control (With Tallow)	<	0.00	0.09	
	Control (No Tallow)	<	0.00	0.09	
Tallow	2 day	0.02[b]	0.00	0.04	0.01
	4 day	0.04[b]	0.02	0.05	
	7 day	0.07[a]	0.05	0.09	
	Control (With Tallow)	0.03[b]	0.01	0.05	
	Control (No Tallow)	0.02[b]	0.00	0.04	
Large Intestine	2 day	3.93[b]	2.14	5.71	0.90
	4 day	4.72[b]	2.93	6.50	
	7 day	7.42[a]	5.63	9.21	
	Control (With Tallow)	0.41[c]	0.00	2.20	
	Control (No Tallow)	0.41[c]	0.00	2.20	

[1]SE denotes pooled standard error.

[abc]Means without a common superscript within a column, by tissue/matrix, differ ($P < 0.05$).

<Denotes below the limit of detection (0.12 ppb).

Table 3.6. Least squares means of total ractopamine concentrations (ppb) in muscle, fat, rendered tallow, and large intestine from steers fed five different treatments: 1) a negative control [fed no ractopamine and no feed-tallow – fed from verified clean feed trucks; Control (No Tallow)]; 2) cattle fed ractopamine hydrochloride (at approximately 250-300 mg/hd/day), but withdrawn from treatment at 7 days before harvest (7 day); 3) withdrawn from treatment at 4 days before harvest (4 day); 4) withdrawn from treatment at 2 days before harvest (2 day); or 5) a control with feed-tallow [fed no ractopamine but fed feed-grade tallow; Control (With Tallow)].

Tissue/Matrix	Treatment	Total			
		LSMean	95% CI Low	95% CI High	SE[1]
Muscle	2 day	0.41$_{c}$	0.25	0.57	0.08
	4 day	0.83$_{b}$	0.67	0.99	
	7 day	1.22$_{a}$	1.06	1.38	
	Control (With Tallow)	<	0.00	0.16	
	Control (No Tallow)	<	0.00	0.16	
Fat	2 day	0.10$_{c}$	0.00	0.22	0.06
	4 day	0.29$_{b}$	0.17	0.42	
	7 day	0.49$_{a}$	0.36	0.61	
	Control (With Tallow)	<	0.00	0.12	
	Control (No Tallow)	<	0.00	0.12	
Tallow	2 day	0.05$_{ab}$	0.02	0.07	0.01
	4 day	0.05$_{ab}$	0.03	0.08	
	7 day	0.06$_{a}$	0.04	0.09	
	Control (With Tallow)	0.03$_{b}$	0.00	0.05	
	Control (No Tallow)	0.03$_{b}$	0.00	0.05	
Large Intestine	2 day	4.76$_{b}$	2.83	6.69	0.97
	4 day	5.69$_{b}$	3.76	7.62	
	7 day	8.45$_{a}$	6.52	10.38	
	Control (With Tallow)	0.69$_{c}$	0.00	2.62	
	Control (No Tallow)	0.52$_{c}$	0.00	2.45	

[1]SE denotes pooled standard error.

$_{abc}$Means without a common superscript within a column, by tissue/matrix, differ ($P < 0.05$).

<Denotes below the limit of detection (0.12 ppb).

Table 3.7. Limit of detection (LOD) and limit of quantitation (LOQ) upon ractopamine concentration residue analysis via liquid chromatography mass spectrometry for rumen fluids, rumen solids, duodenal solids, and fecal fluids from ractopamine- treated and untreated fistulated steers following a three-day treatment and 13-day withdrawal period.

Fluid Type	LOD (ppb)	LOQ (ppb)
Rumen Fluid	0.67	2.22
Rumen Solid	0.64	2.14
Duodenal Fluid	1.28	4.27
Fecal Fluid	1.57	5.23

Figure 3.1. Tagging a carcass for identification of tissues throughout the harvest facility.

Figure 3.2. Tagging a rumen/reticulum sample for identification post-commercial processing.

Figure 3.3. Identifying and collecting omasum samples post-commercial processing.

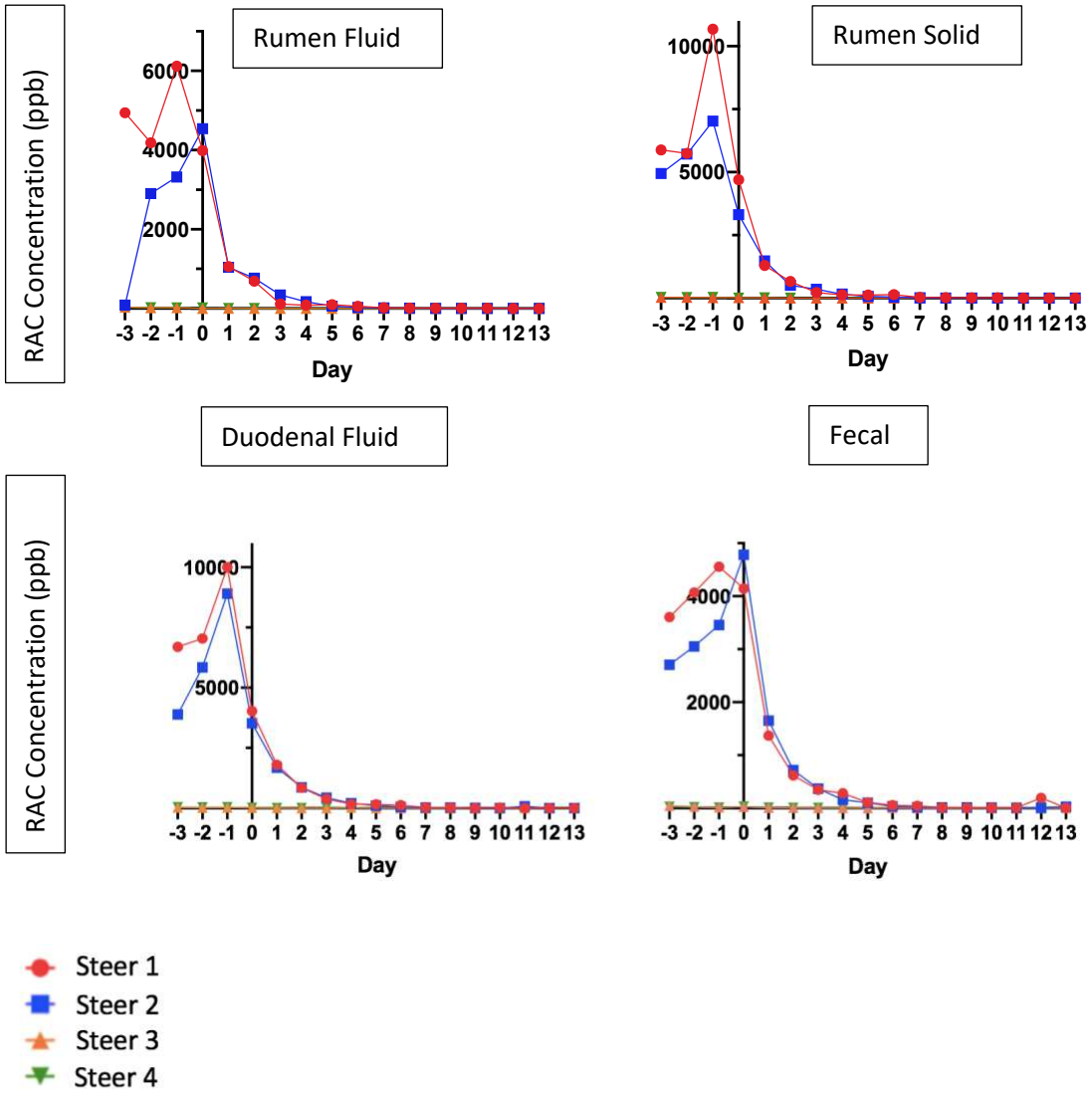

Figure 3.4. Rumen fluids, rumen solids, duodenal fluids, and fecal fluids from fistulated steers either fed (treated) or not fed (untreated) ractopamine over 3-day treatment and 13-day withdrawal. Day -3 denotes treatment day 1 while days 0-13 are post-treatment. Steers 1 and 2 (treated) exhibited an increase in parent ractopamine during treatment and a sharp decrease in parent ractopamine following removal of ractopamine from diets. Steers 3 and 4 (untreated) exhibited a much more stable parent ractopamine residue concentration, however, the compound was present in untreated (control) animals, although it is difficult to see due to such a steep y-axis for ractopamine concentration.

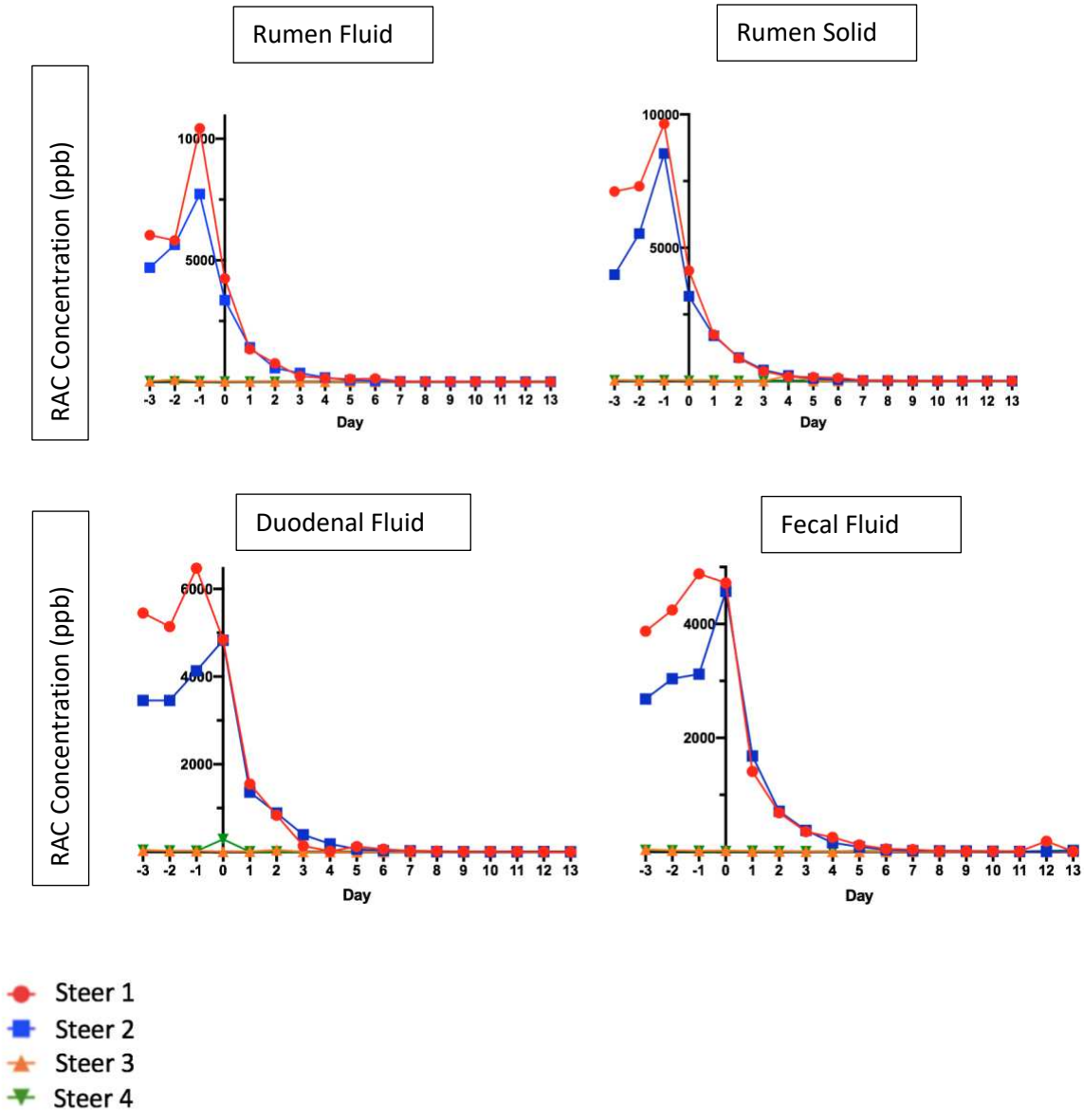

Figure 3.5. Rumen fluids, rumen solids, duodenal fluids, and fecal fluids from fistulated steers either fed (treated) or not fed (untreated) ractopamine over 3-day treatment and 13-day withdrawal. Day -3 denotes treatment day 1 while days 0-13 are post-treatment. Steers 1 and 2 (treated) exhibited an increase in total ractopamine during treatment and a sharp decrease in total ractopamine following removal of ractopamine from diets. Steers 3 and 4 (untreated) exhibited a much more stable total ractopamine residue concentration, however, the compound was present in untreated (control) animals.

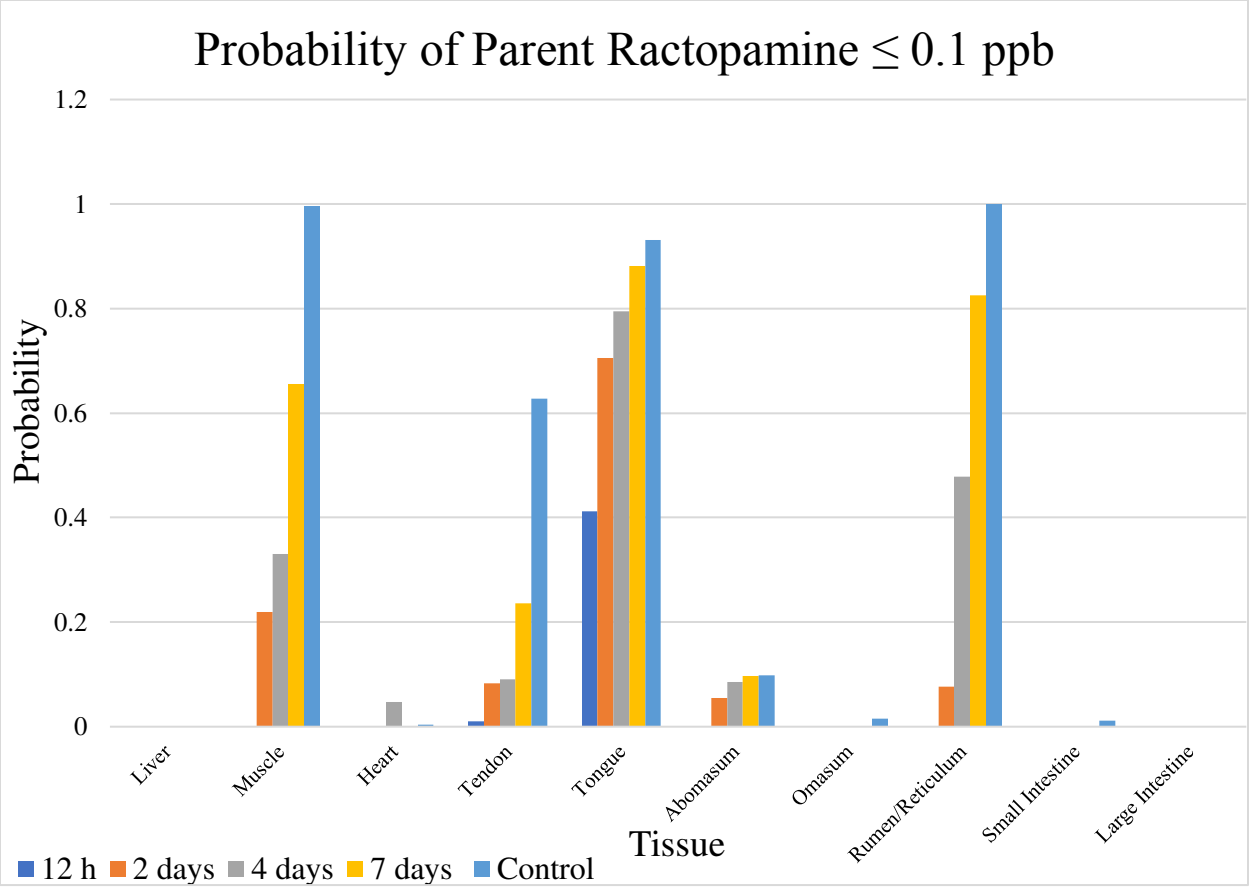

Figure 3.6. The probability of parent ractopamine in tissues (after various withdrawal times) being equal to or below 0.1 ppb, an arbitrary cutoff near the limit of detection for liquid chromatography mass spectrometry analysis.

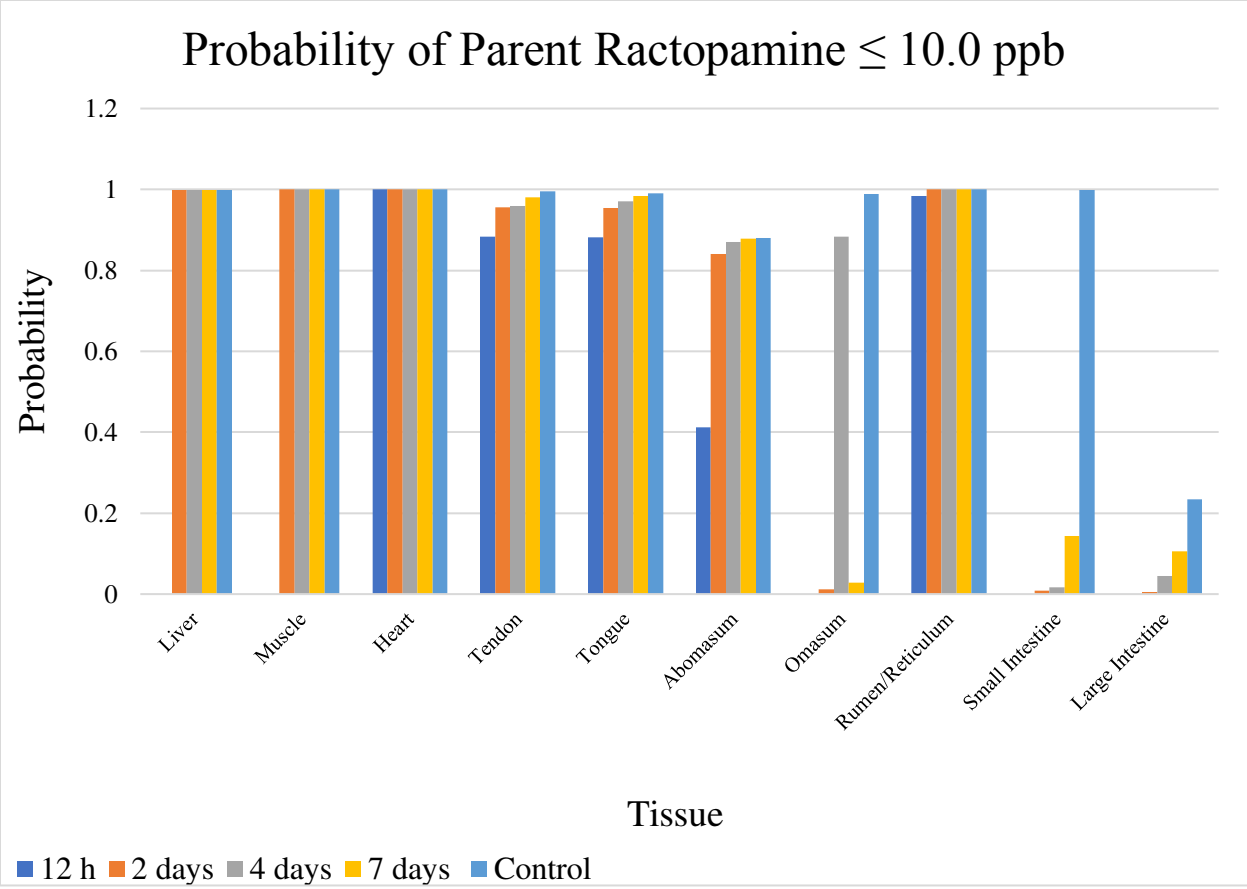

Figure 3.7. The probability of parent ractopamine in tissues (after various withdrawal times) being equal to or below 10.0 ppb, the muscle MRL by the Codex Alimentarius Commission.

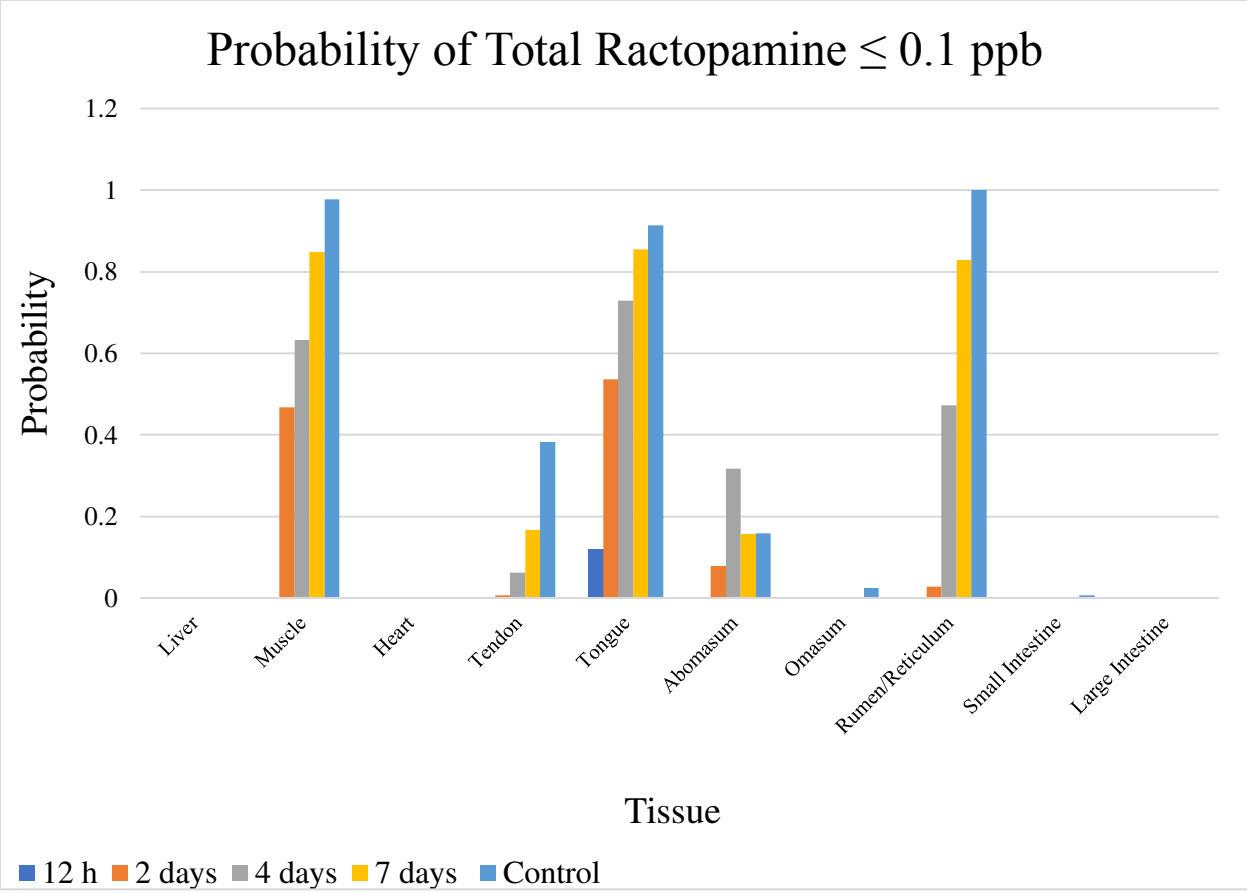

Figure 3.8. The probability of total ractopamine in tissues (after various withdrawal times) being equal to or below 0.1 ppb, an arbitrary cutoff near the limit of detection for liquid chromatography mass spectrometry analysis.

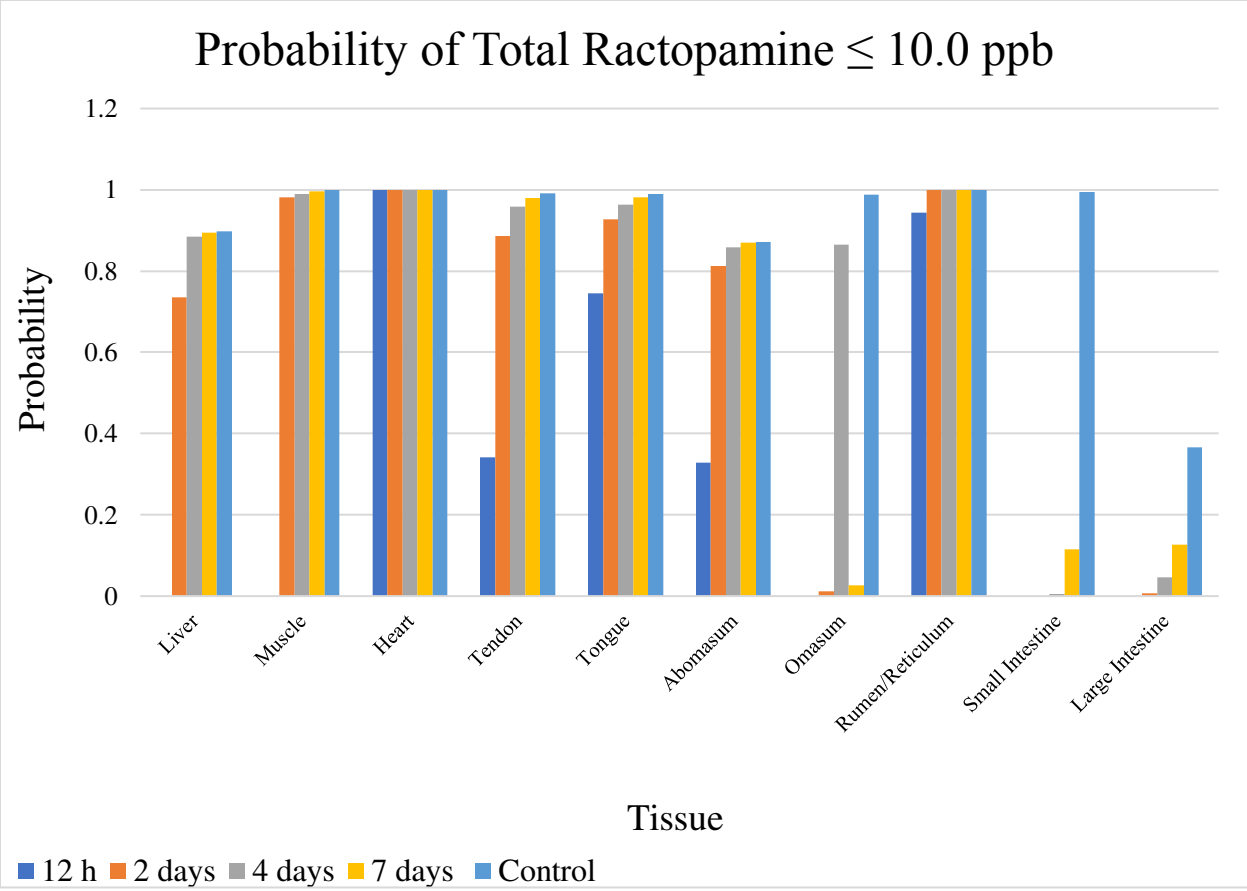

Figure 3.9. The probability of total ractopamine in tissues (after various withdrawal times) being equal to or below 10.0 ppb, the muscle MRL by the Codex Alimentarius Commission.

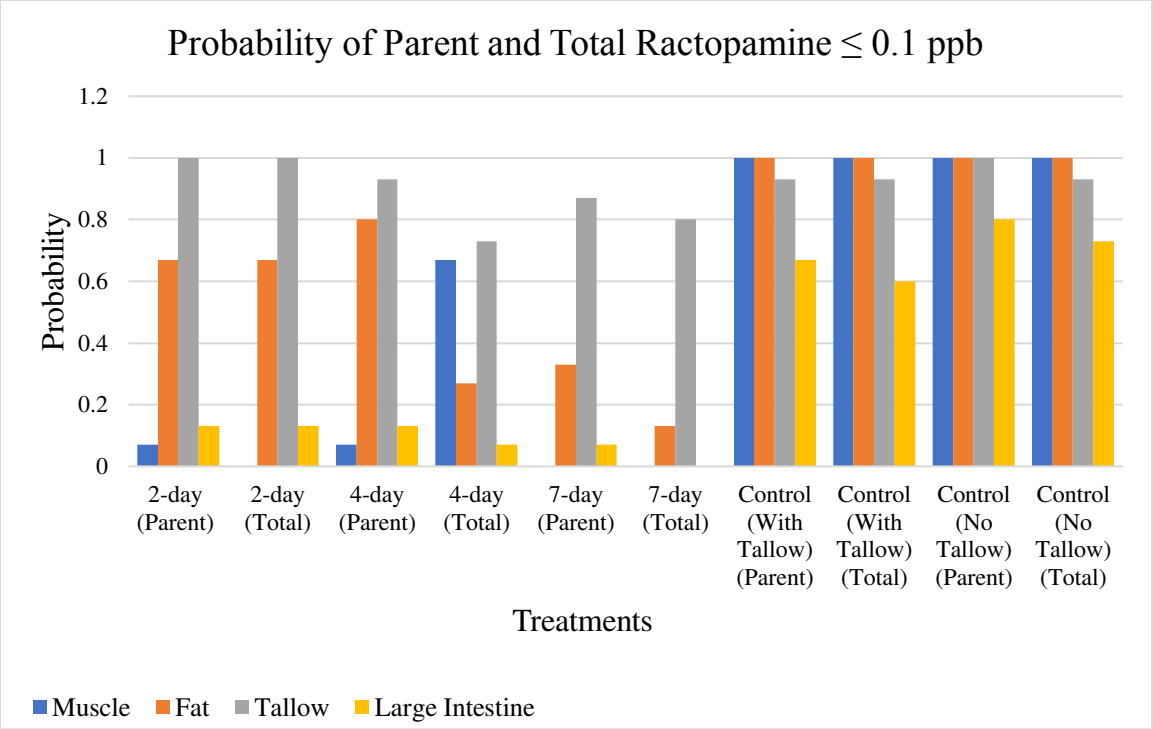

Figure 3.10. The probability of ractopamine in tissues and tallow (after various withdrawal times and with or without tallow) being equal to or below 0.1 ppb, an arbitrary cutoff near the limit of detection for liquid chromatography mass spectrometry analysis.

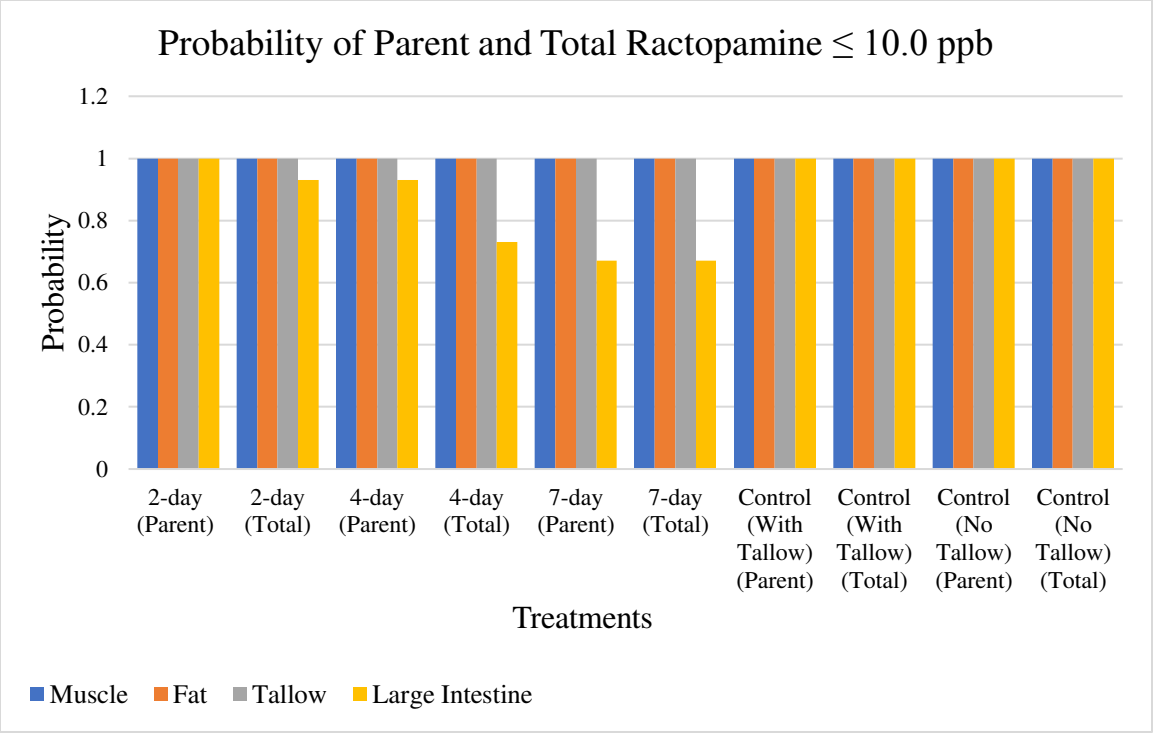

Figure 3.11. The probability of ractopamine in tissues and tallow (after various withdrawal times and with or without tallow) being equal to or below 10.0 ppb, the muscle MRL by the Codex Alimentarius Commission.

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CHAPTER 4

Feed contamination and implications to the U.S. beef industry

Summary

As meat trade issues surrounding use of ractopamine hydrochloride continue to develop and zero-tolerance requirements and standards are implemented, so too do concerns regarding contamination of livestock feed and thus tissues, tallow, and even meat and bone meal. Therefore, these studies were designed to investigate the impact of contamination on ractopamine residues upon ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) analysis. Tallow, meat and bone meal, and tissues collected upon feeding various combinations of tallow rendered from conventional and natural cattle were collected and analyzed for parent and total ractopamine concentrations (ppb). In many cases, both parent and total ractopamine concentrations were low or non-detectable; however, there were exceptions to this proving that contamination could be extremely problematic to U.S. beef exporters.

Introduction

Beef tallow has been commonly included finishing rations of beef cattle for decades at approximately 5% of the diet DM to reduce diet costs and improve energy consumption and cattle performance (Matsushima and Dowe, 1954). However, interest in possible contaminants that remain in the tallow following the rendering process, and the possibility of recycling of such contaminants, has increased due to zero-tolerance standards mandated by countries importing beef and beef products from the United States. Additionally, meat and bone meal rendered from cattle is frequently fed to poultry and swine due to highly digestible protein availability (Eagleson et al., 2018; Traylor, Cromwell, and Lindemann, 2005). Due to concerns regarding

zero-tolerance policies, use of meat and bone meal in these diets has become contentious because of potential for re-circulation of ractopamine in the environment (Aroeira et al., 2019).

Ractopamine hydrochloride, a beta-adrenergic agonist, is commonly used as a feed additive in feedlots as a repartitioning agent to stimulate muscle hypertrophy and decrease the amount of energy used for fat deposition (Aroeira et al., 2019; Johnson and Chung, 2007; Johnson, Smith, and Chung, 2014). Including orally-active ractopamine in the diets of feedlot steers has well-documented effects including: improved carcass conformation score (Quinn et al., 2016), heavier carcass weights, increased average daily gain, and improved lean mass area (Vogel et al., 2009). For all of these reasons, U.S. beef producers rely heavily on ractopamine, but contamination issues are continuing to raise concerns.

Recent analytical testing (via liquid chromatography mass spectrometry) of tallow rendered from conventionally fed and certified antibiotic-free cattle and meat and bone meal from commercial beef harvest facilities determined that residual ractopamine concentrations are possible. In general, when ractopamine is used with extended withdrawal practices, remaining concentrations in muscle and liver tissues are below United States and global standards (Codex, 2015; Elanco, 2003). However, ractopamine contamination continues to threaten exports of U.S. beef to countries with zero-tolerance policies such as China and the European Union (EU). Thus, several studies were conducted to further understand the impact of such contamination.

Tallow and meat/bone meal as potential sources of ractopamine in cattle feed

Materials and Methods

Sample collection. Ractopamine hydrochloride [i.e., parent and total (hydrolyzed with β-glucuronidase) concentrations in tallow destined for feed was evaluated by obtaining a minimum of n = 10 replicate feed-grade tallow samples (approximately 50 g) from each of four

commercial packing plants that allowed for us to control tallow generated from ractopamine-treated (as presented to the plant) versus ractopamine-untreated cattle – dispersed geographically and over various seasons to reflect a wide cross-section of the industry (N = 80 samples). In several cases, collecting tallow rendered from natural cattle (TNAT) from the rendering tank at the commercial beef harvest facility was not plausible; therefore, it became necessary to collect subcutaneous fat from untreated cattle (having not received ractopamine, as presented to the plant). Fat samples were transported to Colorado State University (Fort Collins, CO, USA), and stored at -20°C before rendering in-laboratory.

Tallow rendering. Three of the commercial beef harvest facilities that were used did not have the capacity to separate tallow rendered from conventional cattle (TCONV) versus TNAT. Thus, 10 subcutaneous fat samples (> 100 g each) were collected from each of these facilities and rendered following the Essential Rendering Overview by Meeker and Hamilton. Each sample was cut into small (~ 3 cm x 3 cm) pieces, placed in a sterile 600 ml Pyrex beaker (Corning, Inc.; Corning, NY), and microwaved for 6 min in a Panasonic Countertop Microwave (The Genius Sensor 1250W; Panasonic Corp.; Kadoma, Osaka Prefecture, Japan). After cooking, the remaining fat solids were removed using sterile forceps, and a measure of the temperature was obtained using an infrared thermometer. The average temperature after cooking was 116.6°C ± 8.4 (standard deviation). The liquid portion was carefully poured into two 50 ml centrifuge tubes (VWR International, LLC; Radnor, PA). The first tube was filled to 30 ml and centrifuged for 20 min at 2000 RPM (20°C; Beckman Model TJ-6 Centrifuge; Beckman Coulter, Inc.; Indianapolis, IN). This portion was used for ractopamine analysis of TNAT samples.

Meat and bone meal collection. At each of the four processing plants, meat and bone meal was also collected, although it could not be collected for ractopamine-treated versus

untreated as originally intended. In this case, random samples from production on collection days were obtained and analyzed in laboratory for prevalence of ractopamine hydrochloride. Samples were collected (> 100 g) in Whirl-pak bags (Nasco; Fort Atkinson, WI), transported to CSU, and stored at -20°C until processing/sample analysis.

Analytical method. This process also required that ractopamine extraction and diagnostic procedures for tallow using liquid chromatography mass spectrometry (LC-MS/MS) were validated (which allowed subsequent testing to occur for producers wishing to diagnose issues or prevent positive residue events). Core ractopamine liquid chromatography tandem-mass spectrometry procedures (as described previously and adapted from the AOAC official method 2001.23) were utilized to conduct these analyses.

Statistical analysis. Parent and total ractopamine concentrations were analyzed separately, and a treatment by plant interaction was investigated using a general linear mixed model in SAS (version 9.4; Cary, NC). Analysis of variance included a fixed effect of treatment (conventional versus natural) and a random effect of plant. Data are reported as least squares means using a significance level of $\alpha = 0.05$.

Results and Discussion

Tallow was collected from four different commercial beef harvest facilities across the U.S. during different seasons from both ractopamine-treated (conventional) and ractopamine-untreated (natural) production lots. Because of the lack of ability to track and source tallow rendered from natural cattle (TNAT) in most cases, subcutaneous fat was collected in three of the four plants, and rendered in laboratory, which is depicted below (Table 4.1).

For both parent and total ractopamine, residue concentrations quantified in both TCONV and TNAT samples were less than 2 ppb, falling far below the muscle MRL set by Codex and

the U.S. FDA (Table 4.1) (Codex, 2015; Elanco, 2003). Least squares mean estimates from natural samples for the three plants which required in-laboratory rendering were higher ($P < 0.05$) than the mean estimate from the already-rendered natural samples (Table 4.1). It was unclear whether this was due to the subcutaneous fat samples themselves or whether the temperature during rendering was not high enough, although this was not observed in another set of samples that were processed at the same time. Overall, there were several samples for both TCONV and TNAT that were below the limit of detection (0.1 to 0.3 ppb), which led to the large number of ND (i.e., below the limit of detection) residues represented (Table 4.1). While observable and quantifiable levels were exceedingly low with low standard error of only 0.03 and 0.05 for parent and total ractopamine, respectively (Table 4.1), there could be potential for cross-contamination of cattle feed from tallow at low amounts. Additionally, there could be challenges with residual protein in rendered tallow from commercial plants, and certainly with the in-laboratory rendered tallow due to centrifugal force without steam.

Meat and bone meal (MBM) also was collected at random from the commercial beef harvest facilities that tallow was collected from. As discussed previously, it was a challenge to separate ractopamine-treated from untreated MBM because this is not generally done. Therefore, on a given collection day, a random sample of MBM product was collected and analyzed using liquid chromatography mass spectrometry for both parent and total ractopamine, and the results varied significantly (Table 4.2). This variation could potentially be due to differences in production at the harvest facilities, as the collections were completed in different facilities across four states, a result of regional differences, or caused by different lots of cattle (i.e. conventional versus natural that were not sorted during MBM production). Regardless, even the “high” levels observed in this study were relatively low in comparison to ractopamine residues observed in

problematic tissues from previous experiments (Table 4.2). However, because of the zero-tolerance markets, this could become a greater issue for U.S. producers where exports are concerned if concentrations continue to rise for any number of potential reasons. For that reason, it is increasingly important to understand how tissues could be affected by tallow contamination, so an additional study was conducted to assess inclusion of tallow on ractopamine presence quantified in various tissues collected from feedlot steers.

Differential ractopamine residue content and depletion among tissues

Materials and Methods

Background and objective. As listed on the USDA-FSIS Export Library, "Effective May 24, 2017 deboned beef, bone-in beef including heart, kidney, liver, omasum, tendons, outside skirt, inside skirt, hanging tender, oxtails, beef bones, feet, cheek meat, head meat, oxlips, backstrap, tunic tissue, [ground beef (made strictly in conformance with FSIS regulations excluding weasand meat); were not evaluated in this project], and tongues derived from cattle less than 30 months of age" are eligible for export to the People's Republic of China. Furthermore, "All beef must be derived from cattle that were either (a) born, raised, and slaughtered in the United States, (b) imported from Canada or Mexico and then raised and slaughtered in the United States, or (c) imported from Canada or Mexico for direct slaughter in the United States" which imparts additional need for controls of feeding in Canada and Mexico. Lastly, "Eligible beef products can be scalded, heat-treated, or smoked but not fully cooked" and "No additives will be used in any of these processes." The initial focus of this study was on the likelihood of rare ractopamine violations in the tissues listed. As a consequence, we developed low-dose exposure levels at which ractopamine (parent and glucuronidation metabolites) may be detected in off-target tissues that are eligible for export to China.

Beef tallow is commonly included in finishing beef cattle diets at approximately 5% of the diet DM to reduce diet costs and improve energy consumption and cattle performance. However, interest in possible contaminants that remain in the tallow following the rendering process has increased due to a zero-tolerance requirement for certain contaminants mandated by countries importing beef and beef products from the United States. Recent analytical testing (via liquid chromatography mass spectrometry) of tallow rendered from conventionally fed and certified antibiotic-free cattle, referred to from here on as TCONV and TNAT, respectively, confirmed that low concentrations of parent and total ractopamine can be detected in TCONV. However, neither parent nor total ractopamine were detected in TNAT. In this analytical testing, total ractopamine concentrations in TCONV ranged from 1.22 to 31.67 ppb, with a standard deviation of 10.07, which simulates a low-dose contamination event. Because ractopamine hydrochloride, a beta-adrenergic agonist, is commonly used as a feed additive in feedlots prior to the finishing period to stimulate muscle hypertrophy and decrease the amount of energy used for fat deposition (Johnson and Chung, 2007; Johnson, Smith, and Chung, 2014), it was important to analyze it in this study. Including orally-active ractopamine in the diets of feedlot steers has well-documented effects including: improved carcass conformation score (Quinn et al., 2016), heavier carcass weights, increased average daily gain, and improved lean mass area (Vogel et al., 2009). When ractopamine is used with responsible withdrawal practices, remaining concentrations in muscle and liver tissues are below United States and global standards (Codex, 2015). However, ractopamine contamination of TCONV presents an issue that threatens exports of U.S. beef to countries with zero-tolerance policies such as China and the European Union (EU).

This experiment was conducted to determine the effect of including feed-grade tallow (containing residual ractopamine) in feedlot diets fed to finishing beef cattle on feedlot cattle

performance and tissue residue concentrations post slaughter. The overarching hypothesis was that low concentrations of ractopamine contained in TCONV fed to finishing cattle would not impact growth performance or carcass characteristics but would be detectable in selected tissues post slaughter. Therefore, the objective of the experiment was to include TCONV in the diet at various inclusion rates to investigate the influence of tallow containing residual ractopamine on feedlot performance, carcass characteristics, and ractopamine concentrations in selected tissues post slaughter.

Institutional Animal Care and Use Committee. Prior to the initiation of this experiment, all animal care, handling, and sampling procedures described herein were approved by the Colorado State University Animal Care and Use Committee (Approval #17-7584A).

Cattle processing and weighing. Thirty-five steers (initial BW = 486 kg) were selected from the Colorado State University Agriculture, Research, Development, and Education Center (ARDEC; Fort Collins, CO) from the 2017 calf crop for use in the current experiment. Two purebred Angus, eight purebred Hereford, and twenty-five Angus x Hereford steers were selected based on yearling body weight data obtained on April 19, 2018. Upon selection for the experiment (May 25, 2018), steers were identified by their ARDEC assigned visual ID ear tag, weighed, vaccinated with Bovi-Shield Gold 5 (bovine rhinotracheitis-virus diarrhea-parainfluenza respiratory syncytial virus Zoetis, Parsippany, NJ) and UltraChoice 7 (clostridium chauvoei-septicum-novyi-sordellii-perfringens types C and D bacterin-toxoid, Zoetis, Parsippany, NJ), dewormed with Bimectin (ivermectin, Bimeda, Oakbrook Terrace, IL) and Safe-Guard (fenbendazole, Merck Animal Health, Madison, NJ), and implanted with Component TE-S with Tylan (Elanco Animal Health, Greenfield, IN). Steers were inspected during processing by a veterinarian for castration site abnormalities prior to being enrolled in the study.

As a result, two steers selected for the experiment were replaced because of castration site abnormalities. Replacement steers received vaccinations, deworming compounds and implants two days after processing the aforementioned steers.

Randomization of steers for this experiment occurred as follows: a mean BW for the thirty-five steers was calculated from the initial processing weights. All steer BW were within two SD of the mean. Steers were then blocked by BW (the heaviest five steers were assigned to block 1, the next five heaviest steers were assigned to block 2, etc. until 7 blocks containing five steers each were compiled) and were randomly assigned a number from 1 to 1,000 within block using the random number function in Excel 2013 (Microsoft Corporation, Redmond, WA). Within each weight block, steers were ranked from the greatest to the smallest random number and then randomly assigned to 1 of 5 treatments. With this randomization scheme, 7 weight blocks with all 5 treatments represented in each block were assembled. On day 0 of the experiment (June 13, 2018), all steers were weighed and then randomly sorted into in individual dirt-surfaced feedlot pens (6.1 m x 40 m) containing a 6.1 m x 3 m concrete bunk pad, automatic waterers (shared between adjacent pens), and a 6.1 m concrete feed bunk.

Pens were checked daily to ensure that cattle were in the appropriate pens, that all cattle had *ad libitum* access to feed and water, and that all gates were secure. Furthermore, all cattle were monitored for health and locomotion problems daily. Steers exhibiting significant symptoms of respiratory disease were removed from the pen and rectal body temperatures were recorded. Steers with body temperatures greater than 39.4°C were considered morbid. All morbid steers were treated according to the appropriate treatment schedule and immediately returned to their original pen and allowed a chance to recover. If problems persisted concerning the health status of a specific steer, the steer was removed from the experiment. If a steer was

removed from the experiment, the steer was weighed, the feed in the feed bunk was weighed and placed back into the feed bunk, a feed sample was obtained for DM determination, and the feed delivery was adjusted accordingly for that pen the next day. Two steers were treated for respiratory disease and recovered within three days after being treated. There were no mortalities during the experiment.

Diets. Diets, found below (Tables 4.3 and 4.4) were formulated to meet or exceed the National Academy of Science, Engineering, and Medicine (NASEM, 2016) requirements for finishing cattle. Upon selection for the experiment, steers received a series of transition diets of over a 22-day period. Diets were manufactured daily, and all dietary transitions were simultaneous until the final finishing diet was reached (Table 4.3). On day 41, the finishing diet was reformulated due to a change in corn silage source. Finishing diets were formulated to provide 12.5% crude protein, 5.5% fat, 0.71% calcium, 0.33% phosphorus, 0.73% potassium, 0.14% magnesium, 0.17% sulfur, 65 mg Zn/kg DM, 10.0 mg Cu/kg DM, 0.10 mg Co/kg DM, 0.28 mg Se/kg DM, 33 g per metric ton of monensin (Rumensin; Elanco Animal Health; Greenfield, IN), and 11 g per metric ton of tylosin on a DM basis. Vitamins A and E were included in the diets at 2,200 and 40 IU/kg of DM, respectively, and were added to the diet in a pelleted supplement using wheat midds meal as the carrier.

Feed deliveries were made once daily at 0700h in amounts to allow for *ad libitum* access to feed for a 24 h period. Feed bunk observations were conducted at 0630 daily. If any remaining feed was left in the feed bunk, the amount of feed remaining was visually estimated, recorded, and adjustments to daily feed deliveries were made to ensure *ad libitum* access to feed for the next 24 h period. Dietary treatments were delivered separately from the basal diet as a top dress and mixed by hand in the bunk. Orts were determined for each pen on the days cattle were

weighed. Subsamples of orts for each pen were analyzed for DM and used to calculate the DM weight of the orts for a given period. This value was then subtracted from the total DM delivered to a given pen of cattle to calculate DMI for a given period. Weekly samples of the basal diet were collected and stored at -20°C for dry matter and diet nutrient analysis.

Dietary treatments were the addition of: 1) 100% tallow rendered from conventional cattle (100TCONV); 2) 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle (66TCONV); 3) 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle (33TCONV); 4) 100% tallow rendered from natural cattle (100TNAT); and 5) 100% tallow rendered from natural cattle for 80 days and 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (100TNAT+CONV; days = 90). Steers in treatments 1, 2, 3, and 4 were individually fed for the entire feeding period. Steers in treatment 5 were group housed for 80 days and then individually fed for the last 10 days of the feeding period. Inedible and technical tallow used in the experiment were collected in 250 L food-grade steel drums (Uline; Pleasant Prairie, WI) from a USDA inspected commercial beef harvest facility on May 15th and June 2nd, 2018. All drums were subsampled in triplicate and samples were analyzed for parent and total ractopamine concentrations prior to cattle receiving any tallow for diet adaption and dietary treatment. All tallow was stored in the 250 L food-grade steel drums in a freezer at -20°C when not in use for the experiment to avoid oxidation. The tallow drums were maintained at 60°C with tank heaters when in use during the experiment to keep tallow in a liquid state for diet manufacturing. On days 90 and 91, all steers were weighed, and all 35 steers were transported to a commercial beef harvest facility the morning of day 91.

Carcass data. All steers were transported to a USDA inspected commercial beef harvest facility on day 91 of the experiment. Tag transfer was performed by Colorado State University

Center for Meat Safety & Quality personnel. Hot carcass weight was determined at the time of slaughter. Carcasses were allowed to chill for approximately 36 h before carcass camera data were obtained. Carcass data collected included: dressing percentage (DP), hot carcass weight (HCW), calculated USDA yield grade (YG), and quality grade (QG).

Post-harvest tissue collection. Tissues collected upon harvest included: hanging tender (also known as diaphragm), abomasum, liver sampled from the left lobe, kidney, and subcutaneous adipose tissue [kidney, pelvic, and heart (KPH) fat]. Samples were collected from the carcasses or their respective removed organ sets as they move throughout the beef harvest facility. All samples were collected aseptically, using a new pair of gloves to prevent cross-contamination. Carcasses and respective organ sets were identified with extra plant gang tags placed at each collection station for the above listed tissues as they moved throughout the facility. Each viscera set was marked liberally with a different colored carcass ink (3 colors used in total) to properly identify viscera samples on the evisceration table of the harvest facility. Before collection, livers were washed, and then a cross-section was trimmed on the viscera table, separately from the remaining viscera. Hanging tender, kidney, and subcutaneous fat samples were trimmed by plant personnel on the carcass rail as carcasses proceeded down the line. Abomasum samples were rinsed with hot water and then placed in a cold-water bath before being bagged.

Collected samples were placed in individual sterile Whirl-Pak bags (Nasco; Fort Atkinson, WI) and positioned in direct contact with ice in boxes or coolers. Samples were transported to Colorado State University (Fort Collins, CO) where they were stored in a -20°C freezer until processing for ractopamine quantitation.

Analytical Procedures. Basal diet and individual ingredient samples were sent to at commercial laboratory (SDK Laboratories; Hutchinson, KS) for chemical analysis. All tissues were processed as follows: collected tissues were cryogenically homogenized prior to ractopamine extraction procedures. Approximately 100 g of each tissue were cut into small (~ 3 cm x 3 cm) pieces, flash frozen in liquid nitrogen (N_2), and homogenized using a Nutribullet food processor (Capital Brands, LLC; Los Angeles, CA). Two subsamples of tissue homogenate, each weighing 1 ± 0.5 g, were placed in separate 5 ml conical tubes and stored at -80°C until extraction. An additional sample of each tissue homogenate was stored in sterile Whirl-Pak bags at -80°C in the event that an additional analysis became necessary for any reason.

The tissue homogenate samples were extracted in 4 ml/g MeOH containing 25 ng/mL of the internal standard (IS) ractopamine-d_6. Samples were quickly vortexed to suspend, mixed on a shaker plate for 10 min, sonicated for 30 min, and incubated at -80°C for 30 min. The samples were then centrifuged at 21,000 x G for 15 min to separate the solid from supernatant. One aliquot of 1 ml supernatant was transferred into a 1.7 ml Denville tube and stored at -80°C for analysis of PRAC. A second aliquot of 1 ml supernatant was transferred into a separate 1.7 ml tube for TRAC concentrations. The second aliquot was evaporated to dryness using a nitrogen dryer set at 50°C. The samples were resuspended in 200 µl of a master mix made of 10 ml of 25 mM NaOAc buffer (pH 5.2) and 200 µl β-glucuronidase from Helix pomatia (Sigma Aldrich). Samples were gently vortexed to mix and then incubated at 65°C for 2 h to activate the enzyme. Then, 800 ml of MeOH was added and the samples were mixed thoroughly by vortex and stored at -80°C until analysis. On the day of analysis, samples were taken out of the freezer, centrifuged at 21,000 x G for 30 min to remove any remaining particulates and transferred into microcentrifuge vials for UPLC-MS/MS analysis as described below.

Ultra-performance LC-MS/MS. Ractopamine concentrations were analyzed on a PerkinElmer UHPLC system equipped with a PerkinElmer QSight LX50 Solvent Delivery Module (PerkinElmer, Inc., Waltham, MA, USA). Two µl of sample were directly injected onto a reverse phase 1.0 mm x 50 mm Waters Acquity UPLC HSS T3 column (1.8 µm particles; Waters Corporation, Milford, MA, USA) for chromatographic separation. Mobile phase A consisted of LC-MS grade water (Fisher Scientific) with 2 mM ammonium formate, buffer B consisted of LC-MS grade acetonitrile with 0.1% LC-MS grade formic acid (Thermo Scientific). Elution gradient was initially set at 1.0% B for 0.2 min, which was increased to 30% B at 2.2 min and further increased to 99.0% B at 3 min until 4.5 min when B was decreased to 1% B until 6.5 min for a total run time of 6.5 min. The flow rate was set to 400 µl/min and the column temperature was maintained at 50°C. The samples were set held at 15°C in the autosampler. Detection was performed on a PerkinElmer QSight triple quadrupole tandem mass spectrometer (MS/MS) operated in selected reaction monitoring (SRM) mode. Prior to analysis, SRM transitions were optimized for ractopamine using an authentic standard. The samples were detected in positive mode. The quantitative transition for RAC was 302.5 → 164.10 at a collision energy of 20; the confirmatory ion 302.5 → 284.16 at a collision energy of 16; and finally, the internal standard 308.0 → 168.17 at a collision energy of 22.

Data analysis. Peak picking and integration were performed using Simplicity 3Q software (Version 1.5, PerkinElmer, Inc., Waltham, MA, USA). Peak areas for each sample were normalized to the peak area of the internal standard in that sample. Quantification of the analytes and QCs were performed using a weighted linear regression against an external standard curve. The Limit of Detection (LOD) was calculated by multiplying three times the analytical noise and the Limit of Quantitation (LOQ) was calculated by multiplying ten times the analytical noise.

Statistical analysis. Feedlot performance data were statistically analyzed for treatments 1 through 4 only. Since steers in treatment 5 were group housed for 80 days and then individually fed for the last 10 days of the feeding period, only raw live animal performance means are reported for this treatment. Carcass characteristics [dressing percentage (DP), hot carcass weight (HCW), calculated USDA yield grade (YG) and quality grade (QG)] were analyzed for all five treatments. Animal was considered the experimental unit. Data were analyzed as a randomized complete block design using R (R Foundation for Statistical Computing; Vienna, Austria). Treatment was classified as a fixed effect, weight block was classified as a random effect, and initial BW was used as a covariate for all response variables. A type three ANOVA table was constructed using the Kenward-Roger method of computing denominator degrees of freedom. Significance was determined at $P \leq 0.05$. The USDA quality grade (QG) was analyzed using a chi-squared test of independence using R (R Foundation for Statistical Computing; Vienna, Austria).

Parent and total ractopamine concentrations were analyzed separately for each tissue type [abomasum, liver, kidney, subcutaneous fat (KPH), and hanging tender (muscle)] using a general linear mixed model in SAS (version 9.4; Cary, NC). Analysis of variance included a fixed effect of treatment (five different feed combinations of inedible and technical tallow). Data were reported as least squares means using a significance level of $\alpha = 0.05$.

Results and Discussion

The effect of TCONV and TNAT concentration on live performance is presented in Table 4.5. The TCONV concentration had no effect on final BW, average daily gain, dry matter intake, or feed efficiency for treatments 1 through 4. Raw means reported for treatment 5 of the previously mentioned live performance parameters were numerically similar to those of

treatments 1 through 4. Carcass characteristics ($P < 0.23$) did not vary among all 5 treatments and are presented in Table 4.6. Increasing TNAT inclusion to 100% did not have an effect on live performance parameters.

Raw data points are presented in Tables 4.7 and 4.8, while least squares mean estimates (SAS v. 9.4; Cary, NC) are displayed in Tables 4.9 and 4.10. The CONV concentrations had minimal effect on residual ractopamine in tissues (Tables 4.9 and 4.10). With the exception of muscle, total ractopamine was detectable in all tissues as presented in Table 4.8. Values below the limit of detection (LOD) were reported as non-detectable (ND) and corresponding LOD values for specific tissue types were reported. Of particular interest, abomasum and subcutaneous fat (KPH) were two tissues in which residual ractopamine concentrations were the highest. Even such, these levels are below Codex and U.S. FDA maximum residue limits (MRL) (Codex, 2015; Elanco, 2003; Table 4.11). Upon statistical analysis, differences ($P < 0.05$) were only observed in total ractopamine residues in kidney samples, which likely only existed because one treatment had only two detectable levels of ractopamine (0.05 and 0.06 ppb) and the other four treatments had only non-detectable ractopamine levels, thus resulting in ND displayed (Table 4.10). For all other tissues, ractopamine level least squares mean estimates were either non-detectable or were not different ($P \geq 0.05$) for both parent and total ractopamine (Tables 4.9 and 4.10).

Conclusions. Zero-tolerance policies are becoming increasingly more common, causing some countries in the U.S. and globally to move forward with that change and away from the use of ractopamine as a feed additive (Aroeira et al., 2019; Centner, Alvey, and Stelzleni, 2014). This could create economic hardships for U.S. producers, but it is challenging to associate an actual numeric value on the removal because there are already issues with withdrawal times of the compound among other concerns. While ractopamine use may not be a food safety concern

due to extensive research to develop MRL based on no adverse effect levels, its use remains extremely contentious (Centner, Alvey, and Stelzleni, 2014; Elanco, 2003). Contamination of livestock feed, and therefore tallow, meat and bone meal, and domestic tissues as well as those intended for export is a very real possibility. Although these levels often fall below the U.S. and global MRL for muscle, the lowest of the acceptable limits, the use of ractopamine and any residue in general challenges zero-tolerance policies.

Table 4.1. Parent and total ractopamine concentrations (ppb) in tallow collected from four different commercial beef harvest facilities across the U.S. from both ractopamine-treated (conventional) and untreated (natural) cattle. Tallow rendered from conventionally raised cattle is denoted as TCONV while tallow from naturally raised cattle is TNAT.

Treatment	**Parent Ractopamine (ppb)**			
	LSMean	95% CI Low	95% CI High	SE
TNAT*	1.05[a]	0.98	1.12	0.03[1]
TNAT*	0.99[ab]	0.88	1.02	
TNAT*	0.95[ab]	0.92	1.06	
TNAT	ND	0.00	0.07	
TCONV	0.92[b]	0.85	0.99	
TCONV	0.01[c]	0.00	0.08	
TCONV	ND	0.00	0.07	
TCONV	ND	0.00	0.07	

Treatment	**Total Ractopamine (ppb)**			
	LSMean	95% CI Low	95% CI High	SE
TNAT*	1.15[a]	1.06	1.24	0.05
TNAT*	1.15[a]	1.06	1.24	
TNAT*	0.97[b]	0.88	1.06	
TNAT	ND	0.00	0.09	
TCONV	1.00[b]	0.91	1.1	
TCONV	ND	0.00	0.09	
TCONV	ND	0.00	0.09	
TCONV	ND	0.00	0.09	

[1]Pooled standard error.

[abc]Means without a common superscript within a column are different ($P < 0.05$).

*Rendered in-laboratory from subcutaneous fat.

ND = non-detectable; less than the limit of detection.

Table 4.2. Meat and bone meal samples collected from four different commercial beef harvest facilities across the U.S. during different seasons, analyzed using liquid chromatography mass spectrometry.

Tissue	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Meat and Bone Meal	28.98	27.84
Meat and Bone Meal	ND	ND
Meat and Bone Meal	1.77	1.62
Meat and Bone Meal	1.24	1.70
LOD	0.08 ppb	
LOQ	0.25 ppb	

ND = Non-detectable; less than the limit of detection.

Table 4.3. Dry matter ingredient composition of the basal diet.

Ingredient	Initial finishing diet[1]	Final finishing diet[1]
Steam Flaked Corn, %	61.40	61.19
Original Corn Silage, %	10.00	---
New Corn Silage, %	---	10.30
Alfalfa, %	10.00	10.00
Dried distillers grains, %	10.00	10.00
Pellet Supplement, %	3.60	3.61
Tallow, %	4.95	4.85
Analyzed nutrient composition		
DM, % as-fed	87.08	84.69
CP, % DM	12.86	12.84
ADF, % DM	11.39	11.18
NDF, % DM	19.31	19.01
NEm, Mcal/kg[2]	1.98	1.99
Neg, Mcal/kg[3]	1.28	1.27
Ether extract, % DM	5.67	5.78
Calcium, % DM	0.65	0.64
Phosphorus, % DM	0.36	0.34
Potassium, % DM	0.86	0.74
Magnesium, % DM	0.13	0.14
Sodium, % DM	0.10	0.10
Sulfur, % DM	0.19	0.18
Cobalt, mg/kg DM	0.10	0.10
Copper, mg/kg DM	10.97	10.64
Iron, mg/kg DM	51.98	48.27
Manganese, mg/kg DM	30.15	27.67
Molybdenum, mg/kg DM	0.39	0.35
Zinc, mg/kg DM	62.00	59.73

[1]On day 41 of the experiment the finishing diet was reformulated due to the change in corn silage source.

[2]NEm = ((1.37 x (TDN x 0.0361)) - (0.0138 x (TDN x 0.0361) x (TDN x 0.0361)) + (0.0105 x (TDN x 0.0361) x (TDN x 0.0361) - 1.12)) ÷ 2.205.

[3]NEg = ((1.42 x (TDN x 0.0361)) - (0.174 x (TDN x 0.0361) x (TDN x 0.0361)) + (0.0122 x (TDN x 0.0361) x (TDN x 0.0361) x TDN x 0.0361) - 1.65)) ÷ 2.205

Table 4.4. Dry matter nutrient composition of basal dietary ingredients.

Item	Ingredient				
	Corn Silage	Steamed flaked corn	Alfalfa	Dried distillers grains	Supplement
DM, % as-fed	57.79	89.15	93.68	89.44	91.8
CP, % DM	7.03	8.08	11.36	32.92	60.15
ADF, % DM	24.76	3.01	49.6	19.04	5.72
NDF, % DM	40.91	8.57	59.52	33.93	16.95
NEm, mcal/kg DM	0.81	1.02	0.45	0.85	0.91
Neg, mcal/kg DM	0.49	0.69	0.12	0.52	0.59
Ether extract, % DM	3.20	3.02	1.31	7.18	1.89
Calcium, % DM	0.17	0.03	0.74	0.04	9.42
Phosphorus, % DM	0.19	0.16	0.22	1.05	0.54
Potassium, % DM	1.22	0.38	2.78	1.43	2.29
Magnesium, % DM	0.18	0.09	0.19	3.28	0.31
Sodium, % DM	0.01	0.01	0.03	0.28	1.8
Sulfur, % DM	0.1	0.11	0.17	0.84	0.24
Cobalt, mg/kg DM	<0.02	<0.02	<0.02	<0.02	2.77
Copper, mg/kg DM	3.49	1.47	6.67	6.64	233
Iron, mg/kg DM	117	16.8	72.8	134	258
Manganese, mg/kg DM	25.39	3.93	12.3	21.2	607
Molybdenum, mg/kg DM	0.47	<0.03	2.29	0.98	0.57
Zinc, mg/kg DM	23.7	11.4	17.9	69.2	1220

Table 4.5. Live performance of steers receiving tallow rendered from conventional or natural cattle (TCONV and TNAT, respectively) for 90 days prior to harvest as a carrier of ractopamine in low doses (TCONV) or simply as a control (TNAT).

	Treatments						
Item	100TCONV[a]	66TCONV[b]	33TCONV[c]	100TNAT[d]	100TNAT+CONV[e,f]	SEM	*P* <
Initial BW, kg	481.4	475.0	480.0	496.0	487.0	21.23	0.22
Final BW, kg	656.0	652.0	646.0	650.0	650.0	5.89	0.67
ADG, kg·hd^{-1}·d^{-1}	1.65	1.62	1.56	1.60	1.60	0.06	0.70
DMI, kg·hd^{-1}·d^{-1}	10.1	9.8	9.8	10.4	11.02	0.25	0.26
FE, (g/f)	0.16	0.16	0.16	0.15	0.15	0.006	0.48

[a]100TCONV: 100% tallow rendered from conventional cattle.

[b]66TCONV: 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle.

[c]33TCONV: 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle.

[d]100TNAT: 100% tallow rendered from natural cattle.

[e]100TNAT+CONV: 100% tallow rendered from natural cattle for 80 days + 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (days = 90).

[f]Raw Means reported.

Table 4.6. Carcass characteristics of steers receiving tallow rendered from conventional or natural cattle (TCONV and TNAT, respectively) for 90 days prior to harvest as a carrier of ractopamine in low doses (TCONV) or simply as a control (TNAT).

	Treatments						
Item	100TCONV[a]	66TCONV[b]	33TCONV[c]	100TNAT[d]	100TNAT+CONV[e]	SEM	*P* <
HCW, kg	387	385	377	388	388	3.65	0.26
DP, %	58.8	59.0	58.2	58.8	59.9	0.51	0.23
QG							
Select, %	0.0	28.6	28.6	14.3	14.3	-	0.59
Choice, %	100.0	71.4	71.4	71.4	85.7	-	0.58
Prime, %	0.0	0.0	0.0	14.3	0.0	-	0.39
YG	3.0	3.2	2.9	3.0	3.3	0.16	0.32

[a]100TCONV: 100% tallow rendered from conventional cattle.
[b]66TCONV: 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle.
[c]33TCONV: 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle.
[d]100TNAT: 100% tallow rendered from natural cattle.
[e]100TNAT+CONV: 100% tallow rendered from natural cattle for 80 days + 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (days = 90).
[f]Raw Means reported.

Table 4.7. Parent ractopamine concentrations (ppb; raw data for reference of contamination potential) from liquid chromatography mass spectrometry in abomasum, liver, kidney, subcutaneous fat (KPH), and hanging tender (muscle) from steers receiving various combinations of inedible and technical tallow to simulate low dose ractopamine contamination at a commercial beef harvest facility.

		Parent Ractopamine (ppb)			
Treatment	Abomasum	Liver	Kidney	KPH	Muscle
100TCONV	ND	ND	ND	ND	ND
100TCONV	0.09	ND	ND	ND	ND
100TCONV	0.10	ND	ND	0.01	ND
100TCONV	0.14	ND	ND	0.17	ND
100TCONV	0.16	ND	ND	0.18	ND
100TCONV	0.19	ND	ND	0.19	ND
100TCONV	0.21	ND	ND	0.01	ND
66TCONV	0.06	ND	ND	0.14	ND
66TCONV	0.13	ND	ND	ND	ND
66TCONV	0.07	ND	ND	0.00	ND
66TCONV	0.33	ND	ND	0.00	ND
66TCONV	0.12	*	ND	0.01	ND
66TCONV	0.07	ND	ND	0.14	ND
66TCONV	0.24	2.58	ND	0.03	ND
33TCONV	0.17	ND	ND	0.17	ND
33TCONV	0.10	ND	ND	0.00	ND
33TCONV	0.09	ND	ND	0.12	ND
33TCONV	0.09	ND	ND	0.18	ND
33TCONV	ND	ND	ND	0.02	ND
33TCONV	0.07	ND	ND	ND	ND
33TCONV	0.10	ND	ND	0.01	ND
100TNAT	0.07	ND	ND	0.00	ND
100TNAT	0.16	ND	ND	0.14	ND
100TNAT	0.13	ND	ND	0.00	ND
100TNAT	0.07	ND	ND	0.03	ND
100TNAT	0.09	ND	ND	0.00	ND
100TNAT	0.08	ND	ND	0.18	ND
100TNAT	0.13	ND	ND	ND	ND
100TNAT+CONV	0.12	ND	ND	0.03	ND
100TNAT+CONV	0.07	ND	ND	0.00	ND
100TNAT+CONV	3.63	ND	ND	0.17	ND
100TNAT+CONV	0.11	ND	ND	ND	ND
100TNAT+CONV	0.07	ND	ND	0.18	ND
100TNAT+CONV	0.18	ND	ND	ND	ND
100TNAT+CONV	0.09	ND	ND	0.01	ND
LOD	0.06	0.16	0.06	0.28	0.06
LOQ	0.2	0.54	0.2	0.93	0.19

*No data for this sample due to an injection issue.

ND = Non-detectable; less than the limit of detection.

100TCONV: 100% tallow rendered from conventional cattle.

66TCONV: 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle.

33TCONV: 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle.
100TNAT: 100% tallow rendered from natural cattle.
100TNAT+CONV: 100% tallow rendered from natural cattle for 80 days + 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (days = 90).

Table 4.8. Total ractopamine concentrations (ppb; raw data for reference of contamination potential) in abomasum, liver, kidney, subcutaneous fat (KPH), and hanging tender (muscle) from steers receiving various combinations of tallow rendered from conventional and natural cattle (TCONV and TNAT) to simulate low dose ractopamine contamination at a commercial beef harvest facility.

	Total Ractopamine (ppb)				
Treatment	Abomasum	Liver	Kidney	KPH	Muscle
100TCONV	0.07	ND	ND	0.01	ND
100TCONV	0.09	ND	ND	0.01	ND
100TCONV	0.10	ND	ND	0.03	ND
100TCONV	0.07	ND	ND	0.34	ND
100TCONV	0.10	ND	ND	ND	ND
100TCONV	0.12	ND	ND	0.01	ND
100TCONV	0.08	ND	ND	0.25	ND
66TCONV	0.11	ND	ND	0.26	ND
66TCONV	0.09	ND	ND	0.02	ND
66TCONV	0.09	0.24	ND	0.01	ND
66TCONV	0.28	ND	ND	0.24	ND
66TCONV	0.29	*	ND	0.05	ND
66TCONV	0.07	ND	ND	0.00	ND
66TCONV	0.12	8.00	ND	0.02	ND
33TCONV	0.13	ND	ND	0.04	ND
33TCONV	0.24	ND	ND	0.00	ND
33TCONV	0.13	ND	ND	0.00	ND
33TCONV	0.10	ND	ND	0.01	ND
33TCONV	0.24	ND	ND	0.23	ND
33TCONV	0.21	ND	ND	0.01	ND
33TCONV	0.10	ND	ND	0.02	ND
100TNAT	ND	ND	ND	0.28	ND
100TNAT	0.17	ND	ND	0.00	ND
100TNAT	0.08	ND	ND	0.30	ND
100TNAT	0.23	ND	0.05	0.02	ND
100TNAT	0.10	ND	ND	ND	ND
100TNAT	0.10	ND	0.06	0.00	ND
100TNAT	3.83	ND	ND	ND	ND
100TNAT+CONV	4.22	ND	ND	0.00	ND
100TNAT+CONV	ND	0.40	ND	0.01	ND
100TNAT+CONV	4.19	ND	ND	ND	ND
100TNAT+CONV	0.07	ND	ND	0.04	ND
100TNAT+CONV	0.21	ND	ND	0.00	ND
100TNAT+CONV	0.08	ND	ND	0.01	ND
100TNAT+CONV	0.07	0.60	ND	0.02	ND
LOD	0.06	0.16	0.06	0.28	0.06
LOQ	0.20	0.54	0.20	0.93	0.19

*No data for this sample due to an injection issue.

ND = Non-detectable; less than the limit of detection.

100TCONV: 100% tallow rendered from conventional cattle.

66TCONV: 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle.

33TCONV: 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle.

100TNAT: 100% tallow rendered from natural cattle.

100TNAT+CONV: 100% tallow rendered from natural cattle for 80 days + 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (days = 90).

Table 4.9. Least squares means of parent ractopamine concentrations (ppb) in abomasum, liver, kidney, subcutaneous fat (KPH), and hanging tender (muscle) from steers receiving various combinations of tallow rendered from conventional and natural cattle (TCONV and TNAT) to simulate low dose ractopamine contamination at a commercial beef harvest facility.

Tissue	Treatment	Parent Ractopamine (ppb)			
		LSMean	95% CI Low	95% CI High	SE[1]
Abomasum	100TCONV	0.13[a]	0.00	0.59	0.226
	66TCONV	0.15[a]	0.00	0.61	
	33TCONV	0.09[a]	0.00	0.55	
	100TNAT	0.10[a]	0.00	0.57	
	100TNAT+CONV	0.61[a]	0.15	1.07	
Liver*					
Kidney*					
KPH	100TCONV	0.08[a]	0.02	0.14	0.030
	66TCONV	0.05[a]	0.00	0.11	
	33TCONV	0.07[a]	0.01	0.13	
	100TNAT	0.05[a]	0.00	0.11	
	100TNAT+CONV	0.06[a]	0.00	0.12	
Muscle*					

[1]SE denotes pooled standard error.

[a]Means with a common superscript within a column, by tissue, do not differ ($P \geq 0.05$).

*No statistics presented because all values were below the limit of detection (abomasum – 0.06 ppb, liver – 0.16 ppb, kidney – 0.06 ppb, KPH – 0.28 ppb, and muscle – 0.06 ppb).

100TCONV: 100% tallow rendered from conventional cattle.

66TCONV: 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle.

33TCONV: 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle.

100TNAT: 100% tallow rendered from natural cattle.

100TNAT+CONV: 100% tallow rendered from natural cattle for 80 days + 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (days = 90).

Table 4.10. Least squares means of total ractopamine concentrations (ppb) in abomasum, liver, kidney, subcutaneous fat (KPH), and hanging tender (muscle) from steers receiving various combinations of tallow rendered from conventional and natural cattle (TCONV and TNAT) to simulate low dose ractopamine contamination at a commercial beef harvest facility.

Tissue	Treatment	Total Ractopamine (ppb)			
		LSMean	95% CI Low	95% CI High	SE[1]
Abomasum	100TNAT+CONV	1.26[a]	0.41	2.11	0.415
	100TNAT	0.64[a]	0.00	1.49	
	66TCONV	0.16[a]	0.00	1.01	
	33TCONV	0.15[a]	0.00	1.00	
	100TCONV	0.09[a]	0.00	0.94	
Liver	66TCONV	1.37[a]	0.24	2.50	0.512
	100TNAT+CONV	0.14[a]	0.00	1.19	
	33TCONV	ND	0.00	1.05	
	100TNAT	ND	0.00	1.05	
	100TCONV	ND	0.00	1.05	
Kidney	100TNAT	0.02[a]	0.01	0.03	0.005
	100TNAT+CONV	ND	0.00	0.01	
	33TCONV	ND	0.00	0.01	
	66TCONV	ND	0.00	0.01	
	100TCONV	ND	0.00	0.01	
KPH	100TCONV	0.09[a]	0.01	0.18	0.041
	66TCONV	0.09[a]	0.00	0.17	
	100TNAT	0.09[a]	0.00	0.17	
	33TCONV	0.04[a]	0.00	0.13	
	100TNAT+CONV	0.01[a]	0.00	0.10	
Muscle*					

[1]SE denotes pooled standard error.

[a]Means with a common superscript within a column, by tissue, do not differ ($P \geq 0.05$).

ND = non-detectable; below the limit of detection (abomasum – 0.06 ppb, liver – 0.16 ppb, kidney – 0.06 ppb, KPH – 0.28 ppb, and muscle – 0.06 ppb).

*No statistics presented because all values were below the limit of detection.

100TCONV: 100% tallow rendered from conventional cattle.

66TCONV: 66% tallow rendered from conventional cattle + 33% tallow rendered from natural cattle.

33TCONV: 33% tallow rendered from conventional cattle + 66% tallow rendered from natural cattle.

100TNAT: 100% tallow rendered from natural cattle.

100TNAT+CONV: 100% tallow rendered from natural cattle for 80 days + 100% tallow rendered from conventional cattle for the last 10 days of the feeding period (days = 90).

Table 4.11. Maximum residue limits for ractopamine for target and off-target tissues based on Codex Alimentarius and U.S. FDA.

Tissue	Codex (ppb)	U.S. FDA (ppb)
Abomasum	N/A	N/A
Liver	40	90
Kidney	90	N/A
KPH	10	N/A
Muscle	10	30

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APPPENDIX

Supplemental Table 3.1. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all liver samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	3.41	3.46
	3.40	3.50
	3.40	3.48
	3.40	3.60
	3.41	3.63
	3.40	3.51
7 days	3.41	3.52
	3.42	3.50
	3.41	3.55
	3.45	3.62
	3.42	3.52
	3.42	3.58
	3.44	3.58
	3.42	3.58
	3.45	3.90
	3.42	3.57
	3.42	3.65
4 days	3.44	4.03
	3.42	3.79
	3.44	3.63
	3.42	3.69
	3.45	3.70
	3.49	4.91
	3.43	4.17
	3.43	4.48
	3.42	3.78
	3.44	3.68
	3.43	4.03
	3.43	3.59
2 days	3.42	7.18
	3.53	12.51
	3.49	6.05
	3.44	6.96
	3.46	14.19
	3.46	13.81
	3.48	12.26
	3.54	7.81
	3.51	7.21
	3.44	4.26
	3.48	6.27

Supplemental Table 3.2. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all muscle samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	0.00*	0.03*
	-0.02*	0.04*
	0.07*	0.09*
	-0.01*	0.04*
	0.00*	0.04*
	0.01*	0.06*
7 days	0.19	0.22
	0.12	0.16
	0.07*	0.09*
	0.34	0.34
	0.19	0.20
	0.29	0.33
	0.60	0.63
	0.16	0.21
	0.26	0.31
	0.17	0.21
	0.12	0.15
4 days	0.51	0.56
	0.36	0.96
	0.73	0.83
	0.94	1.04
	0.44	0.52
	0.68	0.87
	0.24	0.47
	1.07	1.23
	0.92	1.10
	0.26	0.37
	0.21	0.25
	0.34	0.35
2 days	0.81	1.30
	1.13	1.52
	0.77	1.08
	0.68	1.03
	0.87	1.72
	0.69	1.25
	1.05	1.50
	0.58	0.89
	0.86	1.37
	0.35	0.53
	0.34	0.67

* Below limit of quantification (0.12 ppb).

Supplemental Table 3.3. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all heart samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	ND^	0.19
	ND	5.83
	ND	ND
	0.11	ND
	ND	0.20
	8.06	ND
7 days	0.29	3.31
	ND	4.13
	ND	7.28
	0.91	ND
	ND	1.73
	ND	ND
	0.94	1.06
	2.66	ND
	2.11	ND
	10.36	ND
	2.14	0.44
4 days	0.57	0.67
	0.89	0.53
	0.97	0.88
	0.86	1.01
	ND	0.86
	1.70	ND
	0.52	2.95
	0.40	1.89
	2.48	ND
	ND	0.94
	1.67	ND
	ND	1.51
2 days	ND	0.99
	2.18	0.27
	0.78	ND
	1.31	0.22
	2.07	0.87
	1.33	1.23
	1.98	3.11
	1.75	1.45
	7.49	2.57
	8.67	2.23
	ND	ND
12 h	3.21	0.45
	3.36	0.69
	4.45	ND
	4.96	2.93
	4.13	3.29
	4.63	1.63
	ND	9.03

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	0.77	11.99
	ND	9.30
	ND	9.19
	0.16	7.55
	14.01	5.66

^ ND (non-detectable); limit of detection (0.02 ppb).

Supplemental Table 3.4. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all tendon samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	0.50	0.62
	0.34	0.47
	0.99	1.23
	0.60	0.79
	0.55	0.78
	0.47	2.55
7 days	1.46	1.53
	3.52	3.46
	0.54	0.69
	1.06	1.24
	1.22	1.32
	0.93	1.29
	4.11	4.21
	1.13	1.22
	1.03	2.20
	2.65	2.84
	0.96	1.10
4 days	1.70	2.37
	1.13	1.29
	2.22	2.57
	3.97	4.63
	3.16	3.23
	1.85	2.06
	1.58	2.17
	3.82	5.05
	7.83	7.63
	1.25	1.43
	1.49	1.58
	2.38	0.49
2 days	2.12	3.90
	2.94	6.01
	1.76	3.54
	2.34	3.14
	3.13	7.87
	3.32	5.74
	4.35	6.76
	2.83	3.40
	3.39	6.02
	1.84	5.20
	2.67	2.38
12 h	7.19	24.84
	4.18	10.87
	3.25	10.66
	4.71	20.15
	6.73	12.75
	4.06	11.42
	4.96	20.14

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	8.20	15.14
	4.69	14.89
	3.84	8.05
	3.14	9.20
	3.29	6.75

Supplemental Table 3.5. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all tongue samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	0.30	0.18
	0.26	0.31
	0.31	0.36
	0.29	0.36
	0.40	0.46
	0.39	0.46
7 days	0.50	0.57
	0.41	0.45
	0.40	0.44
	0.86	0.92
	0.82	0.83
	0.71	0.78
	0.48	0.55
	0.55	0.56
	0.53	0.61
	0.52	0.53
	0.54	0.61
4 days	1.18	1.44
	1.23	1.42
	1.05	1.30
	1.17	1.26
	0.80	0.92
	1.27	1.51
	0.93	1.27
	1.28	1.64
	0.96	1.15
	0.76	0.93
	0.74	0.94
	1.12	1.16
2 days	1.97	3.18
	2.15	3.21
	1.29	2.04
	1.23	1.73
	1.51	2.99
	1.56	2.67
	2.00	2.98
	1.68	2.41
	1.75	2.69
	0.92	1.25
	1.23	1.59
12 h	3.25	8.64
	3.30	9.81
	4.11	10.19
	4.22	9.98
	4.53	7.46
	3.41	5.96
	4.63	7.71

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	4.23	8.18
	5.56	10.03
	3.77	6.76
	4.01	7.26
	2.28	4.10

Supplemental Table 3.6. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all abomasum samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	4.25	4.26
	4.30	4.29
	4.22	4.23
	4.40	4.43
	4.24	4.24
	4.21	4.21
7 days	4.35	4.36
	4.29	4.30
	4.26	4.27
	4.30	4.34
	4.41	4.48
	4.33	4.33
	4.32	4.33
	4.37	4.42
	4.33	4.37
	4.35	4.32
	4.30	4.31
4 days	4.47	4.59
	4.41	4.43
	4.49	4.54
	4.81	4.87
	4.40	4.43
	4.72	4.86
	4.43	4.49
	4.59	4.77
	4.53	4.62
	4.37	4.44
	4.44	4.62
	4.57	4.64
2 days	5.30	5.80
	5.69	7.01
	4.92	5.25
	8.68	8.48
	5.51	7.04
	5.51	6.26
	4.74	5.25
	4.76	5.14
	4.89	5.50
	4.40	4.45
	4.33	4.46
12 h	17.99	22.23

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	18.18	22.46
	67.64	71.36
	20.38	24.45
	7.09	8.73
	14.69	17.30
	7.65	10.94
	7.45	10.05
	15.62	20.82
	8.44	12.37
	6.89	10.34
	7.08	9.51

Supplemental Table 3.7. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all omasum samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	0.78*	0.58*
	5.50	5.23
	ND	ND
	0.94*	0.96*
	4.18	4.38
	4.04	3.58
7 days	21.96	22.51
	27.47	29.85
	52.09	52.73
	5.22	4.81
	5.18	5.57
	34.94	33.09
	2.09*	2.01*
	0.88	0.91
	30.51	32.15
	36.32	40.42
	2.52	2.57
4 days	24.23	22.75
	2.17	2.24
	3.04	2.94
	2.85	2.93
	2.15	2.29
	2.83	2.93
	7.31	7.83
	6.43	7.45
	7.43	7.49
	3.50	3.82
	6.02	6.54
	3.11	4.38
2 days	14.30	15.28
	32.98	34.16
	15.58	14.79
	21.93	21.01
	23.38	24.22
	17.76	17.98
	14.89	15.13
	48.15	48.86
	38.32	40.00
	8.04	8.03
	8.53	9.12
12 h	87.06	87.89
	86.92	91.86
	66.85	72.08
	86.74	93.17
	51.68	53.89
	56.09	62.54
	63.06	70.40

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	109.70	116.90
	70.91	73.84
	91.92	100.99
	66.35	72.61
	84.32	79.79

*Below limit of quantification (1.67 ppb).

Supplemental Table 3.8. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all rumen/reticulum samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	ND^	ND
	ND	ND
	ND	ND
	ND	ND
	ND	ND
	ND	ND
7 days	ND	ND
	ND	ND
	0.53	0.50*
	0.26*	0.30*
	ND	ND
	ND	ND
	ND	ND
	ND	ND
	ND	ND
	ND	ND
	ND	ND
4 days	0.40*	0.34*
	ND	ND
	0.19*	0.24*
	ND	ND
	ND	ND
	ND	ND
	ND	ND
	0.70	0.88
	1.61	1.59
	ND	ND
	ND	ND
	ND	ND
2 days	0.76	0.79
	1.42	1.63
	0.47*	0.50*
	1.03	1.46
	1.53	1.97
	0.77	1.53
	0.72	1.19
	0.35*	0.42*
	0.35*	0.42*
	ND	ND
	0.11*	0.30*
12 h	6.02	6.34
	6.44	7.74
	9.96	11.02
	7.53	9.93

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	5.50	6.71
	6.47	7.31
	6.56	7.54
	5.49	6.42
	3.66	5.83
	5.22	4.50
	3.45	4.07
	3.45	4.19

*Below limit of quantification (0.51 ppb).

^ND (non-detectable); limit of detection (0.15 ppb).

Supplemental Table 3.9. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all small intestine samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	2.05	2.11
	1.88	2.68
	0.86	1.05
	6.12	7.14
	0.82	1.24
	0.88	1.22
7 days	2.88	3.34
	3.34	3.98
	6.47	7.86
	28.23	29.17
	2.28	2.51
	8.74	10.27
	17.49	18.10
	60.28	62.16
	6.37	7.16
	5.66	6.04
	17.73	19.22
4 days	26.27	32.75
	11.15	12.65
	23.34	24.51
	25.60	25.87
	18.18	18.68
	61.09	70.72
	17.75	19.57
	11.59	16.69
	14.11	15.65
	8.39	10.43
	2.72	3.85
	9.97	10.00
2 days	36.66	40.63
	8.76	15.81
	12.26	66.38
	14.93	20.79
	13.55	17.66
	11.58	14.27
	69.94	72.34
	14.83	19.92
	17.72	26.40
	8.93	11.23
	11.32	11.22
12 h	35.09	42.02
	13.60	45.76
	15.50	36.82
	83.10	131.35
	24.30	16.71
	43.24	72.97
	114.13	156.07

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	61.89	92.08
	23.13	31.27
	16.80	31.84
	142.26	181.91
	71.00	95.36

Supplemental Table 3.10. Parent and total ractopamine concentration residues (ppb), analyzed using LC-MS/MS, present in all large intestine samples collected from cattle that received ractopamine hydrochloride (RH) and underwent different withdrawal times (12 h, 2 days, 4 days, or 7 days) or that did not receive RH (control).

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
Control	14.93	15.22
	17.37	18.09
	15.86	16.02
	24.40	24.78
	14.71	14.99
	34.81	14.79
7 days	27.15	26.85
	36.83	41.42
	20.17	21.04
	25.05	25.74
	25.50	25.17
	18.53	19.23
	22.70	23.30
	34.87	35.93
	30.81	31.63
	31.37	33.79
	20.07	20.28
4 days	34.72	37.19
	45.37	47.46
	23.73	23.76
	22.59	22.86
	33.11	33.80
	47.21	47.31
	36.77	38.55
	37.49	41.10
	36.32	40.08
	31.20	32.40
	29.66	30.66
	14.59	34.78
2 days	64.88	72.05
	79.40	85.87
	41.10	46.36
	16.56	16.71
	39.36	46.38
	53.99	62.00
	53.94	58.92
	43.56	46.80
	35.23	41.21
	32.40	32.31
	31.26	34.70
12 h	143.75	169.88
	182.20	186.18
	260.75	269.95
	160.80	201.47
	31.39	39.75
	164.42	180.76
	152.68	169.35

Treatment	Parent Ractopamine (ppb)	Total Ractopamine (ppb)
12 h	282.40	289.85
	169.06	212.37
	198.86	254.22
	101.78	118.76
	79.24	111.75