DISSERTATION

EXAMINING *IN VITRO* AND *IN VIVO* CHARACTERISTICS OF INTELLIBOND AND SULFATE FORMS OF COPPER, ZINC, AND MANGANESE

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In partial fulfillment of the requirements

For the Degree of Doctorate of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2015

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ABSTRACT

EXAMINING *IN VITRO* AND *IN VIVO* CHARACTERISTICS OF INTELLIBOND AND SULFATE FORMS OF COPPER, ZINC, AND MANGANESE

Trace minerals have long been identified as essential components in the diets of domestic livestock species. The mechanisms underlying Cu, Zn, and Mn absorption are complex, but research has demonstrated interesting opportunities to optimize the involvement of trace minerals in ruminant nutrition. To further our understanding of absorption and retention of modern forms of trace minerals 4 experiments were conducted to examine *in vitro* and *in vivo* characteristics of Intellibond (IB) and sulfate forms of copper (Cu), zinc (Zn), and manganese (Mn). In experiment 1, *in vitro* incubations were used to examine the effects of pH and mineral concentration and source (IB vs sulfate) on Cu, Zn, and Mn solubility. Solubility was measured at elemental concentrations ranging from 0.1 to 10.0 mg of product/ml (0.1, 1.0, and 10.0 mg/ml) at: 1) pH 5.5 (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl-MgSO4*7H2O-urea buffer) and 2) pH 2.0 (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl- MgSO4*7H2O-urea buffer). On average, the overall solubility of Cu, Zn, and Mn, from both trace mineral sources were similar at a pH 5.0 and 2.0. In Experiment 2 eight cross-bred steers were utilized to estimate the duodenal appearance of Cu, Zn, and Mn in steers post ruminal administration of IB and sulfate forms of Cu, Zn, and Mn in steers fed a corn silage and steam flaked corn-based diet. Treatments consisted of 1) 60 mg of Zn/kg DM from ZnSO4; 20 mg of Cu/kg DM from CuSO4; 40 mg of Mn/kg DM from MnSO4, and 2) 60 mg of Zn/kg DM from tetra-basic ZnCl; 20 mg of Cu/kg DM from tri-basic CuCl; 40 mg of Mn/kg DM

from tri-basic MnCl. Individual trace mineral treatments were thoroughly mixed with 0.23 kg of ground corn and administered as a single dose via the rumen fistula. Rumen and duodenal samples were obtained at -4, -2, 0 h, pre dosing and at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, and 42 h post dosing. Duodenal appearance of Cu, Mn, and Zn post ruminal administration of different trace mineral sources were similar across treatments. In experiment 3, eight cross-bred steers were utilized in a 19 d experiment to investigate the effects of trace mineral source on apparent absorption and retention of Cu, Zn, and Mn in steers fed a corn silage and steam flaked corn-based diet. Steers were blocked by BW and randomly assigned to one of the 2 treatments. Treatments consisted of: 1) 30 mg of Zn/kg DM from $ZnSO_4$; 10 mg of Cu/kg DM from CuSO4; 20 mg of Mn/kg DM from MnSO4; and 2) 30 mg of Zn/kg DM from tetra-basic ZnCl; 10 mg of Cu/kg DM from tri-basic CuCl; 20 mg of Mn/kg DM from tri-basic MnCl. Total fecal and urine output was measured daily for all steers during the 5 d collection period. Dry matter disappearance, apparent absorption, and apparent retention of Cu, Zn, and Mn were similar across treatments. In experiment 4, four-hundred cross-bred steers (initial BW 335 ± 9.6 kg) were utilized to investigate the effects of supplemental Zn, Cu, and Mn concentration and source on performance and carcass characteristics of feedlot steers fed a high concentrate steam flaked corn-based finishing diet for 159 d and zilpaterol hydrochloride for the last 21 d prior to slaughter. Treatments consisted of: sulfate) 90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$; IB-1) 30 mg of Zn/kg DM from Zn Hydroxychloride; 10 mg of Cu/kg DM from basic Cu chloride ; 20 mg of Mn/kg DM from Mn Hydroxychloride; IB-2) 45 mg of Zn/kg DM from Hydroxychloride; 12.5 mg of Cu/kg DM basic Cu chloride; 29.5 mg of Mn/kg DM from Mn Hydroxychloride; IB-3) 60 mg of Zn/kg DM from Zn Hydroxychloride; 15 mg of Cu/kg DM from basic Cu chloride; 39 mg of Mn/kg DM from

Mn Hydroxychloride ; and IB-4) 90 mg of Zn/kg DM from Zn Hydroxychloride; 17.5 mg of Cu/kg DM from basic Cu chloride; 48 mg of Mn/kg DM from Mn Hydroxychloride. No differences were observed for final body weight ($P > 0.42$). Overall ADG, DMI, and feed efficiency were similar across treatments. Hot carcass weight, dressing percentage, yield grade, LMA, adjusted fat thickness, KPH, and marbling score were similar across treatments. Concentrations of Zn, Cu, and Mn in liver and blood samples collected on d 112 and at harvest were similar across treatments. Overall data collected from these 4 experiments indicate that under the conditions of theses experiments, supplemental Zn, Cu, and Mn concentration and source had no impact on any of the response variables measured.

ACKNOWLEDGEMENTS

I dedicate this dissertation to my son Ryder Emmanuel Caldera, I love you mijo. Thank you Ryder for making me so proud each and every day and thanks for the happiness that you bring to my life. You are my inspiration to work hard in life and you keep me moving forward. Thank you god for blessing me with a true angel baby (big boy) that keeps me motivated to pursue my dreams.

I'm truly blessed to have so many supportive and wonderful individuals in my life. I wouldn't be where I am today if I didn't have the support system and encouragement that I've received throughout my entire college experience. Thank you Terry Engle, for being the best advisor and I appreciate your kindness and friendship. Thank you Terry, Shannon Archibeque Engle, and Shawn Archibeque for being there when I needed someone to talk to and for being like family to me. Thank you Dr. Wagner and Betty for the great experiences we shared. Thank you Dr. Rollin for challenging me to view and understand agriculture from a different perspective and thank you for all the helpful advice that you've given me. Thanks to each and every one of my committee members for the extremely beneficial knowledge that I acquired from you.

I'd like to thank all of the graduate and undergraduate students that have been a part of my graduate school experience. Thanks to the graduate students who allowed me to help with their research projects, and thanks for all the great friendships that were established. I met a tremendous amount of people in graduate school, and I look forward to continuing those friendships.

Thank you, mom and dad for your love and support throughout my entire life, and especially throughout my years in graduate school. Most importantly, I thank you GOD for this wonderful experience, friends and family, and for all your blessings.

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Chapter I

Literature Review

Trace minerals have long been identified as essential components in the diets of domestic livestock species. Included in the category of essential trace minerals (or microminerals) are chromium (Cr), cobalt (Co), copper (Cu), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo), , selenium (Se), and zinc (Zn). Numerous biochemical reactions have been identified that require trace minerals for proper function. It has been well documented that deficiencies of various trace minerals can result in metabolic diseases. However, the interactions between trace minerals and metabolic processes are extremely complex. For example, trace minerals have been identified as essential components for carbohydrate, lipid, protein, and vitamin metabolism, and have been shown to be involved in hormone production, immunity, and cellular homeostasis.

In general, trace minerals function primarily as catalysts in enzyme systems within cells. Enzymes requiring metals for proper function can be classified into two categories: 1) metal activated enzymes and 2) metalloenzymes. Metal-activated enzymes may or may not have an absolute requirement for a metal; however the presence of a metal is typically required for optimizing enzyme activity. Metalloenzymes are enzymes that contain a tightly bound metal ion at or near the active site. The metal ions bound to metalloenzymes are actively involved in catalysis. Removal of the metal ion renders the enzyme non-functional. Enzymes involved in electron transport, bone metabolism, immune response, gene expression, nutrient metabolism, and protection of cells from oxidative stressors all have been shown to require certain trace elements for proper function.

In order to optimize animal performance, ruminant nutritionists focus primarily on dietary carbohydrate and protein. Depending on the type and source of carbohydrate and protein utilized in rations, supplements are then formulated to contain the balance of nutrients that are not met by the basal ingredients. Trace minerals are typically added to these supplements giving very little value/consideration to the trace minerals contained in the primary dietary ingredients. Although, the minimum concentrations of trace minerals needed to avoid deficiencies have been well documented, there is still a need for further research demonstrating optimal levels and sources of trace minerals for proper immune function and growth (Lineman 2013; Cohen, 2014). Several experiments have focused on trace mineral supplementation during the feedlot phase of beef production, but results have been highly variable (Rhoads et al. 2003, Malcolm-Callis et al. 2000). Identifying the optimal source and dose of trace minerals can be challenging because trace mineral nutrition requires a complete understanding of the animal's mineral requirements as well as an understanding of the mechanisms underlying digestion, absorption, and utilization of the minerals provided in the diets. Regardless of contradicting data or variability in response variables, the impact of trace minerals on growth, reproduction, and immune response has been demonstrated.

The intent of this chapter is to review the literature pertaining to the importance of trace mineral nutrition in beef cattle production, focusing primarily on Cu, Zn, and Mn and to discuss the general functions underlying absorption, transport, and storage and excretion of these trace minerals in ruminants. Another aspect of this chapter will be devoted to discussing chemical characteristics of trace mineral sources of Cu, Zn, and Mn supplemented to beef cattle**.**

Trace Mineral Absorption

Zinc

The trace mineral zinc (Zn) is an essential nutrient for mammals because of its role in enzymes involved in protein synthesis and carbohydrate metabolism. From a structural standpoint, Zn will stabilize the quaternary structure of an enzyme (McDowell, 1992). Throughout the absorption process certain dietary factors can alter the absorption of Zn, but will differ between non-ruminants and ruminants. Certain trace minerals such as Cu and Fe can also have an impact on the absorption of Zn. Other factors involved in Zn absorption include the source and dose of Zn. A thorough understanding of Zn absorption will aid in determining the optimal dietary Zn provided to beef cattle.

The majority of Zn transport research has been conducted in mice and rats with little Zn absorption research conducted in ruminants. Zinc absorption takes place primarily in the first meter of the duodenum. This location is also the main site of Zn re-excretion from the circulatory system (Miller, 1970). Zinc absorption can be divided into four phases according to Cousins (1982). Figure 1 demonstrates the processes involved in Zn absorption across the enterocyte. The first phase involves the solubilization of Zn in the lumen of the intestine. Zinc typically becomes soluble at a low (2-4) pH and therefore the majority of Zn is absorbed prior to the increase of duodenal pH. Once Zn is soluble, it then binds to a Zrt- and Irt- like protein -4 (Zip4) transporter located on the apical membrane of the enterocyte (Cousins et al., 2006). Zrt- and Irt- like proteins are a family of solute-linked carrier 39 (SLC39) proteins responsible for increasing cytosolic Zn concentrations (Cousins et al., 2006). Once Zn is transported to the cytosol of the enterocyte, it binds to a cysteine-rich intestinal binding protein (CRIP) and is transported to the basolateral portion of the enterocyte where a solute-linked carrier 30 protein

(ZnT1; SLC30A) transports Zn out of the enterocyte where it is bound to albumin and transported throughout the body. Although much of the Zn is absorbed from the lumen, Zn within the mucosal cells can also be derived from Zn reabsorbed from the blood stream making Zn absorption bidirectional (Hambidge et al., 1986).

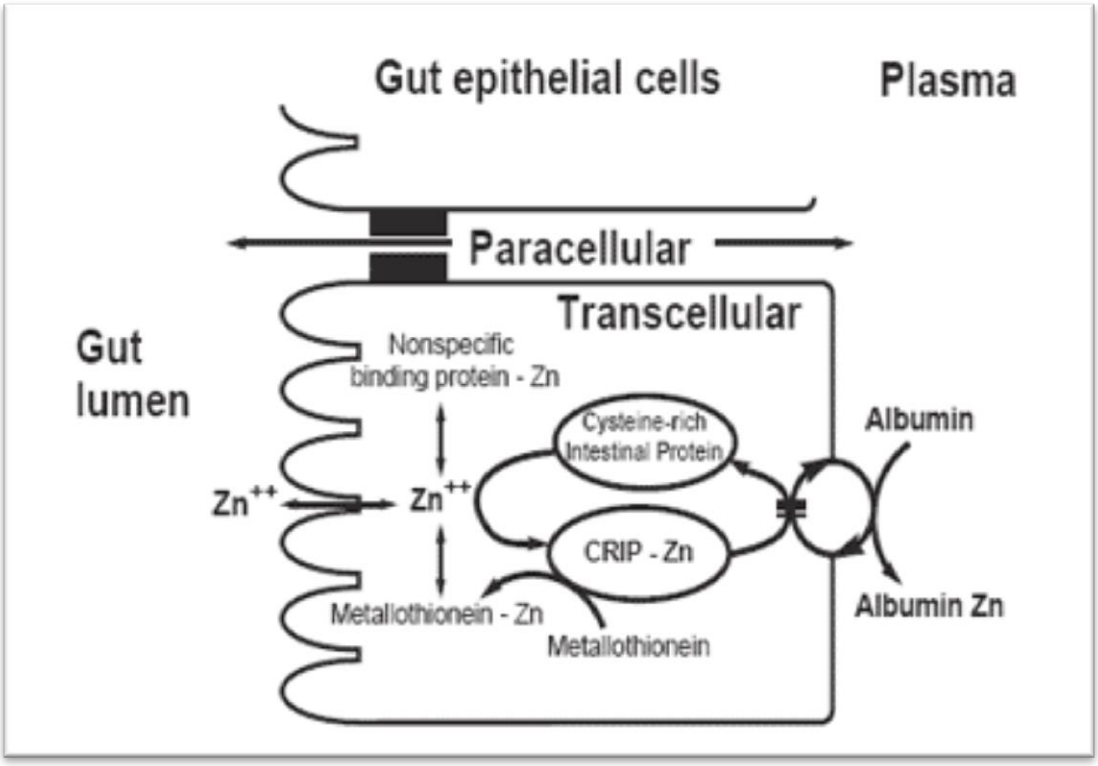

Figure 1. 1. Mechanisms underlying Zn absorption (Adapted from J. NUTR. 122:89-95 (1992))

Absorption of Zn through the small intestine is regulated by a variety of low-molecular-weight binding ligands (McDowell, 1992). The low molecular- weight binding ligands include a low molecular weight protein, such as citrate, EDTA, or amino acids that may not require ATP (Hambidge et al., 1986). Metallothionein (a binding ligand) is a metal binding protein synthesized by hepatic and intestinal tissues, and can be influenced by dietary Zn and plasma Zn concentrations (Mcdowell, 1992). The function of intestinal metallothionein is to limit the absorption of Zn within the intestinal mucosal cells when dietary Zn concentrations are high

(Cousins, 1996; Underwood and Suttle, 1999). When dietary Zn is high, the production of metallothionein increase. Metallothionein binds excess Zn and prevents further absorption into the blood. Also, by binding to excess Zn, the metallothionein is acting as a Zn regulator in achieving homeostasis. However, metallothionein also functions as a regulator of Cu absorption in the epithelial cells of the intestine. Metallothionein production is induced by excess Zn but has a higher binding affinity for Cu. Therefore, high concentrations of dietary Zn would then influence Cu absorption when Cu is at normal concentrations. Furthermore, McDowell (1992) explains that in another study by Cousins (1996), there is evidence of dietary Zn and plasma Zn regulate the quantity of Zn absorbed by the body therefore playing a significant role in Zn homeostasis. When dietary Zn concentrations are low, metallothionein synthesis is decreased thus allowing for increased interactions of Zn and intracellular binding proteins such as CRIP that allows for the transport of Zn to the basolateral surface of the enterocyte. Hempe and Cousins (1991) explain that CRIP moves Zn across the enterocyte to the basolateral side of the cell. However, the exact process of Zn chaperoning has not been fully characterized. Zinc is then attached to a carrier molecule such as albumin. After Zn attaches to albumin it is then transported throughout the body (Hempe and Cousins, 1991). Also, Zn that is absorbed through the rumen wall in ruminants can also be reabsorbed into the lumen of the small intestine. This mechanism of Zn absorption is minimal and secondary to Zn absorption from intestine.

Zinc absorption can also be regulated by other dietary factors with the exception of phytate. For example, inositol hexaphosphates and pentaphosphates are the phytate forms that exert strong negative effects on Zn absorption in non-ruminants (Lonnerdal, 2000). However, in ruminants, with a functional rumen, the amount of phytate found in the diet does not interfere or

limit the amount of Zn absorbed (NRC, 2000). Additionally the amount of dietary protein has been demonstrated to be positively correlated to Zn absorption (Lonnerdal, 2000). Furthermore, fiber can have a negative effect on Zn absorption which could be due to the fact that most fiber-containing foods also contain phytate (Lonnerdal, 2000). However, when dealing with ruminants, the issues with fiber-containing foods would only be an issue in non-functional ruminants such as young calves. The idea that fiber has an influence on Zn digestion is contradicted by the NRC (2000) which states that it's unknown whether zinc's association with fiber reduces absorption. Zinc status should however be considered when dealing with a high fiber diet.

Source (organic or inorganic) of Zn has been reported to influence the absorption of Zn. According to Spears et al. (1989) organic and inorganic forms of Zn are metabolized differently following absorption. Organic forms of Zn have been reported to enhance performance and improve health and reproduction. Data also supports that Zn absorption is similar between Zn methionine and inorganic sources, but evidence exists that Zn provided by Zn methionine is retained in the body more effectively than inorganic Zn (Brown et al., 2004; Spears, 1989). Furthermore, Zn dose has also been of interest when considering the optimum Zn supplementation practices. Interestingly, on average, consulting nutritionist formulate feedlot diets to contain Zn concentrations well in excess of the NRC (2000) requirements (Vasconcelos and Galyean, 2007). Typically the Zn concentrations in feedlot diets formulated by the consulting nutritionist tend to be 3 times the NRC (2000) recommendations (Vasconcelos and Galyean, 2007). This could be counter intuitive since the percentage of Zn absorption increases with decreasing dietary Zn and is reduced with high Zn intake (Church, 1988). Similarly, studies

have shown that high concentrations of Zn have a negative effect on absorption when compared to standard NRC (2000) Zn concentrations.

The mechanisms underlying Zn absorption is complex and further research is needed understand the optimum dose of Zn in ruminant diets. With the current data, enough has been reported in order to avoid toxicities as well as deficiencies in ruminant nutrition. While Zn is involved in complex roles in ruminant metabolism, an optimal nutritional concentration should always be determined in order to continue to allow Zn to perform its role in the diverse metabolic processes.

Copper

Intestinal absorption of Cu can be through both a passive and active process, and is thought to be a similar process in ruminants and non-ruminants (Underwood and Suttle, 1999). Nonetheless, the mechanisms underlying Cu absorption are critical due to Cu being poorly absorbed in most mammals (McDowell, 1992; Underwood, 1977). The absorption of Cu in ruminants is low (<1.0-10%) when compared to non-ruminants (Spears, 2003). As discussed above, Cu absorption, under certain conditions, can be influence by dietary Zn concentrations. Furthermore, the chemical form of Cu can influence the amount of Cu absorbed as explained by McDowell (1992) and Underwood (1977). Supplemental Cu in the form of CuO would be the least available form when compared to Cu sulfate, carbonate, chloride, chelates, and protienates. One of the organic forms of Cu such as a Cu proteinate had a greater bioavailability than $CuSO_4$ when fed to calves receiving diets high in the Cu antagonist Mo (Kincaid et al., 1986; Mcdowell, 1992). The process underlying Cu absorption is controlled through two mechanisms which consist of saturable and an unsaturable mechanisms relating back to the active transport and

simple diffusion process (Mcdowell, 1992). More recently Cu is thought to be absorbed primarily in the duodenum where it is transferred across the brush border into the enterocyte (Hill and Link, 2009; Cater and Mercer, 2006). The majority of research investigating Cu absorption has been conducted in rodents. The exact mechanism(s) of CU absorption is still not well understood (Hill and Link, 2009). Similar to Zn, Cu is solubilized at a low pH (2-4). Once solubilized in the stomach, Cu will enter the small intestine. Briefly, once soluble and in the lumen of the small intestine, Cu will bind to a high affinity copper transport protein (hCTR1) that is expressed on the apical membrane of the enterocyte. Once Cu is transported into the intestinal cell a P-type ATPase MNK protein chaperones Cu to the basolateral surface of the enterocyte where Cu is bound to albumin and transported throughout the circulatory system (Pena et al., 1999). Furthermore, metallothionein also functions as a regulator of Cu absorption in the epithelial cells of the intestine. Much like Zn, when Cu concentrations in the diet are low then Cu absorption is increased. Copper absorption can be negatively influenced by the formation of Cu sulfide in the gut (NRC, 2000). McDowell (1992) explains that in the rumen the formation of thiomolybdates can affect sulfur, Cu, and Mo metabolism. Minimizing the interactions between Cu and other components in the diet would potentially increase the absorption of Cu at the intestinal level (McDowell, 1992).

Manganese

Manganese absorption is similar to that of iron. Although, little is known concerning dietary factors affecting Mn absorption (NRC, 2000), McDowell (1992) explains that Mn absorption occurs in a two-step process which involves the uptake from the gut lumen, and then transfer across the mucosal cells. Also, much like Zn and Cu, when dietary Mn concentrations are high absorption decreases, and when dietary Mn concentrations are low absorption increases.

However, a Mn deficiency can occur in diets composed of normal feed ingredients in swine, poultry, and ruminants (McDowell, 1992). Unlike Zn where there is a regulated absorption and excretion process, Mn homeostasis is primarily regulated through excretion (Thomas, 1970). Manganese is thought to be absorbed from the intestinal lumen via a divalent metal transporter 1 (DMT1) which is located on the apical surface of the enterocyte. The DMT1 transports divalent metal ions such as Mn, Fe, Zn, etc. into the cytosol of the enterocyte.. Once absorbed, manganese is transported throughout the body bound to transferrin (Underwood and Suttle, 1999). However, Mn antagonist in the diet such as Ca and P may cause the Mn requirement in the diet to increase (Olson and Hale, 2001). Typically Mn is thought to be poorly absorbed because of the substantial surplus of manganese provided by most practical rations (Underwood and Suttle, 1999); however, deficiencies have been noted in beef cattle under natural conditions in certain areas of the northwestern United States.

Trace Mineral Transport

Zinc

Once Zn enters the blood circulatory system a variety of factors will dictate its metabolism. Zinc is transported through the blood stream bound to either albumin, □□-macroglobulin, and also as traces of metallothionein (Underwood and Suttle, 1999) and flows to the liver. Once in the liver Zn is primarily bound to metallothionein. Metallothionein in the liver is the major storage form of Zn, and can be mobilized during metabolic need (McDowell, 1992). There are four isoforms of metallothionein present in mammals: metallothionein 1 and 2 which have ubiquitous tissue distribution with particular abundance in liver, pancreas, intestine, and kidney, whereas metallothionein 3 and 4 are found principally in brain and skin (Davis and Cousins, 2000). The binding of Zn to liver metallothionein is

relatively weak, thus giving liver metallothionein the ability to acquire and release Zn. Whereas, the bond between enterocyte derived metallothionein and Zn is extremely tight.

Therefore, once Zn is in the enterocyte the absorption of Zn into the blood stream is dependent on Zn concentrations of Zn in the circulatory system along with the regulation of metallothionein in the enterocyte. When Zn concentrations in the blood are low, Zn transport into the blood will increase (Hambidge et al., 1986). Yet, the reverse effect occurs when Zn concentrations are high in the blood; less Zn is absorbed from the intestinal cells into the blood stream.

Whether the absorption of Zn is up or down regulated, plasma Zn will dictate only part of Zn status within the animal. This implies that in order to make accurate assumptions about Zn status within the animal more should be evaluated than blood status alone. A possible approach would be to identify indicators of Zn status in the serum such as metallothionein or serum alkaline phosphatase activity. Alkaline phosphatase activity has shown to fall during Zn deficiency, but follows a similar time course to serum Zn and is also affected by gut and bone disorders (Underwood and Suttle, 1999).

Since Zn can be transported across the small intestine and transported in the body by albumin, the attachment of Zn to methionine may alter its mode of absorption and transport in the animal's body compared to Zn from Zn oxide (Greene et. al. 1988). Transport of Zn from the intestinal lumen into the blood stream and from the blood stream into the lumen is a bidirectional process which the mechanism remains unknown (Hambridge et al., 1986). The metabolism of Zn in the blood after it is absorbed is affected by the ligands involved which can vary depending on Zn status (McDowell 1992, Underwood and Suttle 1999). Only about two thirds of plasma Zn is bound to albumin in the portal blood stream (Underwood and

Suttle, 1999). The other portion of plasma Zn can be bound to □□-macroglobulin or to metallothionein for example.

Coper

More recent data reports the identification of Cu transporters and chaperones (Hill and Link, 2009). Molecular techniques as well as human metabolic disorders and single-cell organisms have all helped understand the mechanisms underlying Cu metabolism (Hill and Link, 2009). Transporter chaperones are critical for cellular trace mineral homeostasis as well as for the whole animal (Cohen, 2014; Fry et al., 2013). In general, once copper is absorbed and enters the blood stream then it binds to albumin and/or the amino acid histidine and is then transported throughout the body (McDowell, 1992). If Cu binds to metallothionein in the intestinal mucosa then this interaction could restrict the further translocation of Cu (Cousins, 1985; Underwood and Suttle, 1999). While Cu is transported throughout the body there is a good possibility that antagonists such molybdenum (Mo), sulfur (S), and iron (Fe) could induce hypocuporsis by forming insoluble Cu complexes in the digestive tract, bloodstream, and tissues of ruminants (Bailey et al., 2001). Therefore, Bailey et al., (2001) emphasizes that it would be advantageous to develop supplementation strategies that would limit these antagonistic effects.

Manganese

Manganese absorption can occur throughout the length of the small intestine in two steps; uptake from the lumen then transfer across the mucosal cells. Absorbed Mn is transported by transferrin to the liver (Davidson, 1989; Underwood, 1999). Manganese is mainly excreted in the feces via biliary excretion (Thomas, 1970). Manganese being excreted through bile will have a minimal possibility of being reabsorbed (Underwood, 1999). Since bile is a major route for

excess Mn; then attempting to understand flow rates of Mn through the digestive tract post oral or ruminal dosing is challenging due to endogenous losses of Mn. Also, Mn excretion via the feces is proportionally greater as the amount of manganese absorbed increases (Underwood, 1999). Nonetheless, Genther and Hansen (2014) report the importance of Mn superoxide dismutase which is an antioxidant found in the mitochondria that catalyzes the conversion of the superoxide radical to less reactive hydrogen peroxide. Beef steers were utilized in a trace mineral repletion study and demonstrated that trace mineral injection of Cu, Mn, and Zn increased red blood cell lysate Mn superoxide dismutase activity (Genther and Hansen, 2014). However, Genther and Hansen (2014) did report there is still a lack for a good biomarker of Mn in cattle, but Mn superoxide dismutase activity did reflect Mn supplementation via injection in their study and by dietary intake in other animal studies, suggesting it has a potential to be used as a biomarker of Mn status.

Trace Mineral Storage and Excretion

Zinc

Even though the mechanisms of tissue uptake of Zn have not yet been characterized, Zn complexed with albumin is readily available for uptake by tissues (McDowell, 1992). Yet, not all tissues that uptake Zn, make the Zn available to other tissues. For example, the uptake of Zn in bone and the central nervous system is relatively slow and firmly bound once acquired by these tissues, making the Zn unavailable to other tissues (McDowell, 1992).

The distribution of Zn throughout the body is well understood, but the mechanisms involved in Zn uptake by other tissues beyond the liver are not well known (Cousins, 1996, Underwood and Suttle, 1999). Subsequently Zn is released back into the blood stream after

approximately 30-40% of the Zn entering through the hepatic venous supply is extracted by the liver (McDowell, 1992). The circulating Zn enters various extrahepatic tissues at differing rates, which consist of different rates of Zn turnover (Underwood and Suttle, 1999). Body tissues will exhibit different accumulation and turnover rates following oral administration and subsequent absorption (Miller et. al. 1970). After an oral dosing, plasma Zn concentrations reach their peak within 1 to 3 d followed by a rapid decline for 3 to 4 weeks and a subsequent very slow decrease (Miller et. al. 1970). Even though Zn tends to accumulate very slowly in some tissues, the amount in red blood cells, muscle, and bone continues to increase for several weeks after a single oral dose (Miller et. al. 1970). In the blood stream 80% is present in the erythrocytes, which contain about 1mg Zn per 10^6 cells (Underwood and Suttle, 1999). The pancreas, liver, kidney, and spleen have the most rapid accumulation and turnover of retained Zn (McKinney et al., 1962).

Most of the intracellular Zn is found in the cytosol (60-80%), with some Zn found in the crude nuclear fraction (10-20%) and small amounts in the microsomal and mitochondrial fractions (Saylor and Leach, 1980, McDowell, 1992). Zinc found in the cytosol is primarily bound to proteins whereas other fractions of Zn may be found on the cell membrane (McDowell, 1992). However, Hempe et. al (1991) identified a low molecular mass, intracellular constituent from rat intestinal mucosa that binds Zn during transmucosal Zn transport. The low molecular mass was not metallothionein, based on the Cd-hemoglobin affinity assay (Hempe et al. 1991). Indicating the possibility of other cellular homeostatic mechanisms for Zn.

Zinc storage within an animal is minimal, leading to complications during a dietary Zn deficiency. Although Zn is widely distributed throughout the body, animals have limited capacity for storing Zn in a form where it can be mobilized rapidly in order to prevent a deficiency

(McDowell, 1992). Along with its importance in Zn absorption, metallothionein is also involved with being the major storage form of Zn within the liver (Richards and Cousins, 1976). Spears and Samsell (1986) reported that Zn retention was greater for lambs fed Zn methionine compared with those fed a control or Zn oxide-supplemented diet. Even though the absence of recognized stores exist, Zn may be redistributed from large pools found in bone and muscle during a deficiency (Underwood and Suttle, 1999). Also, when Zn is fed in large amounts the Zn content greatly increases in some tissues including blood, pancreas, kidney, bone, hair, and liver, but may have little impact on other tissues such as muscle (Miller et. al. 1970).

Cooper

The liver is the major storage organ for Cu where it is then released for the synthesis of various of enzymes (McDowell, 1992). While other organs contribute to the storage of Cu, approximately 20% of the animal's Cu supply is stored in the liver and remains as the main site for storage (Mcdowell, 1992). Copper is typically stored in the liver in the form of mitochondrial cuprein (McDowell, 1992). Within the body, Cu will be stored in the liver in ruminant animals to a greater extent when compared to non-ruminants. This indicates that ruminants are at a greater risk of a Cu toxicity (Underwood and Suttle, 1999). Again much like Zn metallothionein will bind Cu and aid in Cu storage as well as an aid in providing Cu during a Cu deficiency (McDowell, 1992).

Copper can be excreted through urine, bile, sloughed intestinal cells but the main excretory route is through feces (McDowell, 1992). Non-ruminants will excrete Cu at a greater extent when compared to ruminant animals because they tend to not be as susceptible to a Cu deficiency, and will excrete more Cu via the bile and maintain lower concentrations of liver Cu (Underwood and Suttle, 1999).

Manganese

Manganese is mainly stored in the lowest concentrations of all trace minerals within tissues (Underwood and Suttle, 1999). Unlike Cu, the storage capacity for Mn in the liver is minimal (Mcdowell, 1992). Studies have shown fluctuations in liver Mn when dietary Mn is elevated; however, liver Mn seems to resist change when liver Mn reaches a certain concentrations (Watson et al., 1973; Mcdowell, 1992). Bone growth is affected when a Mn deficiency is present resulting in shorter and thicker bones (McDowell, 1992). Reproductive effects were among the first signs of a Mn deficiency to be observed (McDowell, 1992), therefore a good understanding of storage and excretion rates is necessary to maintain optimal reproduction.

Manganese deficiency is most likely a result of limited concentrations of circulating Mn (McDowell, 1992). Manganese is not only excreted through feces with bile being the main source (Thomas, 1970) but also through pancreatic juice as well as secretion from the intestinal wall (McDowell). Also, the majority of Mn found in the body is found in the skeleton and is not readily available during a Mn deficiency. In calves there were bone abnormalities noted when cows were supplemented at 15.8 mg/kg but not when cows were supplemented 25mg/kg (Rojas et al., 1965; NRC, 2000). The NRC (2000) also reports that Mn requirements for growth and skeletal development are less than for reproduction. Therefore it seems that the excretion of Mn is critical in regards to maintaining concentrations within narrow limits (Ahola et al., 2005).

Bioavailability of Trace Mineral Supplements

Bioavailability has been defined as the proportion of the element consumed that is utilized for a biochemical or physiological function (O'Dell, 1997; Hill and Link, 2009). Currently, trace minerals are available in both organic and inorganic forms as well as hydroxyl minerals. Trace minerals defined as inorganic are those that are typically bound to sulfates, carbonates, chlorides, or oxides, while those defined as organic are bound to amino acids or protein complexes, and are referred to as complexes, chelates or protienates (Spears, 1996). Spears (1996) provides a table from the Association of American Feed control officials providing definitions for various types of mineral products.

Table 1.1 Definitions of various organic mineral products according to the Association of American Feed Control Officials

57.150 Metal amino acid complex - The product resulting from complexing of a soluble metal salt with an amino acid(s).
57.142 Metal amino acid chelate - The product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acids to form coordinate covalent bonds. The average weight of the hydrolyzed ammo acids must be approximately 150 and the resulting molecular weight of the chelate must not exceed 800.
57.23 Metal proteinate - The product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein.
57.29 Metal polysaccharide complex - The product resulting from complexing of a soluble salt with a polysaccharide solution.

J.W. Spears/Animal Feed Science Technology 58 (1996) 151-163

The general premise behind increased bioavailability of organic trace minerals is that organic trace minerals are protected from many of the dietary antagonists (as previously mentioned) that can potentially make them unavailable for absorption (Hemken et al., 1996). It has been theorized by some researchers that organic trace minerals remain intact in the gastrointestinal tract, through the sight of absorption, and perhaps beyond absorption. Whereas, the hydroxyl trace minerals (copper hydroxyl chloride, zinc hydroxylchloride, and manganese hydroxyl chloride) belong to a separate group of trace minerals because of their unique chemical

characteristics. Zinc hydroxychloride differs from Zn sulfate because of the covalent bonds located between the Zn ion, multiple hydroxyl groups and the chloride ions, but Zn sulfate contains an ionic bond where the Zn ion is connected with the sulfate group by weak ionic bond (Mn hydroxychloride and Cu hydroxylchloride contain the covalent bonds as well). The covalent bonds are believed to increase the amount of biologically active Zn delivered to the intestine, driving increased Zn absorption (Cohen and Steward). Cohen and Steward (unknown) explain how the covalent bonds with the metals are strong enough to limit parasitic reactions, but weak enough to readily hand off the metal to the escort ligands involved to facilitate absorption, thus positively affecting the bioavailability of the hydroxyl trace minerals (Cohen and Steward, unknown). The sulfate sources of Zn, Cu, and Mn contain the ionic bond and are thought to disassociate once contact with moisture occurs allowing the metal ions to bind with a large number of diet antagonists (Cohen and Steward, unknown). Tribasic Cu chloride is more bioavailable than $CuSO_4$ when added to diets high in the Cu antagonists Mo and S (Spears et al., 2004).

A number of studies have been conducted using *in vitro* and *in vivo* techniques to determine the relative bioavailability of trace mineral sources. These experiments typically use an inorganic mineral as a benchmark (100%) and compare other mineral sources to it. Results have been variable, however under certain circumstances organic mineral sources have centered around the theory that they are more bioavailable, which suggests that organic trace minerals may be more similar to forms that occur in the body (Spears, 1996). Since trace minerals that are present in the body function as organic complexes or chelates and not as free inorganic ions then a stable metal chelate that keeps from forming complexes with other dietary components that inhibit absorption would allow for a greater absorption and utilization by the body (Spears,

1996). Furthermore, it is challenging to interpret data from different experiments because researchers may have used various methods of supplementation, different sources of trace minerals, a variety of different cattle types, and a variety of environmental variable may exist among experiments. Moreover, breed of cattle, antagonists present in the diet, as well as physiological status of the animal must be taken into consideration when comparing the results from trace mineral source experiments.

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Chapter II

Preliminary *in vitro* solubility testing - Examining the effects of pH and mineral concentration and source on Cu, Zn, and Mn solubility.

Summary

This descriptive experiment was conducted to estimate the solubility of InteliBond (IB) and sulfate forms of copper, zinc, and manganese. *In vitro* incubations were used to examine the effects of pH and mineral concentration and source on Cu, Zn, and Mn solubility. Solubility was measured at elemental concentrations ranging from 0.1 to 10.0 mg of product/ml (0.1, 1.0, and 10.0 mg/ml) at: 1) pH 5.5 (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl- MgSO4*7H2O-urea buffer) and 2) pH 2.0 (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl- MgSO4*7H2O-urea buffer). Each trace mineral compound was weighed and mixed with 100 ml of the appropriate buffer to achieve the desired elemental concentration and pH and then filtered through Whatman 541 filter paper (20-25µm). Metals that passed through the filter were assumed to be soluble. On average the overall solubility of Cu, Zn, and Mn, from both trace mineral sources (Intellibond and Sulfate) was almost completely soluble at the pH of 2 when compared to a pH of 5. However, Intellibond C was less soluble when compared to CuSO4 at the pH of 5.

Introduction

Since the introduction of organic trace minerals, and hydroxy trace minerals a better understanding on the mode of action, solubility, and the overall bioavailability of these innovative trace mineral sources is needed. Spears (1996) reported that in regards to organic trace mineral sources, a metal chelate or complex that remains in its original form throughout the digestive tract, would be protected from interactions with other dietary compounds that could compromise absorption in the small intestine. Furthermore, Spears et al. (2004) compared the solubility of tribasic copper chloride to copper sulfate in water and acid solutions. The findings demonstrated that tribasic copper chloride was less soluble than copper sulfate at a neutral or slightly acidic pH (Spears et al., 2004). The lower solubility of tribasic copper chloride in comparison to coper sulfate would be desirable in the ruminal environment with antagonists such as Mo and S (Spears et al., 2004). Antagonists in diets are frequently present and can reduce bioavailability. Therefore, a trace mineral supplement that would interact less with antagonist in the rumen but would become soluble at a lower pH would be desirable (Spears et al., 2004). The objective of this descriptive experiment was to estimate the solubility of InteliBond (IB) and sulfate forms of Cu, Zn, and Mn in an attempt to better understand under which conditions each mineral source will disassociate from its original ligand.

Materials and Methods

The laboratory procedures used to measure solubility were based on methods described by Brown and Zeringue (1994). Solubility was measured at elemental concentrations ranging from 0.1 to 10.0 mg of product/ml (0.1, 1.0, and 10.0 mg/ml) at: 1) pH 5.5 (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl- MgSO4*7H2O-urea buffer) and 2) pH 2.0 (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl- MgSO4*7H2O-

urea buffer). Briefly, each individual trace mineral compound was weighed and mixed with 100 ml of the appropriate buffer (in McDougal's artificial saliva mixture 0.1 M NaHCO3-Na2HPO4KCl- MgSO4*7H2O-urea buffer) to achieve the desired elemental concentration and pH. Adjustments in pH were made with 0.1 M HCI to reach the target pH (volumes were recorded of any solution added to adjust pH). Mineral source/buffer solutions were incubated and agitated at 39℃ for 2 h and then filtered through filter paper (Whatman 541 paper; 20-25µm; Whatman, Clifton, NJ). The overall premise of this procedure was if mineral disassociates from its ligand it would be small enough to pass through the filter paper pores. However, if the mineral didn't dissociate from its ligand then it would remain on the filter paper. After filtration, filter paper was dry ashed at 600°C, dissolved in 5 ml of 25% HCl, and diluted with deionized H_20 before mineral analysis. All metals were quantified via inductively coupled plasma-atomic emission spectroscopy (ICP-AES) methods (Braselton et al., 1997) as described by Ahola et al. (2004) for Zn, Cu, and Mn, concentrations. Metals that passed through the filter were assumed to be soluble, whereas the metals remaining on rinsed filter papers were assumed to be insoluble. Since this experiment was not replicated, only descriptive statistics are presented.

Results

Zinc from IB was greater than 80 percent soluble at a pH of 2 and pH of 5 for all doses measured (0.1, 1.0, and 10.0 mg/ml; Figure 2.1). Similar responses were noted from the IB manganese (Figure 2.2). However, IB Cu was 66.5 percent soluble at a pH of 5 and at a dose of 0.1mg of Cu/ml (Figure 2.3). The sulfate forms of Cu, Zn, and Mn were numerically greater than 80 percent soluble at a pH of 2 and at a pH of 5 for all doses measured (0.1, 1.0, and 10.0 mg/ml). Zinc sulfate was 71.9 percent soluble at a pH of 5 and at a dose of 0.1mg of Zn/ml. Manganese sulfate was 39.2 percent soluble at a pH of 5 at a dose of 0.1 mg of Mn/ml and 34.5

percent soluble at a pH of 5 and at a dose of 1mg of Mn/ml. On average the overall solubility of both trace mineral sources (IB and Sulfate) were greater than 60% soluble at pH 2 when compared to a pH of 5. Also on average, IB Cu was less soluble when compared to $CuSO_4$ at the pH of 5.

Discussion

Solubility characteristics of each trace mineral source can impact absorption. The metabolic processes involved with absorption at the intestinal level would then affect the animal's ability to deploy ligands to escort essential metals through epithelial tissues (Cohen and Steward, unknown). Furthermore, fewer interactions between the trace mineral source presented in the rumen and ruminal contents can have a significant impact on the total tract digestibility of trace minerals provided in the diet. This is similar to what Spears (1996) proposed earlier. Minerals bound to a ligand would not interact with antagonist in the rumen and then would become soluble in the small intestine. A previous solubility experiment demonstrated that tribasic Cu chloride Cu_2(OH)3Cl) has a very low water solubility relative to Cu sulfate (Miles et al., 1998), and may be more resistant to interactions with Mo and S in the rumen (Spears et al., 2004). However, the present experiment focused on the solubility of the hydroxy trace mineral (Intellibond Z, Intellibond C, and Intellibond M) sources compared to the sulfate (ZnSO4, CuSO4, and MnSO4) trace mineral sources in McDougall's solution instead of water and at low and semi-acidic pH's (2 vs 5, respectively). High solubility's of both sources of trace minerals were reported at a pH of 2 and 5. These results are surprising since earlier research by Spears et al. (2004) demonstrated that tribasic copper chloride was less soluble than CuSO4 at a neutral or slightly acidic pH (pH of 6-7) (Spears et al., 2004). The fact that Spears used close to neutral pH may explain the differences in results. However, rumen pH

of feedlot steers could approach 5.0 and therefore allow for release of the metal from its ligand from both sources of trace minerals. It should be noted that these data are preliminary and further research (replication and different pH's) are needed.

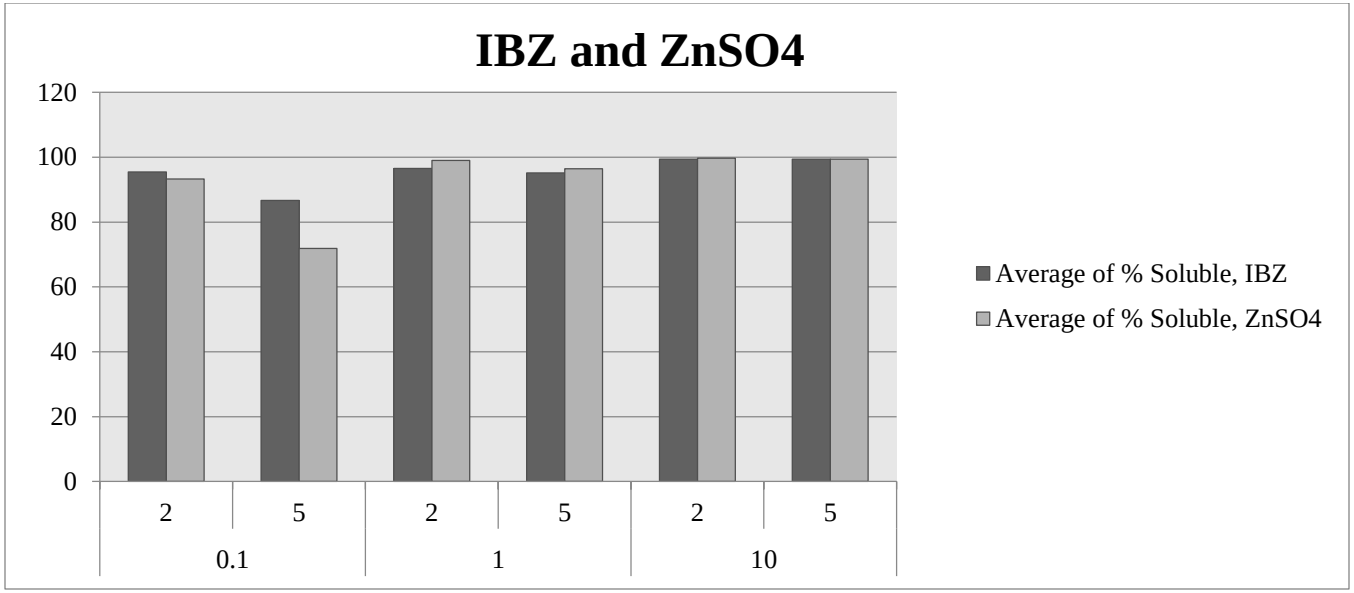

Figure 2.1. In vitro solubility of Intellibond Z and $ZnSO_4$ (raw means)

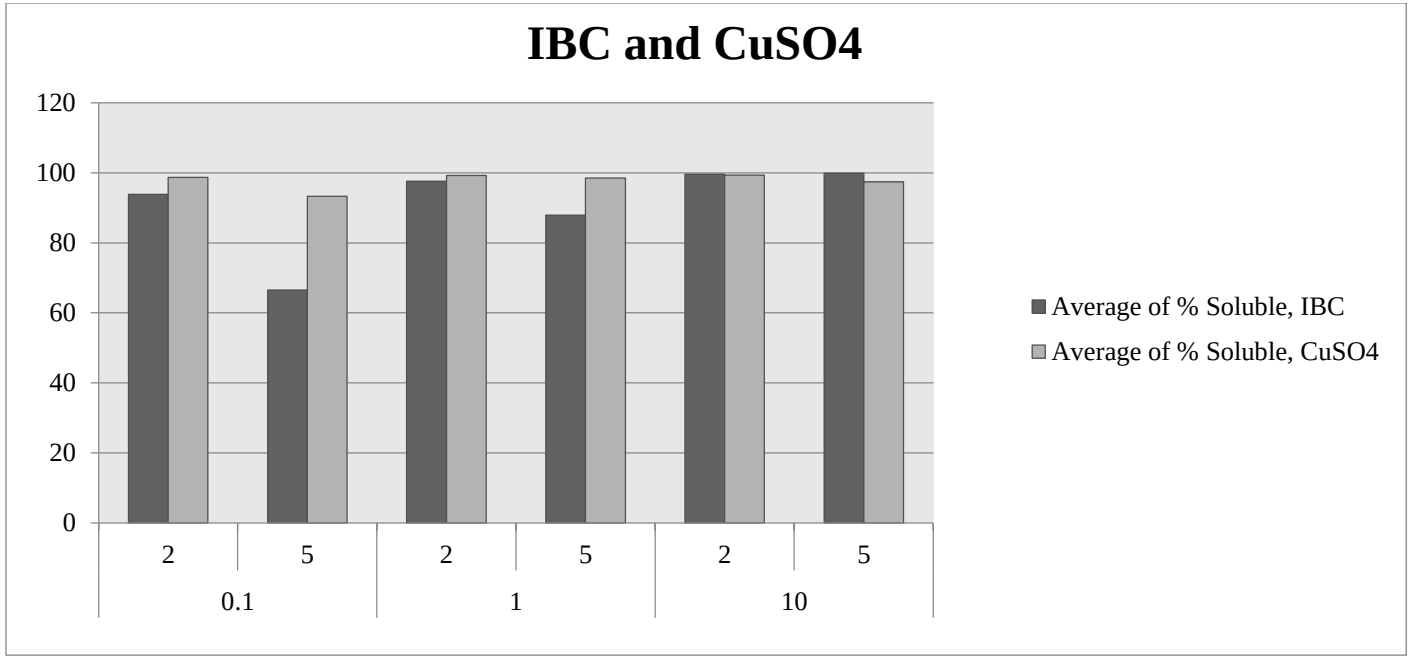

Figure 2.2. In vitro solubility of Intellibond C and CuSO4 (raw means).

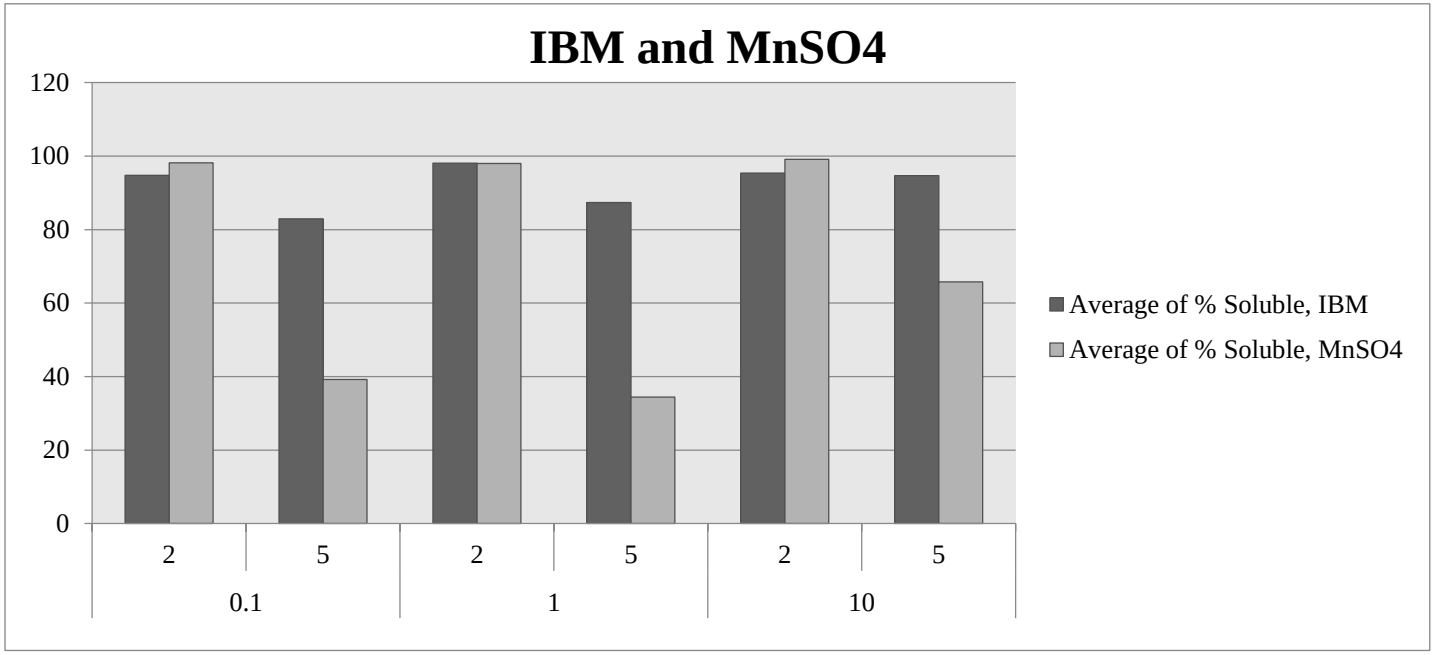

Figure 2.3. In vitro solubility of Intellibond M and MnSO4 (raw means).

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Chapter III

In vivo ruminal pulse-dose administration of different trace mineral sources on duodenal appearance of Cu, Zn, and Mn in steers.

Summary

Eight cross-bred steers (initial BW 718.9 ± 64.9 kg) were utilized to estimate and compare the duodenal appearance of Cu, Zn, and Mn in steers after administration of InteliBond (IB) and sulfate forms of Cu, Zn, and Mn into the rumen in steers fed a corn silage steam flaked corn-based diet. All steers were fed a TMR containing 50% corn silage, 50% steam-flaked corn based diet (on a DM basis) for 14 d. No supplemental Cu, Zn, or Mn was added to the TMR. On day 15, steers were housed in the metabolism barn in individual pens (2.5m x 2.5m). Steers were allowed to acclimate to their new environment for 5 days. Steers with the closet DMI were paired, and randomly assigned to one of the 2 treatments. Treatments consisted of 1) 60 mg of Zn/kg DM from ZnSO4; 20 mg of Cu/kg DM from CuSO4; 40 mg of Mn/kg DM from MnSO4, and 2) 60 mg of Zn/kg DM from tetra-basic ZnCl; 20 mg of Cu/kg DM from tri-basic CuCl; 40 mg of Mn/kg DM from tri-basic MnCl. Individual trace mineral treatments were thoroughly mixed with 0.23 kg of ground corn and administered as a single dose via the rumen fistula. Immediately post administration, the supplement was thoroughly mixed with the rumen contents by hand. Rumen and duodenal samples were obtained at -4, -2, 0 h, pre-dosing and at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, and 42 h post-dosing. During the collection period, steers had access to feed and water. Duodenal appearance of Cu, Zn, and Mn post ruminal administration of different trace mineral sources were similar across treatments.

These data indicate that a single dose of Zn, Cu, and Mn from different sources of trace minerals via the rumen fistula had no impact on duodenal appearance of Cu, Zn, and Mn.

Key Words: Pulse-dose, zinc, copper, manganese

Introduction

Trace minerals are involved in the maintenance of homeostasis, osmotic pressure, acid base balance (RBC and kidney), and in catalytic processes. Zinc is involved in over 200 zinc-dependent enzyme activities which involve catalytic, structural, and regulatory roles. (Aggett, 1985). Furthermore, Cu and Mn function as components or regulators of hormones, vitamins, gene expression, membrane stability, and immunity (Huerta et al., 2002). Due to the importance of trace minerals in animal production, consulting nutritionists will typically fortify feedlot diets with trace minerals because feed ingredients utilized in feedlot diets tend to be inadequate in concentrations of certain essential trace minerals and/or may contain elevated concentrations of known trace mineral antagonists (Ahola et al., 2005).

Duodenal appearance of trace minerals after ruminal administration heavily influences the processes involving absorption at the intestinal level. The metabolic processes involved with absorption at the intestinal level can impact the animal's ability to synthesize ligands to escort essential minerals through epithelial tissues (Cohen and Steward, unknown). Furthermore, fewer interactions between the trace mineral source presented in the rumen and ruminal contents can have a significant impact on the total tract digestibility of trace minerals provided in the diet. Therefore, the objective of this study was to compare the duodenal appearance of Cu, Zn, and Mn in steers following ruminal administration of InteliBond (IB) and sulfate forms of Cu, Zn, and Mn.

Materials and Methods

Prior to the initiation of this experiment all procedures herein were approved by the Colorado State University institutional animal care and use committee.

Eight crossbred steers (BW 718.9 ± 64.9 kg) fitted with ruminal and duodenal canulae were utilized in this experiment. Steers were housed in one common pen (n = 8 steers) and fed once daily a total mixed ration (TMR) containing approximately 50% corn silage and 50% steam-flaked corn (DMB) for 14 d. No supplemental Cu, Zn, or Mn was added to the TMR. On day 15, steers were housed in the metabolism barn in individual pens (2.5m x 2.5m) equipped with automatic waters, individual feeders, rubber matted floors, and a drain. Steers were allowed to acclimate to their new environment for 5 d. At the end of the 5 d acclimation phase, steers with the closest DMI were paired. Once animals were appropriately paired, each individual pair was fed the same amount of feed. Feed delivered to each pair was 90.0% of the steer within the pair with the lowest DMI average during the acclimation period. This ensured equal amounts of feed intake within each pair of steers.

Experimental treatments consisted of: 1) 60 mg of Zn/kg DM from ZnSO4; 20 mg of Cu/kg DM from CuSO4; 40 mg of Mn/kg DM from MnSO4, and 2) 60 mg of Zn/kg DM from tetra-basic ZnCl; 20 mg of Cu/kg DM from tri-basic CuCl; 40 mg of Mn/kg DM from tri-basic MnCl. Trace mineral treatments were weighed in amounts to provide 2 times NRC values for Cu, Zn, and Mn from either IB or sulfate sources. A 10 kg DMI was used to calculate the total amount of trace mineral added to the pulse-dose supplement. Individual trace mineral treatments were thoroughly mixed with 0.23 kg of ground corn and administered as a single dose via the rumen fistula. Immediately post administration, the supplement was thoroughly mixed with the rumen contents by hand. Duodenal samples were obtained at 2 hour intervals starting at -4, and

ending at 42 h post dosing. During the collection period, steers had access to feed and water. Once collected, duodenal samples were centrifuged 1000 x *g* in 50ml graduated conical tubes. Once centrifuged, the volume of supernatant was determined and frozen at -20°C until trace mineral analysis could be performed. All metals were quantified via inductively coupled plasma-atomic emission spectroscopy (ICP-AES) methods (Braselton et al., 1997) as described by Ahola et al. (2004) for Zn, Cu, and Mn, concentrations.

Statistics: Mineral concentrations of Cu, Zn, and Mn from samples collected every two hours for the 42h collection period post ruminal administration of each trace mineral source was analyzed by least square analysis of variance using the Proc Mixed procedure of SAS. Since the cattle were fed on hour 25, hour 25 – 42 data were excluded subsequent analysis. The average of pre-pulse dosing Cu, Zn, and Mn, values were used as covariates for analysis and individual animal was considered the experimental unit. Significance was determined at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Results and Discussion

Table 3.1 describes the impact of trace mineral source on duodenal appearance of soluble Cu, Zn, and Mn, Overall, there was no differences in duodenal soluble Zn appearance between IB Zn when compared to Zn Sulfate throughout the 24hr collection period. However, at 16 h duodenal soluble Zn concentrations form steers receiving IB Zn were greater ($P < 0.002$) than duodenal soluble Zn concentrations from steers receiving Zn Sulfate (0.426 vs. 0.095; respectively; Figure 3.1).

Previous results demonstrate that Cu from tribasic Cu chloride (or IB Cu) could be more available for absorption following solubilization in the acid environment of the abomasum

(Spears et al., 2004). In the present experiment, minimal differences were detected in the soluble concentrations of Cu from either treatment. However, at h 16 IB Cu appeared at a lesser amount ($P < 0.05$) than soluble Cu appearing in the duodenal from Cu sulfate supplemented steers (0.031 vs. 0.069; respectively Table 3.1; Figure 3.2). Smaller amounts of Cu appearing at the duodenal level could be of concern or related to diets high in antagonists such as Mo and S. However, Spears (2004) compared tribasic Cu chloride to sulfate in terms in ability to maintain Cu status when supplemented to steers fed diets high in the Cu antagonists Mo and S, and the two sources demonstrated similar results. Also, in the same experiment tribasic Cu chloride and Cu sulfate were similar ($P > 0.10$) in their ability to increase Cu status in steers receiving diets low Mo, but that had experienced a Cu depletion (Spears et al., 2004). However, tribasic Cu chloride demonstrated a higher bioavailability than Cu sulfate when added to diets high in Cu antagonists (Spears et al., 2004).

Limited experiments have been reported on the solubilization of IB Mn *in vivo*. Soluble Mn appearing at the duodenum from IB Mn and Mn sulfate supplemented steers were similar across treatments at each time point (Figure 3.1; Table 3.3).

In the present experiment, Zn, Cu, and Mn concentrations from samples collected every two hours for 24 h demonstrated duodenal appearance of Cu, Zn, and Mn post ruminal administration of different trace mineral sources to be almost similar across treatments. These data indicate that a single dose of supplemental Zn, Cu, and Mn from different sources via the rumen fistula had minimal impact on duodenal appearance of Cu, Zn, and Mn.

The duodenal appearance of soluble Zn, Cu, and Mn post ruminal dosing of supplemental Zn, Cu, and Mn from different sources provides information as to how much trace mineral might be available for absorption at the intestinal level. Information regarding the influence on

duodenal appearance post ruminal administration of trace mineral supplements is extremely limited. This type of research is similar to previous studies that attempt to better understand passage rates, ruminal particle turnover, or its components which are important to research involving the nutrition of herbivorous livestock (Cochran et al., 1986). Other previous experiments utilizing the pulse dose approach include experiments such as Volden et al., (2002) which attempted to understand apparent ruminal degradation and rumen escape of soluble nitrogen fractions in grass and grass silage administered itraruminaly to lactating dairy cows. The soluble nitrogen utilized in this pulse dose experiment was extracted from first cut consisting of mixture of timothy and metal fescue, grass silage, sources (Volden et al., 2002) . The extracts were administered via the ruminal cannulas to estimate in vivo ruminal degradation rate and rumen escape of soluble nitrogen fractions in grass and grass silage (Volden et al., 2002).

Table 3.1. Effect of *in vivo* ruminal pulse-dose administration of different trace mineral sources on duodenal appearance of Cu, Zn, and Mn in steers.

	Treatment			
Time, h	IB[a]	SO_4[b]	SEM	TRT ($P <$)
Zinc[c]				
0	0.159	0.153	0.089	0.95
2	0.107	0.127	0.089	0.82
4	0.186	0.144	0.089	0.64
6	0.134	0.141	0.095	0.95
8	0.179	0.094	0.096	0.38
10	0.189	0.200	0.089	0.90
12	0.223	0.273	0.101	0.62
14	0.221	0.198	0.101	0.82
16	0.426	0.095	0.101	0.002
18	0.196	0.128	0.101	0.50
20	0.129	0.120	0.101	0.93
22	0.095	0.093	0.101	0.98
24	0.271	0.065	0.101	0.05
Copper[c]				
0	0.021	0.024	0.016	0.86
2	0.016	0.019	0.016	0.86
4	0.029	0.016	0.016	0.40
6	0.030	0.026	0.018	0.81
8	0.021	0.022	0.018	0.99
10	0.034	0.024	0.016	0.55
12	0.028	0.024	0.019	0.82
14	0.031	0.069	0.019	0.05
16	0.026	0.035	0.019	0.62
18	0.041	0.020	0.019	0.26
20	0.016	0.024	0.019	0.69
22	0.034	0.012	0.019	0.23
24	0.028	0.017	0.011	0.56
Manganese[c]				
0	0.026	0.030	0.009	0.67
2	0.026	0.033	0.009	0.41
4	0.041	0.031	0.009	0.27
6	0.036	0.043	0.009	0.44
8	0.037	0.039	0.009	0.83
10	0.040	0.041	0.009	0.91
12	0.038	0.033	0.010	0.56
14	0.042	0.026	0.010	0.13
16	0.042	0.032	0.010	0.28
18	0.037	0.029	0.010	0.42
20	0.023	0.024	0.010	0.99
22	0.022	0.018	0.010	0.67
24	0.023	0.021	0.010	0.82

[a] 60 mg of Zn/kg DM from ZnSO4; 20 mg of Cu/kg DM from CuSO4; 40 mg of Mn/kg DM from MnSO4.
[b] 60 mg of Zn/kg DM from tetra-basic ZnCl; 20 mg of Cu/kg DM from tri-basic CuCl; 40 mg of Mn/kg DM from tri-basic MnCl.
[c] mg of soluble mineral/ kg of soluble DM.

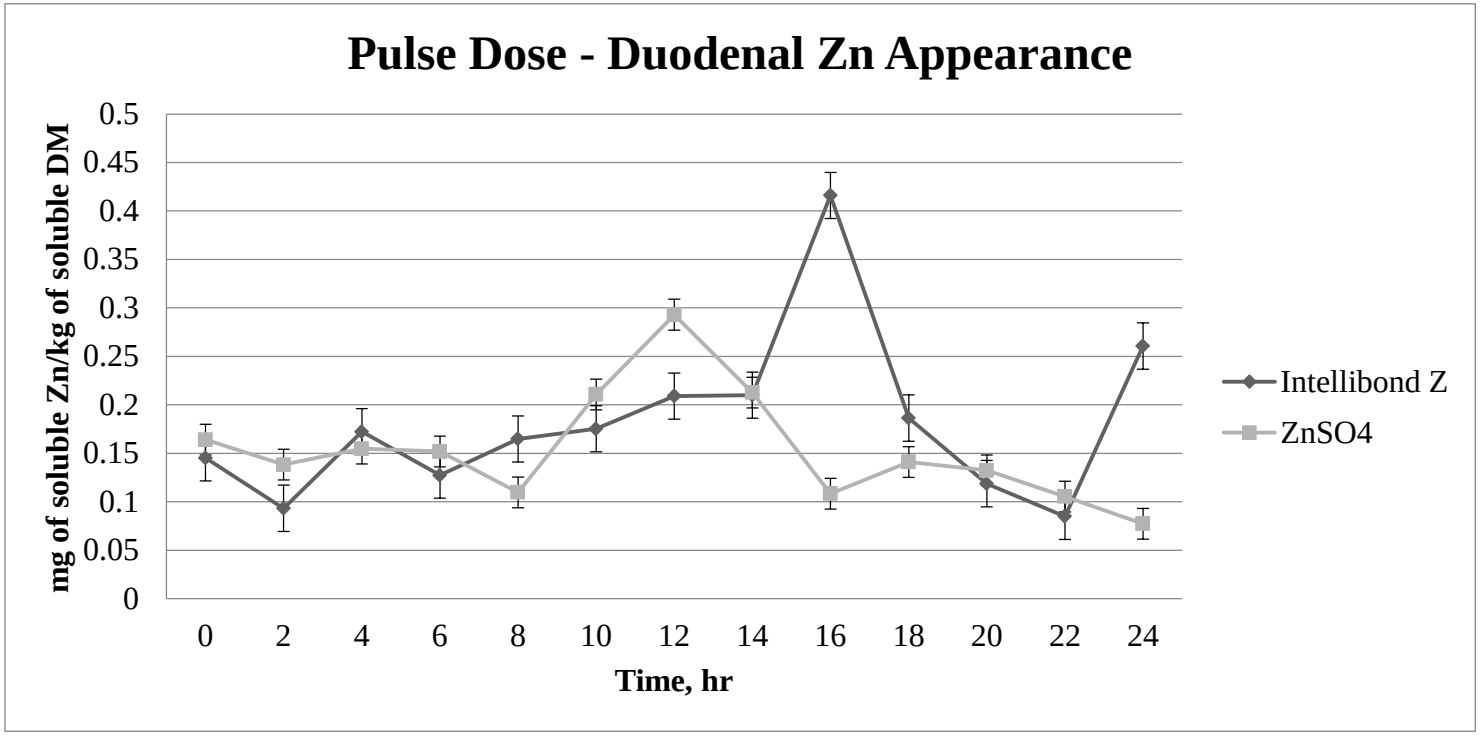

Figure 3.1. Duodenal appearance of Zn post ruminal dosing of Intellibond Z and $ZnSO_4$ supplements.

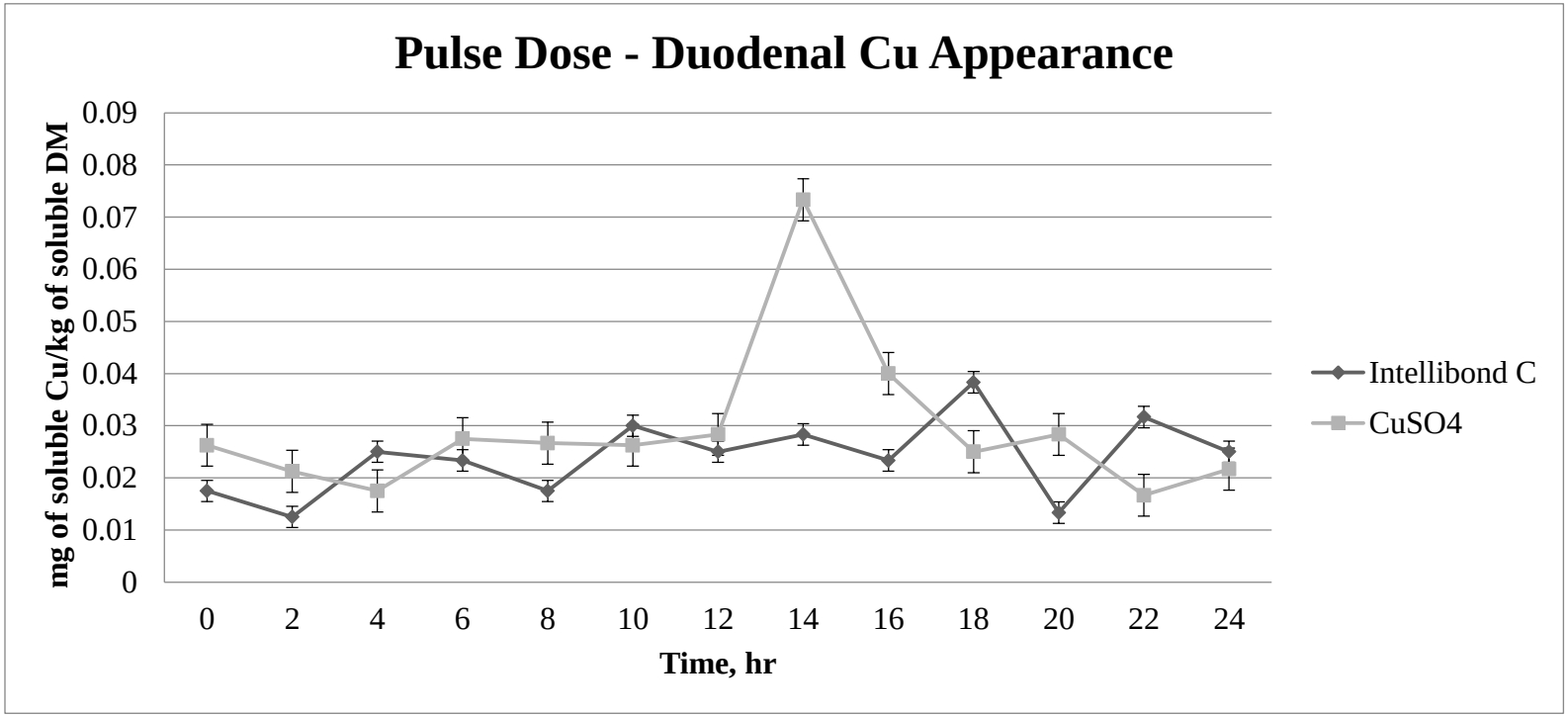

Figure 3.2. Duodenal appearance of Cu post ruminal dosing of Intellibond C and $CuSO_4$ supplements.

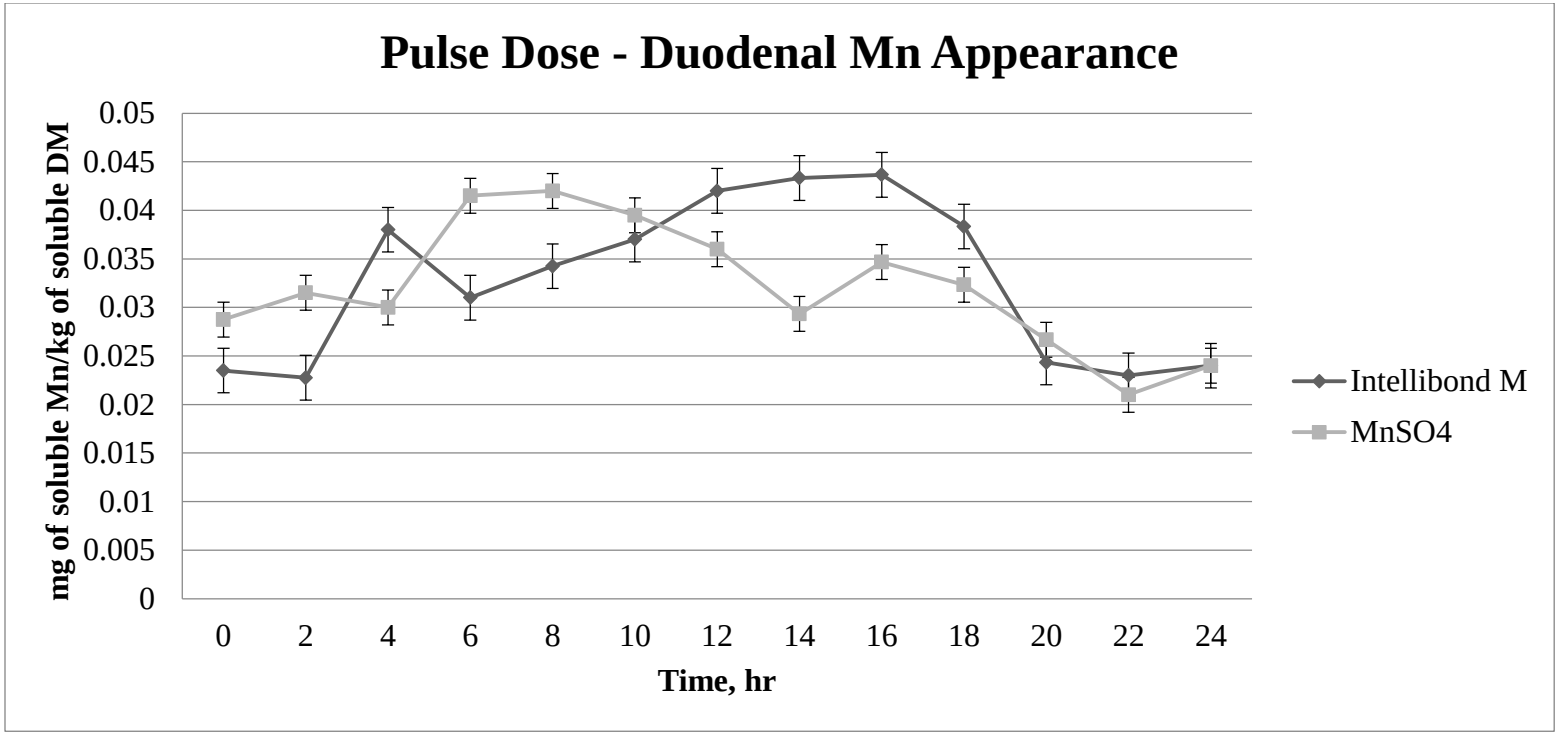

Figure 3.3. Duodenal appearance of Mn post ruminal dosing of Intellibond M and $MnSO_4$ supplements.

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Chapter IV

In vivo apparent absorption and retention of different trace mineral sources in steers.

Summary

Eight cross-bred steers (initial BW 718.9 ± 64.9 kg) were utilized in a 19 d experiment to investigate the effects of trace mineral source on apparent absorption and retention of Cu, Zn, and Mn in steers fed a corn silage and steam flaked corn-based diet. Steers were blocked by BW and randomly assigned to one of the 2 treatments. Treatments consisted of: 1) 30 mg of Zn/kg DM from $ZnSO_4$; 10 mg of Cu/kg DM from CuSO4; 20 mg of Mn/kg DM from MnSO4; and 2) 30 mg of Zn/kg DM from tetra-basic ZnCl; 10 mg of Cu/kg DM from tri-basic CuCl; 20 mg of Mn/kg DM from tri-basic MnCl. All steers were fed a TMR containing 49% corn silage, 49% steam-flaked corn, and 2% supplement. Steers were housed in two pens (4 steers per pen) for 7d and then housed in individual metabolism stalls and fed for the remaining 12d of the experiment. Upon entry into individual stalls, steers were each fitted with a total fecal collection bag and urine harness for separate collections of feces and urine. Total fecal and urine output was measured daily for all steers during the 5 d collection period. Feces and urine collected each day (over a 24 h period) were weighed, thoroughly mixed, and sampled daily (5% of wet weight) and stored frozen (-20ºC). One hundred mL of 6N hydrochloric acid was added to the urine collection containers daily. Steers were fed once per day and orts remaining in the feed bunk the next day were removed, weighed, thoroughly mixed, and sampled prior to the next feeding. Dry matter disappearance, apparent absorption, and apparent retention of Zn were similar across treatments. These data indicate that supplemental trace mineral source had no impact on dry matter disappearance, apparent absorption, and apparent retention of Cu, Zn, and Mn in steers.

Key Words: Apparent absorption, retention, zinc

Introduction

Recently, questions about the absorption and utilization of trace elements have surfaced. Reasons underlying new interest in the use of alternative trace mineral sources rather than the traditional inorganic sources include bioavailability of the modern sources, concerns about environmental contamination, increasing feed costs, and antagonists in the diets (Engle et al., 1997; Spears et al., 2004).

A trace mineral that is absorbed and retained more efficiently will improve the bioavailability of that trace mineral source. Zinc sources have been evaluated in cattle to test the idea that low solubility in the rumen may reduce interactions among Zn and other compounds in the rumen and therefore, increase Zn absorption from the small intestine. Therefore, Zn sources that would not interact with antagonist in the rumen, and thus, remain available for absorption, would be desirable (Spears et al., 2004). This concept would also apply to both Cu and Mn sources. In 1992, Heritage Environmental Services, LLC of Indianapolis, IN launched a research project with the hopes of developing a process to reclaim the copper from byproducts of the manufacturing of printed circuit boards. The findings demonstrated the spent etchants from printed wiring boards that were once considered waste could now be recycled to make trace mineral copper. From this new process Intellibond C (IB Cu), Intellibond Z (IB Zn), and Intellibond M (IB Mn) have been produced and are believed to obtain unique chemical characteristics that will improve the bioavailability of these metals. Therefore the, objective of this study was to estimate the apparent absorption and retention of trace mineral source (Intellibond vs. sulfate) supplemented at NRC (2000) recommendations.

Materials and Methods

Prior to the initiation of this experiment all procedures herein were approved by the Colorado State University institutional animal care and use committee.

Eight ruminal and duodenal fistulated steers were utilized to study *in vivo* apparent absorption and retention of Intellibond and sulfate Cu, Zn, and Mn trace mineral sources in steers. Steers were housed in two pens (4 steers per pen) and fed a TMR containing approximately 50% corn silage and 50% steam-flaked corn supplemented with the appropriate treatments for 7d. At the initiation of week 2, steers were housed in individual metabolism stalls and fed for the remaining 12 d of the experiment. The first 2-5 d of this period served as a metabolism stall acclimation period. During the acclimation period, daily dry matter intakes (DMI) were determined. At the end of the acclimation period, each steer from treatment 1 were blocked/paired with a steer from treatment 2. Steers with the closet DMI across treatments were blocked/paired together. Once animals were appropriately paired, each individual pair was fed the same amount of feed. Feed delivered to each pair was 90.0% of the steer within the pair with the lowest average DMI during the acclimation period. This ensured equal amounts of mineral intake within each block/pair of steers and across treatments during the 5 d total collection phase. The next 5 d served as the sample collection period used to determine apparent absorption and retention of Cu, Zn, and Mn. The final 2 d were used to determine the solubility of Cu, Zn, and Mn in the rumen and duodenum. Diets were fed twice daily (60% of the ration in the morning and 40% of the ration on the afternoon). Diets were manufactured in 250 kg batches using the red Roto-Mix mixer truck at Agricultural Research Development and Education Center. The un-used portion of each batch remained in the truck and mixed into the next batch manufactured.

Appropriate trace mineral treatment supplements were manufactured prior to the initiation of the experiment. Ground corn was used as the carrier. Immediately after feeding the

basal diet, the appropriate trace mineral supplements were top-dressed and mixed thoroughly by hand.

Feed and Orts. The basal diet was sampled daily upon discharge from the feed truck during the 5 d sampling period. Orts remaining in the feed bunk each day during the 5 d sample collection period were removed from the bunk, weighed, thoroughly mixed, and sampled prior to the a.m. feeding. Duplicate 500 g samples were obtained each feed time and orts were sampled. Samples were sealed in a plastic bag, thoroughly labeled, and stored frozen (-20ºC) until analyzed.

Fecal and urine collection: Total fecal and urine output were measured daily for individual steers during the 5 d collection period. The fecal bags and urine collection equipment consisted of fecal collection bags that were connected by straps to the upper girth and a breast collar similar to the equipment described by Tolleson and Erlinger (1989) and a modified version of a urinal harness as described by Border et al., (1963) that allowed for total urine collections. Feces collected each day (over a 24 h period) were weighed, thoroughly mixed, and sampled daily (10% of wet weight). Duplicate, individual samples were sealed in plastic bags, thoroughly labeled, and stored frozen (-20ºC). One hundred mL of 6*N* hydrochloric acid was added to the urine collection containers daily. Urine collected each day (over a 24 h period) was quantified and 10% of the total volume sampled. Duplicate samples of urine were sealed in 250 mL plastic bottles, thoroughly labeled, and stored frozen (-20ºC). Prior to trace mineral analysis, samples were proportionally composited across all collections days for each animal within sample type (feces or urine).

Rumen fluid and duodenal chyme samples were obtained at 4 h intervals over a 48 h period on day 18 and 19. This sampling technique will allow for samples to be obtained every

two hours over a 48 h period. The first 24 h collections began at 0600 h and end at 0400 h the next day. The second 24 h of samples began at 0500 h and ended at 0400 h the following day. At each sampling time point, duplicate 100 mL samples of fluid were removed from the geometric center of the rumen and duodenum. One sample from each of the ruminal and duodenal samples were centrifuged 1000 x g in 50ml graduated conical tubes. Once centrifuged, the volume of supernatant was determined and frozen at -20°C until trace mineral analysis could be performed. The other ruminal and duodenal sample were immediately frozen at -20ºC. Acid insoluble ash was used as the internal particulate matter marker.

The following procedure is commonly used in feedlot experiments; though it didn't necessarily apply to this experiment we still monitored the steers accordingly. Therefore, all cattle were monitored for health and locomotion problems. Steers exhibiting symptoms of respiratory disease were assigned scores of 0 or 1 for each of the following respiratory symptoms: eye discharge, nasal discharge, coughing, rapid breathing, and depressed appearance. Rectal body temperatures were recorded for suspect steers that are removed from the pen. Two additional points were assigned to steers exhibiting body temperatures greater than 39.4°C. Steers with a total of 4 or more points were considered moribund. All moribund steers were treated according to the appropriate treatment schedule, immediately returned to their appropriate pen, and allowed a chance to recover. If problems persisted concerning the health status of specific steers, they were removed from the experiment.

Statistics: Dry matter disappearance, apparent absorption, and apparent retention data were analyzed by least square analysis of variance using the Proc Mixed procedure of SAS. Individual animal was considered the experimental unit. Significance was determined at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

Results and Discussion

The influence of trace mineral source on dry matter disappearance and apparent absorption and retention of Cu, and Zn are shown in Table 4.1. Manganese analysis was excluded from analysis due to an analytical error. Dry matter disappearance, apparent absorption, and apparent retention of Zn and Cu were similar across treatments. Therefore, these data indicate that supplemental TM source had similar apparent absorption, and apparent retention of Zn and Cu in steers.

These data are in contrast to earlier data reported by Nockels et al. (1993). In this experiment eight steer calves were utilized in a crossover design where treatments consisted of either Zn methionine + Cu Lysine or Zn sulfate ($ZnSO_4$) Cu sulfate ($CuSO_4$). Collections consisted of 5 periods: 1) a 5-d baseline period, 2) 3 d of no Cu and Zn supplement, 3) 3 d of stress consisting of feed and water restriction and ACTH (80 IU) injections i.m. every 8 h, 4) 3 d of refeeding with no Cu and Zn supplement, and 5) 4 d of Cu and Zn repletion (Nockels et al., 1993). The results showed that during the repletion period calves receiving Cu Lysine had 53% greater apparent absorption and increased retention ($P < 0.05$) compared with calves fed $CuSO_4$. However, no differences were noted in apparent absorption and retention of Zn between Zn sources, but there was increased percentage of Zn retention when Zn methionine was fed. When Zn methionine was compared to ZnO supplementation to lambs Spears (1989) found that lambs fed Zn methionine had lower urinary Zn excretion which was due to differences in post absorptive metabolism of Zn. In the present experiment no differences were noted in apparent absorption and retention from the Intellibond and sulfate sources.

Table 4.1. Influence of trace mineral source on dry matter digestibility and apparent absorption and retention of copper, manganese, and zinc.

	Source			
Item	IB	Sulfate	SEM	P <
DMD, %	70.7	65.6	2.37	0.18
Zinc				
Absorbed, mg/d	70.6	56.6	11.3	0.32
Retained, mg/d	66.7	51.9	8.9	0.28
Copper				
Absorbed, mg/d	10.1	9.7	1.7	0.87
Retained, mg/d	6.2	5.8	1.04	0.26

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Chapter V

Effects of supplemental zinc, copper, and manganese concentration and source on performance and carcass characteristics of feedlot steers

Summary

Four-hundred cross-bred steers (initial BW 335 ± 9.6 kg) were utilized to investigate the effects of supplemental Zn, Cu, and Mn concentration and source on performance and carcass characteristics of feedlot steers fed a high concentrate steam flaked corn-based finishing diet for 159 d and zilpaterol hydrochloride for the last 21 d prior to slaughter with a 5 d withdrawal. The experimental design was a randomized complete block design. Steers were blocked by weight and randomly assigned within block to one of the 5 treatments (8 pens/treatment; 10 hd/pen). Treatments consisted of: Sulfate) 90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$; IB-1) 30 mg of Zn/kg DM from Zn Hydroxychloride; 10 mg of Cu/kg DM from basic Cu chloride ; 20 mg of Mn/kg DM from Mn Hydroxychloride; IB-2) 45 mg of Zn/kg DM from Hydroxychloride; 12.5 mg of Cu/kg DM basic Cu chloride; 29.5 mg of Mn/kg DM from Mn Hydroxychloride; IB-3) 60 mg of Zn/kg DM from Zn Hydroxychloride; 15 mg of Cu/kg DM from basic Cu chloride; 39 mg of Mn/kg DM from Mn Hydroxychloride ; and IB-4) 90 mg of Zn/kg DM from Zn Hydroxychloride; 17.5 mg of Cu/kg DM from basic Cu chloride; 48 mg of Mn/kg DM from Mn Hydroxychloride. Steers were individually weighed on d−1, 0, 55, 112, and pen weighed two consecutive days at the termination of the experiment. Steers were transported to a commercial abattoir, slaughtered, and individual carcass data and liver samples were collected. Initial pen BW was used as a covariate

in the statistical analysis and significance was determined at $P \leq 0.05$. No differences were observed for final body weight ($P > 0.42$). Overall ADG, DMI, and feed efficiency were similar across treatments. Hot carcass weight, dressing percentage, yield grade, LMA, adjusted fat thickness, KPH, and marbling score were similar across treatments. Concentrations of Zn, Cu, and Mn in liver and blood samples collected on d 112 and at harvest were similar across treatments. These data indicate that under the conditions of this experiment, supplemental Zn, Cu, and Mn concentration and source had no impact on performance and carcass characteristics in feedlot steers.

Key Words: Feedlot, cattle, trace mineral, copper, manganese, zinc

Introduction

Due to the importance of trace minerals in animal production, consulting nutritionists will typically fortify feedlot diets with trace minerals. Fortification is typically done for two reasons: 1) feed ingredients utilized in feedlot diets tend to be inadequate in concentrations of certain essential trace minerals and/or 2) basal diets may contain elevated concentrations of known trace mineral antagonists (Ahola et al., 2005). However, in a 2007 survey of consulting feedlot nutritionsis Vasconcelos and Galyean (2007) reported that on average consulting nutritionist formulate feedlot diets to contain 3 times the NRC (2000) recommendation for certain trace minerals. Furthermore, Vasconcelos and Galyean (2007) reported that some of the consulting nutritionists reported using a combination of inorganic and organic trace mineral sources in feedlot diets.

Several experiments have been conducted examining the impact of the bioavailability of different trace mineral sources (organic vs inorganic) measuring various metabolic, digestibility, and performance variables. (Du et al., 1996; Kegley and Spears, 1994; Spears,

1989; Nockels et al., 1993; Ward and Spears, 1997; Engle et al., 2000; Rust and Schlegel, 1993; Spears and Kegley, 2002). Results from these experiments have been variable. However, sources providing greater availability could help to decrease the amount of mineral added to feedlot diets ultimately decreasing production costs and waste content. Therefore, the objective of this experiment a is to investigate the effects of supplemental Zn, Cu, and Mn concentration and source on performance and carcass characteristics of feedlot steers fed a high concentrate steam flaked corn-based finishing diet for 159 d and zilpaterol hydrochloride of the last 21 of the last 26 d prior to slaughter.

Materials and Methods

Five hundred and five cross-bred steers (initial BW 335 ± 9.6 kg) were transported to the Colorado State University Agriculture, Research, Development, and Education Center. Upon arrival steers were individually weighed, identified with an unique ear tag, vaccinated with Presponse (Pasteurella Multocida Bacterial Extract-Mannheimia Haemolytica Toxoid, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) and Pyramid 2 plus Type II BVD (Bovine rhinotracheitis and bovine virus diarrhea (Types I and II), Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) respiratory vaccines, injected with Promectin (Ivermectin, Vedco, Inc., St. Joseph, MO) drenched with Synanthic (Oxfendazole, Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) for internal parasite control, and implanted with Revalor – XS (120 mg trenbolone acetate and 24 mg estradiol, Merck Animal Health, DeSoto, KS). During processing a breed type was assigned to all cattle based on hair color and phenotype (red, black, black-white face, white, *Bos indicus*, dairy, etc.). Following initial weighing, steers had *ad libitum* access to long-stem grass hay and water overnight and were housed in 10 head pens.

Steers were then ranked by BW and individuals that were beyond ± 2 standard deviations from the mean body weight were eliminated from further consideration for the experiment. Steers exhibiting excessive Brahman, Longhorn, or dairy breed type or if they were found to be bulls, heifers, or displaying symptoms of health problems were eliminated from consideration. The remaining steers were assigned a random number from 1 to 1000 using the random number function in Microsoft Excel 2007®. One hundred and five steers with the lowest random numbers were eliminated from the experiment reducing the number of remaining steers to 400. The 400 eligible steers were ranked by weight and divided into 8 weight block replicates, each one consisting of 50 steers. Each of the successive weight block replicate were labeled as replicates 1 through 8 with the heaviest group of 50 steers considered as replicate 8 and the lightest group of 50 steers considered as replicate 1. Within each weight block replicate, steers were ranked within breed type (red or black) by weight and the heaviest 10 steers were stratified across 5 pens within a weight block so that each pen within a weight block was of similar average bodyweight and breed type. By following this randomization schedule, 8 weight block replicates, each containing 5 pens with 10 steers per pen with similar breed composition (7 black and 3 red steers/pen) were assembled for each of the 5 treatments in the experiment. The following day, steers were returned thru the chute, weighed, and visual tags identifying the experiment, treatment, replicate, and individual steer within the experiment, treatment, and replicate were applied. Steers were then sorted into their respective treatment pens and the experiment was initiated. Initial weights used for the experiment were the average of the 2 weights obtained on day -1 and 0.

Pens were checked daily to ensure the correct 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. Steers exhibiting symptoms of respiratory disease were assigned scores of 0 or 1 for each of the following respiratory symptoms: eye discharge, nasal discharge, coughing, rapid breathing, and depressed appearance. Rectal body temperatures were recorded for suspect steers that are removed from the pen. Two additional points were assigned to steers exhibiting body temperatures greater than 39.4°C. Steers with a total of 4 or more points were considered moribund. All morbid steers were treated according to the appropriate treatment schedule and immediately returned to the pen and allowed a chance to recover. If problems persisted concerning the health status of specific steers, they were 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 to accordingly for that pen.

Diets: All steers were fed a high concentrate steam flaked corn based finishing diet. Steers were adjusted to a high concentrate finishing diet using a series of step-up diets (Starter, Step 1, Step 2, and Finisher; Table 1). Diet changes during the step-up program were simultaneous (every 5-7 d) for all treatments and cattle reached the finishing diet by d 25 of the experiment. Finishing diets were formulated to meet or exceed NRC (2000) requirements for growing and finishing beef cattle. Diets contained 13.5% crude protein, 3.5% crude protein equivalent from non-protein nitrogen, 3.5% added fat, 0.7% calcium, 0.36% phosphorus, 0.7% potassium, 0.25% magnesium, 33 g per metric ton monensin, and 11 g per metric ton tylosin in all 4 rations on a dry matter basis. Vitamins A and E were included in the diets at 2,200 and 40 IU/kg of dry matter, respectively and constituted the protein supplement. Trace mineral test articles appropriate for each treatment were formulated and pelleted separately. Treatments

consisted of: 1) **Sulfate** (90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$); 2) **IB-1** (30 mg of Zn/kg DM from Zn Hydroxychloride; 10 mg of Cu/kg DM from basic Cu Chloride; 15.0 mg of Mn/kg DM from Mn Hydroxichloride); 3) **IB-2** (45 mg of Zn/kg DM from Zn Hydroxichloride and 10 mg of Cu/kg DM basis Cu Chloride; 22.5 mg of Mn/kg DM from Mn Hydroxichloride); 4) **IB-3 (**60 mg of Zn/kg DM from Zn Hydroxichloride and 12 mg of Cu/kg DM from basic Cu Chloride; 30 mg of Mn/kg DM from Mn Hydroxychloride) ; and **IB-4** (90 mg of Zn/kg DM from Zn Hydroxychloride and 17.5 mg of Cu/kg DM from basis Cu Chloride; 48 mg of Mn/kg DM from Mn Hydroxichloride). With this treatment structure direct comparisons between iso-amounts of Cu, Zn, and Mn from sulfate and hydroxychloride mineral sources were made (Sulfate vs. IB-4) as well as a dose response of hydroxychloride Cu, Zn, and Mn (IB-1, IB-2, IB-3, and IB-4).

Weighing and sample collection: Steers were individually weighed on d −1, 0, 55, 112, and pen weights were obtained on 2 consecutive days at the termination of the experiment. Jugular blood samples were collected on d 112 and at slaughter from three animals per pen and analyzed for Zn, Cu, and Mn concentrations. Blood samples were collected into trace mineral free heparinized vacutainer tubes from the same three steers per pen throughout the experiment. Zilpaterol hydrochloride was fed for 21 d of final 26 d finishing period to all cattle with a 5 d withdrawal period. Steers were then transported to a commercial abattoir where individual carcass data was collected. Hot carcass weight was determined at the time of slaughter. Carcasses were allowed to chill for approximately 36 h. Carcass data collected included dressing percentage; rib eye area; subcutaneous adipose tissue thickness; kidney, pelvic, and heart fat; marbling score; quality grade; and yield grade. Liver samples were collected on the day of slaughter from the left lobe of each liver after being inspected by USDA personnel. Following

collection, liver samples were placed in Whirl Pak bags containing the slaughter order number, placed on ice, and transported to the laboratory. Samples were then stored at –20°C until analyzed. At the time of liver analysis, only liver samples pertaining to cattle that were bled were analyzed for Zn, Cu, and Mn concentrations.

Analytical procedures: Liver tissue samples or 1 ml of plasma were dried at 60°C for 24 h in a pre-weighed crucible. Samples were weighed post drying and then dry ashed at 600°C for 12 h. Liver tissues and plasma samples were analyzed for mineral concentrations via inductively coupled plasma-atomic emission spectroscopy (ICP-AES) methods (Braselton et al., 1997) as described by Ahola et al. (2004) for Zn, Cu, and Mn, concentrations. Samples were diluted in distilled H_2O to fit within a linear range of a standard curve generated by linear regression of known TM concentrations. Multielemental analysis was then carried out by simultaneous/sequential ICP-AES analysis with cross flow nebulization.

Statistics: Feedlot performance, plasma and liver trace mineral concentrations, and continuous carcass data were analyzed on a pen mean basis as a randomized block design using PROC MIXED of SAS (SAS Institute, Inc., Cary, NC). Treatment was included in the model as a fixed effect and weight block pen replicate was included in the model as a random effect. The linear and quadratic effects were derived by using orthogonal polynomial coefficients that were applied to the treatment IB-1 through IB-4 and were applied as quantitative factors that are equally spaced levels in order to derive the Type I sum of squares for the increasing doses. Quality grade, yield grade, hot carcass weight category, and carcass maturity distribution data were evaluated as categorical data using PROC GLIMMIX of SAS assuming a binomial distribution. The Link = Logit option of the model statement and the ILINK option of the LSMEANS statement were used to calculate the likelihood ± SEM that an individual within each

pen qualified for a specific category. Significance was determined at $P < 0.05$. Pen initial weight was used as a covariate in the analysis of all response variables.

Results and Discussion

The effect of trace mineral concentration and source on animal performance is shown in Table 2 has been reported to have a positive (Ward and Spears, 1997; Engle et al., 2000c), negative (Engle and Spears, 2000b), or no effect (Engle and Spears, 2000a; Engle and Spears, 2001; Engle et al., 2000b) on performance compared to non-supplemented controls. Engle and Spears (2000b) reported that Cu source had no effect on finishing phase performance. However Lee et al. (2002) reported greater final BW and a tendency for greater ADG in steers receiving organic Cu vs. inorganic Cu.

Variable feedlot cattle performance responses to Zn supplementation have also been reported. Rust and Schlegel (1993) reported that Zn supplementation to finishing cattle diets led to a tendency for greater ADG. Whereas Spears and Kegley (2002) indicate that Zn supplementation had no impact on ADG, DMI, or G:F vs. non-supplemented controls. While Malcolm-Callis et al. (2000) reported no differences in ADG or gain:feed during a 112-d finishing period with added Zn concentrations (as $ZnSO_4$) at 20, 100, or 200 mg/kg of dietary DM. They also reported a decrease in DMI as Zn concentration increased. Relative to Zn source, there was tendency for ADG and G:F to be greater in cattle supplemented with organic Zn compared to inorganic Zn (Spears and Kegley, 2002). However, Nunnery et al. (1996) reported greater ADG in cattle receiving $ZnSO_4$ compared to Zn-methionine . Brown et al. (2004) reported steer DMI, ADG, and ADG:DMI before re-implanting or over the entire feeding period were not influenced by additional Zn from inorganic or organic sources. Several researchers have

also reported that Zn source had no impact on performance (Rust and Schlegel, 1993; Greene et al., 1988; Malcolm-Callis et al., 2000).

Reaching an objective conclusion about the impact of Cu, Zn, and Mn supplementation on finishing cattle performance is difficult. Numerous factors can influence an animal's response to trace mineral supplementation such as disease status, mineral antagonists, environmental factors as well as basal dietary mineral concentrations.

The influence of trace mineral concentration and source on carcass characteristics is shown in Tables 3 and 4. There was a quadratic ($P < 0.02$) response for YG across the IB treatments. As dose of Cu, Zn, and Mn increased YG decreased of IB treatments 2 and 3 and then increased for IB-4 (Table 3). Hot carcass weight, dressing percentage, *longissimus* muscle area, subcutaneous adipose tissue depth and marbling score were similar across treatments. Within the categorical data, yield grades 1 or 2 demonstrated a quadratic response with the greatest percent of yield grades 1 or 2 in treatment IB-3 (Table 4), as well as a quadratic response in the percentage of yield grade 3 with the greatest percentage in treatment IB-4 These finding are similar to those reported by Rhoads et al. (2003) and Ahola et al. (2005) where trace mineral supplementation and source had minimal impact on carcass characteristics of feedlot steers. Although not directly comparable to the present experiment Greene et al. (1988) reported greater USDA quality grades in steers supplemented Zn methionine when compared to control and Zn oxide treatments where the frequency of steers grading USDA choice was 79, 57 and 40%, respectively. Malcolm-Callis et al. (2000) also reported a quadratic responses of added zinc on fat thickness ($P < 0.05$) and yield grade ($P < 0.01$).

Liver and plasma Cu, Zn, and Mn concentrations are presented in Table 5. Copper and Mn concentrations in liver samples collected at harvest were similar across treatments (Table 5).

A quadratic ($P < 0.01$) response was noted in liver Zn concentration with increasing dose of hydroxy trace mineral (IB-1, IB-2, IB-3, IB-4) and the highest Zn liver concentration was in the IB-4 treatment at 108.2 mg Zn/kg DM (Table 5). Wright and Spears (2003) reported liver concentrations in Holstein calves after evaluating the effects of zinc source and dietary level providing basal diet with no supplemental Zn, basal diet plus 20 mg Zn/kg DM from $ZnSO_4$, basal diet plus 20 mg/kg DM from Zn protienate, or basal diet plus 20mg/kg DM from ZnM (50% of the Zn supplied from each source). Liver zinc concentrations were greater ($P < 0.10$) in the Zn supplementation treatments when compared to the control (basal diet plus no supplemental Zn) and the $ZnSO_4$ treatment was greater ($P < 0.05$) when compared to Zn protienate (Wright and Spears, 2003). Concentrations of Zn, Cu, and Mn in blood samples collected on d 112 were similar across treatments (Table 5). In previous studies where organic has been compared to inorganic, plasma Zn concentrations have been greater in ruminants that have been supplemented with an organic source of Zn when compared to an inorganic source (Wright and Spears, 2003; Rojas et al., 1995; Kincaid et al., 1997; Cao et al., 2000). In the present study blood samples collected at harvest had similar Zn and Mn concentrations across treatments (Table 5). However, plasma Cu concentrations from blood samples collected at harvest demonstrated a quadratic ($P < 0.02$) response within the hydroxy trace mineral treatments with the greatest concentrations in the IB-2 and IB-3 treatments (1.38 mg Cu/L; 1.34 mg Cu/L). Previous data supports that steers supplemented with Cu from Cu chloride and Cu sulfate increased plasma and liver Cu concentrations when supplemented to Cu depleted steers for 21 days (Spears et al., 2004).

These data indicate that under the conditions of this experiment, supplemental Zn, Cu, and Mn concentration and source had no impact on performance and carcass characteristics and minimal impacts on liver and plasma Cu, Zn, and Mn concentrations.

Table 5.1. Ingredient composition of basal diet.

Ingredient	Starter	Step-1	Step-2	Finish
Corn silage	16.38	13.38	13.38	13.37
Flaked corn	35	51.51	66.17	78.81
Alfalfa hay	43.93	28.19	12.44	---
DDG[b]	4.69	---	---	---
Liquid Suppl.	---	2.17	3.25	4.33
Pellet Suppl.	---	4.76	4.76	3.50
Theoretical Nutrients				
Dry matter, %	71.00	71.00	71.00	71.00
Crude Protein	13.5	13.5	13.5	13.5
Non-protein nitrogen[b]	3.5	3.5	3.5	3.5
Acid detergent fiber	6.9	6.9	6.9	6.9
Neutral detergent fiber	14.6	14.6	14.6	14.6
Eff-NDF[c]	5.06	5.06	5.06	5.06
ME, Mcal/kg[d]	1.40	1.40	1.40	1.40
NEg, Mcal/kg[e]	0.64	0.64	0.64	0.64
NEm, Mcal/kg[f]	0.95	0.95	0.95	0.95
Calcium	0.70	0.70	0.70	0.70
Phosphorus	0.30	0.30	0.30	0.30
Magnesium	0.17	0.17	0.17	0.17
Zinc, ppm	10.49	10.49	10.49	10.49
Copper, ppm	3.09	3.09	3.09	3.09
Cobalt, ppm	0.10	0.10	0.10	0.10

[a]Percentage of dry matter unless stated otherwise.
[b]Dried distiller's grains plus soluble.

Table 5.2. Effect of supplemental Zn, Cu, and Mn concentration and source on performance of feedlot cattle and feed efficiency of feedlot cattle.

Item	Treatment [a]						Contrasts (P <)				
	Sulfate	IB-1	IB-2	IB-3	IB-4	SEM	TRT	Sulf vs. IB-4	IB-1 vs. IB-2	Linear [b]	Quadratic [b]
Body Wt, kg											
Initial	337.5	336.8	335.3	335.1	334.9	9.57	0.32	0.09	0.61	0.23	0.55
Final	654.6	647.0	647.3	655.6	651.4	4.83	0.42	0.57	0.19	0.24	0.58
DMI, kg/d											
d0- Final	11.3	11.5	11.6	11.6	11.5	0.13	0.31	0.45	0.43	0.96	0.14
ADG kg/d											
d0-Final	2.03	1.98	1.99	2.04	1.99	0.03	0.54	0.37	0.26	0.56	0.33
Gain:Feed											
d0-Final	0.18	0.17	0.17	0.18	0.17	0.003	0.40	0.22	0.15	0.57	0.95

[a] Experimental treatments: Sulfate = 90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$: IB-1 = 30 mg of Zn/kg DM from tetra-basic ZnCl; 10 mg of Cu/kg DM from tri-basic CuCl; 20 mg of Mn/kg DM from tri-basic MnCl; IB-2 = 45 mg of Zn/kg DM from tetra-basic ZnCl and 12.5 mg of Cu/kg DM from tri-basic CuCl; 29.5 mg of Mn/kg DM from tri-basic MnCl; IB-3 = 60 mg of Zn/kg DM from tetra-basic ZnCl and 15 mg of Cu/kg DM from tri-basic CuCl; 39 mg of Mn/kg DM from tri-basic MnCl; IB-4 = 90 mg of Zn/kg DM from tetra-basic ZnCl and 17.5 mg of Cu/kg DM from tri-basic CuCl; 48 mg of Mn/kg DM from tri-basic MnCl.

[b] Statistical analysis of treatments IB-1, IB-2, IB-3 , and IB-4 for linear or quadratic effects.

Table 5.3. Effect of supplemental Zn, Cu, and Mn concentration and source on carcass characteristics of feedlot cattle.

Item	Treatment [a]					SEM	Contrasts (P <)				
	Sulfate	IB-1	IB-2	IB-3	IB-4		TRT	1 vs 5	1 vs 2	Linear [b]	Quadratic [b]
HCW, kg	416.6	412.3	413.0	413.9	418.9	3.07	0.45	0.57	0.29	0.11	0.46
DP, %	63.7	63.7	63.9	63.2	64.4	0.57	0.55	0.32	0.93	0.58	0.28
Yield Grade	2.85	2.87	2.69	2.66	2.90	0.09	0.19	0.63	0.82	0.86	0.02
Rib Eye Area, cm^2	96.9	95.9	98.3	97.5	96.6	1.39	0.78	0.86	0.61	0.85	0.24
Adj. Fat, cm	1.31	1.19	1.16	1.20	1.22	0.07	0.52	0.33	0.19	0.63	0.69
KPH	2.02	2.04	2.03	2.05	2.01	0.02	0.82	0.75	0.69	0.66	0.58
Marbling Score	391.2	389.9	386.9	394.1	392.5	9.93	0.97	0.90	0.90	0.65	0.93

[a] Experimental treatments: Sulfate = 90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$: IB-1 = 30 mg of Zn/kg DM from tetra-basic ZnCl; 10 mg of Cu/kg DM from tri-basic CuCl; 20 mg of Mn/kg DM from tri-basic MnCl; IB-2 = 45 mg of Zn/kg DM from tetra-basic ZnCl and 12.5 mg of Cu/kg DM from tri-basic CuCl; 29.5 mg of Mn/kg DM from tri-basic MnCl; IB-3 = 60 mg of Zn/kg DM from tetra-basic ZnCl and 15 mg of Cu/kg DM from tri-basic CuCl; 39 mg of Mn/kg DM from tri-basic MnCl; IB-4 = 90 mg of Zn/kg DM from tetra-basic ZnCl and 17.5 mg of Cu/kg DM from tri-basic CuCl; 48 mg of Mn/kg DM from tri-basic MnCl.

[b] Statistical analysis of treatments IB-1, IB-2, IB-3 , and IB-4 for linear or quadratic effects.

Table 5.4. Effect of supplemental Zn, Cu, and Mn concentration and source on categorical carcass characteristics of feedlot cattle.

Item	Treatment [a]					Contrasts (P <)				
	Sulfate	IB-1	IB-2	IB-3	IB-4	TRT	1 vs 5	1 vs 2	Linear [b]	Quadratic [b]
n=	77	80	79	80	79					
Quality Grade										
≥ Low Choice	37.5 ± 8.6	36.5 ± 8.6	34.0 ± 8.3	37.3 ± 8.6	33.9 ± 8.2	0.98	0.66	0.98	0.86	0.94
Select	56.0 ± 9.1	60.8 ± 8.8	61.3 ± 8.69	55.5 ± 9.01	64.6 ± 8.42	0.79	0.32	0.60	0.83	0.48
≤ Standard [c]	6.5	2.7	4.7	7.2	1.5	n/a	n/a	n/a	n/a	n/a
Liver Scores										
Normal [c]	96	97	100	98	93	n/a	n/a	n/a	n/a	n/a
Abscesses [cd]	4	3	0	2	7	n/a	n/a	n/a	n/a	n/a
Yield Grade										
1 or 2	50.0 ± 6.04	52.1 ± 5.95	57.5 ± 5.92	68.2 ± 5.47	48.3 ± 5.97	0.10	0.83	0.80	0.99	0.03
3	41.0 ± 5.90	40.0 ± 5.77	28.3 ± 5.31	24.1 ± 4.94	37.6 ± 5.75	0.10	0.67	0.90	0.63	0.02
4 or 5	5.15 ± 2.57	4.98 ± 2.50	7.63 ± 3.09	4.98 ± 2.49	8.91 ± 3.34	0.77	0.37	0.96	0.50	0.86

[a] Experimental treatments: Sulfate = 90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$: IB-1 = 30 mg of Zn/kg DM from tetra-basic ZnCl; 10 mg of Cu/kg DM from tri-basic CuCl; 20 mg of Mn/kg DM from tri-basic MnCl; IB-2 = 45 mg of Zn/kg DM from tetra-basic ZnCl and 12.5 mg of Cu/kg DM from tri-basic CuCl; 29.5 mg of Mn/kg DM from tri-basic MnCl; IB-3 = 60 mg of Zn/kg DM from tetra-basic ZnCl and 15 mg of Cu/kg DM from tri-basic CuCl; 39 mg of Mn/kg DM from tri-basic MnCl; IB-4 = 90 mg of Zn/kg DM from tetra-basic ZnCl and 17.5 mg of Cu/kg DM from tri-basic CuCl; 48 mg of Mn/kg DM from tri-basic MnCl.

[b] Statistical analysis of treatments IB-1, IB-2, IB-3 , and IB-4 for linear or quadratic effects.

Table 5.5. Effect supplemental Zn, Cu, and Mn concentration and source on mineral status of feedlot cattle.

Item	Treatment [a]							Contrasts (P <)			
	Sulfate	IB-1	IB-2	IB-3	IB-4	SEM	TRT	1 vs 5	1 vs 2	Linear[b]	Quadratic[b]
Liver											
Zn, mg/kg	105.0	100.9	97.9	95.0	108.2	3.70	0.02	0.44	0.32	0.15	0.01
Cu, mg/kg	273.5	251.2	261.0	274.2	269.2	14.6	0.78	0.84	0.29	0.31	0.61
Mn, mg/kg	10.4	9.9	10.2	10.2	9.9	0.30	0.74	0.28	0.30	0.95	0.28
Plasma, d 112											
Zn, ug/ml	0.20	0.16	0.22	0.20	0.15	0.03	0.35	0.20	0.28	0.74	0.06
Cu, ug/ml	1.14	1.13	1.27	1.08	1.51	0.35	0.83	0.37	0.98	0.46	0.62
Mn, ug/ml	0.0004	0.019	0.003	0.002	0.0002	0.008	0.47	0.98	0.12	0.13	0.41
Plasma, harvest											
Zn, ug/ml	0.43	0.41	0.48	0.51	0.42	0.08	0.76	0.91	0.81	0.83	0.21
Cu, ug/ml	0.99	1.07	1.38	1.34	0.96	0.15	0.11	0.89	0.67	0.54	0.02
Mn, ug/ml	0.04	0.01	0.02	0.05	0.05	0.02	0.55	0.73	0.36	0.13	0.76

[a] Experimental treatments: Sulfate = 90 mg of Zn/kg DM from $ZnSO_4$; 17.5 mg of Cu/kg DM from $CuSO_4$; 48 mg of Mn/kg DM from $MnSO_4$: IB-1 = 30 mg of Zn/kg DM from tetra-basic ZnCl; 10 mg of Cu/kg DM from tri-basic CuCl; 20 mg of Mn/kg DM from tri-basic MnCl; IB-2 = 45 mg of Zn/kg DM from tetra-basic ZnCl and 12.5 mg of Cu/kg DM from tri-basic CuCl; 29.5 mg of Mn/kg DM from tri-basic MnCl; IB-3 = 60 mg of Zn/kg DM from tetra-basic ZnCl and 15 mg of Cu/kg DM from tri-basic CuCl; 39 mg of Mn/kg DM from tri-basic MnCl; IB-4 = 90 mg of Zn/kg DM from tetra-basic ZnCl and 17.5 mg of Cu/kg DM from tri-basic CuCl; 48 mg of Mn/kg DM from tri-basic MnCl.

[b] Statistical analysis of treatments IB-1, IB-2, IB-3 , and IB-4 for linear or quadratic effects.

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Appendix A

SAS code used to analyze Pulse Dose data.

```
options ls=100 ps=150;
data duodenalmin;
input time2      steerid2 trt NegZn Zn NegCu Cu NegMn Mn;
cards;
;
proc print;
proc sort;
by steerid2 time2;
proc mixed data=duodenalmin;
class time2 trt steerid2;
model Zn= NegZn time2|trt trt/ddfm=kr residual;
repeated time2/ subject=steerid2(trt) type=ar(1);
lsmeans  time2|trt/pdiff;
run;
proc mixed data=duodenalmin;
class time2 trt steerid2;
model Cu= NegCu time2|trt trt/ddfm=kr residual;
repeated time2/ subject=steerid2(trt) type=ar(1);
lsmeans  time2|trt/pdiff;
run;
proc mixed data=duodenalmin;
class time2 trt steerid2;
model Mn= NegMn time2|trt trt/ddfm=kr residual;
repeated time2/ subject=steerid2(trt) type=ar(1);
lsmeans  time2|trt/pdiff;
run;
```

Appendix B

SAS code used to analyze Apparent Absorption and Retention data.

```
options ls=100 ps=150;
data dig;
input strid trt dmd aazn aacu aamn arzn arcu armn;
cards;
;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model dmd = trt /ddfm=satterth;
lsmeans trt /pdiff;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model aazn = trt /ddfm=satterth;
lsmeans trt /pdiff;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model aacu = trt /ddfm=satterth;
lsmeans trt /pdiff;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model aamn = trt /ddfm=satterth;
lsmeans trt /pdiff;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model arzn = trt /ddfm=satterth;
lsmeans trt /pdiff;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model arcu = trt /ddfm=satterth;
lsmeans trt /pdiff;
proc mixed method = reml data=dig covtest cl;
class strid trt;
model armn = trt /ddfm=satterth;
lsmeans trt /pdiff;
run;
```

Appendix C

SAS code used to analyze Performance data.

```
proc sort;
by pen time;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model bw= trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model dmi=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model adg=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model ftg=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
```

```
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model gtf=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
```

SAS code used to analyze Carcass data.

```
proc sort;
by pen;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model hcw=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model dp=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model yg=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model rea=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
```

```
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model pyg=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model apyg=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model adjfat=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model kph=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
```

```
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt;
model ms=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
```

SAS code used to analyze Carcass Categorical data.

```
proc print;
run;
proc sort; by trt rep pen;
proc means noprint sum;
by trt rep pen;
var initial premium choice pc select standard liverabc yg12 yg3 yg45 denom;
output out=carcass sum=initial premium choice pc select standard liverabc yg12 yg3 yg45
denom;
proc print;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model premium/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model choice/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
```

```
class rep trt;
model pc/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model select/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model standard/denom=trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model liverabc/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model yg12/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
```

```
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model yg3/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
proc glimmix data=carcass;
*where rep ne 8;
class rep trt;
model yg45/denom=initial trt/s ddfm=kr error=binomial link=logit;
random rep;
lsmeans trt/cl pdiff ilink;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
```

SAS code used to analyze Liver and Plasma data.

```
proc sort;
by pen time;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model zn=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model cu=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
proc mixed scoring=2;
*where rep ne 8;
class pen rep trt time;
model mn=initial trt/ddfm=kenwardroger;
random rep;
lsmeans  trt/pdiff;
contrast 'trt 1 vs trt 5' trt 1 0 0 0 -1;
contrast 'trt 1 vs trt 2' trt 1 -1 0 0 0;
contrast 'linear' trt 0 -3 -1  1 3;
contrast 'quadratic' trt 0 1 -1 -1 1;
run;
```