Comparison of trace mineral concentrations between liver biopsies of different sizes and durations of storage by inductively coupled plasma mass spectrometry

Scott Louis Radke
Iowa State University

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Comparison of trace mineral concentrations between liver biopsies of different sizes and durations of storage by inductively coupled plasma mass spectrometry

by

Scott Louis Radke

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Toxicology

Program of Study Committee:
Steve Ensley, Co-major Professor
Wilson Rumbeiha, Co-major Professor
Stephanie Hansen

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2018

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>v</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>NOMENCLATURE</td>
<td>vii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Thesis Organization</td>
<td>2</td>
</tr>
<tr>
<td>CHAPTER 2. REVIEW OF THE LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Copper</td>
<td>3</td>
</tr>
<tr>
<td>Background and function of Cu</td>
<td>3</td>
</tr>
<tr>
<td>Sources of Cu</td>
<td>3</td>
</tr>
<tr>
<td>Absorption, metabolism, and excretion of Cu</td>
<td>4</td>
</tr>
<tr>
<td>Toxicity of Cu</td>
<td>7</td>
</tr>
<tr>
<td>Clinical signs of toxicity</td>
<td>7</td>
</tr>
<tr>
<td>Pathogenesis of clinical signs</td>
<td>8</td>
</tr>
<tr>
<td>Gross and microscopic lesions</td>
<td>9</td>
</tr>
<tr>
<td>Deficiency of Cu</td>
<td>9</td>
</tr>
<tr>
<td>Clinical signs of deficiency</td>
<td>9</td>
</tr>
<tr>
<td>Pathogenesis of clinical signs</td>
<td>10</td>
</tr>
<tr>
<td>Gross and microscopic lesions</td>
<td>10</td>
</tr>
<tr>
<td>Mineral interactions of Cu</td>
<td>11</td>
</tr>
<tr>
<td>Diagnostic analysis of Cu</td>
<td>12</td>
</tr>
<tr>
<td>Manganese</td>
<td>13</td>
</tr>
<tr>
<td>Background and function of Mn</td>
<td>13</td>
</tr>
<tr>
<td>Sources of Mn</td>
<td>14</td>
</tr>
<tr>
<td>Absorption, metabolism, and excretion of Mn</td>
<td>14</td>
</tr>
<tr>
<td>Toxicity of Mn</td>
<td>15</td>
</tr>
<tr>
<td>Clinical signs of toxicity</td>
<td>15</td>
</tr>
<tr>
<td>Pathogenesis of clinical signs</td>
<td>15</td>
</tr>
<tr>
<td>Deficiency of Mn</td>
<td>16</td>
</tr>
<tr>
<td>Clinical signs of deficiency</td>
<td>17</td>
</tr>
<tr>
<td>Pathogenesis of clinical signs</td>
<td>17</td>
</tr>
<tr>
<td>Gross and microscopic lesions</td>
<td>18</td>
</tr>
<tr>
<td>Mineral interactions of Mn</td>
<td>18</td>
</tr>
<tr>
<td>Diagnostic analysis of Mn</td>
<td>18</td>
</tr>
</tbody>
</table>
Molybdenum ................................................................................................................. 40
  Background and function of Mo ........................................................................... 40
  Sources of Mo ........................................................................................................ 40
  Mineral interactions of Mo.................................................................................... 41
  Thiomolybdate formation ....................................................................................... 42
  Antagonostic effects of thiomolybdates ................................................................. 43
  Summary of thiomolybdates .................................................................................. 43
Liver biopsies ............................................................................................................. 44
  Background ............................................................................................................ 44
  Development of liver biopsies ............................................................................... 44
  Sample size of liver biopsies ................................................................................ 45
  Aspiration technique for liver biopsies ................................................................. 46
  Biopsy needle technique for liver biopsies .......................................................... 46
  Approach and technique of performing liver biopsies ......................................... 47
Literature cited ........................................................................................................... 49

CHAPTER 3. COMPARISON OF TRACE MINERAL CONCENTRATIONS BETWEEN LIVER BIOPSIES OF DIFFERENT SIZES AND DURATIONS OF STORAGE BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY .... 64
  Abstract.................................................................................................................. 64
  Keywords ............................................................................................................... 65
  Introduction ........................................................................................................... 65
  Materials and methods ......................................................................................... 66
    Sampling procedure ............................................................................................. 66
    Sample processing and analysis ....................................................................... 68
    Statistical analysis ............................................................................................. 69
  Results ................................................................................................................. 69
  Discussion............................................................................................................. 70
  Acknowledgements .............................................................................................. 75
  Declaration of conflicting interests ...................................................................... 75
  Funding ............................................................................................................... 75
  Figures and tables ............................................................................................... 76
  Literature cited ................................................................................................... 78

CHAPTER 4. GENERAL CONCLUSIONS................................................................... 81
  Literature Cited .................................................................................................... 88
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Anatomical landmarks used for percutaneous liver biopsy in cattle.</td>
<td>48</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Anatomical positioning of liver on homemade device. Plastic grid for sample collection is attached.</td>
<td>76</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Collection of 0.02 g biopsy sample.</td>
<td>76</td>
</tr>
<tr>
<td>Figures 4-7</td>
<td>Trace element concentration means for different sample sizes with corresponding storage durations.</td>
<td>77</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Reference guidelines for hepatic concentrations copper, manganese, selenium, and zinc in bovine.</td>
<td>34</td>
</tr>
<tr>
<td>Table 2</td>
<td>Comparison of trace mineral concentration means and resulting p-values.</td>
<td>78</td>
</tr>
</tbody>
</table>
### NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Cp</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>Crt1</td>
<td>Copper transporter 1</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent metal transporter 1</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
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<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
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<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>H₂S</td>
<td>Hydrogen sulfide</td>
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<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>MM</td>
<td>Multimin 90</td>
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<tr>
<td>Mn</td>
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</tr>
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<td>Mo</td>
<td>Molybdenum</td>
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<td>NRC</td>
<td>National research council</td>
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<tr>
<td>P</td>
<td>Phosphorus</td>
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<tr>
<td>PEM</td>
<td>Polioencephalomalacia</td>
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<tr>
<td>S</td>
<td>Sulfur</td>
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<tr>
<td>Se</td>
<td>Selenium</td>
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<tr>
<td>SeMet</td>
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</tr>
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<td>SRB</td>
<td>Sulfur reducing bacteria</td>
</tr>
<tr>
<td>TM</td>
<td>Trace minerals</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Tm</td>
<td>Thiomolybdates</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
</tr>
<tr>
<td>Zrt-Irt-like</td>
<td>ZIP</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
</tbody>
</table>
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“And you, to whom adversity has dealt the final blow

With smiling bastards lying to you everywhere you go

Turn to, and put out all your strength of arm and heart and brain

And like the Mary Ellen Carter rise again”

-Stan Rodgers
ABSTRACT

Consequential adverse health effects in cattle may occur as a result of impaired physiological processes brought on by trace mineral (TM) deficiencies and toxicities. Small amounts of TM are necessary to facilitate these processes, but inadequate or excessive concentrations can result in reduced health and development. Ante mortem sampling of bovine hepatic tissue allows for the evaluation of TM status, specifically copper (Cu), manganese (Mn), selenium (Se), and zinc (Zn), permitting diagnosis of such deficiencies or toxicities. Variation in sample size as a result of differing biopsy instruments and techniques has led to conflict regarding the amount of liver considered adequate for reliable diagnostic interpretation. This study examined the difference of TM concentrations between liver samples of differing sizes and also between samples stored over differing durations of time. Variation among samples of differing storage durations post collection and prior to analytical processing was also evaluated. It was hypothesized that there would be no significant differences in TM concentrations between samples of different sizes stored for different durations. No differences regarding concentrations of copper, manganese, selenium, and zinc were observed between 0.02, 1.0 g, and homogenized samples stored for three and fourteen days. A difference in TM concentrations was observed between 0.02 g samples stored for seven days and all other samples. No difference in TM concentrations between 1.0 g and homogenized samples stored for seven days was observed. Concentrations of these samples did not differ from other samples of different sizes stored for different amounts of time Results at three and fourteen days of storage suggest that trace mineral concentrations did not significantly differ regarding sample size and that 0.02 g of fresh hepatic tissue, dried to approximately
0.005 g is reliable for diagnostic interpretation of toxicities and deficiencies in cattle pertaining to copper, manganese, selenium, and zinc. However, results of the day seven data were inconclusive as differences in TM concentrations were observed in 0.02 g samples while concentrations of different sample sizes and storage duration did not differ significantly.
CHAPTER 1. INTRODUCTION

Trace minerals, Cu, Mn, Se, and Zn serve as constituents of enzymes and proteins that are essential to physiological processes necessary for cattle health and development.\textsuperscript{14} The National Research Council (NRC) has established recommendations of TM for health and growth in beef and dairy cattle.\textsuperscript{17,97} Although rations are designed to supply these TM at established recommended concentrations, bioavailability, antagonists such as iron (Fe), molybdenum (Mo) and sulfur (S), may result in deficiencies. Over supplementation of TM through dietary or parenteral means can result in elevated bodily concentrations that lead to toxicosis.\textsuperscript{16,120,143} Trace mineral deficiencies or toxicities results from impairment of processes such as mineral transport, metabolism, immune system function, and structural development facilitated by TM dependent enzymes.

Evaluation of TM concentrations can aid in gauging nutritional status and herd health while diagnosing and preventing deficiencies and toxicities.\textsuperscript{141} Mn, Se, and Zn in ruminants concentrates in liver. Although the majority of the body’s Mn is stored in bone, concentrations can be evaluated in the liver. Ante mortem evaluation of TM in liver to determine status is a viable option for producers and clinicians. The liver biopsy was adapted for cattle to evaluate TM status in 1950 and has progressively developed in terms of instrumentation and techniques.\textsuperscript{30,145} Controversy regarding an adequate size of sample for reliable diagnostic interpretation persists.\textsuperscript{9,145} Little research has focused on evaluating variation in TM concentrations between hepatic samples of differing sizes for diagnostic purposes. This thesis will focus on the variation in TM concentration between different sizes of liver samples commonly obtained through biopsies to be utilized for diagnostics associated with Cu, Mn, Se, and Zn. It was hypothesized that there would be
no significant differences in TM concentrations between samples of different sizes stored for different durations.

**Thesis Organization**

Chapter II will provide a detailed review of the literature encompassing both the physiological aspects and importance of Cu, Mn, Se, and Zn. Respective toxicities and deficiencies associated with each mineral, and utilization of liver biopsies as a diagnostic tool for determining TM status in cattle will also be covered in chapter II. Portions or the entirety of Chapter II may be used in future publication in *Animal Health Research Reviews*. Chapter III will present research intended for publication in the *Journal of Veterinary Diagnostic Investigation*. Chapter III contains research conducted to study TM concentration variation between hepatic biopsy samples of 0.02 g or less to those of approximately 1.0 g. It will also contain research conducted to evaluate the variation of TM concentrations between samples of differing durations of storage prior to analytical processing for analysis. Chapter IV will conclude the findings of this thesis and present overall findings, conclusions, and implications for future research.
CHAPTER 2. REVIEW OF THE LITERATURE

Introduction

Review of the literature for Cu, Mn, Se, Zn, S, Mo, and liver biopsies is presented in this chapter. Common sources, metabolism, and the role in physiological functions will be introduced for Cu, Mn, Se, and Zn. Pathogenesis of both deficiencies and toxicities of these minerals and the resulting clinical signs and lesions will be described. Trace mineral interactions, diagnostic sampling and testing will be reviewed. Antagonistic effects of Mo and S on TM will be covered following review of Cu, Mn, Se, and Zn. Liver biopsy implementation, development, and procedure is reviewed.

Copper

Background and function of Cu

Copper serves as a component for numerous enzymes that function to sustain cellular respiration, serve as antioxidants, transport Cu, and mobilize iron (Fe) for utilization in hemoglobin (Hb). Energy is generated within tissues as a result of terminal electron transfer through cytochrome C oxidase, a cupric enzyme essential for life. Copper, as well as Zn and Mn, are critical to support super oxide dismutase enzymes that prevent and protect against free radical damage to tissues. Copper is also an essential component for the function of ceruloplasmin (Cp) allowing for transport of Cu. Oxidation and mobilization of Fe from enterocytes is made possible through synthesis of Cu dependent enzyme hephaestin.144

Sources of Cu

Vegetation, soil, and supplements serve as the primary sources of Cu in cattle. Soil type appears to play a critical role in Cu availability in grazing ruminants. Regions
described as having grey soils or bogs with decaying vegetation known as muskeg have been associated with low liver Cu concentrations in dams grazing these areas as well as their corresponding fetuses. More developed soils and peat type soils are considered to be low in Cu content. Speciation of forage material and climate also play a role in Cu content as legumes are expected to possess higher Cu levels than grasses in temperate regions while the reverse occurs in tropic locations due to species and uptake capabilities. However, little difference may be observed if soil Cu is low. Common sources of dietary supplemental Cu utilized in non-grazing cattle include copper sulfate [CuSO₄], copper oxide [CuO], and tribasic copper chloride [Cu(OH)₃Cl]. Multimin 90 (MM; Multimin USA, Fort Collins, CO) is an injectable trace mineral product that contains 15 mg Cu/ml along with the trace minerals Mn, Se, and Zn.

**Absorption, metabolism, and excretion of Cu**

The rumen plays a critical role in the absorption of Cu. Absorption of Cu in functional ruminants is limited to between 1-10%. Decreased absorption of Cu in developed ruminants is a result of formation of insoluble Cu complexes involving both diet and ruminal microorganisms. Sulfur within the rumen may interact with Mo and Cu to form copper thiomolybdate (Tm) complexes. These reduce Cu absorption by causing Cu to become more tightly bound to albumin resulting in insoluble complexes. Sulfur alone can produce insoluble CuS complexes as a result of microbial production of sulfide. This can be due to increased dietary S intake associated with increased intake of protein, S containing amino acids, or low rumen pH.

Young ruminants with undeveloped rumens do not encounter absorption impediments. Absorption of Cu can be as high as 85% in young animals as Cu is able to by-pass the undeveloped rumen and avoid complexing with S or Tm resulting in
unimpeded absorption. Extent of absorption and accumulation of Cu appears to vary by species. Age as well as breed are variables based on a previous study which observed greater hepatic Cu accumulation in Jersey cows in comparison to Holstein cows. Ligands such as glycinate chelated with organic Cu with high dietary S and Mo, can potentially reduce antagonistic absorptive effects that these elements have on Cu. In a prior study, increased liver and plasma Cu concentrations were observed in cattle receiving copper glycinate supplementation when compared with those receiving CuSO₄. No significant difference is observed in bioavailability between different dietary Cu species in Cu depleted individuals suggesting that the availability is similar when there is a depletion of Cu.

Although there is limited absorption of Cu in ruminant species, cattle possess a large liver storage capacity. Copper is accumulated in the bovine liver at a rate of 1.03 mg/kg/day when administered at 175 mg/kg/day. These data can be used to serve as a model regarding Cu accumulation over a long period of time following a change in feed composition or to detect when such an event initially occurred following accumulation of Cu to toxic levels. Use of this data for such a purpose was demonstrated in a previous case to support the theory that dietary Cu intake was too high and could accumulate to toxic levels for the amount of time it was administered in a case involving adult dairy cattle exhibiting clinical signs of Cu toxicity. Copper accumulation within the liver, varies by location. The majority of Cu within the liver is bound to metallothionein and is used for enzyme synthesis. Plasma may be utilized as a measure of both Cu absorption and hepatic mobilization through evaluation of plasma Cu and Cp concentrations which were found to be elevated following Cu supplementation. The caudate lobe in neonates
appears to possess the highest concentration of Cu. In one study, plasma Cu concentrations were shown to correlate with hepatic Cu concentrations below 40 ppm on a dry matter (DM) basis by dropping below 0.5 mg/L. This correlation is questionable as plasma Cu concentrations in a more recent study fell below 0.5 mg/L in five of fourteen individuals when hepatic Cu concentrations were less than 20 mg/kg DM while others remained greater than 0.7 mg/L. No correlation between plasma and hepatic Cu was observed when hepatic Cu exceeded 40 ppm.

Adequate Cu supplementation is necessary in gestating cows. Fetuses are dependent on the dam’s Cu status. Placental transfer of Cu occurs progressively throughout gestation. An exponential increase occurs during the last trimester corresponding to the rapid growth and development of the fetus. Increased fetal hepatic Cu results in progressive loss of hepatic Cu in the dam due to mobilization of Cu to the fetus. Correlation of hepatic Cu concentrations between the cow and fetus based on fetal crown rump length have been established, with fetal liver Cu being approximately 2.6 times higher than that of the dam.

Primary routes of excretion of Cu is in bile and feces. Thiomolybdate complexes form insoluble complexes that impair Cu absorption leading to excess excretion of Cu within the feces. Biliary excretion of Cu in ruminants can be enhanced through Tm through the interference of enterohepatic circulation of Cu. Enhanced biliary excretion of Cu via Tm is important in reducing elevated or toxic Cu levels. Copper in bile is poorly absorbed within the small intestine as it is typically bound to the macromolecule albumin making it fairly insoluble. Urinary excretion of Cu is
minimal and generally occurs in times of excess or through diuretic effects of other elements such as S\textsuperscript{143}.

**Toxicity of Cu**

Increased dietary or parenteral supplementation of Cu, increased Cu bioavailability, or decreased biliary excretion of Cu are all potential causes of Cu toxicity.\textsuperscript{100} Age\textsuperscript{138} and genetic composition may serve as predisposing factors to Cu toxicity.\textsuperscript{26} Free Hb in circulation resulting from hemolysis accumulates in renal tubules to form Hb casts.\textsuperscript{101} Copper toxicity can arise if Cu concentrations within the animal are not evaluated prior to supplementation. Regardless of route of administration or speciation, Cu is cumulative in ruminants.\textsuperscript{141}

**Clinical signs of toxicity**

Clinical signs associated with acute hemolytic crisis include jaundiced tissues accompanied by cyanotic to dark colored mucous membranes. Clinical signs of Cu toxicity may also include darkened scours, poor hair coat, and weight loss. Following onset of clinical signs associated with hemolytic crisis, death may occur within two to four days.\textsuperscript{8,149,150} Preceding hemolytic crisis, anorexia and decreased milk production may be observed. Increased infection rate and mortality in calves born from cows with elevated liver copper concentrations may be observed.\textsuperscript{8,116}

Manifestations of toxicosis were observed in mature dairy cows following over supplementation of dietary Cu at 328 mg/kg DM and 656 mg/kg DM for a period of two and five months. Clinical signs of hemolytic crisis occurred approximately one year after consuming exceedingly high levels of Cu for months. Milk production was reduced by 7 L/cow/day over a year. Conception rates were reduced by 10% over the year. This may
have resulted from negative energy balance. It was reported that affected animals consumed only the necessary amount of feed needed to survive.\\textsuperscript{116}  

The accumulative effect of different Cu sources has been observed in young calves whose dams received an injection of Cu glycinate thirty days prior to parturition. Following parturition, calves were administered 12.5 g CuO boluses. Deaths in treated calves, likely the result of hemolytic crisis as indicated by afebrile individuals with icteric mucous membranes, began within thirty days following administration of the boluses resulting in a mortality rate of 5.2% of 290 calves treated. Hepatic necrosis was evident and accompanied by elevated liver and kidney Cu. The risk for intoxication was increased following bolus administration as cows had received adequate Cu supplementation resulting in normal to high Cu levels in the calves.\\textsuperscript{141} Parenteral administration of a Cu compound more soluble than Cu glycinate resulted in 100% mortality in calves within twelve days of administration with hepatic Cu concentrations as high as 1,190 ppm.\\textsuperscript{96}  

**Pathogenesis of clinical signs**  

Copper toxicity has two distinct phases. The first phase is accumulation of Cu in liver. The second phase is acute hemolytic crisis.\\textsuperscript{150} Excessive Cu accumulation leads to the damaging of hepatocellular membranes. This results in excessive release of Cu into circulation. Depending on its functional state, the liver is able to compensate for excessive release of Cu by absorbing it from serum. Once compensatory effects of the liver are overwhelmed, serum Cu is not controlled. Excess circulating Cu damages erythrocyte membranes resulting in intravascular hemolysis and release of Hb into circulation. Impaired oxygen delivery as a result of destruction of erythrocytes results in hepatic centrilobular necrosis. Centrilobular zones are the most oxygen sensitive.\\textsuperscript{94}
Hemoglobin does not have a direct toxic effect on renal tissue. Following hemolysis, Hb accumulates in the glomerular filtrate eventually resulting in ischemic necrosis of the renal tubules. The classical “gun-metal” coloration of the kidney is due to Hb casts within the renal parenchyma.\textsuperscript{101}

**Gross and microscopic lesions**

Gross lesions of copper intoxication include icteric mucous membranes and organs, particularly the liver, along with darkened metallic colored kidneys.\textsuperscript{150} Gastrointestinal contents, in cases of excessive dietary Cu, may possess a blue to green coloration.\textsuperscript{147} Microscopic lesions in cases of Cu toxicity are generally associated with hepatic and renal tissue characterized by renal tubular damage\textsuperscript{8} and hepatic centrilobular necrosis accompanied by biliary stasis within the canaliculi.\textsuperscript{96,141,150} Renal tubules contain Hb granules and casts as a result of Hb accumulation.\textsuperscript{101}

**Deficiency of Cu**

Cu deficiency is often a sequelae of inadequate dietary supplementation or decreased bioavailability.\textsuperscript{100} Grazing animals in areas of peat soils and muskeg are at risk for deficiency due to low Cu in these soils.\textsuperscript{56} Antagonistic effects of other minerals increase the potential for deficiency. Sulfur and Mo each can independently exert such affects by forming insoluble complexes with Cu impairing Cu absorption. Thiomolybdate complexes increase Cu excretion.\textsuperscript{41} Iron and Mn can also impair Cu absorption through competition for divalent metal transporter 1 transporter.\textsuperscript{41,53}

**Clinical signs of deficiency**

Clinical signs of Cu deficient individuals include decreased fertility and growth\textsuperscript{116}, diarrhea, pica, depigmentation, and anemia.\textsuperscript{59} Increased incidence of infections
or chronic disease problems, though non-specific, may be indicative of a compromised immune system that may be associated in part with Cu deficiency.144

Pathogenesis of clinical signs

Iron deficiency can occur secondary to Cu deficiency due to decreased synthesis of the cupric enzyme hephaestin. To be transferred from the enterocyte and transported through the blood for utilization in Hb synthesis, divalent Fe (Fe²⁺) must be oxidized to the trivalent form (Fe³⁺) by hephaestin. Only the trivalent form of Fe is accepted into circulation. A decrease in hephaestin decreases the capacity for Fe to be oxidized and mobilized from the enterocyte. Reduced Fe results in decreased Hb and erythrocyte synthesis. Anemia ensues due to the lack of Hb and erythrocyte production.144 It has been suggested that Cu deficiency associated anemia may be associated with decreased levels of superoxide dismutase resulting in oxidative damage to erythrocytes.80 Copper deficiency compromises immune system function as phagocytic killing by both lymphocytes and neutrophils is reduced. This reduction in leukocyte killing capacity is the result of deficient levels of the free radical scavengers superoxide dismutase and Cp. Secondary Fe deficiency, as a sequela to Cu deficiency, results from the decrease of the Fe dependent enzyme catalase that is responsible for metabolizing hydrogen peroxide. Coat depigmentation occurs as a result of the lack of tyrosinase which converts tyrosin to melanin giving hair its color.144

Gross and microscopic lesions

Tissues may appear pale as a result of anemia. Hematology may reveal a hypochromic macrocytic anemia.144 Heinz body formation, as a result of oxidative damage, may also be observed. An isolated incident in New Zealand was reported indicated that cows exhibiting post-parturient hemoglobinuria also exhibited Heinz body
formation. This was speculated to be the result of Cu deficiency attributed to elevated Mo consuming in forage. The coat of Cu deficient cattle may have a loss of color and possess a rough appearance. Impairment of endochondral ossification, characterized by epiphyseal widening, may be observed in young calves.

**Mineral interactions of Cu**

Multiple minerals can impair absorption of Cu. The rumen serves as the primary site of Cu antagonism by Mo and S. Environmental sources of Mo and S produced by rumen microorganisms can inhibit Cu absorption through the formation of Tm complexes. Thiomolybdates increase the binding of Cu to albumin resulting in hepatic depletion and increased bile excretion. Absence of Tm due to lack of either Mo or S does not mean that Cu cannot be inhibited by either of the two individually. Both Mo and S can form insoluble complexes with Cu on their own. Forage from Mo rich soils as well as poorly drained flood regions, which have decreased Cu and increased S, Fe, and Mo, can impair Cu absorption.

Excess dietary concentration of Mn and Fe can also interfere with intestinal absorption of Cu. Manganese and Fe both utilize divalent metal transporter 1 (DMT1) to enter the enterocyte. Along with Cu transporter 1 (Crt1), up to 50% of the Cu that is absorbed is through DMT1. Cattle supplemented with Mn were observed to have decreased Cu concentrations, possibly as a result of decreased absorption. Manganese competes with Cu for use of DMT1, and excessive dietary concentrations prevent Cu absorption through this receptor. Decreased hephaestin as a result of Cu deficiency prevents mobilization of Fe from the enterocyte. Feedback as a result of Fe accumulation within the enterocyte results in the degradation and decreased expression of DMT1. Excess Mn may potentiate this feedback effect. Excess dietary Fe at concentrations of
250-1,200 ppm result in impaired absorption of other minerals. Dietary Fe provided at 800 ppm may potentially result in decreased expression of DMT1.0

**Diagnostic analysis of Cu**

Both serum and plasma samples are collected for Cu analysis in bovine in lieu of hepatic samples because of ease of sampling. Although easier to obtain, the diagnostic value of both serum and plasma in determining Cu status and toxicity is limited. Serum Cu homeostasis is regulated by hepatic Cu concentrations, and due to this mechanism, serum Cu decreases when liver Cu becomes depleted. Serum Cu is not an ideal sample in terms of diagnosing Cu status or toxicity. Adult dairy cows exhibited clinical signs of Cu toxicity while serum Cu levels were detected to be low or within established reference limits. Following Cu analysis of liver samples, hepatic Cu was determined to be 381 ppm on a wet weight basis. Both serum and hepatic Cu concentrations were elevated in another case of adult cattle exhibiting signs of toxicity in late gestation with liver Cu ranging between 1,236-2,179 mg/kg DM. Elevation in serum Cu in that case was likely due to mobilization of Cu from the liver to the fetus. There is concern in interpreting Cu in serum as it is sequestered within the clot as Cp. This sequestration of Cu results in significantly lower Cu concentrations than what are present in plasma.

Plasma Cu is maintained through mobilization of Cu from the liver. Plasma Cu may begin to decrease once liver Cu concentrations fall below 40 ppm DM. At that point, there is already a deficiency. Like Se, the analysis of functional enzyme concentrations, such as Cp, to determine Cu status have been evaluated in both serum and plasma. Although serum Cp activity was initially observed to be lower than plasma Cp, Cp appears to correlate well with plasma concentrations. Ceruloplasmin concentrations do not appear to correlate well with hepatic Cu concentrations. Ceruloplasmin is an
acute phase protein, and elevated concentrations within plasma or serum may be a reflection of infection, stress, or parasitism. Ceruloplasmin has limited value in determining hepatic Cu levels.

Other hepatic biomarkers, such as aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), and bilirubin have been examined as indicators of Cu toxicity through hepatic insult. Although these enzymes do indicate hepatic insult, they are non-specific for Cu toxicity. Both serum and plasma Cu concentrations in experimental studies, have remained within normal limits in times of elevated hepatic Cu concentrations. Hepatic Cu is found to be exceedingly high even with normal serum levels in some cases of intoxication. Although decreased serum and plasma levels indicate a potential deficiency, hepatic Cu stores have been depleted at this point. Liver is the optimal sample selection for evaluation of Cu status and diagnosing toxicities and deficiencies.

Manganese

Background and function of Mn

Manganese has a fairly low concentration within the body, but a wide distribution. Highest concentrations reside in the liver, bone, and kidney. Manganese is an essential constituent of multiple enzymatic reactions as it serves an enzyme activator of hydrolases, carboxylases, and transferases. Such enzymes included glycosyl transferases critical in the synthesis of glycoproteins and proteoglycans necessary for cartilage development. Manganese has also been recognized as playing a significant role in bovine reproductive processes. The minimum concentration of dietary Mn in both growing and finishing cattle is established at 20 mg/kg. A minimum of 40 mg/kg is
established for breeding and gestating animals to prevent developmental complications in calves.97

**Sources of Mn**

Manganese is found in both soil and a variety of plants at varying concentrations and valence.61 Divalent Mn, Mn$^{2+}$, is the most efficiently absorbed form.84 This coincides with the DMT1 transporter on the apical surface of enterocytes.50,53 Cattle receiving a diet consisting largely of grasses compared to a ration consisting primarily of legumes may receive more Mn. Grasses tend to have higher concentrations of Mn in comparison to legumes.61,86 Its bioavailability also depends on processing of forage material prior to consumption. These variations were exemplified in a previous study where Mn concentrations were observed to be lower in grass hay rations than in rations of red clover silage and hay silage. Despite the low Mn concentration in the grass hay, serum Mn concentrations following consumption of feedstuffs were higher in animals that consumed the grass hay diet. Calves born to dams on the grass hay diet in conjunction with this study did not exhibit clinical signs such as enlarged joints, domed craniums, and short limbs consistent with Mn deficiency.62,129 The only parenteral source of Mn is MM with 10 mg/ml.120

**Absorption, metabolism, and excretion of Mn**

Absorption of Mn by cattle is poor. Approximately 0.5-1% of Mn that is taken in is actively absorbed through the small intestine, likely via DMT1.50,61 Divalent metal transporter 1 also serves as the primary means of active absorption of dietary Fe.50 Prior to entering the liver, absorbed Mn either remains free or becomes bound to alpha-2 macroglobulin.86 Excretion of Mn is primarily through the bile and feces.61 Ninety-five percent of Mn that is absorbed, is excreted through the bile following first pass through
the liver. Excretion capacity of the hepatic tissue can be overwhelmed following intravenous administration of Mn at a rate of 4000 µg per minute. By this method of administration, biliary excretion of Cu, Fe, and Zn is reduced. Manganese is minimally excreted in milk. Minimal absorption of dietary sources, accompanied with rapid excretion create an efficient homeostatic mechanism for Mn.

Toxicity of Mn

Manganese toxicity, although rare, is most often associated with administration of high Mn diets that may result in adverse growth effects and secondary deficiencies in particular TM as a result of antagonism. Although it has the potential to interfere with the absorption of Cu, Fe, and Zn, the effects are thought to be of little significance due to the aforementioned homeostatic mechanisms that are in place. The tendency to include Mn in rations far below the maximum tolerable levels in feeds also may contribute to this lack of significance.

Clinical signs of toxicity

Decreased feed efficiency characterized by non-specific reduced feed intake and rate of gain can be associated with high dietary intake of Mn. These clinical signs may be observed in cattle that consume diets possessing Mn concentrations exceeding 820 mg/kg DM. A significant reduction in Hb may also be observed in cattle following consumption of rations exceeding 2000 ppm Mn. Similar clinical signs have been observed in calves fed milk replacer containing 1,000 ppm Mn.

Pathogenesis of clinical signs

Complications of growth efficiency associated with high dietary Mn may be the result of a decrease in the production of volatile fatty acids stemming from changes in rumen microflora. Administration of Mn to incubation flasks of microorganisms obtained
from fistulated individuals that either received no Mn or approximately 5,000 ppm Mn, resulted in a decrease in volatile fatty acids.\textsuperscript{20} Volatile fatty acids (VFAs), such as propionate, serve as major sources of energy as substrates for gluconeogenesis.\textsuperscript{29} Investigators observed significant changes in ruminal microflora in incubated samples from cattle fed 5,000 ppm Mn.\textsuperscript{20} Since VFA production depends on the digestion of carbohydrate sources. Changes in rumen microflora may result in microbes with reduced capabilities of producing VFAs leading to decreased substrates for energy production.\textsuperscript{29}

Decreased Hb in cattle that consume greater than 2,000 ppm Mn is believed to be a secondary effect resulting from Fe deficiency due to impaired absorption resulting in less available Fe for Hb synthesis.\textsuperscript{69} Manganese and Fe compete for absorption in the intestine through the transporter DMT1. Both elements also utilize transferrin for transport throughout the body. High dietary Mn consumption may result in tying up transferrin preventing mobilization of Fe from the enterocyte to the body.\textsuperscript{22}

**Deficiency of Mn**

Clinical signs of Mn deficiency can be observed in cattle of any age. Young and developing calves are most often affected. Manganese deficiency in calves is the result of inadequate maternal supplementation.\textsuperscript{140} Calves from Mn deficient cows possess reduced hepatic Mn concentrations. Hepatic Mn levels of calves from Mn deficient cows were 6.24 ppm. Calves from adequately supplemented mothers possessed liver Mn concentrations 11.84 ppm in two instances.\textsuperscript{84,131} Manganese is an activator of enzymes critical in structural development. Calves born to females inadequately supplemented with Mn, may have skeletal structural complications and abnormalities in appearance.\textsuperscript{52,131}
**Clinical signs of deficiency**

Clinical signs of Mn deficiency are most commonly associated with structural abnormalities in calves as the result of inadequate Mn supplementation of cows and heifers during gestation.\(^{52,131}\) Manganese deficient calves may exhibit generalized weakness, enlarged lax joints, and doming of the cranium.\(^{131,140}\) Dwarfism may also be exhibited in calves of Mn deficient mothers.\(^{140}\) Administration of Mn in feed, at a concentration of 15.8 ppm, to gestating heifers is not adequate for fetal development. Calves exhibited previously mentioned structural abnormalities including superior brachygnathism.\(^{52,131}\) Gestating cattle on pasture are susceptible to Mn deficiency depending on type and quality of forage. Calves from cows on pasture during a drought exhibited similar clinical signs observed in previous studies.\(^{52,131,140}\) Increased number of services to conception was observed in cows given Mn supplementation below 20 ppm.\(^{131}\) The affect that Mn deficiency has on reproductive function is debatable as observations in one study\(^{131}\) are contradicted by the findings of another where Mn did not appear to affect pregnancy rate, conception rate, or number of services to conception.\(^{54}\)

**Pathogenesis of clinical signs**

Nutritional chondrodystrophy is believed to be a result of maternal Mn deficiency that manifests in the form of structural abnormalities of the calf.\(^{84,131,140}\) Low to deficient levels of Mn impair activation of glycosyl transferase enzymes. These are critical in the synthesis of glycoproteins as well as proteoglycans for cartilage and joint development. Synthesis of chondroitin sulfate, an important component of cartilage and other connective tissues, is dependent on Mn. A decrease in these components results in the impaired development of cartilage and bone.\(^{76}\)
Gross and microscopic lesions

Shortened and weakened humeri and other long bones may be observed grossly. Bone weakness and fragility can be evaluated by the amount of pressure that is applied to break the bone. Bones of clinically affected calves were more easily broken with less pressure than calves originating from adequately supplemented mothers. Narrowed and irregular growth plates characterized by a reduced zone of hypertrophy containing irregular patterns of chondrocytes with intermittent degenerative chondrocytes may be observed in bone from calves exhibiting abnormalities associated with Mn deficiency.

Mineral interactions of Mn

Dietary intake requirements of Mn may vary due to the amount of Ca and P in the diet. Also both Mn and Fe have the potential to interfere with the absorption of one another as well as interfere with the absorption of Cu. This antagonistic effect was noted in a prior study. Manganese concentrations were observed to be reduced in the duodenum of cattle following fifty-six days of Fe supplemented in a starter diet Liver Mn levels were not found to be decreased in animals supplemented with Fe. It was speculated that fifty-six days was not sufficient time for the liver to become depleted of Mn.

Diagnostic analysis of Mn

Supplementation of Mn does not appear to affect whole blood Mn concentrations in older individuals. Whole blood Mn from a calf at birth may be a good indicator of dam Mn status as calves from cows supplemented with Mn during gestation have been observed to have significantly higher Mn concentrations in whole blood than calves from non-supplemented mothers. Liver has been used as a matrix for identifying Mn deficiencies in calves. Manganese status as it relates to recent dietary history can be
evaluated through serum, but should be used carefully in interpretation of long-term
dietary history of Mn.\textsuperscript{106} There are no analytic tests pertaining to biological samples that
can be used to identify Mn toxicity.\textsuperscript{127}

\textbf{Selenium}

\textbf{Background and function of Se}

Selenium (Se) is critical in preventing oxidative stress and free radical formation
as an essential component of several glutathione peroxidases (GPX). Selenium is often
recognized for its role in the utilization of glutathione (GSH) and its antioxidant
capabilities. Selenium serves as an essential component of deiodinases, selenoproteins,
and other peroxidases.\textsuperscript{132,163} Thirty different selenoproteins have been identified with
functions ranging from antioxidant activity to metabolism of hormones. Selenium
concentrates in endocrine glands, specifically thyroid and pancreas. Selenium
involvement in thyroid hormone synthesis involves protection of thyroid follicular cells
from lipid peroxidation. This occurs through upregulation of the peroxidases GPX1 and
GPX3 during thyrocyte synthesis. Serving as a component of several deiodinases, Se also
assists in the maintenance of blood T3 levels by converting T4 to T3.\textsuperscript{3,163}

GSH is dependent on Se for its ability to prevent lipid peroxidation of sperm cells
that would otherwise decrease sperm motility and reproductive efficiency. Fresh bull
semen was analyzed for Se and GPX levels prior to freezing. Semen possessing higher
levels of Se and GPX were found to have increased motility compared to semen that had
lower levels of Se and GPX. The decreased motility was attributed to increased lipid
peroxidation as lipid peroxidases were elevated in semen possessing low levels of Se and
GPX.\textsuperscript{135} Significance of Se for endocrine function is illustrated by the fact that in times
of deficiency, endocrine organs retain the highest concentration of Se.\textsuperscript{3} Selenocysteine,
the primary form of Se in the body, serves as the UGA codon for protein synthesis while selenoprotein-P both transports Se in plasma and acts as a detoxifying agent.144

**Sources of Se**

Grains, soybeans, legumes, and other forage material consumed by cattle serve as the main source of selenomethionine (SeMet). Concentration of Se in vegetation depends on a multitude of factors. These include plants species, soil Se concentration, moisture, and soil pH.163 Plant species and soil concentration are two important factors affecting Se concentration in plants. Select species of plants are Se accumulators. *Astragalus spp.* can possess exceedingly high levels of Se. There is great variation in Se soil content between different geographical regions as a result of the varying soil types and textures.161 Clay soils tend to possess the highest concentration of Se than sand or silt laden regions.44 Injectable Se products include MM 5.0 mg/ml, BO-SE 1.0 mg/ml (Merck Animal Health, Madison, NJ), and MU-SE 10 mg/ml (Merck Animal Health, Madison, NJ), containing Se in the form of sodium selenite.57,58,120

**Absorption, metabolism, and excretion of Se**

Inorganic forms of Se are not absorbed as readily in ruminants compared to monogastrics and pre-ruminant calves due to the reduction of Se into insoluble forms.65 Due to reduced ability of ruminants to absorb Se, cattle and other ruminants are more tolerant to high Se intake.19 Selenate, an inorganic form of Se, is actively absorbed through the solute carrier superfamily 13A1 (SLC13A1) sulfate transporter. Organic Se in the form of SeMet utilizes methionine transporters. Following absorption, SeMet and selenate are converted to selenocysteine through multiple catabolic and reduction reactions respectively. Utilizing adenosine triphosphate (ATP), selenide is converted to selenophosphate. Through Seryl tRNA and selenocysteine synthase, selenocysteine is
created from selenophosphate that aids in the formation of selenoproteins through its addition to growing peptides of these proteins. Liver and kidney contain the highest concentrations of Se following supplementation. Hepatic concentrations of Se reached nearly 8.0 ppm at twenty-four hours post parenteral administration of MM before steadily decreasing to between 3.0 and 4.0 ppm within fifteen days. Selenium concentrations in fetal and neonate bovine are dependent on the Se status of the dam as Se readily crosses the placenta during gestation. Selenomethionine also accumulates in the fetus of gestation cows as a result of placental transfer. The majority of Se is excreted through the urine. Due to ruminants’ reduced ability to absorb Se, up to 40% may also be excreted in the feces.

**Toxicity of Se**

Selenium intoxication in livestock is often due to natural exposure after consumption of seleniferous vegetation or accidental over supplementation. Natural Se intoxication results from consumption of Se accumulating plant species such as *Astragalus spp.*, *Haploppapus spp.*, *Stanelya spp.*, and *Brassica spp.* Geographic region, soil Se content, pH, as well as season are factors that influence a plant’s capability to absorb Se. Low pH soils promote Se uptake by plants while high pH soils reduce this ability. Periods of drought or arid regions also optimize plant Se uptake. Injectable Se compounds that are misformulated or administered at an incorrect dose, may result in acute Se toxicity.

**Clinical signs of toxicity**

**Acute toxicity**

Clinical signs associated with acute Se toxicity may include depression, increased salivation, respiratory distress, tachycardia, diarrhea, and death. Onset of clinical
signs and death in cases of acute Se toxicity may occur within two hours following
administration of misformulated or exceedingly high doses of an injectable Se
compound. The acute oral dose of sodium selenite for adult cattle is approximately
1.1 mg/kg.

Route of administration of an injectable Se product may be a factor in the lethal
toxic dose of Se. Sodium selenite [Na$_2$SeO$_3$] produced a toxicity in calves when
administered subcutaneously as a result of product misformulation and experimentally
through intramuscular injection. Minimum lethal dose of Na$_2$SeO$_3$, following
subcutaneous (SQ) injection, was determined to be 0.5 mg/kg. The amount injected per
animal was 100 mg, approximately eight times the labeled dose. Treated calves exhibited
clinical signs of acute toxicity followed by death of eighteen, thirty-eight, and seventy-
five calves within twenty-four, forty-eight, and seventy-two hours respectively with
several occurring within the first two hours. Three hundred and seventy-six, out of 557
calves injected died over a period of five weeks. Concentration of hepatic Se forty-eight
hours and one month post treatment were 3.11 ppm and 0.71 ppm. Experimentally, the
lethal dose of Na$_2$SeO$_3$ when injected intramuscularly (IM), is approximately 2 mg/kg.
All four calves died within twelve hours following administration of the 2 mg/kg dose.
Calves given the 1 mg/kg dose exhibit no clinical signs prior to being euthanized on the
seventh day following administration. Hepatic Se concentrations of calves that died
within twelve hours ranged from 7.28-9.20 ppm on a wet weight basis.

**Blind staggers**

Blind staggers is a sub-acute condition believed to be the result of consuming Se
accumulating plant species growing in seleniferous regions. Blindness, anorexia,
dyspnea, ataxia, and abdominal pain are noted in cattle. Blind staggers is thought to
have three stages. Ataxia, anorexia, and blindness characterize the first stage. The second stage involves worsening of present signs accompanied by inability of the animal to support itself. The final stage includes inability to swallow, hypersalivation, and dyspnea.\textsuperscript{152} This particular condition has not been reproduced experimentally. The inability to produce this condition with excessive Se alone suggested that other factors including sulfur may contribute to observable clinical signs.\textsuperscript{107}

\textbf{Alkali disease}

Alkali disease is considered a chronic condition that is the result of long-term consumption of grains or grasses that originate from regions with high soil Se content.\textsuperscript{144} Clinical signs include depression, anorexia, hair loss, lameness, weight loss leading to emaciation, and death.\textsuperscript{69,107} Cows affected by this condition may potentially produce calves that are stillborn or weak.\textsuperscript{164}

\textbf{Pathogenesis of clinical signs}

Mechanisms of action of Se exerts intoxication are not fully understood. Several theories have been proposed.\textsuperscript{48} The first theory suggests that intermediates of Se such as GSH and S-adenosylmethionine, become depleted during acute toxicity. Depletion of Se intermediates and enzymes will result in loss of activity and function.\textsuperscript{155} The second theory is that oxidative damage occurs as result of Se interacting with thiols.\textsuperscript{70} The third theory is that S on amino acids is replaced by Se resulting in change of proteins leading to disruption of function.\textsuperscript{125}

\textbf{Gross and microscopic lesions}

Gross lesions of acute intoxication may include pulmonary edema and congestion, pale cardiac muscle accompanied with petechial hemorrhage, and congested liver.\textsuperscript{134} Microscopic lesions of acute Se intoxication include alveolar edema and hemorrhage
accompanied by centrilobular necrosis of the liver.\textsuperscript{79,134} Lesions identified in alkali disease include cardiac atrophy and cirrhosis of the liver.\textsuperscript{69} Hoof deformities, edema of the coronary band, and hoof sloughing are all potential contributors to lameness.\textsuperscript{48} Loss of hair, commonly in the vicinity of the tail head region, may be observed.\textsuperscript{107}

**Deficiency of Se**

Selenium deficiency may be of concern in cattle located in regions with sandy or silt laden soils due to low Se content resulting in low Se forages.\textsuperscript{69,163} Impaired absorption may also be the result of high S diets due to shared transporters within the intestine.\textsuperscript{11}

**Clinical signs of deficiency**

Selenium deficiency in the bovine is often associated with ill thrift, weakness as a result of muscular dystrophy or white muscle disease, compromised immune system characterized by increased infections, reproductive complications, and lowered milk production. Reproductive deficits believed to be affected by low Se levels include sperm motility and abortions.\textsuperscript{108}

**Pathogenesis of clinical signs**

Decreased Se results in reduced GSH leading to limited neutrophil bactericidal capabilities. Decreased GSH also has been observed to reduce phagocytic killing of microbes as well as increased damage due to hydrogen peroxide formation. Extensive oxidative damage occurs due to the reduced antioxidant capacity of GSH in the absence of Se.\textsuperscript{69,163} Although the current mechanism has not been identified, it is believed that Se deficiency does impair the reproductive system as bull semen with low concentrations of Se compared to semen with higher Se levels showed decreased sperm motility.\textsuperscript{135}
Gross and microscopic lesions

Necrosis of cardiac muscle and signs suggestive of cardiac failure such as pulmonary edema, ascites, and a nodular liver have been observed in aborted fetuses. Lymphoplasmacytic myocarditis and fibrosis was observed in aborted fetuses possessing lower Se concentrations than aborted fetuses in comparison to non-aborted individuals. Se deficiency has been suspected as a potential cause in these instances.\textsuperscript{108}

Mineral interactions of Se

Both Se and Sulfate (SO\textsubscript{4}) share the same sulfate transporter within the small intestine. High sulfate levels competitively inhibit the transport of selenate across the enterocyte reducing absorption. Molybdenum, specifically sodium molybdate, also has the potential to interfere with the absorption of Se as it too utilizes the sulfate transporters.\textsuperscript{11} The heavy metal mercury can also inhibit absorption of Se through the formation of mercury selenide complexes.\textsuperscript{156}

Diagnostic analysis of Se

Liver and kidney are the most useful samples to determine Se status. They are the primary tissues where Se concentrates. Whole blood also serves as a reliable diagnostic sample due to the strong presence of GPX in erythrocytes. Due to the degree of variation of Se concentrations, brain, muscle, and hair, are not the ideal diagnostic samples for interpretation of Se.\textsuperscript{69,79} Use of GPX as biomarkers in evaluation for Se toxicity is of little value. Plateaus of these enzymes are achieved with no further increase even after increased dietary supplementation.\textsuperscript{142} Caution is to be used when analyzing GPX in whole blood and selenoprotein-P in plasma due to leaching of GPX from erythrocytes following hemolysis which can distort plasma results.\textsuperscript{69} Hemolysis of samples can falsely elevate Se, as well as Zn and Cu. If blood samples are not analyzed promptly, there is
potential for Se to diffuse from erythrocytes. It is recommended that liver be obtained for diagnostic purposes when possible.\textsuperscript{45}

**Zinc**

**Background and function of Zn**

Zinc is an essential TM that serves as a component of numerous enzymes. These include carbonic anhydrase and Zn dehydrogenase. These function in maintenance of blood pH, integument, immune system, vision, and transportation of vitamins. Carbonic anhydrase assists to maintain blood pH by converting carbon dioxide (CO\textsubscript{2}) to bicarbonate (HCO\textsubscript{3}\textsuperscript{-}). Bicarbonate is then transported out of erythrocytes resulting in removal of hydrogen protons and a slightly increased pH. Zn dehydrogenase plays a crucial role in vision. It recycles retinal by converting trans-retinal to cis-retinal in order to form rhodopsin for vision in response to light. Zinc is critical for immune system function through antioxidant activity as a component of superoxide dismutase.\textsuperscript{144} Zinc helps in maintenance of integument. Zinc, along with Zn transports and Zrt-Irt-like protein (ZIP), aids in the proliferation and differentiation of keratinocytes that form epidermal layers. Zinc is involved in transport and utilization of vitamin A through the synthesis of retinol binding protein.\textsuperscript{144} Gene expression depends on Zn. Zn serves as a stabilizing component for protein structures that interact with transcription factors, proteins, and genetic material.\textsuperscript{32}

**Sources of Zn**

Forages and grains, milk and colostrum serve as common sources of Zn for ruminants\textsuperscript{144} There is very little variation of Zn concentration between different species of vegetation. Concentration of Zn in both forage and grains depends on Zn levels in soil or surrounding environment.\textsuperscript{93} Zinc concentration also appears to decrease in forage
material following successive cuttings.\textsuperscript{144} Areas in close proximity to smelting factories and production sites that galvanize steel often have increased Zn concentration in plants. Dust and exhaust from these facilities are heavily laden with Zn. These have potential to contaminate surrounding areas. These are areas in which adverse health effects associated with Zn, are observed.\textsuperscript{160} Zinc is also available as an injectable product as MM at 60 mg/ml.\textsuperscript{120}

**Absorption, metabolism, and excretion of Zn**

Bioavailability of Zn is minimally affected by speciation if adequate Zn concentrations are present in the individual and diet. There were no differences in tissue Zn concentrations between groups of cattle receiving either zinc methionine $[\text{C}_{10}\text{H}_{20}\text{N}_{2}\text{O}_{4}\text{S}_{2}\text{Zn}]$, zinc sulfate $[\text{ZnSO}_{4}]$, or zinc oxide $[\text{ZnO}]$. Speciation may be of importance where there are inadequate levels of Zn in cattle. Bioavailability is increased in cases of either inadequate or deficient of Zn concentrations in individuals. Increased bioavailability is illustrated by a significant decrease in excretion of Zn by individuals with low Zn levels than those administered dietary Zn at adequate concentrations.\textsuperscript{130} A hereditary condition in Friesian cattle results in an impaired ability to absorb Zn which has led to cases of thymic atrophy and immunological impairment.\textsuperscript{71,72} Production of T-cells is dependent on the functionality of the thymus. Calves possessing this hereditary condition were observed to have a reduction of T-cells as well as immune response.\textsuperscript{117} The minimum adequate concentration of dietary Zn in calves is 8.6 mg/kg and not to exceed 500 \text{ug/g} in milk replacer or 900 mg/kg in feed.\textsuperscript{42,87,89,109} Young calves have higher absorptive capabilities than older individuals. Most bioavailable Zn is absorbed from the gastrointestinal tract then transported and stored in the liver, kidneys, and pancreas.\textsuperscript{110} Increased consumption of Zn and administration of Zn through intramuscular
injection results in accumulation of hepatic Zn. Elevated serum and renal Zn are also noted. Following injection of a particular Zn compound (Multimin 90, Multimin North America Inc, Fort Collins, CO), Zn concentration in plasma appears to decrease in approximately 24 hours to a level that is comparable to cattle that do not receive additional Zn supplementation. Hepatic concentrations of Zn were found to be significantly higher than control animals. Elevation of Zn in hepatic tissue following supplementation is contradictory to observations in two previous studies which suggested that supplementation did not affect liver concentrations. Rapid decline in plasma Zn concentration occurs as a result of Zn deficient diets, prior to leveling off within two weeks. Like intramuscular injections in one study, plasma concentrations increased prior to plateauing in three weeks. Due to clinical signs of Zn deficiency appearing within weeks of continuous administration of a Zn deficient diet, it is suggested there is a limited storage capability in cattle. The findings made by multiple studies observing plasma and liver Zn concentrations suggest that the liver plays a critical role in maintaining plasma Zn concentrations. Zinc is primarily excreted through the feces.

**Toxicity of Zn**

Zinc toxicity in cattle generally occurs through over supplementation of dietary Zn. Often this is a result of erroneous ration formulation. Intoxication can also occur through consumption of plants adjacent to smelting or galvanized steel production facilities. Although different in clinical presentation, both hepatic and renal tissue Zn concentrations become exceedingly high in cases of both acute and chronic Zn intoxications. Threshold for toxicity in younger animals, is lower and it is recommended that Zn, found in milk replacer, not exceed 500 µg/g or ppm.
Clinical signs of toxicity

Clinical signs associated with Zn toxicity may include anorexia, diarrhea, anemia. Although speciation of Zn does not appear to have a profound effect on bioavailability, high zinc oxide [ZnO], when administered at 2-3 g/kg can lead to reduction in feed consumption and gain in cattle that have adequate Zn concentrations. Cattle fed diets containing Zn at these concentrations were observed to sort through the feed and avoid any finely ground material or premixes. Given the effect this level of Zn has on cattle, it has been suggested that diets containing 1 g/kg or more Zn are considered toxic.\textsuperscript{109} Smelters and galvanizing steel mills serve as potential sources of Zn. Cattle raised on pastures or fed forage material in close proximity to such facilities have the potential to become exposed to high concentrations. A herd of yearlings of undisclosed gender within a lot were affected following consumption of forage material harvested from land within 300 meters of a facility that galvanized steel tubes. Dust and exhaust from the facility had blanketed the area prior to harvesting. Individuals consuming the forage initially exhibited pica and severe diarrhea. A number of individuals appeared emaciated with poor body condition as well as mandibular edema within the following month. Forage was found to contain between 3,400 and 7,300 ppm Zn. Further analysis of hepatic and renal tissue revealed Zn concentrations of 420-1600 ppm and 420-910 ppm. Concentrations were consistent with chronic Zn toxicity.\textsuperscript{160}

Acute Zn intoxications may occur when 150 g of Zn are consumed on a daily basis over a period of several days. Acute intoxications presented differently than the case observed by Allen (1968) where dairy cattle exhibited severe diarrhea accompanied with decreased milk production. Clinical signs were the result of inadvertent addition of approximately 20,000 ppm Zn to the ration. The ration was fed over a period of several
days. Liver and kidney concentrations were exceedingly high in Zn at 2,040 ppm and 240 ppm.

**Pathogenesis of clinical signs**

How Zn exerts its toxic effects in ruminants is not well known. Potential mechanisms resulting in hemolytic anemia include inhibition of erythrocyte enzymes or direct damage to erythrocyte membranes. Due to the caustic potential of Zn, excess concentrations of certain Zn salts can result in gastrointestinal complications. Diarrhea exhibited by cattle may be due to the caustic effects of excess Zn. Fatty degeneration of hepatic and renal tissue was may be the result of cattle being in a negative energy balance as a sequela to malabsorption. Failure of homeostatic mechanisms corresponding to Zn, excess Zn is proposed to form complexes that can result in depression of genes and changes in protein function leading to cell death. Cells possessing high protein synthesis capacity, such as hepatocytes and renal tubular epithelial cells, are likely to be more affected resulting in necrotic lesions.

**Gross and microscopic lesions**

Abomasal and duodenal mucosa may appear darkened and necrotic with green discoloration. Microscopic lesions reveal pancreatic, renal tubular, and hepatic necrosis. Evidence of gastrointestinal irritation and inflammation is characterized by edematous submucosa of the abomasum and duodenum, exfoliation of the glandular epithelium, and regions of necrosis.

**Deficiency of Zn**

Zinc deficiency in cattle has been associated with lesions of skin, reduced feed intake, impairment of reproductive function characterized by delayed sexual maturity and reduced sperm production.
**Clinical signs of deficiency**

Clinical signs associated with Zn deficiency include hyperkeratotic lesions of the integument. Crusts and proliferative scales are noted on limbs, neck, eyes, and face. Periorbital alopecia is often prominent. Deficient individuals also exhibit reduced feed intake. Classic lesions of Zn deficiency is associated with progressive hyperkeratosis that ranges from mild to severe. Correction of a Zn deficient diet will result in the resolution of lesions within several weeks.\(^{89,111}\) Affected animals may also become immunosuppressed resulting in increased infections.\(^{69}\)

**Pathogenesis of clinical signs**

The exact mechanism of Zn deficiency as it relates to its associated clinical signs is not completely understood. As Zn is associated with a large number of enzymes and functions, it is expected that there is a decrease in these. Increased morbidity and infections may be observed since T-cell production and antibody response is reduced as a result of Zn deficiency.

**Gross and microscopic lesions**

Gross lesions may appear as bilateral symmetrical scaly and thickened crusts throughout the epidermis with predilection in the neck, face, limbs, and ventral surface.\(^{111}\) During a previous study, crusts were observed to be thicker in the coronary band region. Microscopic skin lesions include acanthosis, rete peg formation, and parakeratotic hyperkeratosis.\(^{82,111}\)

**Mineral interactions of Zn**

Both Zn and Cu are antagonistic of each other. Increased levels of either element will inhibit absorption and metabolism of the other. Both Cu and Zn competitively bind to metallothionein.\(^{144}\) Increased dietary Zn results in decreased liver Cu. Absorption of
Zn can also be inhibited by high dietary levels of Calcium (Ca) and Cu. Elevated levels of lead can inhibit absorption of Zn. 

**Diagnostic analysis of Zn**

The ideal method of diagnosing Zn deficiency initially was through resolution of clinical signs following Zn supplementation. Although still utilized, the turn-around-time until effects of supplementation are observed, is prolonged. It takes several weeks for deficient cattle to recover. Hair from affected animals may be used to identify Zn toxicity in cattle. This method is generally limited to chronic exposure due to the extended amount of time Zn takes to accumulate in hair. Plasma and serum have been widely used to evaluate both Zn deficiencies and toxicities. There may limited ability to detect Zn deficiency with these matrices alone. Zinc concentrations in plasma level off in cattle within two weeks of consuming a Zn deficient diet. Cattle that received parenteral Zn supplementation had rapid elevation of serum Zn concentrations which tapered off within approximately twenty-four hours. Hepatic and renal Zn concentrations increase substantially when Zn is consumed in exceedingly high to toxic amounts. Liver is the most consistent for detecting elevated to toxic concentrations. The optimal samples of choice to consider for diagnosing Zn deficiency and toxicity in cattle would be serum/plasma and liver. This is due in part to the fairly rapid return of Zn to normal levels within twenty-four hours in cases of high Zn supplementation. It may take up to several weeks for serum and plasma Zn levels to return to normal in cases of deficiency.

**Summary of trace minerals**

Trace minerals are necessary in small concentrations in order to perform a multitude of physiological functions. Adequate dietary concentrations assure production
of healthy growing individuals. Each TM is not independent. Trace minerals exert effects on and are influenced by other trace elements in an interconnected web.

Multiple factors such as geographical region, consumption of additional dietary sources, inadvertent over or under supplementation, age, or genetic predisposition, allow for potential TM deficiencies or toxicities to occur. Guidelines regarding concentrations considered to be adequate, toxic, and deficient for these four trace minerals adopted from a previous publication can be found in Table 1. Although commonly used, these references may be dated as a result of change in species genetics, nutritional practices, and nutritional requirements.

Often clinical signs of deficiency or intoxications appear to be non-specific. Lack or excess of certain trace elements can have detrimental consequences on animal health resulting in further secondary nutritional complications and disease. An equal balance of minerals must be maintained and monitored in order to prevent nutritional toxicities and deficiencies. The absorption, metabolism, and excretion is comparatively different among TM. Samples utilized for diagnostic analysis of TM status in bovine, and ruminant species in general, include serum, blood, plasma, and liver. Multiple enzymes of which these TM are components of have also been evaluated as biomarkers in determining the status of their respective TM. The use of plasma as a diagnostic indicator of toxicosis is difficult as plasma concentrations of Cu, Mn, Se, and Zn decrease and return to normal levels within approximately twenty-four hours while hepatic concentrations remain elevated for several days following supplementation. Hepatic mineral values reflect supplementation over the last thirty days. Liver appears to serve as
the most reliable diagnostic matrix for analysis and interpretation of Cu, Mn, Se, and Zn status in cattle.\textsuperscript{45,74}

Table 1 Reference guidelines for hepatic concentrations copper, manganese, selenium, and zinc in bovine.\textsuperscript{124}

<table>
<thead>
<tr>
<th></th>
<th>Copper</th>
<th>Manganese</th>
<th>Selenium</th>
<th>Zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequate</td>
<td>87.5–350</td>
<td>8.75–21</td>
<td>.875–1.75</td>
<td>87.5–350</td>
</tr>
<tr>
<td>Deficient</td>
<td>35</td>
<td>3.5</td>
<td>.6</td>
<td>70</td>
</tr>
<tr>
<td>Toxic</td>
<td>875</td>
<td>-</td>
<td>4.4</td>
<td>420</td>
</tr>
</tbody>
</table>

Reference values are adapted from \textit{Mineral Levels in Animal Health 2\textsuperscript{nd} Edition}. Values are provided in ppm on a dry weight basis following multiplication of wet weight reference values by 3.5 to account for moisture content.

**Sulfur**

**Background and function of S**

Sulfur is necessary for the growth of rumen microorganisms as well as for protein synthesis in ruminants.\textsuperscript{97} Ruminal microorganisms require S for their growth. They are also able to reduce dietary S in order to utilize amino acids for protein synthesis.\textsuperscript{67} Sulfur is especially critical because it is a component of the start codon methionine necessary for translation and protein synthesis.\textsuperscript{34} Sulfur is an essential component of cysteine as well as thiamine and other B vitamins. Sulfonated mucopolysaccharides such as chondroitin sulfate, are required for cartilage development.\textsuperscript{97} Although the natural dietary supply in plants may be variable\textsuperscript{27,40}, it is recommended that S should be fed to cattle at no less than 0.15\% (1,500 ppm) but not to exceed 0.4\% (4,000 ppm).\textsuperscript{97}

**Sources of S**

Multiple sources of dietary S include water,\textsuperscript{158} forages,\textsuperscript{40} molasses, distillers grains, glutens, and salts.\textsuperscript{97} Sulfate can be present in ground and surface water at concentrations ranging from <250 ppm\textsuperscript{40,158} to 7,600 ppm.\textsuperscript{40} Plants containing high amount of S containing amino acids possess higher levels of S.\textsuperscript{67} Variation of S
concentration within forages is present across plant species. Alfalfa and grasses may contain S concentrations exceeding both 0.3% and 0.4%. Evaluation of 1,190 bovine operations within several regions throughout the United States revealed the greatest risk for S toxicity to be in the north central and northwest. 40

Absorption, metabolism, and excretion of S

Dietary S is utilized for the growth of microorganisms in the rumen.97 Absorption of S following consumption of forage material fertilized with S may vary depending on plant species as shown in a previous study. Sulfur absorption was decreased and increased after S fertilization of orchard grass and tall fescue.139 Rumen microbes also synthesize S containing amino acids for sulfide as inorganic S sources are reduced to sulfide.34,38 Both dissimilatory and assimilatory reduction of sulfate occurs in the rumen. Sulfate is utilized as a terminal electron acceptor by sulfur reducing bacteria (SRB) in the production of hydrogen sulfide (H2S). Assimilatory bacteria utilize dietary S to sulfide for cellular functions.115

Sulfur is excreted primarily in the urine.67 Undegraded organic forms are primarily excreted in the feces.68 Increased excretion of S through the urine results following consumption of forages possessing high amounts of S139 or water high in sulfate. This results in a 45% increase in renal filtration of S.158 In a previous study, an increase in urinary S excretion through decreased renal tubular reabsorption in the presence of high Mo concentrations was observed.5

Toxicity of S

Polioencephalomalacia (PEM) is a major concern in ruminants. It can result from excessive S, lead poisoning, sodium intoxication/water deprivation, or thiamine deficiency. Both total intake of S and fully functioning ruminal microflora are factors in
the development of PEM.\textsuperscript{38} The maximum level of dietary S recommended in beef cattle is 0.4%. However, the maximum tolerable concentration of S in diets containing less than 15\% forage is 0.3 percent. Diets containing greater than 40\% forage have a maximum tolerable concentration of .5\%.\textsuperscript{97} Cattle exposed to forages containing concentrations exceeding 0.4\% are at risk. Consumption of forages possessing S concentrations less than 0.4\% but greater than 0.3\% may be a risk with concurrent consumption of water with elevated sulfate levels.\textsuperscript{40} Elevated level of sulfate in water sources have been shown to decrease both feed and water consumption at levels exceeding 2,814 ppm. Complete refusal was noted at concentrations of 7,500 ppm.\textsuperscript{40,157} Discrimination of water by cattle at sulfate concentrations of 1,450 ppm was described in a prior study.\textsuperscript{157}

**Clinical signs of toxicity**

Clinical signs of S associated PEM may include depression, ataxia, blindness, dysphagia,\textsuperscript{38} head pressing, convulsions, and death.\textsuperscript{83} Cattle may have a detectable S odor on their breath within five days on a high S diet. Changes in mentation and respiratory patterns, characterized by uneasiness and increased rate and depth of respirations, have been observed in cattle maintained on a high S diet following eructation.\textsuperscript{39} Elevated concentrations of S can lead to secondary deficiencies of Cu and Se.\textsuperscript{41}

**Pathogenesis of clinical signs**

Fully functional rumen microorganisms have increased reducing capacity leading to increase in \( \text{H}_2\text{S} \) formation in instances of excessive S intake. Hydrogen sulfide inhibits cytochrome C oxidase. This results in inhibited cellular respiration and reduced energy production for neurons.\textsuperscript{102} Both direct absorption of \( \text{H}_2\text{S} \) through the rumen wall or inhalation following eructation are potential routes of exposure to excessive \( \text{H}_2\text{S} \) resulting in PEM.\textsuperscript{25} Hydrogen sulfide collects in the rumen gas cap. Individuals may become
exposed through inhalation of significant amounts of the gas following eructation.

Inhalation of eructated gas will bypass hepatic detoxification. Breath analysis of cattle on elevated S, while utilizing presence of nitrous oxide as indicator of respiratory insult, detected no such compound. No correlation was observed between H2S in the breath of cattle when compared with H2S concentration or high S diets. Only two cattle within the same study had detectable levels of H2S in their breath. Much of the H2S absorbed directly through the rumen is thought to be detoxified through oxidative processes.

Another theory is that H2S diffuses from the rumen and overwhelms the liver in high concentrations so that it cannot be converted to sulfate, allowing for H2S to directly affect the brain. However, this mechanism is questionable as sheep with closed tracheas did not collapse following eructation in comparison to individuals whose tracheas remained open.

Following disturbance through agitation in conferment feeding facilities, H2S is released and rises to concentrations leading to exposure. Inhaling concentrations of 1,000 ppm or greater results in, rapid onset of neurological clinical signs. These are characterized by lateral recumbence, paddling, and death.

**Gross and microscopic lesions**

Gross lesions of S associated PEM, may include softened and flattened gyri. Cavitation of the cerebral tissue may be present as well. Generalized cerebral edema and hemorrhage within areas of necrotic cavitations may be observed. Lesions may fluoresce. The rumen may appear dark in color due to precipitated sulfide salts. Microscopic lesions for PEM are non-specific for S but include neuronal necrosis, separation of grey and white matter, pyogranulomatous vasculitis, and meningeal edema.
Gross lesions with acute death associated with \( \text{H}_2\text{S} \) through pit gases are not appreciable cortical laminar necrosis accompanied by necrotic hypereosinophilic and shrunken neurons may be observed microscopically in individuals that perish forty-eight hours following exposure.\(^6^3\)

**Deficiency of S**

Sulfur deficiency occurs through decrease in dietary supplementation. This may be brought on by extensive use of non-protein nitrogen sources in rations, low quality protein, diets deficient in protein, or the consumption of forages low in S.\(^3^4\)

**Clinical signs of deficiency**

The typical presentation of S deficiency in ruminants is characterized by anorexia leading to weight loss, emaciation, and eventually death if left uncorrected.\(^9^7,^{14^6}\)

**Pathogenesis of clinical signs**

Sulfur deficiency leads to reduced function of ruminal microorganisms resulting in decreased utilization of protein supplements.\(^6^7\) Decreased intake and impairment of protein synthesis results in, clinical signs associated with S deficiency. Deficiency observed in lambs, is characterized by anorexia and weight loss. This may lead to emaciation and death.\(^9^7,^{14^6}\)

**Mineral interactions of S**

Elevated levels of dietary S can impair both \( \text{Cu} \)\(^2^3\) and Se absorption.\(^1^1\) Reduced S in the rumen can form either insoluble CuS or Tm complexes impairing Cu absorption.\(^2^3,^{4^1},^{1^1^9}\) Thiomolybdates may also induce hypocupremia through increased biliary excretion of Cu resulting in decreased hepatic Cu concentrations when diets high in Mo, S, and Cu are fed to ruminants.\(^1^1^9\) Both excessive Mo and S can interfere with one another in regards to renal reabsorption.\(^5\)
Diagnostic analysis of S

Sulfhemoglobin concentrations in blood was utilized by investigators in a previous study to evaluate sulfate levels in cattle following consumption of water possessing 5,000 ppm sulfate. Hypoxia as a result of sulfhemoglobinemia was not observed. Sulfhemoglobin must exceed 20% within blood to produce effects. Following S supplementation, rumen H₂S and urine thiosulfate concentrations become elevated. Concentrations in which PEM would be induced has not been able to be determined from these parameters. Thiosulfate in the ocular fluid and sulfate urine have been utilized to diagnose H₂S poisoning in confinement cattle (Rumbeiha and Radke unpublished data).

Summary of S

Sulfur is an essential mineral required for both ruminal microorganism growth and protein synthesis. It is essential for translation of RNA to proteins as it is a constituent of the start codon methionine. Excess S concentrations in ruminants is of concern due to absorptive interference of other minerals and as a neurotoxic agent. Sulfur acts in the role of an antagonist to Cu through both independent CuS and Tm complex formation. Sulfur can compete with minerals including inorganic Se which it shares a common transporter in the duodenum.

Inorganic and organic dietary S are readily converted by SRB to sulfide. Hydrogen atoms combine with sulfide to form hydrogen sulfide in a low pH rumen. This compound is utilized in reduction reactions to form of Tm complexes. Excessive production hydrogen sulfide followed by inhalation of eructated gases has been suggested to result in the development of PEM in ruminants. This manifests as neurological signs
that may include depression, ataxia, blindness, dysphagia, head pressing, convulsions, and death. Excessive concentrations of H$_2$S that overwhelm the capacity of the liver following direct absorption from the rumen may also lead to PEM. Hydrogen sulfide is also a concern in any species that is raised above manure pits on slatted flooring as agitation of pits results in excessive release of the gas exposing animals. Rations for beef cattle should contain no more than 0.4% S in order to prevent PEM. Attention should be given to other potential sources of S such as forages and water as the effects of S are cumulative.

**Molybdenum**

**Background and function of Mo**

Molybdenum has been identified as a component of xanthine oxidase, aldehyde dehydrogenase, and sulfite oxidase. Xanthine oxidase is responsible for converting xanthine to uric acid. It was the first observable enzyme in which Mo was identified as serving as an essential component. This was accomplished through the examination of xanthine oxidase concentrations when exposed to individual elements. The conversion of xanthine to uric acid is critical in the degradation of purines. Aldehyde dehydrogenase possesses similar catabolic reactions to that of xanthine oxidase especially in catabolism of pyrimidines. Aldehyde dehydrogenase also possesses the capability to convert retinaldehyde to retinoic acid, the active form of vitamin A. Sulfite oxidase is critical in metabolism of S from methionine and cysteine. There are no recommended concentrations for Mo in feed for cattle.

**Sources of Mo**

Dietary sources of Mo consist mainly of forage material and supplementation through mineral premixes. Molybdenum accumulation and concentration in plants is
dependent on several environmental factors.\textsuperscript{144} Accumulation of Mo in forages is dependent on plant species, soil Mo concentrations, soil moisture\textsuperscript{66}, and pH.\textsuperscript{43} Legumes appear to be more efficient accumulators of Mo than grass species.\textsuperscript{44,66} Certain species, such as alsike clover may possess Mo concentrations between 55-65 ppm.\textsuperscript{59} Forages also tend to possess higher Mo concentrations in alkaline soils. Soil Mo concentration varies in geographical location, soil type, and a region’s drainage capabilities. Peat soils and regions containing black shale tend to possess relatively higher concentrations of Mo. Peat soils may contain between 2.5 and 11 ppm Mo.\textsuperscript{73,148} Areas in which drainage is poor or flooding occurs, tend to have higher Mo levels.\textsuperscript{59,73} Molybdenum trioxide in exhaust originating from steel mills serves as a source of forage contamination.\textsuperscript{1}

**Mineral interactions of Mo**

Molybdenosis is a nutritional disease in cattle as a result of consumption of excessive amounts of Mo resulting in secondary Cu deficiency. Dietary ratios of Cu to Mo for optimal production in cattle is considered to be 6:1-10:1.\textsuperscript{124} Increased Mo in the diet progressively interferes with Cu absorption in which a Cu:Mo ratio of 1:1 results in a threefold reduction in Cu absorption.\textsuperscript{143} The majority of the toxic effects of Mo in ruminants is associated with its interaction with S in forming sulfur thiomolybdate complexes.\textsuperscript{47} Clinical signs associated with high Mo intake include diarrhea related to peat soils high in Mo and low in Cu.\textsuperscript{21,59} Clinical signs in acute intoxication may include anorexia, weakness, and mucoid feces.\textsuperscript{47} Clinical signs are nearly identical to those of Cu deficiency.\textsuperscript{21} Forage material that contains between 10-20 ppm Mo is considered to be potentially toxic for grazing animals.\textsuperscript{73} Elevated Mo intake can also interfere with phosphorus (P) metabolism resulting in structural complications characterized by osteoporosis, fractures, and abnormal joints.\textsuperscript{90}
Thiomolybdate formation

Formation of Tm occurs in the rumen through the combination of Mo with H$_2$S.$^{118}$ The mechanism in which Tm form was proposed following results of a previous study where ruminal microorganisms first reduce sulfates to sulfides followed by sulfide interaction with Mo.$^{23}$ Thiomolybdate formation occurs through a series of reduction reactions$^{14}$ through dehydrolysis of molybdate. Attached oxygens of Mo are progressively replaced by S.$^{55}$ Formation of Tm is dependent on both pH and S:Mo ratio.$^{14}$ It is also dependent on liquid and solid phases in the rumen.$^{122}$ The dependence of Tm formation on S:Mo ratio is dependent on which stage of molybdate is attained in the rumen at a neutral pH. The reduction process did not continue beyond dithiomolybdates in ratios of 5:1-10:1. Trithiomolybdates were formed at S:Mo ratios of $>$10:1. Tetrathiomolybdates formed at ratios of approximately 300:1. The faster conversion to the trithiomolybdate form at a pH of 6.5 in comparison to slower conversion at a pH of 8.0 indicates conversion of Tm during reduction occurs more rapidly at a lower pH.$^{14}$

The liquid and solid phase of the rumen are also believed to play a role in Tm stability as formation of these complexes is reversible through hydrolysis in the liquid phase of the rumen.$^{122}$ Both tetrathiomolybdates and trithiomolybdates are the predominant form of Tm in the rumen.$^{10}$ Following intraruminal injections of tetrathiomolybdate, only dithiomolybdates and trithiomolybdates were detected. The solid phase not only provides stability to teterathiomolybdates but also contains Cu available to form insoluble Tm complexes.$^{122}$
Antagonistic effects of thiomolybdates

Due to the antagonistic effects of Tm on Cu, clinical signs are of Cu deficiency. Clinical signs are a result of loss or decrease in function of cupric enzymes. Inhibition of cytochrome oxidase, superoxide dismutase, Cp, and tyrosinase was observed in vitro in a previous study. Molydenum can independently impair Cu absorption, through the formation of insoluble MoCu complexes. The effect is not as significant as Tm. Previous work observed higher concentrations of insoluble Cu following Tm administration compared to very little insoluble Cu when Mo was administered alone. Due to the stability that the solid phase of the rumen provides for Tm, these complexes are able to bond with Cu that is more available in the solid phase. Higher order Tm, possess greater affinity for Cu. These higher order Tm cause Cu to become tightly bound to high molecular weight molecules such as albumin. This results in an insoluble precipitate. Thiomolybdate complexes further deplete hepatic Cu stores through increased biliary excretion of Cu. Due to the dependence of the calf on the dam’s Cu status, calves from dams on high Mo and S diets tend to possess lower hepatic Cu levels.

Summary of thiomolybdates

Thiomolybdates are complexes of particular concern in ruminants. These complexes are formed through multiple reversible reduction reactions between molybdate and hydrogen sulfide. The pace and state of progression are largely dependent on both ruminal pH and the ratio of S to Mo within the rumen. S:Mo ratios in the rumen less than 10:1 at a neutral pH, do not to result in progression of Tm beyond dithiomolybdate to the higher forms of tri- and tetrathiomolybdate that possess a higher affinity for Cu. Advancement of Tm to the trithiomolybdate form occurs more rapidly at a lower ruminal
pH of 6.5. Progression is slowed at a ruminal pH of 8.0. Both the solid and liquid rumen phases play an integral part in Tm stability. Formation of Tm is a reversible process, through hydrolysis, that occurs in the liquid phase of the rumen. While offering stability to Tm, the solid phase also provides the opportunity for Tm are able to complex with Cu. Copper is widely available in the solid phase of the rumen. Higher order Tm that complex with Cu result in Cu becoming tightly bound to high molecular weight molecules such as albumin. Due to its antagonistic effects on Cu, clinical signs associated with Tm appear as signs of Cu deficiency. It is important to observe the environment for sources of both Mo and S, such as Mo accumulators and water sources with elevated levels of sulfate, which may potentiate Tm formation.

Liver biopsies

Background

Application of liver biopsies in cattle to monitor nutritional status was not implemented until over twenty years after its original development by physicians for use in humans in 1939. Following modifications and procedural adaptations, the liver biopsy technique was first integrated into the veterinary medical profession for monitoring Cu concentrations in sheep. This technique was further adapted and modified due to advances in analytical technology. Multiple techniques regarding approach, ranging from laparotomies to simple stab incisions with a scalpel blade have been used. A variety of instruments have been developed and utilized for biopsy procedures.

Development of liver biopsies

Liver biopsy techniques have been developed in livestock for multiple reasons. First, to evaluate individual and herd nutritional status. Second diagnosing potential issues associated with nutrition since most primary TM accumulate in liver. A third
reason for further development in biopsy techniques and instrumentation is to decrease the deleterious effects caused by tissue damage to both animal health and production. Many early techniques implemented approaches and instruments that were of large size that resulted in significant hepatic trauma. The use of large biopsy instruments are not suited for calves leading to the development of smaller instruments. Liver biopsies are relatively safe as demonstrated in a previous study by the performance 156 biopsies on thirty-three neonatal calves resulting in only two complications.\textsuperscript{145} The fourth reason for further development is acquisition of a liver sample in a timely manner that does not cause undue stress to the animal or impede production practices. Original aspiration and laparotomy techniques take between fifteen to twenty-five minutes and required larger incisions for passage of instruments.\textsuperscript{30,92} Current aspiration and fine needle biopsy procedures, with or without ultrasound assistance, have reduced sample collection time for each animal to as little as five minutes (Ensley and Radke unpublished data). Although there is greater ease with less time required in collecting serum, serum is of little diagnostic value for certain TM, specifically Cu, in determining toxicities and deficiencies.

**Sample size of liver biopsies**

Quantity of liver collected since the advent of performing liver biopsies in cattle has decreased. Samples collected utilizing original developed techniques and instruments ranged in size from 1.0 - 1.25 g while laparotomy procedure samples sizes range from 2.0 – 4.0 g.\textsuperscript{30,92} One of the primary driving forces leading to smaller amounts of liver, besides reducing potential damage to the animal, is improved analytical testing capabilities for TM. There are a number of conflicting views regarding the amount of hepatic tissue that is sufficient enough for thorough interpretation. A prior study determined that as little as
0.005 g DM of liver was enough to determine the status of several TM using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) also known as inductively coupled plasma-optical emission spectroscopy (ICP-OES) with ultrasonic nebulization. Several wedges of liver were collected from various location throughout the liver along with two to three biopsies of approximately 0.02 g taken immediately adjacent to each wedge in this study. The minimum sample size required for reliable interpretation determined by in that study is contradicted by a study performed in the Netherlands using ICP-OES by another. The latter study suggested that liver samples less than 0.15 g on a wet weight basis were considered unreliable and that there was little ability to determine low TM concentrations. Biopsies of greater than 0.15 g were in strong agreement with homogenate TM concentrations.

Aspiration technique for liver biopsies

Aspiration techniques provide larger amount of liver to be collected for additional analytical tests due to the size of instruments. The instruments utilized for aspirated liver biopsies, such as a bone trocar, are robust and are not easily damaged or bent. Collection time is minimal taking several minutes at most from preparation to collection of sample. Problems that may be encountered when utilizing aspiration techniques include pneumothorax, inability to collect or loss of sample due to negative pressure, and traumatization of tissues and vasculature by excessive penetration.

Biopsy needle technique for liver biopsies

Use of a 14ga or 16ga needle biopsy instrument is minimally invasive as it requires a smaller incision and results in less hepatic trauma. Although there is potential for vascular and tissue damage from excessive penetration, due to the small size of the instrument damage is likely to be minimal. Much like the aspiration technique, samples
can be collected efficiently within several minutes. There is concern of the sample collected being too small for reliable analytic testing as well as the fragility of the instrument. The thin nature of the instrument makes it prone to bending as a result of animal movement. This may render the biopsy needle unusable.\textsuperscript{145}

**Approach and technique of performing liver biopsies**

The general approach and location of liver biopsies has remained consistent since implementation of this sampling technique. The vast majority are performed blind, although, ultrasound guidance is utilized. Physical anatomical landmarks are employed to identify the position of the liver and incision site Figure 1. Biopsies are performed on the right side due to the location of the rumen on the left which would impede procurement of the sample. The incision point is identified at intersection of lines originating from the greater trochanter to the dorsal aspect of the shoulder and the tuber coxae to the elbow within either the tenth, eleventh, or twelfth intercostal spaces. Once the incision is made in the skin, the biopsy instrument, may be inserted to collect the sample. If a blunt tipped instrument is used, such as alligator rongeurs, the incision must go through the body wall. There is a degree of uncertainty in regards to position of the liver even when using anatomic landmarks.\textsuperscript{145} Age of the animal, stage of gestation, and rumen fill should be taken into account as each of these aspects may involuntarily displace the liver. The liver may be an additional intercostal space forward in young animals. Due fetal size during late-gestation, the liver of a cow or heifer may be shift the liver dorsal. Low rumen fill may lead to a ventral shift in position (Ensley and Radke unpublished data).
Figure 1. Anatomical landmarks used for percutaneous liver biopsy in cattle. Incision site (blue dot) is determined at the point in which lines originating from the ilium to the elbow (A) and from the greater trochanter through the middle of the scapula (B) and from the intersect at the level of the tenth rib space.
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CHAPTER 3. COMPARISON OF TRACE MINERAL CONCENTRATIONS BETWEEN LIVER BIOPSIES OF DIFFERENT SIZES AND DURATIONS OF STORAGE BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

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Abstract

Trace mineral status plays an integral role in bovine health. Impairment of physiologic processes, due to trace mineral deficiencies or toxicities, serve as potential underlying factors of disease. Acquisition and analysis of bovine hepatic tissue has been utilized for decades, to evaluate critical trace minerals. Variation of copper, manganese, selenium, and zinc concentrations between liver samples of approximately 0.005 g and 0.3 g dried from 0.02 g and 1.0 g samples respectively, was assessed. Differences among samples of differing storage durations post collection and prior to analytical processing was also evaluated. No differences regarding concentrations of copper, manganese, selenium, and zinc were observed between 0.02, 1.0 g, and homogenized samples stored at three and fourteen days. Differences in concentrations of these elements were observed in 0.02 g biopsy samples stored for a period of seven days. No difference was observed between 0.02 g and both 1.0 g and homogenized samples regarding copper and selenium concentrations stored for seven days. No differences were evident between other durations of storage. Results of day three and fourteen samples suggest that trace mineral concentrations do not significantly differ regarding sample size or duration of storage duration and that 0.02 g of fresh hepatic tissue, dried to approximately 0.005 g is reliable
for diagnostic interpretation of toxicities and deficiencies in cattle pertaining to copper, manganese, selenium, and zinc.

**Keywords**

Trace minerals; toxicity; deficiency; liver biopsy; copper (Cu); manganese (Mn); selenium (Se); zinc (Zn)

**Introduction**

Cattle health and growth is dependent on trace minerals (TM) that serve as constituents of enzymes and proteins needed for physiological processes.\(^{14}\) Despite established recommended dietary concentrations, stringent mixing practices, and well-formulated parenteral products, TM deficiencies and toxicities occur.\(^{14,21}\) Contributing factors to deficiencies or toxicities include bioavailability, antagonists, and over supplementation.\(^{5,18,22}\) Predisposing factors to deficiencies and toxicities include age, breed, and environment.\(^{4,15,20,25}\) Unfavorable effects include impaired immune system function, antagonism of other trace minerals, decreased production, and death.\(^{8,9,15,17}\) Economic losses to producers are attributed to decreased production, increased morbidity and mortality, secondary complications, and cost of treatment.\(^{11,12}\)

Utilization of liver biopsies in cattle has continued and developed since its original implementation in 1950.\(^6\) Copper (Cu), manganese (Mn), selenium (Se), and zinc (Zn) concentrate in the liver of ruminants. Antemortem liver biopsies to evaluate trace mineral status, are a viable option for producers and veterinarians.\(^2,6\) Differences in instrumentation and techniques have lead to varying sizes of hepatic tissue obtained during sampling.\(^{24}\) Due to size variation of samples collected through different methods, there is controversy regarding what amount of hepatic tissue can be used to interpret trace
mineral status.\textsuperscript{1,16} It was hypothesized that there would be no significant differences in TM concentrations between samples of different sizes stored for different durations.

Objectives were to first evaluate for differences in Cu, Mn, Se, and Zn between liver samples of approximately 0.02 g and 1.0 g. The second objective was to evaluate differences in concentration of these minerals following differing storage times prior to analytical processing.

**Materials and methods**

**Sampling procedure**

Bovine livers were collected from abattoirs and regional meat lockers from different geographical regions for potential future evaluation of regional differences in TM content in cattle. Geographical origins of the livers included the northwest, northeast, central, and southeast regions of Iowa. Two livers were obtained from southeast Nebraska with the remaining two livers originating from Massachusetts and Texas. Livers, averaging 6.7 kg, were obtained from beef cattle weighing between 454 and 590 kg. Each liver was collected within a one week period preceding sample collection and maintained at -20\degree Celsius. A period of twenty-four hours at room temperature was used to allow thawing of livers prior to sampling.

Each liver was positioned in a manner consistent with its normal anatomical position of the liver within a standing animal using a homemade device prior to sampling Figure 2. Contamination between livers through the leaching of biological tissue and fluids into the device was prevented through the application of Thompson’s Waterseal Clear Multi-surface Waterproofer (The Thompson Company, Cleveland, OH). Contamination was further prevented by disinfecting the device following sampling of each liver. Consistent sampling was achieved through the placement and fixation of a
plastic grid (Prym Consumer USA, Spartanburg, NC) over the dorsal caudal area of the regions designated as D3 and D4 in a previous study. Fixation of the grid was such that the first sampling section laid at 2.5 cm and 3.5 cm cranial to the caudal margin and ventral to the dorsal margin of the right lobe. Modification of the grid prior to sampling allowed for consistency and to ensure that the correct size of samples were excised from their coordinates. A total of eight livers were sampled. Nine sets of paired samples of four different sizes were obtained from each liver. A single large section of liver was obtained for homogenization and trace mineral analysis at the same time. Rows of the grid were identified by the numbers 1-9 indicating each group of paired samples while columns were labeled 1-4 to identify size of samples to be collected. Columns corresponded with the size of sample. Collection of 0.02 g samples was performed using a Surgivet 20mmT 14 gauge biopsy needle (Smiths Medical, St. Paul, MN) Figure 3.

Liver samples of 0.1g to 1.0 g were obtained through excision using a number 11 scalpel blade (Medical Action Industries, Arden, NC) inserted to a depth of 0.5 cm using the graduated scalpel handle. One gram samples, taken 0.6 cm from paired 0.02 g samples, measured 1.5 cm x 1.5 cm x 0.5 cm. Half-gram sections were one-half the size. A liver portion, measuring 5.0 cm x 14.0 cm x 1.0 cm, immediately cranial to the grid was excised to serve as a representative post mortem sample. Size approximations of 0.1, 0.5, and 1.0 g samples were made through comparison to pre-weighed liver samples obtained from a bovine prior to this study. Each set of paired samples was taken at the same time prior to collection of the next set of paired samples in the following row.

Samples were transferred to pre-labeled 5ml polystyrene snap cap tubes (Corning Science, Tamaulipas, Mexico) with toothpicks (MedTech Products, Irvington, NY), for
0.02 g samples from the biopsy needle, and forceps for larger samples. To prevent contamination all collection instruments were rinsed in saline (B. Braun Medical, Irvine, CA) and dried between samples. Toothpicks were disposed of after each use. Prior to analysis, samples were grouped according to their intended storage duration and stored at -20° Celsius.

**Sample processing and analysis**

Samples for processing, were weighed and dried at 3, 7, and 14 d post collection. Liver samples, excluding homogenates, were dried at 100°C for a period of twenty-four hours. All samples were placed in a desiccator at room temperature prior to digestion and analysis to prevent moisture accumulation.

Dried 0.02 g and 1.0 g samples, weighing on average 0.005 g and 0.3 g respectively, were digested in trace metal grade nitric acid for a period of two hours at 65°C on a hot plate (BioExpress, Kaysville, UT). Following homogenization of the large liver section collected, multiple 1.0 g samples were dried at 65°C for approximately 24 hours. Homogenized and 1.0 g liver samples were digested using trace metal grade nitric acid using the methods of a previous study.³ Tenth of a gram and 0.5 g samples did not undergo digestion and were stored for potential future studies. Randomized ordering of samples for analysis was performed using the Microsoft Excel (Microsoft, Redmond, WA) random number generator function. Liver samples were analyzed for Cu, Mn, Se, and Zn, using an Inductively Coupled Plasma Mass Spectrometry (Varian Analytical Industries, Victoria, Australia). A single analytical run was performed on all samples. A National Institute of Standards and Technology (NIST) liver standard was included in the run. An in-house laboratory control liver was also used to ensure quality control and to verify instrument accuracy.
**Statistical analysis**

Data was analyzed using a mixed model repeated measures of analysis of variance (ANOVA) through the SAS product JMP Pro (SAS Institute Inc., Cary, NC). Livers served as the experimental unit (n = 8) and subject of the repeated measures. Experimental design allowed for analysis on two classes: amount of liver collected representative of method (0.02 g, 1.0 g, homogenate) and days in storage prior to analytical processing (3 d, 7 d, 14 d), along with their interaction with TM concentrations as the response. Responses in Cu, Mn, Se, and Zn concentration means as a result of interaction between sample amount and duration of storage were also evaluated. Five samples were determined to be outliers following evaluation of residuals and eliminated from subsequent analysis. Following establishment of any significant difference among the classes, specific comparisons to identify which specific TM concentration means differed between classes were performed using Tukey-Kramer adjustment for multiple comparison. Significance difference was reported P ≤ 0.05.

**Results**

Results for TM concentration means can be found for different samples sizes with corresponding storage durations in Figures 4-7. There was a difference in Cu concentration means regarding the interaction of sample size and duration of storage (P < .01). There was a difference in Se concentration means regarding storage duration (P = .04) and interaction of the variables (P < .01). Manganese concentrations differed regarding sample size (P < .01) and storage duration (P = .02). Zinc mean concentrations differed in sample size (P < .01), duration of storage (P < .01), and variable interaction (P < .01). Results of further evaluation of TM through Tukey-Kramer adjustment for multiple comparisons can be found in Table 2. Analysis of Cu revealed a difference in
concentration means between the 0.02 g biopsy samples stored for 3 d and 7 d (P = .02). Selenium concentration means differed between .02 g samples at 7 d and to same sized samples stored for 3 d (P < .01) and 14 d (P <.01). There was a difference in the mean Mn and Zn concentrations of 0.2 g samples compared to all other samples of different sizes and durations of storage (P < .05). No differences in Cu, Mn, Se, and Zn concentrations were observed for different sized samples stored for three and fourteen days (P > .05). No differences in concentration means were observed between 1.0 g and homogenized samples stored for same and differing lengths of time for any of the elements analyzed (P > .05).

**Discussion**

Cattle health and development is dependent on adequate levels of TM.\(^{14}\) Adequate biological concentrations necessary for physiological functions can be attained through balanced trace mineral supplementation with the aid of established dietary recommendations.\(^{18,23}\) Multiple factors may contribute to inadequate or excessive supplementation resulting in deficiencies or toxicities that may adversely affect health.\(^{18,22}\) Ante mortem testing for trace mineral status in liver can aid in diagnosing and preventing trace mineral associated disease. The amount of hepatic tissue obtained may vary depending on the method and instrument implemented.\(^{1,6,21,24}\) This study was initiated to determine differences in trace mineral concentrations between liver sample of different sizes for diagnosis of trace mineral status. Collection methods, one post mortem and two ante mortem, represented those utilized in veterinary practice. Samples were evaluated to determine difference in trace mineral concentrations and to establish if collection of 0.02 g of liver by biopsy, is reliable for diagnostic interpretation of trace mineral status regarding deficiencies and toxicities in cattle.
A difference in hepatic concentration means for both Mn and Zn (P < .05) was present between samples of different amounts and durations of storage. Further analysis revealed that the difference observed for both Mn and Zn was the result of the difference in 0.02 g biopsy samples stored for 7 d. Concentrations means for these samples differed from all other population means. Copper and Se concentrations were different (P < .05) between 0.02 g biopsy samples stored for 7 d compared to 0.02 g samples for other storage durations.

Excluding the 0.02 g biopsy samples stored for 7 d, no differences in concentration means for Cu, Mn, Se, and Zn among samples of 0.02 g, 1.0 g, and homogenates stored for different durations were observed. No difference was observed in trace mineral concentration of 1.0 g samples dried to 0.3 g and homogenized samples. Results in a previous study that compared TM concentrations of biopsies of approximately 0.175 g in size to homogenized samples were consistent with the findings of this study. Trace mineral concentrations from biopsies did not differ from homogenized samples concentrations. There was a difference in TM concentrations between dried 0.005 g and both 0.3 g and homogenized samples stored at day seven despite no difference in TM concentrations between samples stored for three and fourteen days post collection. Mean TM concentrations of 20 mg samples stored for 7 d were consistently elevated compared to those stored for 3 d and 14 d.

It is uncertain why the TM concentrations of 0.02 g samples stored for 7 d differed from other sample types. Several potential reasons for the differences include instrument variation or error, sample contamination, sample processing error, sample location, and sample size. Trace mineral concentrations of both NIST and control liver
analyzed with the samples were within acceptable ranges. The same instrument analyzed all samples during the same analytical run. The randomization of sample order for analysis precluded an erroneous analysis of a specified group of samples that may have otherwise occurred with multiple runs or samples being analyzed in order according to their groups. Multiple steps were implemented to prevent sample contamination or inadvertent mixing of samples.

The processing procedure for each biopsy sample was identical and was performed in a consistent manner. Although within the same region of the liver, samples were not taken from the exact same location. The similar TM concentrations between samples of different sizes stored for 3 d and 14 d make this appear a less likely cause for the difference. The sample size may have served as a potential cause as variability in TM concentration may increase with decreasing size of sample. In a previous study, samples less than 0.005 g dry weight were considered unreliable for interpretation. A number of biopsy samples in the 7 d group were approximately 0.003 g or less whereas there were very few among the other two groups combined. This finding suggests that moisture content of the sample may play a role in sample amount as all 0.02 g samples collected completely filled the trough of the biopsy instrument. This emphasizes that TM analysis of liver should be performed on a dry weight basis as the moisture content can vary between samples, even those within close proximity, giving inaccurate results if performed on a wet weight basis.

Limited information is available on variation of TM concentrations between samples of different sizes and duration of storage utilizing ICP-MS. Data available from previous studies utilizing inductively coupled optical emission spectrometry (ICP-OES)
are consistent to findings of this study with the exception of 0.02 g biopsy samples stored for 7 days.\textsuperscript{16} The lack of difference in TM concentration variation between 0.005 g and both 0.3 g and homogenized liver samples stored for 3 and 14 days is consistent with a previous study.\textsuperscript{16} Little to no significant difference in Cu, Mn, Se, and Zn concentrations among samples of different sizes stored for three and fourteen days suggests that collection of 0.02 g fresh liver is reliable for diagnostic interpretation of trace mineral status. However, the results may be inconclusive due to the difference observed in TM concentrations observed in 0.02 g samples at day 7 of storage.

The location in which biopsies were taken from in this study correlates to liver that lies in the region of the 10th intercostal space as indicated in a previous study. The reliability for the use of liver biopsies for determining TM toxicities and deficiencies is further exemplified by the findings of that study. The region in which samples were obtained in this study was established to possess higher concentrations of Cu, Mn, and Zn. Biopsies in the previous study were taken more caudal and underestimated the concentration of these elements within the liver. As suggested in the previous study, clinicians should be aware of what portion of liver is collected pertaining to the reason for testing. Since the portion of the liver sampled in this study is believed to be the region containing the highest concentrations of the minerals of focus, it would be the best location to samples to evaluate for deficiencies. If this area is depleted, it is likely that the animal is depleted of the specified mineral.\textsuperscript{10} The fact that this area possesses higher concentrations of Cu, Mn, and Zn suggests that interpretations regarding toxicity be made with caution. Interpretations should be made in correlation to clinical context of the case.\textsuperscript{10}
Establishment of a reliable sample for determining Mn concentration in cattle is difficult due its limited absorption and rapid excretion. Liver is the most appropriate sample, as hepatic Mn corresponds with both increased and reduced Mn intake. Liver Mn concentrations were utilized as an aid in a previous study to identify Mn deficiency in calves originating from mothers provided low Mn diets. Liver Mn of all affected calves was significantly lower than unaffected calves originating from adequately supplemented females.

No difference in Cu, Mn, Se, or Zn dry weight concentrations between 0.005 g, 0.3 g, and homogenized liver samples were observed with the exception of Mn and Zn concentration means in 0.02 g samples stored for 7 d. There was a difference in Cu and Se concentrations between 0.02 g samples of different storage durations. Liver biopsy samples provide a reliable diagnostic technique for evaluation of bovine trace mineral status. The results for samples of different sizes at three and fourteen days of storage suggest that there is little difference in TM concentrations between samples of different sizes stored for different period of time suggesting that 0.02 g of hepatic tissue would be reliable for diagnostic interpretation. Results for day seven data pertaining to 0.02 g biopsies contradict such results and are inconclusive. It is uncertain whether or not storage duration or sample size truly caused a difference in TM concentrations in biopsy samples stored for 7 days. Duration of storage at -20°C does not appear to affect trace mineral concentrations despite differences observed in 7 d samples. Potential factors that may contribute to TM concentration variation during hepatic tissue collection in a field setting include blood contamination or sampling of non-hepatic tissue. Inadvertent
collection of tissue other than liver can be determined through TM analysis. Trace mineral profiles for various tissue were determined in a previous study.¹

Consistent monitoring of TM in cattle is encouraged to prevent inadvertent trace mineral associated disease. Evaluation of trace mineral concentrations from 0.02 g liver biopsies utilizing ICP-MS may offer aid in identifying deficiencies and toxicities.

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Declaration of conflicting interests

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Figures and tables

Figure 2. Anatomical positioning of liver on homemade device. Plastic grid for sample collection is attached. A 45° incline of the device was required for the liver to stay in position during sampling.

Figure 3. Collection of 0.02 g biopsy sample.
Figures 4-7. Trace element concentration means for different sample sizes with corresponding storage durations. Bars represent 95% confidence interval. Figure 4. Copper. Figure 5. Manganese. Figure 6. Selenium. Figure 7. Zinc.

* Significant difference (P < 0.05)
Table 2. Comparison of trace mineral concentration means and resulting p-values. Samples stored for groups 3, 7, and 14 days are indicated by groups A-C, D-F, and G-I respectively. One gram samples are indicate by the letters A, D, and G. Biopsy samples are indicated by the letters B,E, and H. Homogenized samples are indicated by the letters C, F, and I.

* Significant difference (P < 0.05)

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CHAPTER 4. GENERAL CONCLUSIONS

Trace minerals are vital in bovine health and development by serving as components of enzymes involved in numerous physiological processes.\textsuperscript{9,11} Maximization of their affect is achieved when bodily concentrations are adequate. Established dietary recommendations serve as an aid in ensuring that these concentrations within the bovine are met.\textsuperscript{6} If factors impeding adequate dietary supplementation are present, additional parenteral sources of trace minerals are available. These products should be used with caution to avoid unwarranted over supplementation. Excessive or inadequate bodily concentrations of individual TM may cause health complications. The numerous interactions amongst minerals allow for potential secondary disease in association with another mineral to occur. Hepatic tissue has been utilized for diagnostic interpretation of trace mineral status in cattle and to identify TM toxicities and deficiencies.\textsuperscript{4,8} The manner in which liver is collected varies through the continual development of instruments and techniques.\textsuperscript{3,12} The amount of tissue obtained depends on the method implemented.\textsuperscript{2,12} The focus of this thesis was to examine the difference in concentrations of the TM Cu, Mn, Se, and Zn between liver samples of different amounts that were representative of the method used while also examining the variation in concentration of these minerals in samples for different durations of storage prior to analytical processing.

Previous studies utilizing inductively coupled plasma optical emission spectrometry (ICP-OES) offer differing opinions regarding the amount of liver considered to be reliable for interpretation in a diagnostic setting.\textsuperscript{2,7} With the exception of 0.02 g samples stored for 7 d, no difference in variation of TM concentrations for Cu, Mn, Se, and Zn was observed between fresh liver samples of different sizes. Results also
reveal that there is little variation in the concentrations of these trace elements in hepatic tissue when stored at \(-20^\circ\text{C}\) for different lengths of time prior to processing except for 0.02 g samples at 7 d storage. With the exception of 20 mg samples at 7 days of storage, these results are consistent with a previous study in which multiple samples were taken from a single liver. Results of that study suggested that TM concentrations in 0.02 g of hepatic tissue dried to 0.005 g did not differ from those of larger samples collected when analyzed by ICP-OES.\(^2\) Although the biopsy samples of 0.02 g were considerably smaller than the 0.175 g samples collected in a previous study, the findings comparing biopsy and homogenate TM concentrations between the present study and that study are consistent.\(^7\)

Mean TM concentrations of 0.02 g, 1.0 g, and homogenized samples were similar with the exception of 0.02 g biopsies stored for 7 d duration. The lack of difference in duration of storage between samples at days three and fourteen was expected, as TM are stable and not expected to deteriorate within the amount of time provided. The difference exhibited by the day seven 0.02 g samples was unexpected when there was no difference observed between any of the sample sizes stored for three and fourteen days.

It is uncertain why the TM concentrations of 0.02 g samples stored for 7 d differed from other sample types. Several potential reasons for the differences include instrument variation or error, sample contamination, sample processing error, sample location, and sample size. Trace mineral concentrations of both NIST and control liver analyzed with the samples were within acceptable ranges. The same instrument analyzed all samples during the same analytical run. The randomization of sample order for analysis precluded an erroneous analysis of a specified group of samples that may have otherwise occurred with multiple runs or samples being analyzed in order according to
their groups. Steps were taken to prevent sample contamination or inadvertent mixing of samples. During transferring of sample material, identification of samples was verified on both the donating and receiving vessels. Pipet tips that contacted anything but the sample or inside of the vessel were discarded and replaced. During pipetting and transferring, the pipet was not held above or moved over top open vessels to prevent potential leaking of material into other tubes. Samples were sealed within containers.

The processing procedure for each biopsy sample was identical and was performed in a consistent manner. Although within the same region of the liver, samples did not share the exact location. The similar TM concentrations between samples of different sizes stored for 3 d and 14 d make this appear a less likely cause for the difference. The sample size may have served as a potential cause as variability may increase with decreasing size of sample. In a previous study, samples less than 0.005 g dry weight were considered unreliable for interpretation. A number of biopsy samples in the 7 d group were approximately 0.003 g or less whereas there were very few among the other two groups combined. This finding suggests that moisture content of the sample may play a role in sample amount as all 0.02 g samples collected completely filled the trough of the biopsy instrument. This emphasizes that TM analysis of liver should be performed on a dry weight basis as the moisture content can vary between samples, even those within close proximity, giving inaccurate results if performed on a wet weight basis.

With the exception of the 0.02 g samples stored for seven days, there was no difference regarding concentration means of Cu, Mn, Se, and Zn between the different sizes of samples collected. Little to no difference in mineral concentrations between
differing amounts of liver suggests that the TM concentrations of smaller 0.02 g samples are similar to those of both the larger 1.0 g and homogenized samples. This similarity in concentrations amongst different samples sizes stored for 3 d and 14 d suggests that 0.02 g of liver may be an adequate amount for diagnostic interpretation of the TM status of an animal. The difference in TM concentrations observed in 0.02 g samples compared to other samples of different sizes and storage durations suggests otherwise. Established TM concentrations consistent with deficiencies and toxicities extend well beyond hepatic reference intervals considered adequate.\textsuperscript{1,8,10} With little difference in TM concentrations between differing amounts of liver, a 0.02 g sample of fresh liver dried to approximately 0.005 g may reliably serve as a diagnostic sample for detecting such cases. With the difference in the 0.02 g biopsy samples from others at day seven of storage, it is less clear as to how long biopsy samples can be stored for in order to have consistent TM concentrations as larger samples. Provided that liver biopsy samples are stored properly, withholding of samples from testing until necessary, can be done for an extended period of time according to a previous study.\textsuperscript{2} Hepatic tissue serves as a reliable matrix for trace minerals, but not for the interpretation of macro minerals such as calcium, magnesium, phosphorus, potassium, and sodium, as the liver does not serve as a primary storage site for these minerals.\textsuperscript{11}

Variables likely to be encountered during the performance of a liver biopsy of an animal in a field setting that may impact trace mineral concentrations were not factors in this study. These variables include non-hepatic tissue or blood originating from the incision site or vessels that may contaminate the sample. Since the operator is blind to what tissue is being collected until the instrument is extracted, there is potential for
inadvertent sampling of non-hepatic tissue. Analysis of tissue that is not liver will yield TM results that may lead to incorrect interpretation of TM status of the animal. A past study established TM profiles for other tissue. Livers in this study were fully visible with no other tissue types present. This eliminated the chance that non-hepatic tissue was collected. Blood tended to lead to the underestimation of the concentration of Cu, Mn, and Zn in liver in a previous study. Samples were collected from harvested livers of exsanguinated cattle. As such, TM concentrations of biopsies and other samples should have been comparable since the presence of blood was minimal.

When collecting samples, the biopsy needle was used to obtain 0.02 g samples while 1.0 g samples were not collected through the aspiration technique, the respective method for obtaining larger biopsy specimens. The aspiration technique, through the use of a trocar, was not used during this study in order to maintain consistency of sample size and location during collection. With aspiration of the liver requiring the application of negative pressure coupled with simultaneous movements and penetrations, the location and sample size may not be consistent for each paired sample as the instrument may penetrate too deeply or be inconsistently spaced from its smaller 0.02 g counterpart.

It was not the intention of the study to diagnostically interpret the TM status in animals from which the livers originated or diagnose trace mineral deficiencies or toxicities. The clinical history of all individuals remained unknown providing no clinical context to diagnose toxicosis or deficiency. Identification and speculation of potential details as to why TM were at the concentrations detected was not part of the study. As such, rations and mineral supplementation of the cattle whose livers were used, were unknown in this study, so no specific TM concentrations were expected. Although the
analysis performed through ICP-MS analyzed the concentrations of fourteen minerals, focus was limited to the concentrations of Cu, Mn, Se, and Zn that concentrate in the liver. The majority of the remaining minerals analyzed by ICP-MS, although found in the liver, concentrate elsewhere. Analysis of samples by ICP-MS was performed on a dry weight basis to prevent variation attributed to moisture within samples. It is important that hepatic TM are analyzed on a dry weight basis because of this particular factor. The percent moisture of homogenized and 1.0 g samples in this study tended to be within 1-3% of each other for each liver. However, 0.02 g samples appeared to vary in moisture content compared to the both other biopsy and larger samples. This inconsistency of moisture content stresses the importance of analyzing samples on a dry weight basis because not all samples, even from the same tissue, will have the same percent moisture. This can lead to erroneous analytical results because the true moisture content is not known. Although able to utilize the 0.02 g samples for diagnostic purposes in determining trace mineral status of cattle in relation to detecting deficiencies and toxicities, the biopsy method utilizing a fine needle instrument like the one in this study may not be a suitable method for research relating to nutritional studies.

Toxicities and deficiencies, even subtle at times, tend to occur when bodily TM concentrations are well beyond the interval considered to be adequate. The results of diagnostic TM analysis must be taken in the clinical context of each individual case based on signs and lesions in order to definitively diagnose such issues. Diagnostically, the goal is to garner an idea of what the TM status of the animal is. It is unclear if the difference observed is diagnostically significant, but the differences between biopsy samples and
larger 1.0 g samples may be significant as it pertains to nutritional studies that attempt to evaluate the absorption, distribution, metabolism, and excretion on the trace elements.

A portion of the results of this study suggest there is little difference in TM concentrations between 0.02 g biopsies and larger sample sizes and that such biopsy samples would be as suitable for diagnostic interpretation as larger samples. The difference in TM concentrations between biopsies and larger samples observed at 7 d of storage are confounding to results showing no significant difference in TM concentrations. A repeat of the experiment or conduction of a similar study should be considered to confirm these findings.

Additionally, utilization of a small instrument such as the one used in this study to obtain 0.02 g samples is both less invasive and less traumatizing than other procedures and techniques. In a previous study, no observable lesions correlating to ante mortem biopsies were identified in livers during post mortem harvesting and examination. Trace mineral concentrations regarding duration of storage of the sizes of samples examined does not vary following collection if samples are stored properly at 20°C. Although biopsies were initially adapted from human medicine to monitor Cu status in sheep, this sample size can be utilized across different ruminant species. Implementation of a fine needle biopsy instrument has been implemented in a previous porcine study. Properly stored sample can be kept for extended periods of time before testing is initiated as long as they are properly stored. Utilization of such a small sample requires the use of an instrument capable of collecting a sample of this size. Following operational training of the instrument, veterinary practitioners can utilize this method and sample for trace mineral analysis in clients’ cattle. There is potential for samples of 0.02 g in size to be
used in research, but further work, such as nutritional studies comparing trace mineral concentrations of both 0.02 g and 1.0 g samples taken from live animals is needed.

**Literature Cited**


