Dairy cow copper status in molybdenum rich areas

- focus on copper excretion in faeces, urine and milk

Lisa Stensson

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Faculty of Veterinary Medicine and Animal Science
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Kopparstatus hos mjölkkor i områden med molybdenrika jordar - fokus på kopparutsöndring i träck, urin och mjölk

Lisa Stensson

Supervisor:	**Cecilia Kronqvist, Swedish University of Agricultural Sciences (SLU), Department of Animal Nutrition and Management**
Assistant supervisor:	Maria Åkerlind, Växa Sverige
	Torbjörn Lundborg, Växa Sverige
Examiner:	Horacio Gonda, Swedish University of Agricultural Sciences (SLU), Department of Animal Nutrition and Management

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Swedish University of Agricultural Sciences
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Sammanfattning

Koppar (Cu) är ett essentiellt spårämne med flertalet funktioner hos idisslare men både över- och underutfodring kan ha negativa effekter på djurets hälsa och produktion. Utöver det interagerar Cu med andra mineraler i våmmen vilket minskar tillgängligheten av Cu i tunntarmen. En av de mest avgörande antagonisterna för kopparupptag är molybden (Mo) som tillsammans med svavel (S) binder till Cu och bildar olösliga komplex. Molybden överförs från jord till gräs vilket resulterar i höga molybdenkoncentrationer i foder som är skördat från molybdenrika jordar. Högplatåerna kring Falköping är ett sådant område och för att motverka den inhiberande effekten av Mo tillförs extra Cu till mjölkkofoderstater i området. Idag är leverprover den vanligaste metoden för att utvärdera kopparstatus, antingen från leverbiopsi eller från slaktade djur. En metod för att utvärdera Cu status som kan implementeras i rutinskötsel av mjölkkor i områden med molybdenrika jordar hade varit ett viktigt verktyg för att säkerställa korrekt utfodring av Cu. Målet med denna studie var därför att utvärdera och jämföra kopparstatus på mjölkbesättningar i Falköpingsområdet med höga och låga molybdenkoncentrationer i fodret. Fokus i detta masterarbete var på Cu som utsöndras via träck, urin och mjölk då dessa metoder skulle vara möjligt att genomföra som del av rutinskötsel i mjölkkobesättningar.

10 mjölkgårdar i Västra Götaland valdes ut för att delta i studien baserat på molybdenkoncentrationen i hemodlat grovfoder. 5 av gårdarna hade högt (HI) molybdeninnehåll ($\geq$ 5 mg Mo/kg ts) och 5 av gårdarna hade lågt (LO) molybdeninnehåll ($\leq$ 1.2 mg Mo/kg ts) i grovfodret. På varje gård provtogs 5 kor på träck, urin och mjölk. Proverna analyserades för kopparkoncentration. Utöver detta provtogs även allt hemodlat foder för mineralinnehåll och en endagars foderstatskontroll genomfördes för att skatta dagligt foderintag, inklusive mineraler. Kopparkoncentrationen i proverna jämfördes med intag samt analyserades för korrelationer.

Kopparkoncentrationen i både träck (FCu) och urin (UCu) påverkades av intaget av Cu, dock inte av molybdenintaget. Kopparkoncentrationen i mjölk (MCu) påverkades varken av koppar- eller molybdenintag. Inga prover visade någon skillnad mellan gårdar som var HI eller LO, troligen till följd av lägre kopparutfodring än förväntat. Träckprov har i denna studie visat sig har störst potential för att användas som ett verktyg att utvärdera kopparupptag hos mjölkkor men vidare forskning är nödvändig för att bekräfta dess korrelationer med kopparstatus.

Nyckelord: mjölkko, idisslare, koppar, molybden, antagonist, exkretion

Abstract

Copper (Cu) is an essential trace mineral with several important functions in ruminants, but both under- and over feeding of Cu can have negative effects on the animals health and production. In addition, Cu interacts with other mineral elements in the rumen, which decrease the Cu availability in the small intestine. One of the major antagonists of Cu uptake is molybdenum (Mo) which together with sulphur (S) binds to Cu, forming insoluble complexes. Molybdenum is transferred from soil to crops which results in high Mo concentrations in feed harvested from Mo rich soils. The high plateaus around Falköping, Sweden, is an area with high Mo soils. To compensate for the inhibitory effect of Mo, extra supplementary Cu is included in the rations to dairy cows in the area. At present, the most common method for assessing Cu status is analysis of liver samples, either as liver biopsy or from slaughtered animals. A method for monitoring Cu status which could be implemented into routine management of dairy herds in high Mo areas would be an important tool to ensure correct supplementation of Cu. The objective of this study was therefore to evaluate and compare Cu status in dairy herds located in the area around Falköping, Sweden, with high or low Mo concentrations in the feed. Focus in this master thesis was on Cu excreted in faeces, urine and milk as these methods could be relatively easy to implement in routine management of dairy herds.

10 dairy farms in Västra Götaland, Sweden, were chosen for the study based on the Mo levels in their home grown forage. 5 farms had high (HI) levels of Mo (≥ 5 mg Mo/kg DM) and 5 farms had low (LO) levels of Mo (≤ 1.2 mg Mo/kg DM) in their forage. On each farm 5 lactating cows were sampled for faeces, urine and milk and the samples were analysed for Cu concentration. In addition, feedstuffs produced on the farm were analysed for mineral content and a one day feeding control was performed to estimate daily feed intake, including mineral intake. Sample Cu concentrations were compared with intake and analysed for correlations.

Both faecal (FCu) and urine (UCu) Cu concentration was affected by intake of Cu but not intake of Mo. Milk Cu concentration (MCu) was not affected by intake of either Cu or Mo. In none of the samples a difference between HI or LO farms could be observed, probably because of lower Cu supplementation than expected on HI farms. Faecal samples had the highest potential for being a useful tool in monitoring of Cu status but further research is needed to confirm its correlations with Cu status.

Keywords: dairy cow, ruminant, copper, molybdenum, antagonist, excretion

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Abbreviations

ADG	Average daily gain
BW	Body weight
Ca	Calcium
Co	Cobalt
Cu	Copper
DM	Dry matter
Fe	Iron
FCu	Cu concentration in faecal samples
HI	High levels of Mo in forage (≥ 5 mg Mo/kg DM)
HOL	Swedish Holstein
I	Iodine
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-SFMS	Inductively Coupled Plasma Sector Field Mass Spectrometry
JER	Swedish Jersey
K	Potassium
LO	Normal/low levels of Mo in forage (≤ 1,2 mg Mo/kg DM)
MCu	Cu concentration in milk samples
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
Na	Sodium
NASEM	The National Academies of Sciences, Engineering and Medicine
NIR	Near Infrared Reflectance
NRC	National Research Council
P	Phosphorus
RDC	Swedish Red and Swedish Ayrshire Cattle
S	Sulphur
SD	Standard Deviation
Se	Selenium
SKB	Swedish Polled Cattle
SNDRS	Swedish National Dairy Herd Recording Scheme

TM	Thiomolybdates
UCu	Cu concentration in urine samples
Zn	Zink

1. Introduction

Copper (Cu) is a trace mineral with an important role in several physiological functions in the ruminant, such as synthesis of blood cells, enzymes and antioxidants (NASEM 2021; Suttle 2022). Copper deficiency in cattle have been shown to relate to reduced fertility, growth and immune function as well as various clinical symptoms, for example anemia, if severe hypocuprosis occurs (Suttle 2022). In addition, Cu have toxic properties and long term supply of excess Cu can lead to liver damage, haemolysis and even death from Cu toxicosis (National Research Council 2005).

The bioavailability of Cu in the small intestine of ruminants is low, approximately 5 % of ingested Cu, because of interactions with other minerals in the rumen, leading to a reduced supply of available Cu to the intestine (López-Alonso & Miranda 2020). The most substantial inhibitory effect of Cu absorption is caused by molybdenum (Mo) in combination with sulphur (S) which forms the complex molecule thiomolybdate (TM) and bind dietary Cu. Other minerals, for example iron (Fe) and zinc (Zn) have similar effect (Spears 2003; Espinoza et al. 2012; Minervino et al. 2018). These interactions in the rumen makes feeding of Cu complex and Cu antagonists in the feed need to be considered in ration formulation.

Previous studies have shown that the transfer of Mo from soil to grass is linear, meaning that in areas with Mo rich soils the concentration of Mo in home grown feed is generally high (Axelson et al. 2018; Lätt 2019). The high plateaus in Skaraborg, Sweden, is an area with high Mo in the soil and the farmers and advisors in the area have a history of dealing with the issue of increased Cu supplementation to avoid deficiency. At present, the recommended Cu:Mo ratio in feed to counteract the inhibitory effect of Mo and avoid Cu deficiency is 3-4:1 (Lundborg 2023). In the region around Falköping, Sweden, there are farms with Mo concentrations far above the average in their home grown feed and they therefore supplement with additional Cu to reach an adequate Cu:Mo ratio. By doing so, the risk of Cu deficiency in dairy herds is minimized but there could possibly be a risk of exceeding maximal inclusion of Cu in the diet. When absorbed above the requirement, Cu is stored in the liver until saturation in the hepatocytes occurs, after which excess Cu can be excreted via the bile (López-Alonso & Miranda 2020). Although there are mechanisms to regulate Cu excretion, these mechanisms are limited. Too high compensatory inclusion of Cu in the diet could therefore possibly have a negative impact on both animal health and production.

Today, there is no routine method for assessing Cu status in dairy cows in use on farm level. The existing methods are liver samples taken after slaughter and liver biopsy, which both have their limitations (Puls 1988). Collecting samples after slaughter, although giving an indication of the overall status of the herd, does not provide information about status of an individual dairy cow in production. Using biopsy as sampling method, in addition to being an invasive procedure on the animal, entails a risk of the sample not being representative because of uneven storage of Cu in the liver (Miranda et al. 2010). The concentration of accumulated Cu in the liver can also show a large variation within the herd (Grace et al. 2010), increasing the risk of incorrect conclusions on herd Cu status from liver samples. A simple method of monitoring Cu status which could be implemented into routine management of dairy herds in risk prone areas would be an important tool to ensure correct supplementation of Cu. As milk analysis, and to some extent analysis of faeces and urine, is already in use on most dairy farms there is potential to extend the area of use to include monitoring of Cu status as well.

1.1 Objectives

The primary objective of this study was to evaluate and compare Cu status in dairy herds located in the Västra Götaland area with high or low Mo concentrations in the feed, resulting in a diet with high levels of both Mo and supplemented Cu to compensate for the inhibitory effect of Mo. By doing so I hope to find answers to the following research questions:

- How is Cu excretion distributed in faeces, urine and milk and how well does this reflect the Cu intake of the animal?
- Is there a difference in Cu excretion depending on Mo concentrations in the feed?
- Would analysis of Cu concentrations in faeces, urine or milk be an effective tool to monitor Cu status on dairy cows in production?

2. Literature review

2.1 Physiological functions of copper

Copper is an essential trace mineral with an important role in many different functions of the body. One of these is through Cu dependent enzymes which drives several physiological processes such as cellular respiration, iron (Fe) transport for synthesis of red blood cells, formation of connective tissues and pigmentation of hair and wool (Suttle 2022). In addition to enzyme formation, Cu interacts with antioxidants and takes part in protecting the cells from free radicals and oxidative stress (Suttle 2022). Copper also seem to have an important role in normal growth, fertility and immune function although the exact mechanisms are not yet clear (National Research Council 2005; Suttle 2022).

2.2 Copper metabolism in cattle

Copper metabolism is governed by a complex system in the gastro intestinal tract, blood circulation, liver and other tissues in the body. In ruminants, Cu absorption is low but can range from <1 – 10 % of ingested Cu depending on several factors (National Research Council 2005).

2.2.1 Copper metabolism in the rumen

The main factor affecting Cu absorption is interactions occurring in the rumen. In studies on young lambs the availability of Cu decreased from approximately 47.2 % before weaning to 10.8 % 15 days after weaning, indicating a significant effect on Cu availability from ruminal processes (Suttle 1975a).

In the rumen, other dietary components interact and bind to Cu, hence decreasing the availability. Molybdenum in the feed have a key role in this through formation of the molecule thiomolybdate (TM) (Suttle 2022). TM have long been known to have an effect on Cu metabolism and injections of TM have been used as a tool to treat Cu toxicosis in sheep (Van Ryssen 1994). The formation of TM occurs in the rumen through a reaction between molybdate and sulphide which bind to free Cu creating very strong insoluble complexes (Suttle 1991; Spears 2003). The most significant effect of TM takes place in the rumen although evidence suggest that the molecule can be absorbed and bind to Cu on a cellular level as well (Suttle 1991). Inside the cell TM can bind to Cu already bound to transport molecules or stored in the cell, increasing the biliary excretion of Cu. In addition, the molecule can enter the blood circulation and bind Cu carried by plasma proteins, making it unavailable for absorption from the blood into the cells of other tissues. In beef cattle fed a diet containing high levels of Mo (10 mg/kg DM) and S (5 g/kg DM) a significant increase in Cu excretion was seen in both bile and urine in comparison to the control diet of 1 mg Mo/kg DM and 2 g S/kg DM, indicating a decreased Cu availability with increased intake of dietary Mo and S (Gooneratne et al. 1994, 2011). In these studies, when cows were fed a diet with high dietary Mo but low S, no difference in Cu concentration in bile and urine compared to the control diet was observed. These results agree with previous studies arguing that the inhibitory effect of Mo on Cu absorption depends on dietary S (Suttle 1975b). In a study on sheep fed a low concentration of S (1 g/kg diet) an increase of Mo in the diet from 0.5 to 4.5 mg/kg diet had no significant effect on the availability of Cu (Suttle 1975b). Sulphur on the other hand, have the ability to decrease Cu availability without available Mo through the formation of insoluble copper sulphides (Spears 2003; López-Alonso & Miranda 2020). Even so, this inhibitory effect is estimated to be less significant than that of TM (Gooneratne et al. 1994, 2011; Spears 2003). The amount of S available in the rumen can also be effected by several other factors apart from intake of dietary S, making its effect more unpredictable (López-Alonso & Miranda 2020). When the ruminal pH is lowered due to for example a starch rich diet, the microbial degradation of S compounds increases, resulting in a higher availability of sulphides (López-Alonso & Miranda 2020).

In addition to Mo and S, other metals such as Fe and Zn can affect Cu metabolism and absorption through several pathways (Clarkson et al. 2020; López-Alonso & Miranda 2020). Like Mo, Fe can together with S form complexes with Cu in the rumen (López-Alonso & Miranda 2020). The Cu-Fe interaction is not additive to TM because of the competition of available S, which inclines that it does not provide additional inhibitory effect on Cu availability if dietary Mo is high (Suttle 2022). Copper absorption can also be inhibited by metals, for example Zn, through competition of binding sites to metal transporters in the cell and on the cell membrane (Espinoza et al. 2012; López-Alonso & Miranda 2020), although, it seems as very high Zn concentrations need to be present in the feed to be able to observe a clear effect on Cu status. In sheep fed a diet with supplementary Cu of 450 mg/kg DM and Zn of 35, 150 and 300 mg/kg DM a significant decrease in hepatic Cu as well as biliary Cu excretion was observed only in the group supplemented with 300 mg Zn/kg DM (Minervino et al. 2018).

The availability of Cu can differ between different feed types because of the effect of nutritional composition on ruminal fermentation and pH and in turn its effect on the antagonistic processes in the rumen (McCaughern et al. 2020; Suttle 2022). In silage Cu availability is approximately 5 %, which is higher than in pasture but lower than in hay (Suttle 2022). See average mineral concentrations in forage in table 1.

Table 1. Mineral concentrations in grass and legume forage in Sweden. Low = lowest 25 %, average = mean, and high = highest 25 %. Copper (Cu), molybdenum (Mo), iron (Fe) and zinc (Zn) expressed in mg/kg DM and sulphur (S) expressed in g/kg DM. Values from (Lätt 2019).

Mineral	Low	Average	High
Cu	< 5.6	7.0	> 7.8
Mo	< 0.9	1.9	> 2.3
S	< 1.8	2.1	> 2.4
Fe	< 93	190	> 201
Zn	< 25	34	> 35

2.2.2 Cu trafficking

In the small intestine, before being absorbed into the enterocytes, Cu^{2+} is reduced to Cu^{+} and bound to transporters in the intestinal villi. Two types of transporters are responsible for Cu transportation into the cell, of which one, is Cu specific and one can transport all metal ions (Clarkson et al. 2020; López-Alonso & Miranda 2020). Cu in its free ionized form is highly reactive and can cause cellular damage. As a result several Cu transporters exist to safely traffic Cu through the cells (La Fontaine & Mercer 2007). Once transported into the cytosol of the enterocyte Cu chaperone proteins transport Cu to different sites in the cell, one of them being the golgi apparatus. In the golgi, Cu is used to synthesise several enzymes and proteins and excess Cu is bound to the protein metallothionein which stores Cu in the lysosomes (Clarkson et al. 2020). Once saturation occurs in the enterocyte a Cu carrier protein transports Cu to the cell membrane and into the blood circulation where it is bound to plasma proteins (López-Alonso & Miranda 2020). Cu is then transported in the blood to the liver.

Once in the liver similar events as in the intestine occurs. Cu is reduced, transported into the hepatocyte, bound to Cu chaperone proteins and transported to the golgi (Clarkson et al. 2020; López-Alonso & Miranda 2020). In the hepatocyte golgi, Cu trafficking can take two routes. It can either be bound to metallothionein and stored in the lysosome or incorporated into caeruloplasmin (cp), which is the main Cu transporter in the blood (López-Alonso & Miranda 2020). Cp bound Cu is released back into the blood circulation where it is distributed to the body tissues (López-Alonso & Miranda 2020). Cp which returns to the liver, and thus have not been absorbed into the body tissues, is absorbed and transported via the golgi, proteolysed and excreted into the biliary route (Clarkson et al. 2020). This way excess Cu is excreted from the liver to the bile. In addition to being excreted in the bile Cu can, while the blood passes through the kidneys, be filtered to the urine and excreted (Sjaastad et al. 2010).

As total copper concentration in the hepatocyte increases, as a result of increased dietary intake, the proportion of stored Cu in the cell shifts from the lysosome to the cytosol and nucleus, indicating maximum storage capacity of the lysosome has been reached (López-Alonso et al. 2005). In ruminants, the binding of Cu to metallothionein and storage capacity in the lysosomes are limited. In the cytosol and nucleus of the hepatocyte, free Cu can lead to intracellular damage and eventually cell death, resulting in damage of the liver (López-Alonso & Miranda 2020). When instead there is insufficient supply of Cu to the liver the reserves in the hepatocyte gets depleted and the Cu concentration in the blood is reduced. This leads to an inadequate Cu supply to Cu dependent enzymatic processes in the body, for example iron transportation and synthesis of connective tissues (Suttle 2022). The liver Cu concentrations to indicate Cu toxicity is thought to be approximately 300 – 400 mg/kg DM (National Research Council 2001, 2005; NASEM 2021; Suttle 2022).

2.2.3 Pathways for copper excretion

Copper in faeces

The Cu present in faeces originates from several different pathways. The main proportion, on average 95 % of ingested Cu, is in an indigestible form and have been passed through the gastro intestinal tract without being absorbed (Buckley 1991). After absorption, the main pathway for Cu excretion is through the bile (Buckley 1991; Gooneratne et al. 2011). This consists of either excess absorbed Cu or released Cu from storage in the liver. Once excreted in the bile Cu is not reabsorbed in the small intestine due to its chemical form (National Research Council 2005). The final part of the Cu in faeces is Cu bound to metallothionein in dead enterocytes from normal intestinal cell turnover (National Research Council 2005). The exact ratios from the different pathways is not established but (Buckley 1991) suggests 62 % of Cu entering the liver is excreted through the bile which would mean that approximately 2.85 % of total ingested Cu exits via the biliary route (Table 2).

To some extent biliary excretion of Cu can increase with increased supply of dietary Cu to avoid overload in the liver. In cattle, an increase in both biliary flow and Cu concentration in the bile has been observed when shifting the diet from 5 mg Cu/kg DM to 40 mg Cu/kg DM (Gooneratne et al. 1994). However, in comparison to non-ruminant species, the capacity for excretion of excess Cu through bile is limited. This makes ruminants more sensitive to Cu toxicity due to oversupply of dietary Cu (National Research Council 2005; López-Alonso & Miranda 2020). Gooneratne et al. (1994) also saw that Cu concentration in bile increased significantly from 0.08 to 0.24 mg Cu/L when changing from a high Cu diet (5 mg Cu/kg DM, 1 mg Mo/kg DM and 2 g S/kg DM) to a high Cu, Mo and S diet (40 mg Cu/kg DM, 10 mg Mo/kg DM and 5 g S/kg DM). This shows that Cu intake, as well as intake of Mo and S, have an effect on Cu concentration in bile and by extension in the faeces.

Copper in urine

The urinary pathway is a relevant route for Cu excretion, although it seems to be less significant than the biliary pathway (Gooneratne et al. 2011). In comparison to the Cu concentrations in bile, the urine contains very low concentrations of Cu (Buckley 1991). In a study by Buckley (1991) of Cu metabolism in dairy cows, Cu concentration in the majority of urine samples were below the detection level of 0.024 mg Cu/L and assessed to be approximately 0.075 % of ingested Cu. Gooneratne et al. (2011) observed a slightly higher urinary Cu concentration which reached 0.081 mg Cu/L when fed a low Cu diet of 5 mg Cu/kg DM which increased numerically but non-significantly to 0.099 mg Cu/L when fed a high Cu diet of 40 mg/kg DM. Chase et al. (2000) deemed Cu excretion in urine as insignificant as it in their trial corresponded to less than 1 % of total excreted Cu.

Like in bile, diet seems to have an effect on Cu concentration in urine. In the study by Gooneratne et al. (2011) a significant increase in urinary Cu from 0.081 mg/L to 0.116 mg/L was observed when changing from a low Cu, Mo and S diet (5 mg Cu/kg DM, 1 mg Mo/kg DM and 2 g S/kg DM) to a high Cu, Mo and S diet (40 mg Cu/kg DM, 10 mg Mo/kg DM and 5 g S/kg DM). No significant change in urinary Cu excretion was seen with the diets of high Mo or S alone.

Copper in milk

The concentration of Cu in milk from ruminants is generally low (Buckley 1991; Castillo et al. 2013; Suttle 2022). In a study on 39 dairy farms the average concentration of Cu in milk was 0.036 mg Cu/kg (Castillo et al. 2013), which agrees with the concentration in milk used to calculate dairy cow Cu requirement (Table 3) (NASEM 2021). The concentration of Cu in milk could also have seasonal and lactational variations (Wang et al. 2014).

Research suggests that dietary Mo have little effect on Cu concentration in milk. Beef cattle fed a diet of 0, 20 or 40 mg Mo/kg DM had no significant decline in milk Cu, even though a reduction in plasma Cu and Cp activity could be observed (Wittenberg & Devlin 1987).

Table 2. Pathways and distribution of ingested copper (Cu). Based on model by Buckley (1991).

Pathway		% Cu from pathway	% of total ingested Cu
Ingested Cu in feed to			
	Absorbed	5	5
	Indigestible (faeces)	95	95
Absorbed Cu to			
	Liver	92	4.6
	Urine	1.5	0.075
	Milk	3.5	0.175
	Circulation (Cu^{2+}*)	3	0.15
Liver to			
	Circulation (Cu^{2+}cp**)	38	1.75
	Bile (faeces)	62	2.85
Circulation (Cu^{2+}cp) to			
	Milk	19	0.33
	Body	81	1.42
Total % of ingested Cu in			
	Faeces		97.85
	Urine		0.075
	Milk		0.51

* Free Cu, before entering liver.

** Cu bound to caeruloplasmin (cp), after being released from liver.

2.3 Requirements and dietary recommendations

2.3.1 Requirements of copper and molybdenum

Table 3. Requirements and maximum tolerance of copper and molybdenum in lactating dairy cows. Cells with "-" is not stated in the referenced literature

Reference	Requirement Cu	Max tolerance Cu	Max tolerance Mo	Cu:Mo ratio
Suttle[1]	6 mg/kg DM	50 – 300 mg/kg DM	Mainly Cu:Mo ratio	>2:1 – 3:1
INRA[2]	10 mg/kg DM	-	-	-
NASEM[3]	8 - 10 mg/kg DM	20 – 40 mg/kg DM	-	-
NRC[4]	11 mg/kg DM	40 mg/kg DM	10 mg/kg	3:1 - 20:1
CVB[5]	11.1 mg/kg DM	-	-	-

[1] Based on DMI. <1,5 mg Mo/kg DM in forage (Suttle 2022).

[2] Based on DMI (Institut national de la recherche agronomique et al. 2018).

[3] 650 kg mature Holstein cow, 8 mg/kg DM in mid lactation and 10 mg/kg DM in peak and late lactation *(NASEM 2021).*

[4] 680 kg mature Holstein cow. 0,15-0,25 % DM S and 1-2 mg Mo/kg DM in TMR (National Research Council 2001).

[5] Based on DMI. Mature dairy cow, 40 kg milk/day *(Centraal Veevoederbureau 2022).*

Requirements for copper

Different references states slightly different Cu requirements for dairy cattle (Table 3). The National Academies of Sciences, Engineering and Medicine (2021) (NASEM, previously NRC) states that the requirements for a holstein dairy cow of 650 kg body weight (BW) which produces 35 kg milk/day will be met with an intake of 8 – 10 mg Cu/kg DM depending on stage of lactation (NASEM 2021) which is a slight reduction from the recommendations of 11 mg Cu/kg diet from 2001 (National Research Council 2001) and which is still recommended by CVB (Centraal Veevoederbureau 2022). The recommendations set by NASEM (2021) corresponds to the 10 mg/kg DM recommended by INRA (Institut national de la recherche agronomique et al. 2018). Suttle (2022) on the other hand recommends a lower dietary Cu concentration of minimum 6 mg Cu/kg DM in forage.

According to Suttle (2022) requirements for minerals should be calculated solely on dry matter intake (DMI) instead of BW. Suttle (2022) argues that the Cu content in milk and the additional Cu needed to support the foetus during gestation is very low and does not make a significant difference in the total dietary need of Cu. NASEM (2021) on the other hand does provide individual formulas for calculating the Cu requirement for maintenance, growth, gestation and lactation, where requirements for maintenance and gestation is based on BW (Table 4).

Table 4. Formulas for calculating Cu requirements for dairy cattle (NASEM 2021). Average daily gain (ADG) and milk is based on kg/day and body weight (BW) on kg.

Requirement for:	Formula daily Cu requirement (mg Cu)
Maintenance	0.0145 x BW
Growth	2.0 x ADG
Gestation (90-190 days)	0.0003 x BW
Gestation (>190 days)	0.0023 x BW
Lactation	0.04 x milk

Setting an exact maximum tolerance level for Cu in cattle is complex as several factors affect the absorption of Cu. It is somewhat agreed that exceeding 40 mg Cu/kg DM increases the risk of copper toxicity in cattle if antagonists in the feed is low (National Research Council 2001, 2005; NASEM 2021; Suttle 2022). However, in presence of high levels of antagonists in the diet the tolerance can be significantly higher due to a lower Cu absorption.

Requirements and tolerance for molybdenum

Molybdenum is an essential mineral for the synthesis of several enzymes but no minimum requirement is established based on existing research (NASEM 2021). Symptoms of Mo deficiency is unknown and has not been observed in any animal species. However, there is evidence that Mo toxicity is a problem easily misinterpreted as Cu deficiency (Suttle 2022). Maximum tolerable levels of Mo is not yet clear although decreased fertility and growth have been observed as Cu status was reduced through a diet of 5 mg Mo/kg DM when the same decline in Cu status caused by other antagonists had no effect (Phillippo et al. 1987a; b). Usually maximum levels of Mo is expressed in relation to Cu levels in the feed. When the Cu:Mo ratio is below 3:1 the risk of copper deficiency related disorders increases (López-Alonso & Miranda 2020; Suttle 2022).

2.3.2 Dietary recommendations

In some areas of Västra Götaland, where there is an existing issue of high levels of Mo in the soil and thus in the feed, the advisors use the recommendation of having a Cu:Mo ratio of 4:1 in the feed rations (Lundborg 2023). This recommendation is based on literature which states that having a ratio of less than 3:1 increases the risk of Cu deficiency (Eriksson 2005; López-Alonso & Miranda 2020; Suttle 2022). In contrast, the inhibitory effect of Mo on Cu absorption seems to reach a plateau at which it does not have additional effect. In sheep fed various diets with Mo levels varying from 0 to approximately 24.4 mg Mo/kg DM the Cu absorption had stabilized at 1 % in all types of feed at 8 mg Mo/kg DM and higher (Suttle 1983). This is perhaps a result of depletion of available S to form TM in the rumen

2.3.3 Imbalanced copper supplementation

Copper deficiency

When fed a diet with inadequate levels of Cu, or if Cu absorption is low due to presence of antagonists, the Cu stores in the cells can be depleted resulting in an inadequate supply to the tissues of the body. Copper deficiency, or hypocupraemia, can have several effects on the animals health and production but clinical symptoms require relatively severe deficiency. A first symptom is change in hair structure and dullness in hair colour, followed by decreased immune function, ataxia and skeletal abnormalities (Suttle 2022). Cu deficiency have been thought to cause reduced fertility and growth but studies have later shown that this effect is actually caused by Mo or thiomolybdate toxicity (Phillippo et al. 1987a; b).

Copper toxicity

Cu toxicosis can occur as acute or chronic. Acute toxicosis is caused by a single high dose of Cu and cause severe toxicological symptoms such as vomiting, excessive salivation, convulsions and even death. In cattle the dose needed to cause acute toxicosis is 200 mg Cu/kg BW, which corresponds to 120 g Cu for an average dairy cow with a BW of 600 kg (National Research Council 2005). The high required dose makes acute Cu toxicosis rare.

Chronic Cu toxicity, which is more common, is caused by long term exposure to dietary Cu levels which exceeds the requirements of the animal (National Research Council 2005). As previously mentioned, excess Cu is safely stored bound to metallothionein in the lysosome of the hepatocytes. As saturation levels of the lysosome is reached, free Cu will start accumulating in the cytosol and nucleus (López-Alonso et al. 2005). This accumulative state is characterized by increased liver Cu and liver necrosis but not necessarily clinical symptoms (National Research Council 2005). If the animal with high liver Cu is exposed to stress a haemolytic crisis can occur. During a haemolytic crisis Cu is rapidly released from the hepatocyte into the blood causing severe toxicological symptoms such as diarrhoea, vomiting, convulsions and even death due to blood cell damage (National Research Council 2005).

3. Material and methods

3.1 Study design

Data collection for this study occurred on 10 dairy farms, all in the Västra Götaland region in Sweden in January 2023. Farms were chosen based on the level of Mo in their home grown forage. This study was conducted as a case-control study and five farms were therefore chosen with high levels of Mo in home grown forage and five farms as a control with normal levels. High levels (HI) was in this study set to ≥ 5 mg Mo/kg DM and normal levels (LO) to ≤ 1.2 mg Mo/kg DM based on previous data (Lätt 2019). On each farm, samples were collected from five lactating dairy cows of various breeds. On each farm lactating dairy cows were sampled for milk, urine and faeces. In addition, all cows were measured for chest girth to assess their individual body weights. The majority of cows in this study was of the breed Swedish Holstein (HOL) but other breeds included was Swedish Red and Swedish Ayrshire Cattle (RDC), Swedish Polled Cattle (SKB), Swedish Jersey (JER) and crosses between these breeds. All sampling methods were tested before data collection to minimize sampling error.

3.1.1 Contact with farmers

In the present study, participating dairy farmers were all members and customers of Växa Sverige. First contact with the farmers was made by a consultant working for Växa Sverige during which the farmers agreed to participate in the study. After this initial contact remaining communication was made by me and my co-worker, together with whom sampling and practical components of this study was carried out. 2 – 3 weeks before sampling telephone contact with the farmers was initiated to confirm and provide more information about the project. Approximately 1 week before the farm visit an email was sent to all farmers with detailed information and identification numbers of the individual cows chosen for sampling. Warrants to use farm data from the Swedish National Dairy Herd Recording Scheme (SNDRS) was provided by Växa Sverige and signed by the farm owner.

3.1.2 Animal selection

From each farm 10 cows were randomly selected using Microsoft Excel. Lists of all cows on each farm was provided by a consultant from Växa Sverige. Each individual cow from 1 - 250 days in lactation was provided with a number. The selection was limited to maximum 250 days in lactation to ensure all cows selected would be lactating during the sampling period. The excel function =RANDBETWEEN was used to get a random selection of 10 numbers matched with the numbers assigned to the cows. 5 of these cows were selected for sampling on farm based on which were able to be located and fixated first. The reason for selecting 10 cows were to have extra individuals in case a cow had been culled, sold or dried off before our visit or if we encountered any issues during sampling.

3.2 Sampling methods

3.2.1 Faeces

Faeces were collected through four different methods depending on events during sampling. If a cow defecated spontaneously this was the preferred method to avoid invasive sampling methods. If this was not possible we entered the anal opening to stimulate the ventral side of the spine which activates emptying of the bowel. If this was not effective, we did a manual rectal collection. If a cow defecated spontaneously while we were occupied with sampling another cow and therefor was not able to collect the sample, we took a small sample from the top of the faecal pile immediately after defecation. This method of sampling was only used if we assessed that faeces could be collected without risk of contamination. Independent of sampling method, approximately 100 – 200 ml of faeces was collected in a 90 cm long rectal examination glove which was tied and labelled with the ID-number of the cow. When all sampling was complete, faeces was transferred from the examination glove to two 50 ml sampling containers per sample, labelled with animal ID and farm ID. Samples were stored in -18°C until analysis.

3.2.2 Urine

Urine was collected either when the cow urinated spontaneously or was manually induced by stroking the skin underneath the vulva. To avoid contamination of the sample it was collected mid-stream when possible. The urine sample was collected in a 250 ml cup and immediately transferred to two 50 ml test tubes per sample labelled with animal ID and farm ID. Samples were stored in -18°C until analysis.

3.2.3 Milk

Milk samples was in nine of the ten farms made by the farmer in a period of maximum five days before and 14 days after the farm visit. On one of the farms we assisted with sample collection. The samples were collected from an entire milking to ensure a representative sample (Bermejo-Barrera et al. 1997). On seven of the ten farms the farmers separated the milk from an entire milking in the robot into a separate container and collected the sample from the separated milk. On the remaining three farms the farmers took the sample in connection to a regular test milking. Those samples contained bronopol, a preservative used when taking routine milk samples on farm, which after research was concluded to not interfere with the Cu content in the milk or effect our analytical results. After collection, the milk samples were stored cold, alternatively frozen if pick up would be later than 5 days after sampling. Samples were then stored in -18°C until analysis.

3.2.4 Feed

Feed samples were collected from both silage and grain produced on the farm. Individual samples were collected from the different batches used in the TMR fed to the lactating cows at the time before and during the farm visit. Silage stored in bunker silos were sampled by drilling from the top down through all the layers of feed. Silage stored in bales were sampled by drilling from the side and straight in to the middle of the bale. The selected methods was used to ensure a representative sample. Samples of the grain mixture were taken from the silos, either vertical or bunker silo, on all farms. Samples of silage was stored in -18°C and grain mixtures in room temperature for maximum 5 days until sent for analysis.

3.3 Analytical methods

3.3.1 Sample preparation and analysis

Faeces, urine and milk

Samples of faeces, urine and milk were sent frozen for analysis to ALS Global (Luleå, Sweden). All samples were analysed for Cu concentration using isotope analysis, inductively coupled plasma sector field mass spectrometry (ICP-SFMS) (*ICP laboratorium | ALS Scandinavia* n.d.). Faeces was also analysed for DM. Measure uncertainties for analysis of Cu concentration was in urine 14 % and in both faeces and milk 10 %.

Feed

Both silage and grain mix was sent for analysis to Eurofins Agro Testing Sweden (Kristianstad) and was tested for full nutritional composition including the 14 macro- and micro minerals calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), sodium (Na), S, cobalt (Co), Cu, iodine (I), Fe, manganese (Mn), selenium (Se), Zn and Mo. Nutritional composition was analysed using Near Infrared Reflectance (NIR) based on reference methods recommended by NorFor (Volden 2011). Mineral content was analysed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Nutritional composition, including minerals, of commercial feed was acquired from NorFor and the feed companies.

3.3.2 One-day feeding control

Daily mineral intake was calculated through a one-day feeding control. All information on feed composition and the quantities fed daily to the lactating cows was provided from the farmers. In addition, the farmers provided us with the number of animals in the lactating dairy cow group, including any dry cows or heifers consuming the same feed ration. Average body weights were calculated using the values for chest girth acquired at the farm visits and used in the one day feeding controls. See table 5 for equations used to calculate body weights. Average daily nutrient intake, including minerals, was calculated in the one-day feeding control function in Typfoder (Växa Sverige; NorFor Plan 1.24.0.690, FST version 2.09, FRC version 2.13, OFC version 1.41). Faecal output was also estimated in the one-day feeding control as undigested feed.

Table 5. Equation used to calculate body weights from values for chest girth.

Breed	Equation for calculating body weight	Reference
Holstein (HOL) incl. crosses*	kg = (167.5 – 3.7682cm) + $0.0295cm^2$	Coburn (2000)
Swedish Red and Ayrshire (RDC) incl. crosses	kg = (239.2 – 4.4cm) + $0.031cm^2$	Pönniäinen (1989)

*HOLxRDC was included in HOL

An estimation of daily Cu absorption as g absorbed Cu/g of total ingested Cu based on levels of Mo and S in the feed ration was calculated in Typfoder using the following equation by Suttle & Mclauchlan (1976):

$$\text{Cu Absorbtion} = 10^{(-1.153 - 0.0019 \text{ x Mo} - 0{,}076 \text{ x S} - 0.0131 \text{ x S x Mo})}$$

Where Mo is mg Mo/kg DM and S is g S/kg DM in the feed ration.

Total daily Cu excretion through faeces was calculated using the analysed values for Cu concentration in faeces (mg Cu/kg faecal DM) and the estimation of total daily faecal output per cow generated in Typfoder. This estimation of faecal production is calculated as the remainder after total tract digestibility estimated by NorFor (Volden 2011).

Daily urinary excretion of Cu was calculated using the analysed values for Cu concentration in urine (mg Cu/kg) with the assumption of the urine specific gravity being 1.030 kg/L (Megahed et al. 2019) and an estimation of total daily urinary output per cow. This estimation was made using the following equation by Bannink et al. (1999):

$$\mathrm{Uvol} = 0{,}1153\mathrm{I}_{\mathrm{Na}} + 0{,}0577\mathrm{I}_{\mathrm{K}}$$

Where U_{vol} = daily urine volume produced per cow in kg/d, I_{Na} = daily intake of Na per cow and I_K = daily intake of K per cow, both expressed in g/d. Urine volume is highly dependent on mineral intake, especially intake of Na and K, which is the reason for using this equation (Bannink et al. 1999).

Daily Cu excretion through milk was calculated using the analysed values for Cu concentration in milk (mg Cu/kg) with the assumption of the milk specific gravity being 1.030 kg/L (Parmar et al. 2021) and individual daily milk yield. Data on individual milk yield was taken from existing herd data from the test milking closest in time to our farm visit. On 9 of the 10 farms this test milking was performed in January 2023. On one farm no test milking was performed in January and data from February 2023 was used instead.

3.4 Statistical analysis

Initial simple correlations and associations between variables were visualized using scatter plots and t-test in Microsoft Excel (2016). The excel function =AVERAGE was used to calculate mean values and =STDEV.S to calculate standard deviations (SD). Further statistical analysis on relevant correlations between Cu concentrations in samples and daily intake of Cu and Mo as well as correlations between the three sampling methods and interactions between variables was performed using the statistical programme R (4.2.3). Linear regression models with fixed factors and two way interactions was used. Distribution of variation in the outcome variables between and within herds was analysed in the statistical programme SAS (9.4), using a mixed model with herd as random factor.

Level of significance was in all models set to $p < 0.05$.

4. Results

Herd size on the farms in this study were between 52 and 320 lactating cows. All farms had the cows in loose housing systems. The cows were milked in automatic milking systems or in a parlour. Three of the farms had organic production, all of which were in the HI group. See appendix 2 for individual milk yield and estimated faecal and urine output.

4.1 Feed composition

Feed rations had a Cu concentration from 9.8 to 18.6 mg/kg DM and Mo concentration from 0.39 to 7.65 mg/kg DM (Table 6). The S concentrations were from 1.4 to 2.9 g/kg DM.

As expected, the cows from HI farms had a higher daily Mo intake than cows from LO farms ($p < 0.001$). The Cu intake was higher in HI farms than in LO farms ($p = 0.05$) although the Cu:Mo was lower than expected in some of the HI farms (Table 6). HI farms had a lower Cu:Mo ratio than LO farms ($p < 0.001$) which was mainly caused by the difference in Mo concentration (Table 6).

From the feed rations in this study, a negative correlation between Mo in feed and S in feed could be observed ($p < 0.001$), meaning that in the feed ration from farms with higher dietary Mo, the dietary S was generally lower.

Table 6. Composition of the feed ration, including relevant minerals (copper (Cu), molybdenum (Mo), sulphur (S), iron (Fe) and zinc (Zn)), fed to the lactating cows on the ten farms included in the study. Home grown % includes both roughage and grain mix produced on the farm on a DM basis. Farm 1 to 5 was in the LO group (≤ 1.2 mg Mo/kg DM in forage) and 6 to 10 in the HI group (≥ 5 mg Mo/kg DM in forage).

Nutrient	Unit	Farms									
		1	2	3	4	5	6	7	8	9	10
Feed intake	kg DM/d	24.6	27.5	27.1	26.6	26.2	18.4	23.6	24.8	28.1	23.3
Roughage	%	54.7	59.0	58.8	48.1	50.4	61.0	63.4	56.3	61.2	72.7
Home grown	%	85.1	87.9	83.5	83.0	89.9	82.9	89.4	80.1	79.7	89.4
Net energy	MJ/kg DM	6.93	6.50	6.77	6.97	6.58	7.18	6.34	6.86	6.74	4.50
Crude protein	g/kg DM	184	173	172	173	170	172	166	154	184	136
Cu	mg/kg DM	13.2	12.4	16.4	9.8	11.3	15.7	10.9	18.6	16.5	9.8
Cu intake	mg/d	325	339	444	261	296	289	259	462	463	227
Mo	mg/kg DM	0.67	0.39	0.83	0.81	0.53	3.93	7.65	6.16	2.66	6.93
Mo intake	mg/d	16	10	22	20	14	72	181	153	75	161
Cu:Mo		20:1	32:1	20:1	12:1	21:1	4:1	1.4:1	3:1	6.2:1	1.4:1
Cu absorption*	%	4.5	4.7	4.1	4.3	4.1	3.7	3.1	3.9	3.8	4.1
S	g/kg DM	2.3	2.1	2.6	2.5	2.9	2.1	1.9	1.6	2.4	1.4
Fe	mg/kg DM	94.9	114	171	237	196	201	156	242	462	158
Zn	mg/kg DM	64.1	55.6	85.1	60.5	50.0	77.7	49.1	46.1	77.7	39.5

*(Suttle & Mclauchlan 1976)

4.2 Sample Cu content

Table 7. Mean copper (Cu) concentration in faeces, urine and milk from all ten farms included in the study and mean Cu concentration in LO (farm 1 - 5) and HI (farm 6 - 10) farms. Standard deviation (SD) for all mean values included. P-values for difference in Cu concentration in samples from HI or LO farms. Complete results from analysis in appendix 1.

	Faeces (mg/kg DM)		Urine (mg/kg)		Milk (mg/kg)	
Farm	Mean	SD	Mean	SD	Mean	SD
1	60.1	7.11	0.018	0.006	0.031	0.020
2	72.6	19.8	0.026	0.009	0.097	0.036
3	75.7	5.98	0.025	0.016	0.084	0.032
4	41.9	4.99	0.018	0.007	0.155	0.046
5	38.6	2.70	0.008	0.003	0.052	0.028
Mean LO	57.8	18.1	0.019	0.011	0.084	0.053
6	59.6	7.62	0.023	0.009	0.064	0.012
7	32.8	6.96	0.011	0.005	0.069	0.037
8	82.3	12.9	0.028	0.013	0.083	0.075
9	63.2	10.6	0.018	0.006	0.043	0.024
10	32.0	4.55	0.013	0.003	0.043	0.013
Mean HI	54.0	21.3	0.019	0.010	0.061	0.040
P	0.50		0.89		0.087	

4.2.1 Faeces

The Cu concentration in collected faecal samples ranged from 25.0 to 106 mg/kg DM (appendix 1). There were no differences in FCu between samples from HI and LO farms (Table 7) but a significant herd dependent variation where 78 % of the variation could be explained by herd. Estimated daily faecal output ranged from 3.95 to 10.5 kg DM/day which resulted in a total daily Cu excretion through faeces between 194 to 771 mg/day (appendix 2). A correlation was seen between FCu and total daily Cu excretion through faeces ($p < 0.001$) (Figure 1).

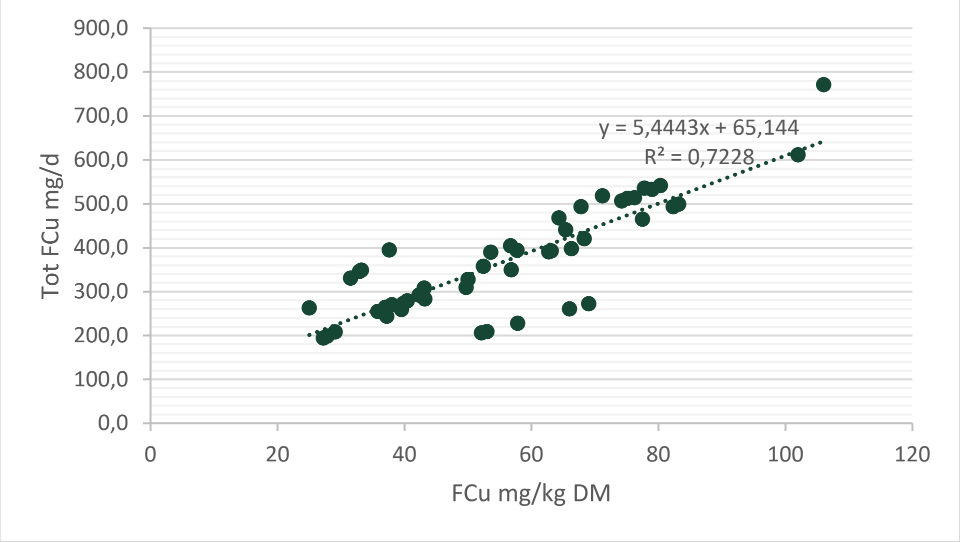

Figure 1. Correlation between faecal copper (Cu) concentration (FCu) and estimated total daily Cu excretion through faeces (Tot FCu) ($p < 0.001$).

4.2.2 Urine

The Cu concentration in collected urine samples ranged from 3.49 to 49.4 µg/L which corresponds to 0.004 to 0.051 mg/kg (appendix 1). There were no differences in UCu between samples from HI and LO farms (Table 7). UCu showed a large variation within farms, both when including only Cu concentration in the model and when adding total urine output as a variable, which indicates a low degree of similarity between cows within a farm. Calculated daily urine output ranged from 23.2 to 36.2 kg/day which resulted in a total daily Cu excretion through urine between 0.12 to 1.81 mg/day (appendix 2). A correlation was observed between UCu and total daily Cu excretion through urine ($p < 0.001$) (Figure 2).

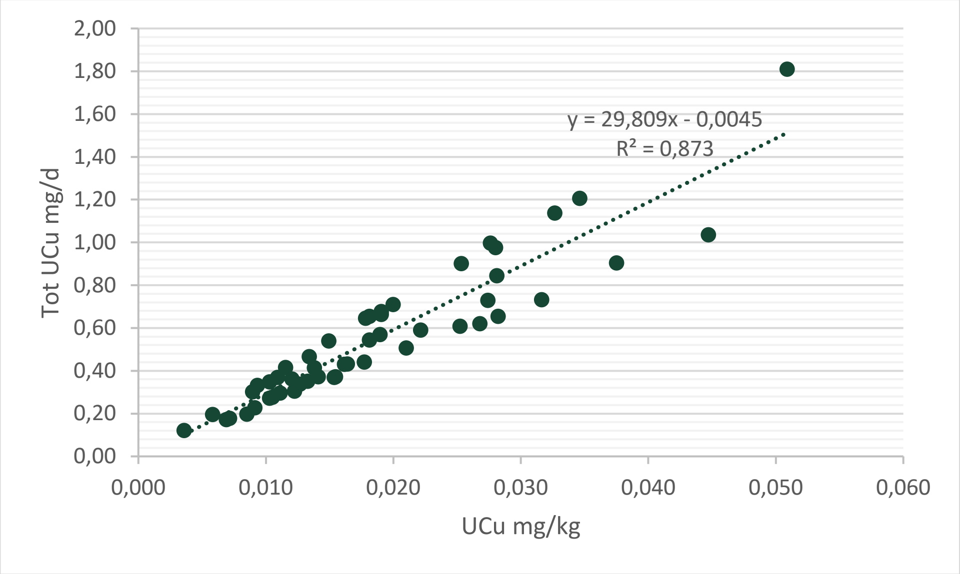

Figure 2. Correlation between urine copper (Cu) concentration (UCu) and estimated total daily Cu excretion through urine (Tot UCu) ($p < 0.001$).

4.2.3 Milk

The Cu concentration in collected milk samples ranged from 14.5 to 206.0 µg/L which corresponds to 0.015 to 0.212 mg/kg (appendix 1). There were no differences in MCu between samples from HI and LO farms (Table 7). Daily milk yield at the time of data collection ranged from 22 to 64 kg/day which resulted in a total daily Cu excretion through milk between 0.54 to 8.91 mg/day (appendix 2). No correlation was observed between MCu and milk yield, indicating that the Cu concentration does not decrease with increased milk yield. Correlation was observed between MCu and total daily Cu excretion through milk ($p < 0.001$) (Figure 3).

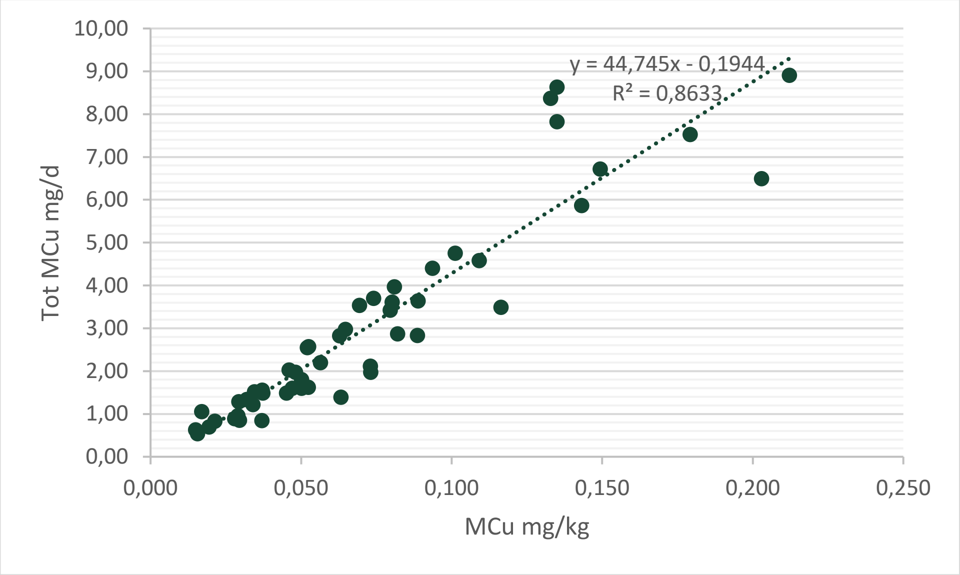

Figure 3. Correlation between milk copper (Cu) concentration (MCu) and estimated total daily Cu excretion through milk (Tot MCu) ($p < 0.001$).

4.2.4 Distribution of excreted Cu

In this study, the largest proportion of excreted Cu took the faecal route. Average proportion of ingested Cu excreted through faeces, urine and milk was110 %, 0.16 % and 0.95 % respectively. Due to a high faecal Cu excretion the total Cu excreted through faeces, urine and milk exceeded daily Cu intake in several sampled cows (Figure 4). An individual variation was seen within all three variables (Table 7).

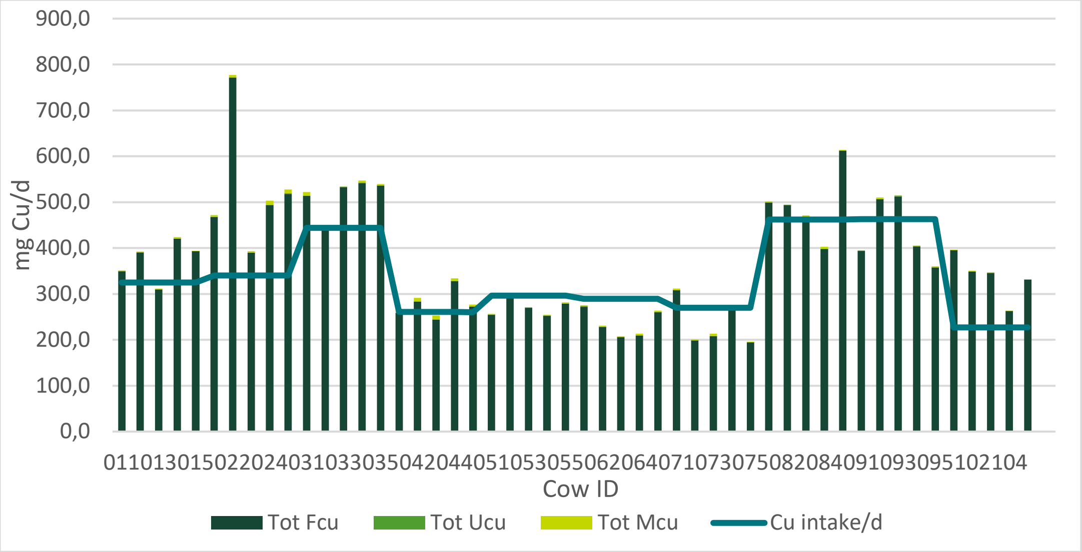

Figure 4. Daily copper (Cu) intake in comparison to total excreted Cu through faeces (FCu), urine (UCu) and milk (MCu).

A positive correlation between Cu concentration in faeces and urine was observed in these data (p = 0.020) (Figure 5) but not between any of the two variables and Cu concentration in milk (p (FCu) = 0.70, p (UCu) = 0.43).

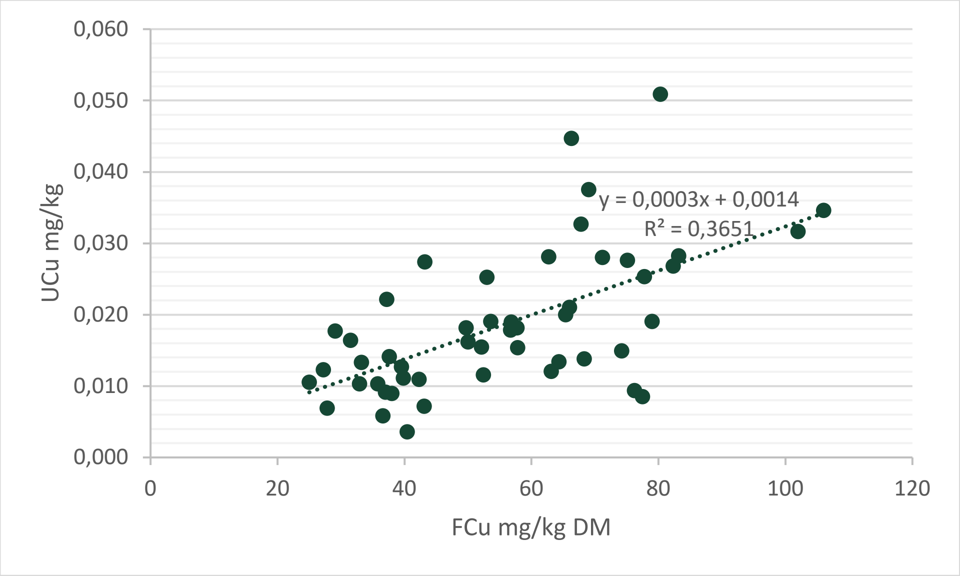

Figure 5. Correlation between faecal copper (Cu) concentration (FCu) and urine Cu concentration (UCu) ($p < 0.001$).

4.3 Effect of feed composition on excreted Cu

Dietary Cu, as Cu concentration in feed and total daily Cu intake, had an effect on both FCu ($p < 0.001$ on both) and UCu ($p < 0.001$ and $p = 0.005$ respectively) (Figure 6 and 7). FCu was not affected by dietary Mo, neither expressed as Mo concentration in feed ($p = 0.067$) or total daily intake ($p = 0.078$). Neither was the proportion Cu excreted through faeces different between HI and LO farms ($p = 0.116$). However, when including both Cu and Mo concentration in the statistical model, both minerals had an effect on FCu ($p < 0.001$ and $p = 0.0025$ respectively). UCu had a correlation with daily Cu absorption ($p = 0.001$) but not dietary Mo, expressed either as dietary concentration ($p = 0.428$) or total daily intake ($p = 0.471$). Neither was the proportion Cu excreted through urine different between HI and LO farms ($p = 0.095$).

Intake of Cu, expressed as concentration in feed ($p = 0.384$) and total daily intake ($p = 0.703$), or Mo ($p = 0.271$) had no effect on MCu (Figure 8). MCu had no correlation with daily Cu absorption ($p = 0.953$) but the proportion of Cu excreted through milk was higher in HI farms than in LO farms (Figure 9).

None of the samples was effected by dietary S concentration (p(FCu) = 0.89, p(UCu) = 0.42, p(MCu) = 0.48) nor were they effected differently by dietary Cu concentration depending on if the cow was from a HI or a LO farm.

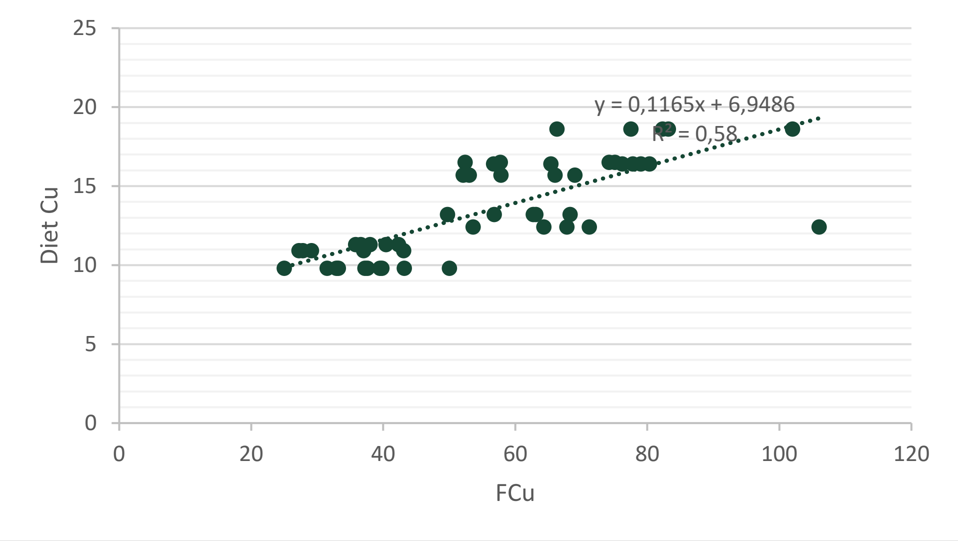

Figure 6. Effect of dietary Cu concentration (mg/kg DM) on Cu concentration in faeces (FCu) ($p < 0.001$).

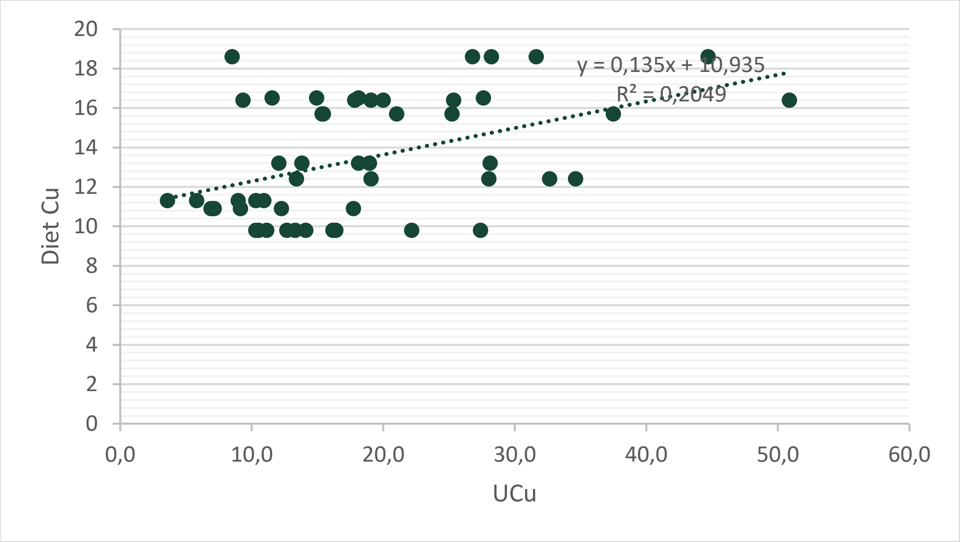

Figure 7. Effect of dietary Cu concentration (mg/kg DM) on Cu concentration in urine (UCu) ($p < 0.001$).

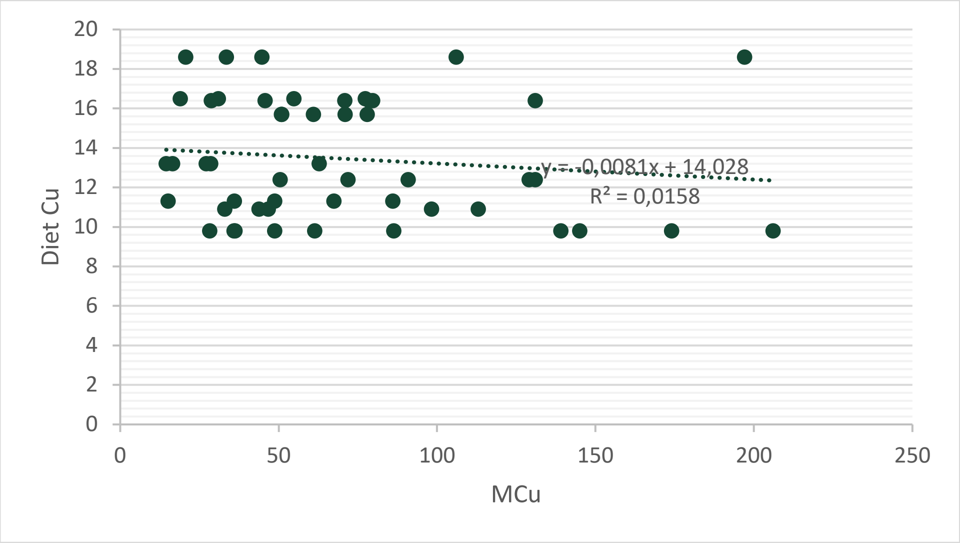

Figure 8. Effect of dietary Cu concentration (mg/kg DM) on Cu concentration in milk (MCu) ($p = 0.38$).

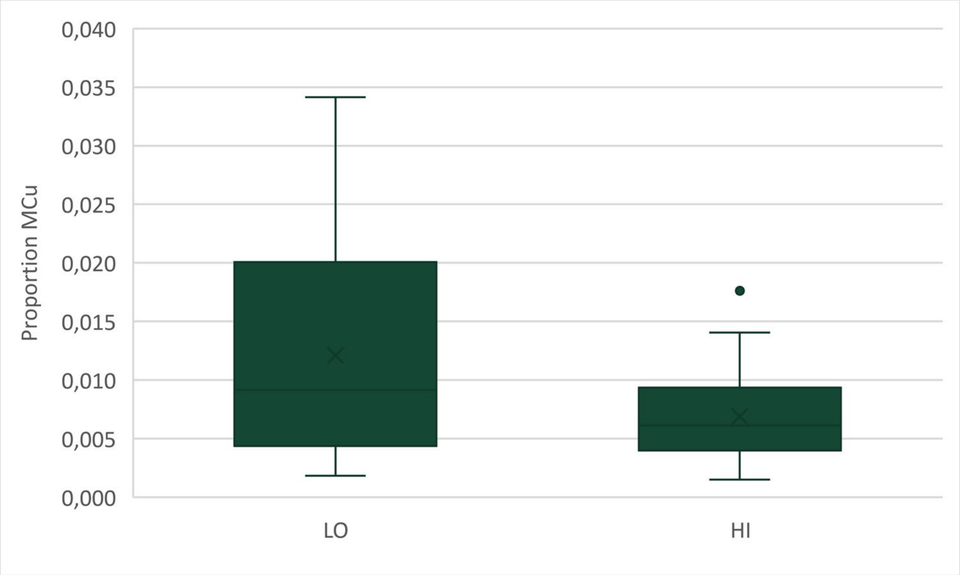

Figure 9. Proportion of Cu excreted through milk in farms with low (LO ≤ 1.2 mg Mo/kg DM) and high (HI ≥ 5 mg Mo/kg) molybdenum (Mo) concentration in their forage.

5. Discussion

The Mo concentration in the feed rations of the HI farms were all higher than the LO farms, although some did not exceed the original set limit of ≥ 5 mg Mo/kg DM. This was caused by a lower Mo concentration in bought feed as well as grain mix produced on the farm than in the forage, which were the values used when selecting farms. One of the HI farms had lower Mo concentration in the ration than expected due to the farmer buying forage from another farm which had a low Mo concentration. On another farm we encountered big differences in Mo concentration between two silos of the same harvests. This shows the importance of taking full mineral analysis on all harvests and batches in use. Being in an area rich of molybdenum is not sufficient information to base a feeding plan on as the Mo concentration can vary between fields and harvests.

All of the LO farms were well below the criteria of ≤ 1.2 mg Mo/kg DM. All diets were within the recommended Cu concentrations according to NASEM (2021), which was the reference with the lowest set limit, and if excluding potential effect of other antagonists the risk of both Cu deficiency and toxicity was low. However, on some of the HI farms the Cu concentration in the feed ration was lower than initially expected. This, in combination with high Mo concentrations, resulted in a very low Cu:Mo ratio of 1.4:1 on these farms (farm 7 and 10), which could indicate an increased risk of the cows on these farms suffering from chronic Cu deficiency or possibly TM toxicity. Interestingly, the two farms do not have the same calculated Cu absorption even though they have the same Cu:Mo ratio. Farm 7 have a lower absorption (3.1 %) than farm 10 (4.1 %) which reaches the same Cu absorption values as some of the LO farms in despite of the large differences in Cu:Mo ratio. This is caused by a very low S content in the diet (1.4 g/kg DM) in comparison to the other farms. This provides a clear view of the importance of not only taking the Cu:Mo ratio into account, but also other antagonists. It is worth mentioning that the Cu absorption is assessed using the equation from Suttle & Mclauchlan (1976) which was formulated using data on sheep and it has not yet been evaluated how well it represents Cu absorption in cattle.

In addition, the low Cu supplementation levels of the HI farms most likely caused the insignificant differences between HI and LO farms in statistical analysis of the results. For example, FCu had a strong herd dependent variation but no difference was observed in FCu between HI and LO farms. As FCu had a strong correlation with Cu intake, the faecal excretion had had probably been higher if the Cu supplementation would have been higher in the HI farms.

The Cu:Mo ratio is a good tool to assess a risk of Cu deficiency but it has limitations in ration formulation. As previously stated, the Cu:Mo ratio does not take into account the effect of other antagonists nor feed characteristics with potential effect on Cu availability. In this study we found a negative correlation between Mo and S concentration in the rations, which would be positive for Cu absorption in regards of the dependence of S in the formation of inhibitory TM. The recommendation of a Cu:Mo ratio of 3 – 4:1 could be used, but under the circumstances that the S concentration is low, as S in combination with Mo have a strong antagonistic effect on Cu absorption (Gooneratne et al. 1994, 2011). In addition, to avoid over supplementation of Cu, it could be reasonable not to use this recommendation infinitely due to the probability that the inhibitory effect of Mo reaches a peak (Suttle 2022). If taking this into consideration a Cu:Mo ratio of 3 – 4:1 could be used up to a Mo concentration of approximately 8 mg Mo/kg DM in the feed ration, at which point the Cu concentration would not need to be compensated further. None of the farms in this study exceeded the maximal tolerance levels of Cu and the risk of Cu toxicity, both acute and chronic, is therefore assumed to be small. This would be confirmed with liver samples with Cu concentrations of less than approximately 300 mg/kg DM (NASEM 2021).

Contradictory to the advisors aim of having a Cu:Mo ratio of 4:1 in the ration of these high Mo farms, the one-day feeding controls performed in this study showed that this was not the reality. Three out of the five HI farms had a Cu:Mo ratio below 4:1 and there could be several reasons for this. One possibility is that different advisors calculate the rations differently and give the farmers different recommendations than their colleagues. The other possibility is that the farmers do not feed according to the calculated feed ration, either by mistake or as a conscious action. Because of different infrastructure on the farms, a gap between the feeding plan and what is actually fed and consumed by the cows is a possibility. On farm 8 (Cu:Mo of 3:1) this is possible but on farm 7 and 10 (Cu:Mo of 1.4:1) it is unlikely due to the considerable low ratio.

The levels of Zn in the feed rations on the farms included in this study were most likely not high enough to have any measurable effect on Cu concentrations in collected samples. The same conclusion can be made from the total Fe concentrations which were all except one (farm 9) within or in close proximity to average Fe concentrations in forage (Table 1). At farm number 9 the total dietary Fe concentration was quite high (462 mg/kg DM) but as the Cu concentration also was high (16.5 mg/kg DM) and the Cu:Mo above the recommended lower limit (6.2:1) the risk of Cu deficiency can be considered low.

When comparing the sample Cu concentrations analysed in this trial (FCu, UCu and MCu) with values from literature, the results are reasonable. The mean proportions of dietary Cu intake in faeces, urine and milk are 110, 0.16 and 0.95 % respectively. Buckley (1991) presented the same proportions as 97.85, 0.075 and 0.51 %. On the other hand, FCu from several cows either greatly exceeded or fell below the Cu intake of the cow which can indicate multiple things. The Cu intake is a calculated average based on the feed intake by the whole herd which means that individual cows can have a higher or lower actual Cu intake. It is also possible that the faecal samples were not representable as it was taken at a single point in time and the analysis have a measure of uncertainty. Lastly, the excess could be caused by a release of Cu from storage in the liver. This could for example be possible on the one sample from farm 2 that is almost double in total Cu excretion from some of the other samples (Figure 4).

Interestingly, in accordance to the results of Gooneratne et al. (1994, 2011), Cu concentration in both faeces and urine had a strong correlation with Cu intake. None of the samples were directly affected by dietary Mo concentration but other analytical results could give an indication of an indirect effect. For example, the correlation between FCu and Mo in feed had p-values close to significance ($p = 0.067$ and $p = 0.078$) and when including the effect of dietary Cu concentration in addition to Mo in the model, both mineral concentrations (Cu and Mo) in the feed had a significant effect on FCu. There were no correlation between UCu and Mo intake but on the other hand a strong correlation between UCu and absorbed Cu estimated from the previously mentioned equation by Suttle & Mclauchlan (1976).

The Cu concentration in milk was not affected by intake of Cu, Mo or S. This agrees with the results of Wittenberg & Devlin (1987) who saw no effect of Mo on milk Cu concentration. A large variation in MCu was observed both within and between farms and do not seem to give an accurate reflection of Cu intake or effects of antagonists on Cu absorption and Cu status.

There were no difference in Cu excretion through faeces, urine or milk between HI or LO farms. In urine and milk this was probably due to a large in herd variation. Because of this it would be important to have many samples per farm to accurately reflect Cu intake on farm level. In faeces on the other hand, the variation was larger between farms than within. This means that if taking faecal samples to analyse Cu status on herd level, the number of sampled individuals within a herd is of less significance than the number of herds.

When taking both dietary intake of Cu, Mo and S as well as Cu concentration in faecal and urine samples in consideration some farms could be considered as high-risk farms, in regards to Cu deficiency. The most prominent high-risk farms are farm 7 and 10. They both had high Mo concentrations in their ration as well as a very low Cu:Mo ratio. They both had a high roughage share and 89 % home grown feed. This means that the high Mo soils in their area have a large impact on the final Mo concentration in their TMR which can be hard to avoid. When looking at the Cu concentrations in faeces and urine collected at these farms their mean values are numerically lower than the means from the other farms, both from the HI and the LO groups. This could be an indication that both farms could benefit from additional supplementary Cu to decrease the risk of Cu deficiency in their herds. The risk of Mo toxicity however remains and should be considered. Three farms (farm 7, 8 and 10) had high Mo concentration in their total ration which results in a high Mo intake (181, 153, and 161 mg/d, respectively). This could indicate a potential risk of Mo toxicity, even if the provision of Cu is adequate.

There were a few aspects that could have interfered with our results. The collection of milk samples proved to be more difficult than we had anticipated as they were collected by the farmers themselves during regular milking. Due to the possibility that the Cu concentration could vary over the period of a milking a sample taken from the whole milking was required to ensure a representative result (Bermejo-Barrera et al. 1997). There were some issues with communicating this to the farmers which resulted in some samples taken as strip samples. These samples were resampled correctly but the possibility of other samples being collected incorrectly without our knowledge remains. On the other hand, the variation in MCu explained by herd was small which indicates that if the samples were collected differently on different farms, it did not have a significant effect on the results.

Information about daily feeding was also provided by the farmers which had different possibilities of accurate measurement on the farm. Some had an on farm scale or automatic feeding systems and thus were able to get accurate weights of all feedstuffs as well as the left-overs removed from the feed table daily. Others were not able to weigh the feed but for example knew the number of bales or number of scoops added to the ration and from that could make an estimation. On the farms that used bales to ensile their forage instead of bunker silos we encountered a large difference in DM between bales. This can result in differences in kg DM fed between days and feedings when feeding a specific number of bales per day. Because of these factors, errors in calculating the nutritional intake per cow is a possibility.

As well as error in sampling there are uncertainties in the laboratory analyses. For the analysis of Cu concentration in urine the measure uncertainties were 14 % and 10 % in faeces and milk. This means that there is a possibility the actual values were slightly higher or lower than the received results.

6. Conclusions

The largest proportion of ingested copper was excreted through faeces and Cu concentration in urine and milk was low. Copper concentration in faeces and urine correlated with the Cu intake but not Mo intake. Milk Cu concentration was not affected by either dietary Cu or Mo and its potential use in assessment of Cu status is therefore low. HI farms were not fed as high Cu concentrations as expected which resulted in very low Cu:Mo ratios on some of the farms. The same farms had numerically lower Cu concentration in faeces and urine than the other farms which could indicate a higher risk of Cu deficiency. A Cu:Mo ratio of 3 – 4:1 could be an acceptable tool in ration formulation but concentrations of other antagonists, especially S, need to be considered in addition to the Cu:Mo ratio. According to the results of this study, faecal Cu concentration, and possibly urine Cu concentration, seem to have the highest potential for being a useful tool in monitoring of Cu status on animals in production. Further research on the topic is needed, for example to examine a possible correlation between the parameters examined in this study and liver Cu concentrations.

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Popular science summary

Copper supplementation to dairy cows can be challenging as it can have negative effects on the cows health as well as production capacity if administered incorrectly. Too low supplementation can lead to deficicency symptoms such as anemia and rugged fur and too high supplementation can have toxic effects. What further complexes the issue is that other minerals in the feed can effect how well the cow can use the dietary copper.

The main mechanism for this antagonistic effect takes place in the rumen, where the minerals molybdenum and sulphur interact with copper. This interaction binds the copper strongly and makes it unavailable for the cow. The largest proportion of the bound copper then passes through the digestive system and exits through the faeces. The interaction can also have some effect on copper inside the cells and blood circulation, effecting copper being released from the liver through bile, in urine and even the copper content of the milk.

Molybdenum is present in all crops but the concentration depends on the concentration in the soil, which can vary between regions, farms and even fields. One region with molybdenum rich soils is the area around Falköping, Sweden, where the farmers deal with the issue of copper supplementation. The ability to monitor the copper supplementation and its effect on copper status with a tool that is easy to use on the farm would be very useful in areas like these. In this study we therefore chose to investigate the levels of copper supplementation in connection to dietary molybdenum and how this was reflected in samples of faeces, urine and milk. In total, 50 dairy cows from 10 different farms were sampled for faeces, urin and milk and all samples were analysed for copper concentration. The study was conducted in January 2023 and all farms were located in the Västra Götaland region of Sweden.

Our results showed that copper content in the feed had an effect on copper present in both faeces and urine but not in milk. The molybdenum content of the feed had no effect on copper in any of the samples. From this study we can make the conclusions that it is very important to be aware of the molybdenum content of all feeds fed to the cows to be able to make a well balanced feeding plan and avoid both copper deficiency and toxicity. Faecal and urine samples could possibly be a good tool to monitor copper status on dairy cows and we hope for more research in the area.

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Appendix 1

Complete results from analysis of copper (Cu) concentration in faeces (FCu), urine (UCu) and milk (MCu) and analysed faecal dry matter (DM) from laboratory. Farms 1 – 5 is from the LO group and farms 6 – 10 is from the HI group. Mean values and standard deviations (SD) in table 6.

		Sample Cu concentration			Sample DM content
Farm	Cow	FCu mg/kg DM	UCu mg/kg	MCu mg/kg	Faeces DM
1	1	56,8	0,019	0,017	14,1
1	2	62,7	0,028	0,028	15,2
1	3	49,7	0,018	0,029	13,4
1	4	68,3	0,014	0,065	8,3
1	5	63,1	0,012	0,015	13,6
2	1	64,3	0,013	0,074	15,1
2	2	106	0,035	0,094	16,2
2	3	53,6	0,019	0,052	12,3
2	4	67,8	0,033	0,135	13,5
2	5	71,2	0,028	0,133	10,8
3	1	76,2	0,009	0,135	14,0
3	2	65,4	0,020	0,073	12,7
3	3	79,0	0,019	0,047	15,1
3	4	80,3	0,051	0,081	16,5
3	5	77,8	0,025	0,082	14,0
4	1	39,5	0,013	0,149	14,2
4	2	43,2	0,027	0,179	13,3
4	3	37,2	0,022	0,212	12,4
4	4	50,0	0,016	0,143	13,3
4	5	39,8	0,011	0,089	12,8
5	1	35,8	0,010	0,037	12,5
5	2	42,3	0,011	0,069	10,5
5	3	38,0	0,009	0,016	10,3
5	4	36,6	0,006	0,050	10,6
5	5	40,4	0,004	0,089	14,2
6	1	69,0	0,037	0,073	15,3
6	2	57,8	0,015	0,053	12,6

6	3	52,1	0,015	0,052	16,1
6	4	53,0	0,025	0,080	11,2
6	5	66,0	0,021	0,063	11,5
7	1	43,1	0,007	0,116	12,6
7	2	27,8	0,007	0,048	14,7
7	3	29,1	0,018	0,101	13,4
7	4	37,0	0,009	0,045	13,1
7	5	27,2	0,012	0,034	14,7
8	1	83,2	0,028	0,046	14,2
8	2	82,3	0,027	0,021	12,9
8	3	77,5	0,009	0,203	12,1
8	4	66,3	0,045	0,109	13,5
8	5	102	0,032	0,035	12,6
9	1	57,7	0,018	0,019	14,3
9	2	74,2	0,015	0,080	10,7
9	3	75,1	0,028	0,032	13,6
9	4	56,7	0,018	0,030	15,3
9	5	52,4	0,012	0,056	13,1
10	1	37,6	0,014	0,063	12,1
10	2	33,2	0,013	0,050	12,4
10	3	32,9	0,010	0,037	12,4
10	4	25,0	0,011	0,029	13,3
10	5	31,5	0,016	0,037	14,6

Appendix 2

Calculated individual output of faeces and urine per day and individual milk yield per day at the time of data collection. Estimated excreted copper (Cu) per day in faeces, urine and milk based on these outputs. Farms 1 – 5 is from the LO group and farms 6 – 10 is from the HI group.

		Output/d			Total Cu output mg/d		
Farm	Cow	Faeces kg DM	Urine kg	Milk kg	Faeces	Urine	Milk
1	1	6,2	30,1	62	349	0,57	1,05
1	2	6,2	30,1	32	390	0,85	0,89
1	3	6,2	30,1	44	310	0,54	1,29
1	4	6,2	30,1	46	420	0,41	2,98
1	5	6,2	30,1	42	393	0,36	0,63
2	1	7,3	34,8	50	468	0,47	3,70
2	2	7,3	34,8	47	771	1,21	4,40
2	3	7,3	34,8	49	390	0,66	2,55
2	4	7,3	34,8	64	493	1,14	8,64
2	5	7,3	34,8	63	518	0,98	8,37
3	1	6,7	35,6	58	514	0,33	7,83
3	2	6,7	35,6	29	441	0,71	2,12
3	3	6,7	35,6	34	533	0,68	1,60
3	4	6,7	35,6	49	541	1,81	3,97
3	5	6,9	35,6	35	536	0,90	2,87
4	1	6,6	26,7	45	259	0,34	6,72
4	2	6,6	26,7	42	283	0,73	7,53
4	3	6,6	26,7	42	244	0,59	8,91
4	4	6,6	26,7	41	328	0,43	5,87
4	5	6,9	26,7	41	273	0,30	3,64
5	1	7,1	33,8	42	255	0,35	1,56
5	2	6,9	33,8	51	292	0,37	3,54
5	3	7,1	33,8	35	270	0,30	0,54
5	4	6,9	33,8	32	253	0,20	1,61
5	5	6,9	33,8	32	279	0,12	2,83
6	1	3,9	24,1	27	272	0,90	1,97
6	2	3,9	24,1	49	228	0,37	2,57
6	3	3,9	24,1	31	206	0,37	1,63

6	4	3,9	24,1	45	209	0,61	3,61
6	5	3,9	24,1	45	260	0,51	2,83
7	1	7,1	24,9	30	308	0,18	3,49
7	2	7,1	24,9	41	199	0,17	1,97
7	3	7,1	24,9	47	208	0,44	4,75
7	4	7,1	24,9	33	264	0,23	1,49
7	5	7,1	24,9	36	194	0,31	1,22
8	1	6,0	23,2	44	499	0,65	2,03
8	2	6,0	23,2	39	494	0,62	0,83
8	3	6,0	23,2	32	465	0,20	6,49
8	4	6,0	23,2	42	398	1,04	4,59
8	5	6,0	23,2	44	612	0,73	1,52
9	1	6,8	36,1	36	394	0,65	0,70
9	2	6,8	36,1	43	506	0,54	3,42
9	3	6,8	36,1	42	513	1,00	1,34
9	4	7,1	36,2	29	404	0,65	0,86
9	5	6,8	36,1	39	358	0,42	2,20
10	1	10,5	26,4	22	395	0,37	1,39
10	2	10,5	26,4	36	349	0,35	1,81
10	3	10,5	26,4	40	346	0,27	1,49
10	4	10,5	26,4	33	263	0,28	0,96
10	5	10,5	26,4	23	331	0,43	0,85

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