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Review: Bioavailability of trace elements in farm animals: definition and practical considerations for improved assessment of efficacy and safety

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ABSTRACT

Currently, the authorisation procedure of trace elements as feed additives in the European Union according to Regulation (EC) No. 1831/2003 does not consider the bioavailability of trace element sources. This manuscript provides framework conditions for in vivo experiments that aim to estimate differences in the relative bioavailability between supplements of essential trace elements. Framework conditions encompass necessary technical information on the test substance, the experimental design and diet composition as well as the suitability of status parameters that allow for relative comparisons of regression variables. This manuscript evolves recommendations for researchers to conduct solid and reliable experiments on the matter as well as decision makers to interpret the value of studies submitted with authorisation applications regarding a certain trace element supplement.

Implications

Trace element supplements for animal nutrition must be approved as feed additives within the European Union. However, the applicable Regulation (EC) No. 1831/2003 does not define specific requirements for experiments addressing trace element bioavailability from different sources. Therefore, bioavailability could not be taken into account when assessing the safety and efficacy of such additives. This work proposes a framework that can help to better define the conditions for the procedural method and the evaluation of studies on the bioavailability of essential trace elements.

Introduction

Trace elements are essential dietary components that organisms need to ingest only in very small amounts. In contrast to macro elements, concentrations of specific trace elements within the organism are usually <50 mg/kg BW, with the exception of iron (National Research Council, 2005). Current recommendations for feeding farm animal species include total dietary concentrations of trace elements, because concentration in and bioavailability from plant-based feed can be quite variable and are often not high enough to meet the animals' needs (Suttle, 2010). Feeding recommendations are defined for: Fe, Zn, Cu, Mn, I and Se (National Research Council, 1994; 2012). For ruminant species, there are also recommendations for Co to address the specific requirement of the rumen microbiota and to promote ruminal cobalamin synthesis (National Research Council, 2001; 2016). All supplemented elements are generally mixed into compound feed in the form of inorganic or organic sources. During the past years, also special physically processed forms have been put on the market, the most prominent of which are nano particles of common sources (e.g. nano ZnO) (Anonymous, 2021a). In the European Union, feed additives require the authorisation in accordance with Regulation (EC) No. 1831/2003. The authorisation procedure should ensure that a feed additive is safe for the animal, the personnel handling the product, the consumer of animal-derived products as well as for the environment. This implies a trace element supplement is sufficiently utilised in order to minimise necessary total dietary concentrations and, thereby, excessive accumulation in animals, edible products and the environment. Changes in feeding efficiency

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with respect to the choice of different trace element sources arise from differences in their bioavailability. Hence, trace element bioavailability should be assessed as proof of effectiveness, when comparing trace element sources *in vivo*. In order to ensure a harmonised authorisation procedure, Regulation (EC) No. 429/2008 regulates the requirements for the studies to be submitted by the applicant for the authorisation of feed additives including trace elements (Anonymous, 2008). Neither this legal framework nor the European Food Safety Authority guidance document on the assessment of efficacy of feed additives (Rychen et al., 2018) defines specific requirements for experimental studies addressing trace element bioavailability from different sources. This also applies to countries outside the European Union. For example, the governments of the United States of America as well as Canada have installed complex workflows in the registration process of novel feeds and feed additives. Neither of these codes defines bioavailability as a necessary information to be provided by the applicant when claiming increased efficiency and safety of a trace element product in regard to the feeding of animals (Anonymous, 1983; 2021b). Consequently, no framework exists that provides applicants with the necessary information to define and measure bioavailability concerning trace element supplements in farm animal species. Until now, it has therefore not been possible to consider bioavailability when assessing the safety and efficacy of such additives.

The objective of the present work is to propose a framework, which may help to better define conditions for the procedural method and evaluation of studies on the bioavailability of essential trace elements in farm animal species. These basic methodological prerequisites arise from the fundamental principles of trace element metabolism, which are therefore also addressed. It is noteworthy that we did not intend to provide detailed "cooking recipes" for the application of experiments on trace element bioavailability. Instead, our goal was to highlight a principle of thought for the design of such experiments, which involves the understanding of the principles of quantitative trace element metabolism that should be acknowledged when considering dose ranges and status parameters for the experimental design. Exemplarily, we reviewed specific aspects of Zn, Cu and Mn metabolism, also with respect to different species of farm animals, to identify potential gaps in the current knowledge, which may have to be filled prior to the design of bioavailability studies. This literature review does not claim to be complete and largely represents our professional perspective. Definitions of certain terms and parameters were summarised in alphabetical order in a glossary that is available for download as Supplementary Material S1. This file also includes a detailed explanation and discussion of the framework highlighted in Table 1.

Table 1
Checklist for requirements of studies on trace element bioavailability in farm animals.

Category	Recommendation
A	Precise description of the test substance Chemical structure of the potentially absorbable compound Particle size distribution (especially with respect to nano-sources) Purity of the product formulation Coating yes/no (if yes, what kind of coating)
B	Application of a proper experimental and dietary design Apply sufficient statistical power Reduce bias by applying randomisation and blinding Explain the chosen animal category Explain the chosen phenotype Apply a finely graded dose–response study that spans the range between deficient and sufficient dietary dosages Avoid clinical deficiency as well as supranutritional dosing Apply a suitable composition of the basal diet Apply and prove sufficient dietary homogeneity Comprehensively analyse and communicate the dietary conditions
C	Determination of bioavailability by relative regression comparison between the test substance and reference source(s) Explain the chosen reference source(s) Avoid the application of blends of trace element sources Apply the same dosages for the test substance(s) and reference source(s)
D	Applying a proper combination of appropriate status parameters Quantitative metabolic data Trace element concentrations in tissues, body fluids and animal products Physiological parameters (enzyme activities, protein and gene expression patterns)

Definition of the term 'bioavailability' in regard to trace elements

The term bioavailability is often used as a synonym for absorbability or retention. However, this is misleading as it implies that trace elements are 100% bioavailable as long as they are present as an available chemical compound within the gastrointestinal tract (**GIT**). Especially for essential transition metals, this is not the case (Suttle, 2010). Furthermore, the retention of trace elements does not necessarily mean that they are actually used in metabolic processes. This accounts especially to the high potential of I and Se to accumulate in tissues and edible animal products (EFSA, 2012; 2013). Whole body contents of essential trace elements are actively regulated already at the gut barrier through specific absorption mechanisms as well as various absorption and excretion mechanisms throughout the body in response to its metabolic status (Windisch, 2002). Therefore, the bioavailability of a trace element source can be defined as its potential to supply the physiological functions that depend on the respective element within the animal's metabolism (O'Dell, 1984). These physiological functions are tightly related to the total requirement of the organism and the kinetics of the element within the body. With regard to animal nutrition, the estimate of bioavailability therefore relates to the estimate of the gross requirement, which is commonly expressed as the minimum concentration in complete feed providing sufficient metabolically available quantities under *ad libitum* feeding conditions. In other words, every attempt to prove the superiority of a certain supplement compared to a reference source must demonstrate that it can promote the same physiological response with lower total amounts or concentrations of the respective element in the diet. This means that the superior trace element source should yield a reduced need for supplementation.

Basic concepts of essential trace element metabolism and consequences for the experimental determination of trace element bioavailability

Essential trace elements predominantly circulate in biological systems in their ionic form bound to suitable chemical ligands. The role of these ions in biochemical reactions as co-factors of peptide structure and activity is considered to be irreplaceable by other substances and marks the foundation of their essentiality for animal organisms (Maret, 2016). Windisch (2002) reviewed the basic regulation kinetics of essential trace elements within the animal organism and pointed out differences when comparing the essential transition metals with Se and I. The former appear to be primarily regulated by absorption from and/or excretion into the GIT. Thereby, truly absorbed amounts of essential transition metals reveal saturation kinetics with further increase in dietary

supply levels above the point of satisfied requirements. True absorption is defined as the amount of total disappearance of the ingested element alongside the GIT corrected for endogenous metal losses into the GIT (Weigand and Kirchgessner, 1980). At the same time, excretion of excess amounts of already absorbed essential transition metal ions in response to passive influx is increasing dose-dependently when the supplementation exceeds the actual requirements. These basic principles have been so far reproduced in a diverse set of animal models, for example for Zn in rats, pigs, chickens and cattle (Schwarz and Kirchgessner, 1975; Weigand and Kirchgessner, 1980; Linares et al., 2007; Brugger et al., 2014). In contrast, selenium and iodine have been demonstrated to be readily absorbed from the GIT of rats at levels of >90% of dietary intake and subsequently regulated by excretion into urine (Kirchgessner et al., 1997; Kirchgessner et al., 1999). Data obtained from farm animals (laying hens, dairy cows) paint a comparable picture and further highlight the quite efficient transfer of absorbed amounts into eggs and milk (EFSA, 2012; 2013). In conclusion, a stepwise increase in dietary supply levels of trace elements will inevitably lead to a further saturation of their uptake and/or excretion, and therefore their retention, to counteract intoxication as far as possible.

Using appropriate status parameters allows for mapping the dose range within which this transition from active retention to excretory behaviour occurs (Figs. 1 and 2). This statistically defined threshold represents the gross requirement under given experimental conditions (Robbins et al., 2006). Such non-linear response curves indicate clear differences in the homeostatic adaption of an organism depending on whether the supply with a given essential trace element was deficient or sufficient. Hence, comparing differences in the utilisation of trace elements from varying sources solely at recommended supply levels above the requirement and within the authorised dietary concentrations, to avoid situations of deficiency and to reflect practice, could yield false results. Under such conditions, either active absorption from the gastrointestinal lumen is reduced (i.e. essential transition metals) or absorbed surplus amounts are quickly directed towards excretion pathways (i.e.

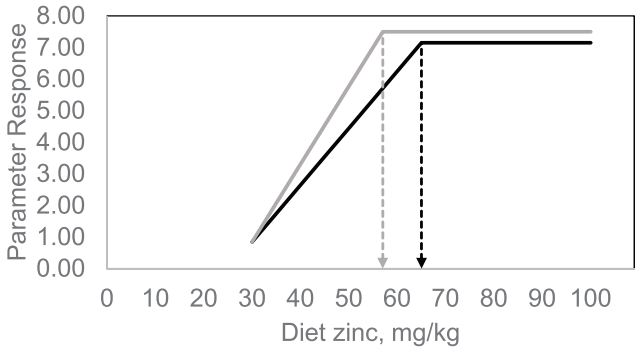

Fig. 1. Comparative broken-line regression of a fictous status parameter on changes in dietary Zn supply to farm animals from a fictitious test and reference zinc source. Notes: In this example, the parameter exhibits a plateau in response above dietary thresholds of 65 and 57 mg Zn/kg diet when feeding the reference zinc source A (black) and the test zinc source B (grey), respectively. It is noteworthy that the response above the requirement threshold must not necessarily yield a plateau. Depending on the parameter of choice and the applied experimental conditions (e.g. subclinical vs clinical conditions, short-term vs long-term challenge), a less steep increase or complete turnaround in the response patterns are other possible scenarios. Below the respective dietary threshold, parameter response to sources A and B decreases/increases by 0.18 and 0.25 units/mg reduction/rise in dietary zinc, respectively. In conclusion, feeding the test zinc source B results in a decreased gross zinc requirement (-8 mg zinc/kg diet). Based on the slope comparison, test zinc species B promoted a 1.39-fold higher relative Zn absorption compared to the reference source A.

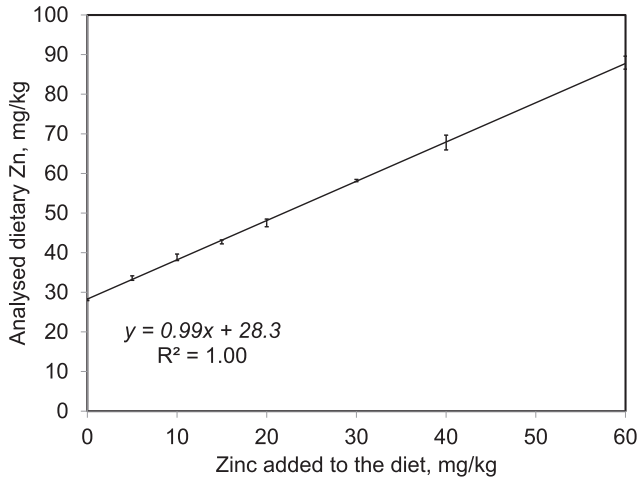

Fig. 2. Relationship between supplemental Zn from $ZnSO_4 * 7H_2O$ and analysed Zn in complete feed for weaned piglets (Brugger, 2018). Notes: The slope of +0.99 indicates a high analytical recovery rate of 99%. Low SDs (error bars) around respective mean values indicate high dietary homogeneity. Values represent the means of a triplicate analysis of three independent samples per diet (9 single values per mean; a total of 72 data points).

Se, I). Hence, differences in retention from varying sources under such conditions just reflect passive transfer but not differences in the availability of ions for the active transport systems (Windisch, 2002).

Another important aspect refers to the severity of the induced phenotype of dose-dependent trace element malnutrition. In intensive or semi-intensive farm animal production systems, clinical events of trace element deficiencies are rarely occurring and the majority of malnutrition events are of a subclinical nature. A subclinical compared to a clinical deficiency is defined by the total absence of visible symptoms of pathophysiological adaption, and just becomes evident by changes on the metabolic level. It appears rational to simulate subclinical events due to their higher practical relevance. Furthermore, inducing a dose–response model of gradual differences in clinical trace element deficiency until the point of sufficient supply appears inappropriate, as it compares clinically impaired with healthy individuals. Such an approach provides limited information due to the fact that clinical deficient individuals experience degenerative processes (Suttle, 2010) and may therefore utilise far more of the trace element pool as under conditions without any visible symptoms of deficiency.

Framework conditions for experimental studies on bioavailability

Table 1 contains a checklist for the framework conditions that should be met when *in vivo* studies on bioavailability of trace elements are planned and conducted. In addition, the online supplementary material S1 contains detailed background information on the application of said framework conditions. The basic principles apply to *in vivo* studies on feed-associated substances in general, including macro minerals, pharmacologically active substances, and contaminants. The framework conditions were subcategorised into four different points, which are discussed in more detail in the online supplementary material S1 together with the consequences/pitfalls arising from not following certain recommendations:

A Precise description of the test substance
B Application of a proper experimental and dietary design

Table 2
Basic, complementary and potentially beneficial status parameters to assess the bioavailability of the essential trace elements zinc, copper and manganese in feeding trials with farm animals.

Item	Zinc	Copper	Manganese
Basic	[1]Apparently absorbed amount of dietary Zn	[1]Apparently absorbed amount of dietary Cu	[2]Apparently retained amount of Mn (if applicable)
	[2]Apparently retained amount of Zn (if applicable)	[2]Apparently retained amount of Cu (if applicable)	Liver Mn
	[3]Bone zinc (trabecular bone tissue)	Liver Cu Mo and S in ruminant diets	[4]Bile Mn (if applicable)
Complementary	Serum or plasma Zn [5]Free Zn-binding capacity in serum or plasma Liver Zn Hepatic MT1 gene expression Milk Zn (if applicable) Egg Zn (if applicable) [7]Jejunal + colonic relative SLC39A4 gene expression	Serum or plasma Cep activity Serum or plasma Cu Hepatic MT2 gene expression	Kidney Mn Bone Mn ([6]*Phalanx proximalis*)
Potentially beneficial	Zn-enzyme activities (e.g. alkaline phosphatase, carbonic anhydrase) [8]Cell stress signalling pathways (transcription, post-transcription)	Cu-enzyme activities (e.g. erythrocyte Cu/Zn-SOD) Iron status parameters (in case of reduced plasma Cep activity) [8]Cell stress signalling pathways (transcription, post-transcription)	Mn-enzymes (e.g. Mn-SOD) [8]Cell stress signalling pathways (transcription, post-transcription)

Cep, ceruloplasmin; MT, metallothionein; SOD, Superoxide dismutase; SLC39A4, Solute Carrier Family 39 Member 4.
[1] Total dietary intake of the respective element (DI) corrected for the total faecal losses. Alternatively, in studies assessing the percentage apparent absorbability (D) via the indicator dilution method, the apparently absorbed amount equals D/100 * DI as mg/kg dietary intake.
[2] Apparently retained amount of the respective element defined as the total dietary element intake corrected for the total faecal and urinary element losses.
[3] Trabecular bone tissue can be found for example in the femoral head or the tibia.
[4] Applicable for all species with a bile bladder.
[5] percentage amount of free Zn-binding sites in blood plasma according to Roth and Kirchgessner, 1980).
[6] According to Pallauf et al., 2012).
[7] It is noteworthy that some data suggest a shift of the main absorption side for Zn from the small intestine to lower intestinal segments under subclinical conditions (discussed in detail in Brugger et al., 2021)).
[8] The regulation of cell stress pathways may provide useful additional information on the physiological adaption to trace element deficiency as well as accumulation in target tissues.

C Determination of bioavailability by relative regression comparison between the test substance and reference source(s)
D Applying a proper combination of appropriate status parameters

Exemplary considerations regarding special features when carrying out studies on zinc, copper and manganese

This section intends to address special features of certain trace elements, which may affect the experimental modelling of their metabolism in different livestock species. These include their kinetics of absorption, distribution and excretion, special features concerning different livestock species as well as suitable status parameters under experimental conditions. We selected Zn, Cu and Mn because these elements were the subject of many recent studies on different sources of trace elements in livestock feeding. Our subsequent review on status parameters for these elements is summarised in Table 2, which subcategorises respective measures as ‘‘basic”, ‘‘complementary” and ‘‘potentially beneficial”.

Further essential trace elements exist but it would have increased the size of this already very extensive article to also include particular information on these. However, the general concept of understanding the quantitative principles of an element’s metabolism based on earlier data to decide on suitable parameters applies to all of these.

The available literature is very much dominated by metabolic studies on pigs, followed by chicken and considerably less information on ruminants. The subsequent subsections directly reflect this imbalance, yet the information provided in our manuscript is intended to be of use for research projects on all types of animal models.

Zinc

Kinetics and mechanisms of absorption and excretion

The total content of Zn inside the body is regulated at the level of absorption from the GIT and through faecal excretion of secreted endogenous amounts. These fluxes show clear saturation kinetics under conditions of varying dietary Zn concentrations in the range between deficient and sufficient supply. Thereby, true and apparent absorption increases significantly with further increase in dietary Zn supply on top of a deficient diet, until the point of satisfied requirements is reached. Every further increase in the dietary supply above the requirement still yields some passive Zn uptake; however, the efficiency is significantly reduced compared to the energy-dependent active absorption process. These principles have so far been demonstrated in various animal models, including rats, pigs, chickens and cattle (Schwarz and Kirchgessner, 1975; Weigand and Kirchgessner, 1980; Brugger et al., 2014; Boerboom et al., 2021). Basic research on mice revealed the status-dependent regulation of Zn absorption by a finely tuned interplay between the main active transporter at the apical membrane (solute carrier family 39 member A4 (**SLC39A4**)) (Liuzzi et al., 2009) as well as Solute Carrier Family 30 Member A1 (**SLC30A1**) at the basolateral membrane of enterocytes (Liuzzi et al., 2001). In parallel to the upregulation of GIT absorption capacity, the excretion of endogenous Zn is reduced to an inevitable minimum under conditions of dietary Zn deficiency and increases with additional Zn supplied above the requirement threshold as shown in ^{65}Zn-labelled rats (Weigand and Kirchgessner, 1980). Later studies revealed this to be due to changes in the expression of Solute Carrier Family 39 Member A5 and SLC30A1 on the basolateral side of pancreatic acinar cells (Liuzzi et al., 2004) as well as Solute Carrier Family 30 Member A2 on the membranes of zymogen granules (Guo et al.,

2010) in rodents. The expression of these transporters alongside an intestinal-pancreatic axis of Zn homeostasis, originally proposed by Liuzzi et al. (2004) in mice, has so far been partially reproduced in pigs (Brugger et al., 2021) but is supposed to be a well-conserved trait in mammal species in general (Lichten and Cousins, 2009). Open questions with respect to farm animals refer to the role of the forestomach system of ruminants in zinc metabolism and a mapping of the genetic architecture of Zn transport in chickens.

In summary, the quantitative kinetics of Zn metabolism highlight the organism's attempt to maintain homeostasis under varying supply conditions.

Special features concerning different livestock species

Differences between animal species regarding the susceptibility to Zn deficiency are predominantly related to their ability to deal with antagonists of Zn absorption within the digestive tract.

Native phytic acid in cereal-based diets may impair the Zn status in young growing pigs without sufficient dietary Zn supplementation and in the absence of native and/or exogenous phytase activity (Schlegel et al., 2010; Schlegel et al., 2013). In maize grain-soybean meal-based diets without the addition of exogenous phytase and crystalline amino acids, native phytic acid concentrations can go up to levels >8 g/kg (Selle and Ravindran, 2007). Such amounts have been shown to literally deactivate Zn absorption from the gut of rodents (addition of 9 g/kg Na-phytate to a semi-purified diet) and pigs (native phytic acid at 9 g/kg) (Windisch and Kirchgessner, 1999; Brugger et al., 2014), making it an effective dietary challenge for Zn intervention studies.

Poultry and especially broiler chickens could also experience some negative effects from phytic acid in their diet but recent data suggest that significant phytate degradation happens alongside their GIT by mucosal as well as microbial phytase activity (Zeller et al., 2015; Sommerfeld et al., 2019). To which extent this affects their Zn requirements or the need for supplementation, respectively, has yet to be quantified but it could be assumed that this positively affects the Zn utilisation capacity in broiler chicks. The interaction of phytic acid, P and Zn in poultry diets deserves more attention in future, latest since the observation that a reduced dietary supply with mineral P may temporarily impair the Zn status of laying hens (Brugger et al., 2020). The same study also suggests that the Zn status of layers may be impaired by native phytic acid under comparable conditions as applied earlier in weaned piglets (Brugger et al. (2014). This is in strong contrast to studies on broiler chickens which were more efficient than pigs with respect to the utilisation of native Zn (Schlegel et al., 2010; Schlegel et al., 2013), which is presumably due to their reportedly relevant mucosal and microbial phytase activity (Zeller et al., 2015; Sommerfeld et al., 2019). Future studies should compare the layers' ability for phytate breakdown with that of broilers.

Cattle and other ruminants harbour significant activity of microbial phytase in their ruminal ecosystem (Humer and Zebeli, 2015). Hence, their susceptibility to phytate-induced Zn malnutrition should be lower than in non-ruminants, especially pigs. However, reports of Zn deficiency in high-yielding dairy cows indicate certain dietary situations may have the potential to impair Zn absorption also in ruminants (Cope et al., 2009). The ruminal microbial breakdown of phytic acid is usually not complete and could decrease significantly under conditions where dietary passage through the rumen increases, e.g. under conditions of high DM intake (Colucci et al., 1990; Humer and Zebeli, 2015). Furthermore, soil contamination of grassland forage (especially silage) may yield high Fe concentrations (up to 1 500 mg/kg DM) in high-forage diets, which have been shown to impair feed Zn utilisation (Standish et al., 1969). Studies in pigs highlighted a competition of both ions for transport mechanisms in the GIT mucosa (Bertolo et al., 2001), which may explain this phenomenon. Finally, recent data indicate that also certain clay minerals from such contaminations could impair the solubility of Zn alongside the GIT of cows (Schlattl et al., 2021).

Status parameters under experimental conditions

The apparently absorbed amount of Zn can be easily measured applying standard procedures for the estimation of percentage nutrient digestibility and using these values to calculate the apparent absorption based on the ingested amount of the element (Zhang and Adeola, 2017). This measure shows a strong positive correlation to the true Zn absorption (as well as the apparent and true Zn retention), as demonstrated earlier in rats (Weigand and Kirchgessner, 1980), which represents the apparently absorbed amount of feed Zn corrected for the endogenously secreted amount of Zn. It is noteworthy that partial digestibility data reflecting the disappearance until the end of the small intestine do yield limited information since the caecum and colon have been shown to absorb significant amounts of dietary Zn (Hara et al., 2000). Monitoring apparent absorption or retention under high-resolution dose–response conditions allows an estimation of the gross-requirement threshold, marked by a clear decrease in the magnitude of the response to a further increase in dietary Zn. This satiation in Zn absorption (and retention) when comparing the response between sufficient and deficient Zn dosages is well established in the available literature across species (e.g. rats, pigs, chicken and cattle (Schwarz and Kirchgessner, 1975; Weigand and Kirchgessner, 1980; Brugger et al., 2014; Boerboom et al., 2021)). In chickens, where measurement of whole-tract absorption is difficult due to mixed excretion of faecal and urinary losses, the apparent Zn retention should be measured instead (Linares et al., 2007). The ability of a test substance compared to a reference source to provide sufficient Zn to the animal at a significantly lower total dietary Zn concentration can be proven with these comparably simple measurements. Therefore, recording these basic parameters is important for studies on Zn metabolism and bioavailability. In addition, the gene expression of the major active Zn transporter in the gut mucosa (SLC39A4) follows a response that is indirectly related to the apparent total tract absorption of Zn. It is upregulated with a further decrease in dietary Zn supply below the gross requirement, which has been shown in pigs and mice (Liuzzi et al., 2009; Brugger et al., 2021). Simultaneous measurements of apparent total tract Zn absorption and mucosal SLC39A4 gene expression combine information of cause and effect and strengthen interpretations on the change in Zn status as affected by different feeding interventions. Earlier data from rats and pigs suggest that the main Zn absorption site may shift towards distal intestinal segments, especially under conditions of an early subclinical Zn deficiency (Pfaffl and Windisch, 2003; Brugger et al., 2021). Therefore, it should be considered to assess the SLC39A4 gene expression in both, the small and large intestines. For chickens as a non-mammal species, there is yet no confirmation of the function of SLC39A4, therefore, data on its expression should be used with caution.

Most Zn absorbed from the GIT is subsequently transported to the liver via the portal vein. Liver total Zn concentration can be used as a marker for the endogenous Zn status. This has been exemplarily demonstrated under dose–response conditions in a short-term model of subclinically Zn-deficient weaned piglets (Brugger et al., 2014). Based on these observations, surplus amounts of Zn above the gross-requirement threshold are linearly accumulating in liver tissue, whereas in animals fed below the requirement, liver Zn plateaus. The latter reflects the tendency of the liver to release its stored amounts into the periphery in times of limited supply to benefit other tissues and just keeping the necessary minimum to maintain tissue integrity and metabolic activity. The linear accumulation of surplus Zn in the liver of animals

fed above gross requirements has been also shown in chickens (Shyam Sunder et al., 2008) and should apply to farm animals in general (Suttle, 2010). Parallel analysis of Metallothionein 1 (**MT1**) gene expression could provide additional information on liver Zn accumulation, since it directly followed the response of liver Zn over a physiological dose range supplied to weaned piglets (Brugger et al., 2014; Boerboom et al., 2022). However, a reproduction of this observation in other farm animals has yet to be done. It is noteworthy that the above-described response patterns of liver Zn may change with the severity of Zn depletion and under conditions of longer experimental durations. For example, Revy et al. (2006) observed a linear response pattern of liver Zn in piglets with different slopes depending on the addition or absence of exogenous phytase, after 7 d of experimental Zn depletion followed by a 19 d feeding period with varying dietary Zn levels (33–113 mg/kg diet). Overall, liver Zn can be used to discriminate between the deficiently and sufficiently supplied animals under short-term experimental conditions and, in general, is suitable to compare effects on the endogenous Zn status between groups of animals. However, using it to estimate the gross requirement should be done with caution, as it may tend to underestimate the true gross Zn requirement (Brugger et al., 2014) or does not show a breakpoint in response (Revy et al., 2006), depending on the experimental conditions. In addition, because the response of liver Zn and MT1 gene expression to inadequate Zn doses in the diet for weaned piglets was quite restrained in the pig studies on short-term subclinical Zn deficiency (Brugger et al., 2014; Boerboom et al., 2022), their suitability for estimating relative differences in bioavailability by means of statistical slope comparisons is questionable under such conditions.

Tissue Zn accumulation beyond the point of satisfied requirements has been associated with oxidative stress, e.g. in the pancreas of weaned piglets (Pieper et al., 2015); hence, such measures could add useful information from a feed safety perspective.

The majority of mobilisable Zn is present in the skeleton. This pool is subject to targeted depletion and replenishment under the terms of a deficient and subsequent sufficient supply with dietary Zn, as demonstrated in adult rats (Windisch, 2001; 2003). However, it is important that the appropriate part of the skeleton is sampled for total Zn analyses because it appears to be predominantly mobilised from trabecular bone tissue, as demonstrated in rats (Windisch et al., 2002). Such can be found e.g. in the tibia or the femoral head, which have been proved sensitive also in farm animals like pigs and chicken (Brugger et al., 2014; Boerboom et al., 2021). It is noteworthy that the response patterns of bone Zn may not necessarily correlate to those already described for Zn absorption and liver Zn. Indeed, experiments $\geq$2 weeks in chickens revealed also a breakpoint for bone Zn which allowed a discrimination between deficiently and sufficiently supplied animals (Wedekind et al., 1992; Boerboom et al., 2021). However, this is not the case under short-term conditions. A gradual reduction in dietary Zn for weaned piglets for just 8 d initiated a straight linear response of bone Zn over all dose levels (Brugger et al., 2014; Boerboom et al., 2022), which was also observed after 19 d repletion of clinically Zn-deficient weaned piglets (Revy et al., 2006). This also comprised dose levels greater than the gross-requirement threshold. Overall, the interpretation of status parameter response must always occur under consideration of the experimental duration and the experimental set up in general. The first is especially true for certain tissue Zn concentrations, which may respond quite differently in short-term compared with long-term experiments (Brugger and Windisch, 2019).

Plasma and serum Zn parameters (Zn concentration, Zn-binding capacity, enzyme activities) can be useful additional markers to assess the animal status. However, the interpretation of circulatory Zn must occur under consideration of potential acute-phase events (Galloway et al., 2000), which can occur under conditions of Zn deficiency as well as excess (Maret, 2019). In addition, as mentioned for bone Zn also blood parameters show different responses in short- compared to long-term experiments. For example, Zn concentration, alkaline phosphatase activity and percentage Zn-binding capacity in blood plasma directly followed the linear response of bone Zn in short-term experiments on pigs (Brugger et al., 2014; Boerboom et al., 2022) whereas long-term experiments in chickens showed a non-linear response of both parameters (Wedekind et al., 1992; Boerboom et al., 2021). Finally, the response of Zn-dependent enzymes alone yields little information on the body's Zn status because the addition and removal of Zn from the active centre are the measures of active regulation of the enzyme's activity under basal physiological conditions (Maret and Li, 2009).

Dose-response studies in dairy cows and laying hens revealed a tight regulation of Zn secretion via milk and eggs (Schwarz and Kirchgessner, 1975; Paulicks and Kirchgessner, 1994). Therefore, these measures may serve as additional status parameters to diagnose the successful induction of Zn deficiency under specific experimental conditions comprising layers and lactating animals.

Copper

Kinetics and mechanisms of absorption and excretion

Copper absorption occurs via two pathways, either paracellular between enterocytes or transcellular by translocation through the enterocytes (Goff, 2018). Solute Carrier Family 31 Member A1 (**SLC31A1**) was identified as the main regulated Cu transporter in mammals (Lee et al., 2002). In addition, Solute Carrier Family 11 Member A2 (**SLC11A2**) has been proposed as a passive Cu transporter (Arredondo et al., 2014), however, data obtained by ectopic expression studies in cell lines may not directly translate to the situation *in vivo*. The Cu transport via SLC31A1 occurs as monovalent Cu^{+}, involving membrane-associated reductases to reduce Cu^{2+} to Cu^{+} (Ohgami et al., 2006). The SLC31A1-dependent transport decreases significantly in animals supplied with sufficient copper, which means that primarily non-regulated secondary Cu transporters facilitate Cu uptake under practical feeding conditions. This response has been shown in various animal species including mice, rats and pigs (Kuo et al., 2006; Nose et al., 2010). A cell-specific intestinal epithelial SLC31A1$^{-/-}$ knockout in mice promoted severe Cu deficiency and death three weeks *postpartum,* which could be circumvented by intraperitoneal Cu administration (Nose et al., 2006). This suggests the remaining secondary transport mechanisms for Cu were not able to maintain sufficient feed Cu utilisation, which underlines the role of SLC31A1 as the major active Cu transporter in the gut.

The second line of defence against uncontrolled passive Cu uptake is the association of Cu to intracellular MT within the enterocyte to inhibit its transfer to the circulation in times of sufficient or excess supply (Cousins, 1985). In contrast, during times of marginal Cu supply, SLC31A1 activity is upregulated and intracellular Cu transfers through a specific Cu chaperon (Antioxidant Protein 1) to the responsible transporter at the basolateral membrane (ATPase Cu^{2+} transporting alpha polypeptide) (Hamza et al., 1999; Banci et al., 2008).

Liver Cu represents the primary Cu storage in the organism (Suttle, 2010). Here, it is either stored bound to MT or subjected to Cu-protein synthesis (especially Ceruloplasmin (**Cep**)), biliary excretion or directly transferred to other tissues for the purpose of Cu-peptide synthesis. The particular target of Cu transport depends on the Cu status and needs of the animal (Bremner, 1993). Under conditions of dietary Cu excess, liver Cu accumulates (mostly associated to MT2) and is increasingly subject to biliary

excretion. How fast the liver is able to translocate accumulated amounts to biliary secretion as a measure to prevent intoxication appears to be species dependent (Suttle, 2010).

Special features concerning different livestock species

Cu can be chelated by native phytate and thereby blocked from absorption especially in non-ruminating farm animals. Phytase addition to pig diets increased feed Cu utilisation on some occasions (Adeola et al., 1995), but datasets on the matter are quite variable and a meta-analysis of 14 studies yielded no effects of exogenous phytase on Cu digestibility and plasma Cu of growing pigs under practical feeding conditions (Bikker et al., 2012). Indeed, native phytate has a much higher affinity for other ions like Ca^{2+} and Zn^{2+}, hence, in the presence of considerable amounts of these ions, the Cu association to phytate is less likely (Humer et al., 2015). In addition, the total daily Cu requirements of pigs are considerably low (National Research Council, 2012). Therefore, dietary Cu deficiency is a rare event in pigs when feeding common ingredients (Suttle, 2010). Dietary Cu is subject to a strong antagonism with dietary Zn. A supplementation of 100 mg Zn/kg complete feed effectively decreased the apparent Cu digestibility from 22 to 13% in piglets fed a maize grain-soybean meal-based diet with a total dietary Cu concentration of 14 mg/kg (Adeola et al., 1995). However, the overall Cu supply would still be sufficient when following nowadays feeding recommendations (National Research Council, 2012).

There seem to be no practical observations of Cu deficiency in poultry. The Cu availability from different ration ingredients has been thoroughly tested. Although Cu availability for chickens is reduced in the presence of high phytate concentrations, the apparent absorption is not dropping to zero or even negative values (Funk and Baker, 1991; Baker and Ammerman, 1995), like it has been reported for Zn in rodents and pigs at $\geq$8 g phytate/kg complete feed (Windisch and Kirchgessner, 1999; Brugger et al., 2014).

Ruminants are the predominant risk group for Cu deficiency. Increased intake of sulphur (S) and molybdenum (Mo) mainly from roughage leads to the formation of thiomolybdates under the chemical conditions within the rumen. These chelate Cu and shift the Cu distribution more to the rumen particulate matter, extremely lowering apparent Cu absorption to <1%, e.g. in sheep (Suttle, 1983; Allen and Gawthorne, 1987). Comparisons between Cu supplements in ruminant trials should consider this antagonism as it could significantly affect the outcome of a study. Spears et al. (2004) demonstrated that Cu supplemented as tribasic Cu chloride to growing cattle has a superior bioavailability compared to Cu sulphate under conditions of high dietary Mo and S levels. This appeared to be due to a lower rumen solubility of the tribasic Cu chloride and a thereby reduced susceptibility to the thiomolybdate antagonism. In strong contrast, Hanauer (2017) suggested that this superiority may shift to the opposite when dietary Mo and S are moderate, because the lower solubility may then become a disadvantage in terms of bioavailability, as demonstrated in non-lactating cows. Negative effects of high Fe intake (e.g. through soil contamination in forage) have been already mentioned in regard to Zn absorption in cattle and are also true for Cu (Standish et al., 1969). Dietary Mn excess may further promote a molybdenum-associated hypocupraemia, as demonstrated in growing cattle (Hansen et al., 2009).

Status parameters under experimental conditions

Apparent total tract Cu absorption is a relevant status parameter especially under conditions of dose–response studies investigating the range from deficient to sufficient supply levels. Due to the very stable and low amounts of renal Cu excretion across species (Suttle, 2010), apparently absorbed values directly correlate with the retained amounts of Cu from feed. Adaptions in homeostatic regulation of absorption and/or endogenous secretions of Cu culminate in the apparent whole-tract absorption, which can thus be used to assess the status of whole body Cu homeostasis and to map the gross Cu requirement. Again, in chickens with their mixed excretion of faecal and urinary losses, the apparent retention is the parameter of choice. Across species, the already mentioned antagonistic interactions between trace elements make a precise dietary control necessary.

One of the first responses to dietary Cu deprivation is a drop in liver Cu (Suttle, 2010). Liver Cu represents the primary storage pool, which should always be measured in addition to the apparently absorbed or retained amounts. In addition, bile Cu could add valuable information on the Cu status. In both cases, Cu accumulation is reflected in increasing amounts within these compartments, as shown e.g. in sheep (Grace et al., 1998), whereas variations in Cu supply levels below the gross Cu requirement are only leading to restrained adaptions (Suttle, 2010). This allows a very clear discrimination between deficiently and sufficiently (oversupplied) animals if a high-resolution dose–response study was conducted. In addition to liver Cu, assessing MT expression (especially MT2) can yield valuable additional information as a marked parallel rise of both parameters indicates liver Cu accumulation and excess dietary Cu intake, which has been exemplarily shown in rats (Evering et al., 1991).

The limitations of using plasma and serum Cu as a status parameter have already been mentioned in the respective section on Zn status parameters. The abundance of Cu-dependent proteins could be used as a status parameter, but these values should always be measured in addition to quantitative parameters and not as a replacement for such. In fact, their levels and activities in a biological system are not solely affected by dietary Cu supply. For example, Cep is considered an acute-phase protein (Hellman and Gitlin, 2002), hence, times of increased stress and inflammation can affect this parameter independent of variations in dietary Cu supply.

Several Cu-proteins are involved in Fe metabolism. In fact, one leading symptom of Cu deficiency is an Fe-deficiency anaemia due to impaired transfer of Fe out of the cells into the circulation especially in response to reduced Cep activity (Prohaska, 2012). This brings the necessity to control the iron concentration in the diet. Furthermore, it might prove beneficial to monitor the Fe status when performing Cu metabolism trials in order to get a complete view of the physiological adaption to reduced dietary Cu supply especially if the Cep activity was negatively affected by reduced dietary supply levels.

In case of ruminant studies, a determination of S and Mo concentrations in the ration appears indispensable to allow an interpretation of feeding trials, especially when different sources of dietary Cu are compared (Spears et al., 2004; Hanauer, 2017).

Manganese

Kinetics and mechanisms of absorption and excretion

Manganese is one of the least abundant essential transition metals in animal tissues (National Research Council, 2005; Suttle, 2010). Given the narrow range of concentrations of Mn fulfilling its biochemical purpose, its body pools must be tightly regulated. A radio-Mn measurement in rats showed satiation kinetic of Mn absorption with increasing dietary intake (Weigand et al., 1986), thereby supporting the general notion of absorption being a cornerstone of homeostatic regulation of essential transition metals (Windisch, 2002). However, data obtained in farm animal models suggest that absorption relative to intake may not always be regulated so precisely according to status. For example, Pallauf et al. (2012) observed a linear adaption of apparent Mn absorption over the whole dose range (0.24 to 32 mg/kg to a semi-purified

diet), whereas endogenous pools (like bone, liver, kidney or pancreas) responded in a non-linear fashion. Comparable response patterns could be found also in broilers (liver, pancreas, heart) (Li et al., 2011), suggesting that Mn retention is indeed tightly regulated across species but absorption appears to be not in every case predictive for it. This obvious contrast to other metals including Zn may suggest that the renal excretions are of greater quantitative importance for the homeostatic regulation of Mn. A hypothesis that appears to be supported by the non-linear adaption of kidney Mn in pigs, as reported by Pallauf et al. (2012). It is yet unclear, why homeostatic downregulation of Mn absorption in case of sufficient Mn intake is much less consistent than e.g., for Zn, and which nutritional situations cause such deviations from generally expected metabolic reactions. Manganese exhibits a close interaction with proteins that are associated with Fe metabolism. It has been shown in rodent studies that Mn is transported in relevant amounts by SLC11A2 and subsequently processed by ferritin (Arredondo and Munez, 2005), transferrin (Davidson et al., 1989), and Cep (Jursa and Smith, 2009). Furthermore, based on data obtained in rhesus monkeys, Mn absorption from milk appears to be largely facilitated by lactoferrin receptors (Davidson and Lonnerdahl, 1989). Although a direct translation of these mechanistic data into farm animals has yet to be done, a clear antagonism between dietary Mn and Fe has also been reported for these species (Suttle, 2010). Feeding simultaneously high amounts of Mn and Fe to rats promoted a much higher decrease in Mn absorption efficiency than was evident from the feeding of high Mn alone (Davis et al., 1992). This reflects a combined regulation of SLC11A2 activity by Fe as well as Mn, with Fe being the more dominant regulative stimulus. It may explain the aforementioned lack of regulation with respect to Mn absorption in certain farm animal trials, but targeted studies on the matter have yet to be performed. The regulation of Mn homeostasis gained a further level of complexity with observations in mice regarding certain Zn transporters also being Mn transporters, responsible for its uptake from the gut and its redistribution in and between tissues like liver and brain (Felber et al., 2019; Taylor et al., 2019). The practical consequences of this interaction for the feeding of farm animals are still unclear.

Special features concerning different livestock species

There are reports of clinical Mn deficiency under field conditions mostly from high-intensity poultry production systems. It appears that especially chickens are less efficient than, e.g. pigs in the absorption of Mn from the GIT. Therefore, their gross requirements under practical conditions are higher and, hence, there is a higher risk of suboptimal dietary supply. High phytate and fibre concentrations are the most relevant antagonisms in this regard and this could be used to create a dietary challenge under experimental conditions (Suttle, 2010). Another antagonism exists between excessive dietary P levels and the gastrointestinal Mn absorption. Respective data have been obtained in chickens fed ~8 g/kg DM of excess mineral P to either a semi-purified diet with low Mn at 12 mg/kg diet (Baker and Odohu, 1994) or a maize grain-soybean meal-based diet with excessive Mn at 1 000 mg/kg diet (Wedekind et al., 1991). In both cases, Mn absorption was strongly impaired (≥50%) by the P overload. The above-described interactions between Mn and other nutrients in feed and metabolism strengthen the need to ensure moderate and stable dietary levels of these factors, close to their recommended levels, during Mn intervention studies.

Reports on Mn deficiency in commercial pig herds are scarce. The most recent study on Mn requirements of piglets estimated a necessary dietary minimum of 16 mg/kg under semi-purified dietary conditions (Pallauf et al., 2012). Flaws in the dietary design could impair dietary Mn supply. Excessive dietary Fe loads are a prominent example in this regard and can cause Mn deficiency in all livestock species (Suttle, 2010). Overall, the relevance of Mn feeding efficiency from different sources for pigs under practical feeding conditions appears questionable.

The native Mn content in herbage is quite variable depending on its geographical origin. In addition, it is also dependent on other factors, such as botanical composition, growth stage, harvest number and conservation, which have been quantified and thus explain part of the variability (Schlegel et al., 2016; 2018). Roughage produced on alkaline soils may be affected by low Mn uptake of plants (Suttle, 2000) and otherwise, acidic soils can harbour so much available Mn for plants that intoxication of animals might become an issue (Grace and Lee, 1990). Hence, the determination of Mn in feedstuffs is especially important for the feeding of ruminants.

Status parameters under experimental conditions

The aforementioned lack of consistent regulation observed for Mn absorption from certain farm animal studies suggests that this parameter should be used with caution when aiming to monitor the homeostatic adaption to varying supply levels. Pallauf et al. (2012) suggested using certain endogenous Mn pools instead, which reflected the homeostatic adaption of Mn retention. They mapped the Mn requirement of piglets using Mn in liver, kidney and bone but found even more tissue pools that showed a homeostatic response pattern. Overall, this suggests that Mn apparent retention should be in any case a suitable status parameter for Mn response studies. It is noteworthy that data from several chicken studies are in agreement with Pallauf et al. (2012). Li et al. (2011) reported homeostatic response patterns for Mn pools in liver, pancreas and heart of broiler chickens when supplying Mn in dosages between 18.5 and 158 mg/kg. The same was observed for bone Mn in broilers and layers receiving dietary dosages between 12.2 and 150 mg/kg as well as 16 and 56 mg/kg, respectively (Attia et al., 2010; Saldanha et al., 2020). This appears to contradict the general notion that bone Mn in chickens is not homeostatically regulated and therefore should respond in a linear fashion to the dietary Mn intake. However, this seems to be an artefact from studies with an excessive dose range and a rather crude variation in dosing steps, e.g. in broilers receiving between 93 and 2 200 mg Mn/kg diet (Henry et al., 1989). This shows again the importance of considering a high-resolution dosing regimen within physiological ranges when aiming to evaluate trace element feeding strategies to meet endogenous requirements. Taken together, these findings suggest that the most suitable parameter to map the homeostatic response to varying Mn supply in farm animals is the apparent Mn retention. Alternatively, the above-proposed tissue pools could be used when a quantitative collection of excreta is not possible.

The activity of Mn-enzymes like arginase or Mn-superoxide dismutase could also yield valuable information, e.g. based on observations in pigs (Pallauf et al., 2012). However, as mentioned before for Zn and Cu, such measures must always be interpreted in context to the quantitative measures of Mn homeostasis.

Conclusions

The fluxes of essential trace elements are actively regulated in response to the dietary supply levels ranging below vs above metabolic requirements. This means that oversupplied amounts are less efficiently retained and surplus amounts behind the gut barrier temporarily accumulate and are subject to rapid excretion soon after. Otherwise, in a deficiently supplied individual, its gastrointestinal absorption capacity is upregulated and/or the endogenous excretory losses are reduced as far as possible. This adaption must be mapped by parallel monitoring of quantitative parameters as well as endogenous markers of trace element status in sufficient

resolution, to evaluate the efficiency of trace element feeding interventions e.g., the use of different sources of trace elements under conditions of insufficient supply levels. This directly reflects relative differences in bioavailability in response to the supplementation of different dietary trace element sources, with higher efficiency being reflected by a markedly reduced necessity for supplementation on top of complete feed. This study proposes framework conditions to design and communicate studies that aim in testing such differences in the bioavailability of dietary trace elements. These may be useful for researchers that are in the stage of study design, but also for decision makers to interpret earlier studies on trace element feeding in the context of respective authorisation applications.

Supplementary material

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Ethics approval

The present work required no ethics approval.

Data and model availability statement

The data/models were not deposited in an official repository. Access to data can be provided by the authors upon request.

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Declaration of Interest

The authors have no competing interests to declare.

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