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# The impact of physicochemical properties of different sources of ZnO used in animal feeding in zinc bioavailability in broilers

Denise de Senna Cardoso

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N°d'ordre NNT : 2019LYSE1085

## **THESE de DOCTORAT DE L'UNIVERSITE DE LYON**

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### **The impact of physicochemical properties of different sources of ZnO used in animal feeding in zinc bioavailability in broilers**

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***In loving memory of my dear friend, Isa***

*Pra quem tem fé, a vida nunca tem fim*

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*Three years have passed. I have learnt about chemistry, about zinc, about nutrition, I have learnt about France - not French, though. I have visited beautiful places, châteaux, mountains, lakes. I have enjoyed wines, cheeses – it was a full French experience indeed. But nothing of it would be worth if wasn't accompanied by people. I am only luck for those whom crossed my path in this journey. People who inspired me and left their marks. Stephane, I am grateful for hooking me into this project, with your 'obsession' for zinc that made me join this adventure and for teach me about commitment and passion ever since. Yves, your generosity towards us, your patience to teach me from the simplest steps, with humility and a good smile is a real inspiration, a true professor, who teaches the chemistry of a joyful work life. Agnes, thank you for your input in my work, to adjust my fences and help me to go through all the analysis and writing. I truly admire your organization and always valuable feedback, your tough-sweet touch was essential to make this manuscript real. Nathalie Meme, thank you for kindness and French lessons. You are light and organized, it is just delightful to be around you. My dear Animine colleagues, for encouragement and advice during the trials. INRA and Lyon University staff to give me all the support so my goals could be accomplished. My friends, the cherry of life. I have the luck and the sorrow to have them spread all over the globe - literally. My life friends back home, that no matter where I am or how many letters I add to my name, always see me as a loud clumsy torch. The friends 'animal nutrition' brought into my life, even spread around, we always find time to meet and remember that good things never change. The new valuables ones, that France added to my collection and is safely stored in my heart. Isa, thank you for continually inspiring me, wherever you are, you will always be my 'best-i' and my example of a real fighter, I miss you. My family, that always have been my safe shore, to be present, away from home. My loving mom and dad, to endure by absence and support my choices. My dear brother, the one whom I share my best laughs and funniest fights. My sweetheart Jan, for bear with me in these final moments, to not quit, even when I did - many, many times. Hard work can lead one to great accomplishments, but are people who give purpose and make hard times worth of go on. Three years have passed. What a bumpy road. I discovered an amazing new country. I made friends. I learned home is where your heart is and the miss of loved ones is the comfort of the joyful moments shared.*

## ABSTRACT

The bioavailability of a trace mineral source is related to its *in vivo* solubility, which in turn is determined by its physicochemical properties. It is still not clear which characteristics are more relevant in affecting solubility and bioavailability of feed compounds. Zinc Oxide (ZnO) is a common feed additive used to supplement zinc in the diet of monogastrics animals. However, different sources have shown different responses in animal bioavailability. This project aims to characterize different feed grade ZnO to better clarify their fate in the digestive tract and explain differences in bioavailability. It was hypothesized that the different sources of feed grade ZnO have various physicochemical properties that lead to distinct dissolution kinetics. Over 40 samples of ZnO have been collected from the feed industry worldwide. Samples were analyzed for density, tapped density, particle size, shape, specific area and dissolution kinetics. A principal component analysis (PCA) was performed to define the most relevant characteristics and categorize the samples into groups. The results showed that there is not only one variable influencing the kinetics of dissolution. It suggests a dissolution mechanism in 2 steps, in which there is the dissolution of ZnO into Zn ions in the surface of the particle, followed by a diffusion to the bulk solution. The speed of dissolution can determine the fate of zinc oxide products in the gastrointestinal tract and therefore may explain the different results in animal performance. Representative products from each family were selected for an *in vivo* trial to measure the effect of their characteristics on the zinc bioavailability. Male Cobb broilers were used in a dose-response experiment from 8 to 23 days of age. Treatments consisted of a basal diet with 23 ppm of zinc and 14 diets supplemented with 6 or 12 ppm of Zn in the form of different oxides or sulfate. Bone zinc was used to determine zinc bioavailability. The bioavailability of the different sources varied from 49 to 160% considering zinc sulfate as the reference. Aggregate size and specific surface area explain a large part of the variability observed on values of bioavailability. In conclusion, physicochemical properties of ZnO can partly explain the variability observed in terms of Zn biological value. However, the complexity of physiological processes makes difficult the prediction of Zn bioavailability based only on these characteristics.

**Key words:** Physicochemical properties, Dissolution kinetics, Bioavailability, ZnO, Broilers

## RESUME

La biodisponibilité d'un composé minéral dépend de sa solubilité in vivo contrôlée par ses propriétés physicochimiques. Cependant, les caractéristiques physicochimiques qui influencent la solubilité et la biodisponibilité des produits utilisés en nutrition animale ne sont pas clairement connues. L'oxyde de zinc (ZnO) est un additif alimentaire couramment utilisé pour satisfaire le besoin en zinc (Zn) des animaux monogastriques. L'objectif de ce projet était de caractériser plusieurs types de ZnO utilisés en nutrition animale pour mieux appréhender leur devenir dans le tractus digestif et leur biodisponibilité. Les différentes sources de ZnO ont des propriétés physicochimiques variables qui influencent leurs cinétiques de dissolution. Une quarantaine d'échantillons de ZnO collectés à travers le monde auprès d'industries de la nutrition animale ont été caractérisés en termes de densité, densité tassée, taille de particules, forme, aire spécifique et cinétiques de dissolution. Une analyse en composantes principales (ACP) a été réalisée pour identifier les caractéristiques les plus pertinentes et classer les échantillons par familles. La nature des paramètres les plus pertinents suggère un mécanisme en deux étapes : dissolution de ZnO en ions  $Zn^{2+}$  à la surface des particules, suivie d'une diffusion vers la solution. La vitesse de dissolution détermine probablement le devenir de ZnO dans le tractus gastro-intestinal et pourrait donc expliquer les différences de performances. Une étude in vivo de produits représentatifs de chaque famille a permis de déterminer les effets des caractéristiques physicochimiques sur la biodisponibilité. Une étude dose-réponse a été réalisée sur des poulets Cobb en croissance (8 à 23 jours d'âge) soumis à un régime de base contenant 23 ppm de zinc ou à 14 régimes supplémentés à raison de 6 ou 12 ppm de ZnO (6 sources différentes) ou de sulfate de Zn. La teneur en zinc du tibia a été utilisée pour estimer la biodisponibilité relative des différentes sources de ZnO par rapport au sulfate de Zn utilisé comme référence (100%). La biodisponibilité des différentes sources variait entre 49% à 160%. La taille des agrégats et la surface spécifique expliquent une large part de la variabilité sur la biodisponibilité. Les propriétés physicochimiques de ZnO permettent d'expliquer partiellement la variabilité observée des biodisponibilités du zinc car la complexité des phénomènes physiologiques rend difficile une telle prédiction.

**Mots clés :** Propriétés physicochimiques, Cinétiques de dissolution, Biodisponibilité, ZnO, Poulets

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## LIST OF PUBLICATIONS AND COMMUNICATIONS

### Publications

Cardoso, D., Narcy, A., Durosoy, S., Bordes, C., Chevalier, Y.. 2019. "Physicochemical characterization and dissolution kinetics of zinc oxides sources used in animal feeding." *Animal Feed Science and Technology* (preparation)

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Cardoso, D., Durosoy, S.. 2018. "Zinc and Copper oxides: The best combination." *Pig Progress*; December 2018.

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## LIST OF ABBREVIATIONS

<b>ADG</b>	Average Daily Gain
<b>BET</b>	Brunauer-Emmet-Teller
<b>BW</b>	Body Weight
<b>Cu</b>	Copper
<b>DM</b>	Dry matter
<b>FCR</b>	Feed Conversion Ratio
<b>Fe</b>	Iron
<b>g</b>	Gram
<b>GIT</b>	Gastrointestinal Tract
<b>H<sub>2</sub>O</b>	Water
<b>HCl</b>	Hydrochloric Acid
<b>I</b>	Iodine
<b>ICP-OES</b>	Inductively Coupled Plasma – Optical Emission Spectrometry
<b>IP</b>	Inositol phosphate
<b>ISO</b>	International Organization for Standardization
<b>kg</b>	Kilogram
<b>L</b>	Liter
<b>mg</b>	Milligram
<b>Mg</b>	Magnesium
<b>mL</b>	Milliliters
<b>Mn</b>	Manganese
<b>MT</b>	Metallothionein
<b>PCA</b>	Principal Component Analysis
<b>RBV</b>	Relative Bioavailability Value
<b>RPM</b>	Rotations Per Minute
<b>Se</b>	Selenium
<b>SE</b>	Standard Error
<b>SEM</b>	Scanning Electron Microscopy
<b>SSA</b>	Specific Surface Area
<b>TEM</b>	Transmission Electron Microscopy
<b>XRD</b>	X-Ray Diffraction
<b>Zn</b>	Zinc
<b>ZnCl<sub>2</sub></b>	Zinc Chloride
<b>ZnO</b>	Zinc Oxide
<b>ZnSO<sub>4</sub></b>	Zinc Sulphate

## INTRODUCTION

The exponential population growth, estimated to be at 9 billion people by 2050, is followed by the need to increase food production by 70% until then. Animal production plays an important role in human nutrition as a source of protein, and poultry meat is most likely to lead this growth due to its lower production cost, absence of religious restrictions and others advantages over other meat species. Nevertheless, the environmental footprint left by these productions impact the climate change, ocean health, deforestation, water cycle, which push animal production into strict regulations and towards a more sustainable and efficient production (Santoma, 2017).

Trace minerals, are those nutrients present in little amounts in the body but essential for a proper body function. The trace elements Iron (Fe), Zinc (Zn), Copper (Cu), Manganese (Mn), Selenium (Se) and Iodine (I), are of primary concern in poultry diets and supplementation is recommended. Zn, as an essential trace mineral, is present in many physiological pathways in the body (Park *et al.*, 2004). The limited storage of this element requires a daily supply in the diet (Yu *et al.*, 2017). Additionally, native Zn in cereal based diet of poultry it is not enough to meet animals' requirement, therefore, supplemental sources are often used (Schlegel *et al.*, 2013). Although, there is a gap in the literature between the requirement of Zn from scientific bodies and genetic company recommendations and the European Legislation, supplementation above the requirements is a current practice in poultry production (Naz *et al.*, 2016). Yet, the excess of Zn in the diet also lead to an increased excretion of this mineral causing environment concerns, especially in areas where there is intensive production (Schlegel *et al.*, 2013).

Bioavailability is the proportion of a certain element consumed that is utilized for a biochemical or physiological function, in other words, are those elements that are absorbed, reaching systemic circulation and act on functions in the animal. The main factors influencing the Zn bioavailability are: (1) Interactions with feed compounds, such as phytate, that bind to divalent cations forming insoluble complexes, and (2) the supplemented source. Several studies evaluating different supplemented sources have claimed variable bioavailability values (Brooks *et al.*, 2013; Brugger and Windisch, 2017; Yan *et al.*, 2016), however it is still not clear the mechanism underlying the bioavailability of these different sources. Additionally, different methodologies used in animal studies to assess bioavailability can justify the different outcomes.

The most common supplemented sources used in broiler diets are inorganic forms (Sulfate and Oxide) and chelates, also known as 'organic' forms. Chelates forms are variable, as they present different properties in respect to their complexation. Zinc sulfate and oxide are the most commonly used sources. Zinc sulfate is considered to be 100% bioavailable, since it is a water soluble source. On the counterpart, zinc oxide, a non-water-soluble source have shown great variability within different sources of this powder.

Bioavailability is habitually related to solubility, as to be absorbed the nutrient has to be soluble. However, studies have shown that some sources of non-water-soluble ZnO can have a bioavailability equal or higher in relation to the water-soluble source, Zn sulfate (Edwards and Baker, 1999; Narcy *et al.*, 2015). Better knowledge of supplemented sources can widen the understanding of how these sources behave in gastrointestinal tract of animals and how affect their bioavailability.

Dry powders, like ZnO, have various characteristics depending on the method by which they are manufactured. Color, flowability and structure are some of the properties visually observed in powders and also play a role in the handling and manipulators' safety. However, intrinsic properties such as: size, shape, specific surface can also affect their dissolution behavior in biological tissues. Characterization of powders and *in vitro* observation of dissolution kinetics of sources can provide information on the fate in the gastrointestinal tract and elucidate the bioavailability of supplemented Zn.

The first part of this thesis purposed to analyze the recent literature of the effect of different sources of Zn in broiler bioavailability. A compilation of data from the last 8 years was done to present the physiological dose-response of Zn in broilers and the effect of sources on Zn bioavailability. An *in vitro* study was performed to characterize the different sources of feed grade ZnO and categorize them according to their physicochemical properties. Samples were analyzed for crystal shape, size, particle size, specific surface area, density and flowability. To categorize them, a principal component analysis was performed to identify clusters of samples and which characteristics are more relevant affecting the *in vitro* dissolution kinetics of ZnO powders. It is hypothesized that different powders characteristics lead to different dissolution speeds that will affect the interactions with feed compounds, their dissolution into zinc ions and at last their bioavailability. An *in vivo* study was performed to assess the effect of the different physicochemical properties and dissolution kinetics of ZnO samples in bioavailability of broilers. The experiment investigated the dose-response of different levels and sources of feed grade ZnO and Zn sulfate. Bioavailability was assessed in bone Zn of birds. Additionally, the gastric and

intestinal content was collected to observe the solubility of the experimental sources in the different compartments of the gastrointestinal tract of the animals.

# CHAPTER 1

# 1 LITERATURE REVIEW

## 1.1 Poultry production and the environment

In a FAO report from October 2018, the forecast for total world meat production for 2018 was 335.048 million tons, in which 121.6 million tons corresponded to poultry meat, a rise of 1.4% compared to 2017. In terms of volume, poultry meat is the second rising grow behind only pig meat. By 2050, it is expected a rise of 121%, surpassing pig meat. The affordability, short rearing time, limited greenhouse emissions, no religious restrictions are some of the advantages of this production over other meats, which most likely put poultry as leading meat source for human nutrition in the future (Santoma, 2017).

The world is facing climate change, a decrease in biodiversity, compromise of ocean health, deforestation, changes in the water, carbon and nitrogen cycle, in which animal production also has its impact and leaves an important environment footprint, contributing to the threat to Earth's system. The effects of poultry production in the environment start at the poor management of manure and litter to contamination of streams, emission of greenhouse gases, acidification and eutrophication (Rodic *et al.*, 2011).

Feed corresponds to 70% of carbon footprint in animal production. Monteiro and Dourmad (2018), calculated the contribution of feed ingredients to environmental impact. Cereal and oilseed, that are the base of animal feed have the most impact in acidification, eutrophication and terrestrial toxicity (Figure 1). Even though trace minerals represent less than 1% of total feed ingredients, the analysis showed trace minerals the highest threat to freshwater and marine ecosystems.

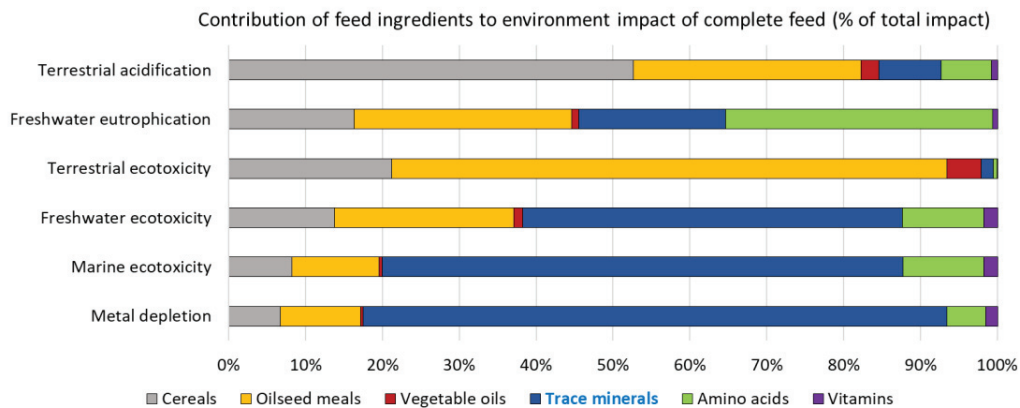


Figure 1: The proportion of environmental impact caused by feed ingredients in animal feed (Monteiro and Dourmad, 2018).

## 1.2 The element Zinc

Zinc (Zn) is a transition metal with the atomic number 30, and an atomic mass of 65.38 u. This element makes up to 75 ppm of Earth's crust, making it the 24<sup>th</sup> most abundant element. Zinc is the fourth most widely used metal in the world after iron, aluminum and copper. Zn exhibits only one state of oxidation, the divalent form,  $Zn^{+2}$  being the main soluble species. The main use of Zn in the industry is directed to the galvanizing of iron and steel to protect them from oxidation – in a process that Zn oxidizes to protect the other metals. Zn sulfate is a compound of Zn used mainly in the textile industry and for soil enrichment. ZnO is the most known and industrially used form of Zn. More than half of the ZnO produced in the world is used in the rubber industry, as a vulcanizing activator, for motor car tires - one tire has approximately 100 g of ZnO. Followed by the rubber industry, ZnO is largely applied in ceramics and concrete, plastics, pigments and coating, cosmetics, medical, dental, etc. A wide range of cosmetics products contains ZnO. This substance aid cosmetics to adhere to the skin and also have a broad-spectrum ultra-violet absorption. Clinically, ZnO promote wound healing and keep wound moist and clean.



### 1.3 ZINC IN ANIMAL NUTRITION

#### 1.3.1 SOURCES OF ZINC IN ANIMAL FEED

Chelates, sulfates and oxides are the most common source of zinc supplemented in poultry diets. There is a poor characterization of these sources and they are classified basically as 'organic' or inorganic source and by the zinc percentage and solubility.

##### 1.3.1.1 ZnO

In poultry nutrition, ZnO is well used: the non-hygroscopic properties, low reactivity with other feed compounds and higher concentration of zinc (70 – 75% Zn), which occupies less space in the premixes factory, associate to the relatively low price of this commodity, also make ZnO a feasible option as a Zn source in poultry diets (Edwards and Baker, 1999). However, studies with ZnO have shown a wide range in bioavailability values. Studies have shown a ZnO bioavailability ranging from 37 to 105% in relation to ZnSO<sub>4</sub> (Edwards and Baker, 1999; Narcy *et al.*, 2015).

ZnO powders are variable in appearance, color, according to process by which it is manufactured (Edwards and Baker, 1999). Figure 2 illustrates some different feed grade ZnO sources.



Figure 2: Different sources of feed grade ZnO. Powders vary in color, structure according to the manufactured process. Left to right, powder produced with different methods (1) French method, (2) hydrometallurgical and (3) American method.

Zinc oxide can be obtained by many industrial processes, each of them resulting in a final product with distinct characteristics. The most used methods and properties are described on Table 1. The variations within methods regarding specific surface area, porosity, morphology and purity are related to process conditions, like temperature and quality of raw materials (Moezzi *et al.*, 2012).

Table 1: Description of most used methods for ZnO fabrication and their properties according to Moezzi *et al.* (2012); SSA = specific surface area.

Method	Process	Properties
French (indirect) pyrometallurgical	Reduction of Zn metal followed by re-oxidation	- SSA up to 12 m <sup>2</sup> ·g <sup>-1</sup> - rod-like shape - wide size range
American (direct) pyrometallurgical	Oxidation of Zn metal	- SSA < 3 m <sup>2</sup> ·g <sup>-1</sup>
Spray pyrolysis	Thermally decomposition of Zn-bearing salts	- SSA > 12 m <sup>2</sup> ·g <sup>-1</sup> - Uniform particle shape - Narrow size distribution
Hydrometallurgical	Precipitation of the oxide from an aqueous solution followed by calcination	- "Active ZnO" - High SSA - Irregular morphology

#### 1.3.1.2 Zn Sulfate

Zn Sulfate, along with ZnO are the most used sources in animal production. The low price and assumed bioavailability of 100%, due to its water solubility property are some of the advantages of this source. This white powder, have a zinc percentage of 37%. It has a hygroscopy characteristic that can be a problem in hot and humid countries. In the feed, the water soluble

properties cause reactive metal ions to promote free-radical formation, causing reactions with vitamins and degradation of fat and oils, decreasing the value of the diet (Park *et al.*, 2004).

#### 1.3.1.3 Chelates/Organic sources

Chelates are complex species resulting from the complexation between metal and a certain ligand. When this ligand is a non-metallic compound, it is then called 'organic' (Vieira, 2008). These zinc-ligand complexes, that varies from weak to strong complexes according to the molecule to which they are attached, supposedly avoid interactions with phytate (Schlegel *et al.*, 2013).

The main claimed difference of these sources from sulfate is the stability of the complex species. In contrast with their inorganic counterpart, ligands remain complexed to Zn, even when solubilized in aqueous medium, – due (1) to the stability of their structure and (2) these complexes are resistant to interactions with other feed nutrients in the gastrointestinal tract. Zn sulfate allows the released ions ( $Zn^{+2}$ ) to interact with feed compound forming insoluble complexes that are unavailable for absorption (Zhao *et al.*, 2015).

Chelates can have different chemical characteristics which are poorly characterized (Zhao *et al.*, 2015). As chelates forms are highly variable according to the complex to which it is attached, it is challenging to compare effects and elucidates mechanisms of action.

#### 1.3.2 FUNCTIONS IN THE BODY

Trace elements are those nutrients with a concentration in the body lower than 50 mg/kg (Fisher, 1975). Zinc is the second most abundant micromineral, followed by iron (Fe). Although, it is in such small amount in the organism, Zn, is virtually existing in the whole body as almost every

signaling and metabolic pathway contains of one or more zinc-required protein. This essential metal is present in the body through structural and functional integrity of over 2000 transcription factors, responsible to regulate gene expression. Zn is required for the maintenance of their structural integrity and binding to DNA (Underwood, 2010) and is a catalyst of more than 300 metalloenzymes (Murakami and Hirano, 2008; Park *et al.*, 2004). The main functions of zinc are related to: (1) gene expression (2) animal growth, (3) antioxidant defense of cell membranes and (4) immune system (Murakami and Hirano, 2008; Underwood, 2010).

Additionally, Zn also seems to play a role in the maintenance of the barrier function in the intestine. A study using a Zip-14 knockout mouse showed this Zn transporter in the intestine is necessary for the uptake of Zn in the basolateral membrane of the enterocytes and the maintenance of the barrier function in gastro intestinal tract, through the stabilization of occludin phosphorylation, a tight junction protein (Guthrie *et al.*, 2015).

There are several symptoms described in the literature related to zinc deficiency in animal production: anorexia, skeleton disorders, poor bone mineralization, skin problems (Park *et al.*, 2004; Underwood, 2010), impairment of growth, reproductive and immunological functions (Sunder *et al.*, 2008; Yu *et al.*, 2017). In poultry, zinc deficiency showed reduced hatchability, embryonic malformation, weak chick with poor feathering and higher mortality (Hudson *et al.*, 2005).

Keratin and collagen are two keys structural proteins that require zinc for their synthesis. Keratin is the major component of feathers, skins, beaks and claws. Collagen, is present in extra cellular matrix and connective tissues in internal tissues, like cartilage and bones. Zinc deficiency

decreases the synthesis rate of these structural proteins that can lead to bone abnormalities, poor feathering, dermatitis and decreased tissues strength (Richards *et al.*, 2010). Zinc deficiency is related to an increase in the oxidative damage of cell membranes caused by free radicals. The mechanism by which this happens is still not completely elucidated, however, it is thought that Zn upregulates metallothionein, a cysteine-rich protein that acts as a free-radical scavenger (Sahin *et al.*, 2009).

### 1.3.3 DIGESTIVE BIOAVAILABILITY OF ZN

#### 1.3.3.1 *An overview of the digestive tract of birds*

In the digestive tract of poultry, feed is ingested, moisturized, ground into small particles, acidified and cleaved into simpler molecules by endogenous enzymes that can be absorbed. Figure 3 illustrates the different segments of the digestive system of a broiler chicken.

The feed is ingested in the mouth without any process of moisturize nor grounding. The crop is a transient store for ingested food, where moisturizing takes place that can help in further steps along the digestive tract.

The gizzard and proventriculus are the true stomach of birds. The gizzard contains strong muscles that grind the feed and the proventriculus secrete hydrochloric acid and pepsinogen. The poultry gastric juice has a pH of 2, but this value is variable according to the diet, retention time, that often results in a higher pH (average value of 3.5). The lower pH in the stomach is important for the activation of pepsin, enzyme responsible for the cleavage of feed protein. The small intestine is the site where most digestion and absorption of nutrients take place. The duodenum is the first segment, with transit time estimated to be less than 5 minutes. The duodenum ends at the

pancreatic and bile ducts, which quickly rises the pH above 6 and the digestion begins (Svihus, 2014).

The jejunum is a key segment as all major nutrients are digested and absorbed here. Ileum is the last segment of the small intestine. This segment is mainly responsible for water and mineral absorption. Ceca is a pair of elongated blind sacs at the junction of the ileum and the colon, responsible for the fermentation and electrolyte and water absorption.

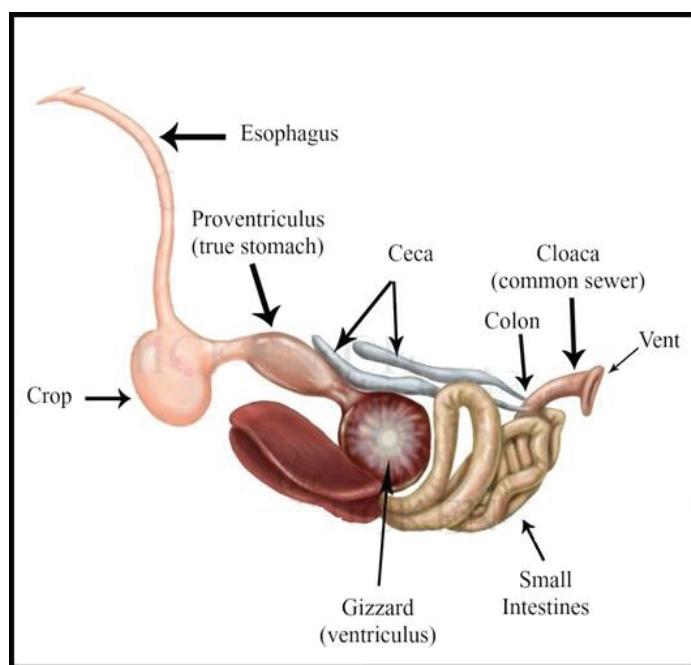


Figure 3: An overview of the digestive tract of a chicken.

### 1.3.3.2 Dissolution process

Mineral absorption can occur throughout the whole length of the intestine, but the duodenum is the main site of metals absorption because the ions are still soluble after the gastric treatment. As the ionization of metals is pH dependent, the acidic environment of the proventriculus of the

bird favors the dissolution, as the gastric juice secreted by broilers has a pH of 2 (Svihus, 2014). The low pH in the upper part of the intestine favors absorption, before the pancreatic secretions increase the pH (Schlegel *et al.*, 2013; Vieira, 2008).

The source of Zn which is supplemented can have different behavior in the gastrointestinal tract, and affect further bioavailability. Water soluble sources, such as sulfates and some chelate forms, are solubilized within a second in an aqueous medium. The release of zinc ions is essential for the micro-element to be absorbed in the body. Zn may also be available as a complex with other feed compounds and becoming unavailable for uptake by the body. Some chelate forms were claimed to be solubilized in complexed forms. This complexation varies from weak to strong bounds, that avoid interactions with phytates, providing a higher bioavailability (Vieira, 2008). Zinc oxides, non-water soluble compounds, have shown variable bioavailability according to the different sources (Edwards and Baker, 1999). Although this source is not water soluble, under the low pH of gastric conditions, it can solubilize.

One can hypothesize the ideal source as the one which solubilizes in the stomach, but has no interactions with feed compounds for it is absorbed in the first stages of the intestine.

#### 1.3.3.3 Phytate

The myo-inositol-phosphate (IP-1, -2, -3, -4, -5 and -6), also known as phytic acid is, as free acid, a hexa phosphorus acid ester of the 6-hydroxyl group cyclic alcohol myo-inositol. When chelated, it forms salts denominated phytate (Pallauf and Rimbach, 1997).

Phytic acid is the main form of phosphorus storage in grains, that are the base for poultry diets, and its presence cannot be avoided (Li *et al.*, 2016; Schlegel *et al.*, 2013). Strategies to reduce

phytate can be challenging, as the main diet of poultry are cereal based, either soy or corn that have 40 – 60 % of their phosphorus as phytates. Phytates are the major dietary factor limiting Zn bioavailability in monogastrics (Schlegel *et al.*, 2013).

Phytic acid forms insoluble complexes with divalent cations ( $\text{Ca}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Mg}^{+2}$  and  $\text{Fe}^{+2}$ ) and proteins inhibiting their absorption and therefore reducing the bioavailability in chickens (Humer *et al.*, 2015; Mohanna and Nys, 1999; Schlegel *et al.*, 2013). Zinc appears to be the mineral most affected by phytic acid due its greatest affinity for it and the formation of more stable and insoluble complexes (Pallauf and Rimbach, 1997; Selle *et al.*, 2009). The presence of phytate in the diet can also capture up to 80% of endogenous Zn, avoiding its reabsorption (Adeola *et al.*, 1995).

Calcium has one of lowest affinities for phytic acid, but it has the greatest impact, because it is the mineral present at the highest concentration (Tamim and Angel, 2003). In addition, Ca level in the diet increases the antinutritional effect of phytate on zinc bioavailability due to the formation of complexes phytate-Ca-Zn (Pallauf and Rimbach, 1997), that can render more than 90% of dietary Zn (Adeola *et al.*, 1995). These complexes are resistant to enzymatic hydrolysis by phytase (Maenz *et al.*, 1999; Selle *et al.*, 2009).

These complexations are more relevant for high esters IP-6 and IP-5, as lower esters seem not to affect as much zinc bioavailability. Solubility of 10 mmol/L of  $\text{ZnSO}_4$  ranged from 93% in the presence of 100 mmol/L of IP-3 to only 8% in the presence of 100 mmol/L of IP-6 in *in vitro* studies, in which, also the rate of Zn-transport in a human cell line declined in the direct proportion of the level of esterification of IP (Han *et al.*, 1994).



#### 1.3.3.4 *Phytase*

Phytase is an exogenous enzyme responsible for the break of phytic acid and it is commonly added to the diet (Li *et al.*, 2016). This enzyme catalyzes the hydrolysis of phosphorus from the phytic acid molecule, breaking the IP-6 into IP-5, -4, -3 -2 and -1, in descending order of preference (Dersjant-Li *et al.*, 2015). There are extensive literature elucidating improvements in the utilization of phosphorus, minerals and proteins (Mohanna and Nys, 1999; Schlegel *et al.*, 2010; Selle *et al.*, 2009; Tamim *et al.*, 2004; Walk *et al.*, 2012a; Walk *et al.*, 2012b).

In a meta-analysis performed by Schlegel *et al.* (2013), the use of phytase improved bone Zn concentration in broiler, although to a lesser extent when compared to piglets. The study concluded that broilers have the ability to use native Zn, even those bound to phytate, due to the low pH of the gizzard that allows to release part of the Zn which, is rapidly absorbed in the beginning of the duodenum before the rise of pH.

The use of a phytase source with an optimal activity at the low pH of the stomach can degrade phytic acid in the early stages of the digestion, would prevent minerals and proteins to be complexed with phytic acid and therefore unavailable to absorption (Dersjant-Li *et al.*, 2015). Fast degradation of phytic acid by phytase is essential as complexations by metal ions prevents the break by phytase in the gut.

#### 1.3.3.5 *Absorption of zinc in the body*

Zinc is tightly regulated and has a small storage in the body, therefore a daily supply is required to maintain body activities (Yu *et al.*, 2017). The rapid onset of clinical symptoms in the case of depletion corroborated the lack of storage in the body for this mineral (Shames *et al.*, 2000). Zinc

homeostasis is maintained mainly at the gastrointestinal level according to dietary levels of zinc, through both absorption and excretion of zinc in the feces (Park *et al.*, 2004; Shames *et al.*, 2000). The homeostasis is established through the synergistic action of the absorption of Zn in the intestine and the endogenous excretion, in which the latter have an immediate response to the dietary zinc intake but in a rather small amount, as absorption has a slower but greater response to the fluctuation of Zn in the diet (Shames *et al.*, 2000) .

A lower dietary zinc intake increases the efficiency of absorption of Zn. As a low absorption is also associated to a lower endogenous excretion. Endogenous excretion is due to metabolic, hence obligatory loss, and by the excess of Zn that controls the retention of absorbed Zn. The pancreas and the bile are the major endogenous excretion route for endogenous zinc, followed by the intestine (Cousins, 2010).

Zinc, like other metals, is absorbed in its ionic form:  $Zn^{+2}$  by transporters in the enterocytes membranes. ZnT-1 to ZnT-10 and ZIP-1 to ZIP-14 are protein “families” of transporters responsible for zinc uptake at the cell level. The ZIP transporters, unlike the ZnT, are not Zn exclusive and are also involved in the homeostasis of other metals: Cd, Mn, Fe and Ca (Beegam *et al.*, 2016). These transporters are not only responsible for the uptake of Zn from the lumen of the intestine to the cell cytoplasm, but also inside the cell, into and out compartments (Figure 4), helping to maintain the homeostasis of Zinc in the body (Murakami and Hirano, 2008).

Zip transporters are located at the apical part of the enterocyte, responsible to the uptake of Zn from the lumen. ZnT-2, -4, -5, -6 and -7 are located intracellularly. Their function is to lower intracellular Zn, either through the efflux of Zn or to move the mineral into vesicles, Golgi

complex, etc. ZnT-1 is the major transporter controlling the efflux of Zn and it is located in the basolateral membrane. It is a zinc-responsive transporter, so a reduction of this transporter would limit the zinc transfer in circulation (Cousins, 2010).

Methallothionein (MT) also plays an important role in Zn regulation. When the dietary of Zn in the diet increases the mRNA levels of MT also increase, decreasing the absorption of Zn. MT complexes with cytosolic Zn avoiding the exit of Zn from the enterocyte to the portal circulation (Liu *et al.*, 2008).

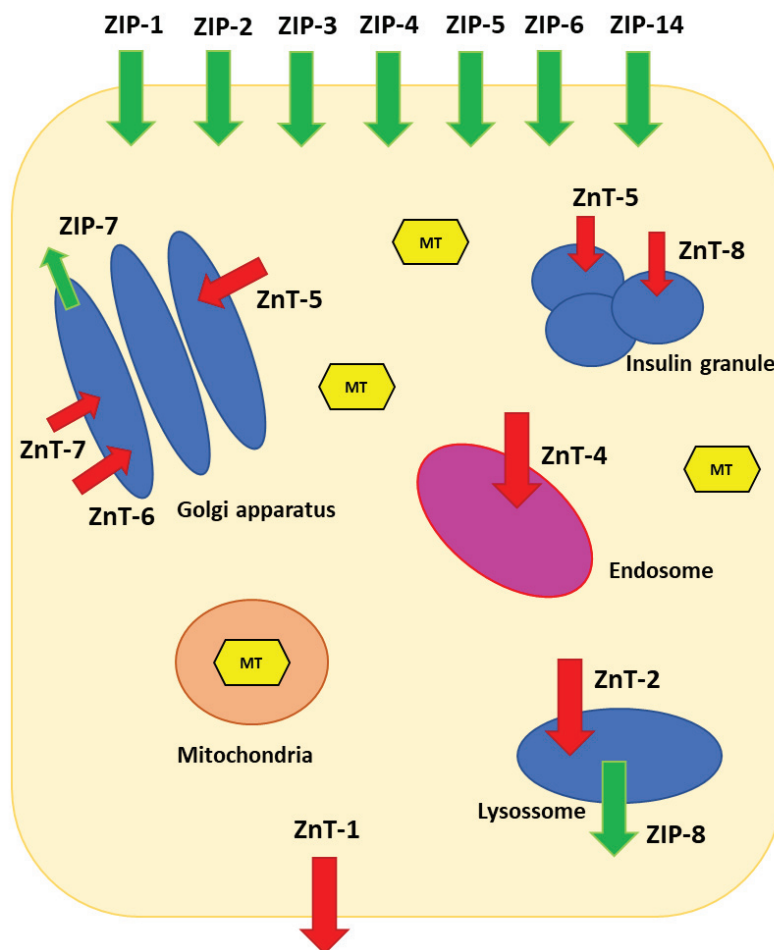


Figure 4: Location of Zn transporter at the cell level. The arrows indicate the direction of Zn mobilization (Murakami and Hirano, 2008).

There is some controversy in the literature regarding the site of the most Zn absorption in the small intestine. The duodenum, seems to be favorable by the low pH from the stomach, which favors the solubilization of mineral like Zn (Vieira, 2008). However, in a study with broilers Liu, *et al.* (2008) found the ileum to be the site with the highest absorption of Zn. The authors also suggest distinct mechanisms of absorption in each segment. The absorption of Zn in the duodenum and jejunum is through a saturable carrier-mediated pathway, influenced by ZnT-1, ZnT-5 and MT and a non-saturable diffusive process in the ileum.

Once absorbed by the enterocytes, zinc is transported through the blood to organs and tissues.

The storage of zinc is more relevant in the bone, small intestine, liver, skin and feathers. The liver receives a great amount of zinc, due to high activity in enzyme production and zinc-dependent proteins. Emmert and Baker (1995) observed when Zn was supplemented in an excess, the highest accumulation of the mineral was in the bone and to a lesser extent in small intestine (Figure 5). This zinc can be easily mobilized in case of a deficiency of Zn in the diet.

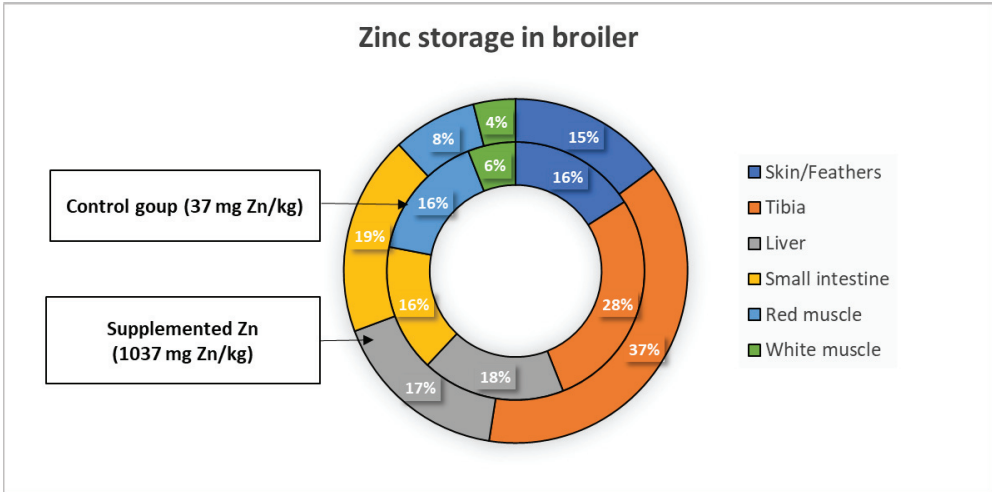


Figure 5: Zinc storage in body in broilers fed either a control diet with 37 mg Zn/kg or 1037 mg Zn/kg. The main site of Zn storage is bone and small intestine (Emmert and Baker, 1995).

#### 1.3.4 ZINC REQUIREMENTS AND RECOMMENDATIONS

The amount of zinc recommended in broiler diets is 40 mg/kg (NRC, 1994). It is a current practice in poultry production to add Zn above NRC recommendations to assure a proper supply and avoid deficiency (Naz *et al.*, 2016). Additionally, studies have shown improvement on growth performance (Azad *et al.*, 2018; Ibrahim *et al.*, 2017; Yan *et al.*, 2016), mineral retention, immune response and alleviation of stress (Sunder *et al.*, 2008), when Zn was supplemented above the requirements.

The gap between requirement and recommendation might be due first to earlier studies (1994). In the current poultry production, especially in intensive breeding, the want for faster growth rate increased requirements for nutrients to achieve optimal animal performance. Studies should be updated in a shorter period, following the bird's genetic development.

There is innumerable published work in bioavailability of Zn in broilers. However, results from a single study cannot be a basis for definite conclusions of wide breadth due to the narrow conditions the experiment is designed. Therefore, there is a need, from time to time, to summarize findings across different studies and take a better look into the data. For that, a collection of studies was selected to evaluate Zn bioavailability in broilers.

##### 1.3.4.1 *Collection of bioavailability studies in broilers*

Data were collected from broilers studies published between 2010 to 2018. In each experiment, the data is represented by the respectively treatment mean value. In total, 7 experiments with 24 treatments were used to assess performance as average daily gain (ADG) and 6 experiments with

14 treatments were used to assess bioavailability from Zn in tibia at 21 or 22 days of age, as summarized on Table 2. Descriptive statistics on Table 3 summarizes the values observed in the set of data.

#### *1.3.4.2 Analysis of bioavailability studies*

Table 2 describes experiments based on diets, Zn content and levels of supplementation. Supplemented sources are classified as: chelates, sulfates and oxides. Table 2 also shows which treatment was used for each analysis (bone Zn or/and ADG). Figure 6, 7 and 8 show bone and ADG response for each treatment. Values are expressed in percentage, in which the maximum response is set at 100% and the other treatments levels in relation to it.

Table 2: Summary of Zn bioavailability studies in broilers.

Reference	Treatment	Diet type	Native Zn (mg/Kg)	Total Zinc (mg/Kg)	Zn Source	ADG	Bone Zn
Ao et al. (2011)	1	Maize/Soy	31	31, 67, 45	ZnSO <sub>4</sub>	x	
	2	Maize/Soy	31	31, 45, 45	Chelate	x	
	3	Maize/Soy	34	34, 69, 114	ZnSO <sub>4</sub>	x	
	4	Maize/Soy	34	34, 47, 62	Chelate	x	
	5	Maize/Soy	31	31, 81, 101	ZnSO <sub>4</sub>	x	
	6	Maize/Soy	31	31, 45, 57	Chelate	x	
	7	Maize/Soy	31	31, 81, 101	ZnSO <sub>4</sub>	x	
	8	Maize/Soy	31	31, 45, 57	Chelate	x	
Star et al. (2012)	9	Wheat/Maize/Soy	34	34, 37, 42, 47 52, 71	ZnSO <sub>4</sub>	x	x
	10	Wheat/Maize/Soy	34	34, 36, 42, 46	Chelate	x	x
Brookset al. (2013)	11	Maize/Soy	21	21, 27, 33	ZnSO <sub>4</sub>	x	x
	12	Maize/Soy	21	21, 24, 30	Chelate	x	x
Liu et al. (2013)	13	Maize/Soy	27	27, 62, 69, 94, 131	ZnSO <sub>4</sub>	x	x
	14	Maize/Soy	27	27, 61, 70, 91, 128	Chelate	x	x
Zhao et al. (2015)	15	Maize/Soy	9	30, 45, 60, 75	ZnSO <sub>4</sub>		x
	16	Maize/Soy	9	30, 45, 60, 75	Chelate		x
Yan (2015)	17	Maize/Soy	35	35, 55, 75, 95, 115	ZnSO <sub>4</sub>	x	x
	18	Maize/Soy	35	35, 55, 75, 95, 115	Chelate	x	x
	19	Maize/Soy	36	35, 55, 75, 95, 115	ZnSO <sub>4</sub>	x	
Tang (2015)	20	Maize/Soy	36	35, 55, 75, 95, 115	Chelate	x	
	21	Maize/Soy	29	30, 104	ZnSO <sub>4</sub>	x	
	22	Maize/Soy	29	30, 59, 81, 98	Chelate	x	
Narcy (2015)	23	Maize/Soy	22	22, 50, 57, 64	ZnSO <sub>4</sub>	x	x
	24	Maize/Soy	22	22, 52, 58, 66	ZnO	x	x
	25	Maize/Soy	22	22, 51, 56, 65	ZnO	x	x
	26	Maize/Soy	22	22, 49, 57, 69	ZnO	x	x

The data represented the treatment mean value within an experiment. Treatments were included in the database according to: maximum of 100 mg/kg of Zn supplemented. Only one source of Zn was fed in the same treatment. Sources of Zn used were classified in three categories: (1) Zn Sulphate, (2) Zn Chelate and (3) ZnO. Bone zinc was assessed in mg/kg DM basis. Bone ash was considered 450 g/kg when bone Zn was in ash basis.

Table 3: Descriptive statistics of variables.

		ADG (g/day)	Bone Zn (mg/kg DM)
<b>Data</b>			
No of observations		92	60
<b>Dependent variable</b>			
	Min	13.9	54
	Max	87.2	230
	Mean	46.1	125
<b>Independent variables</b>			
Native Zn (mg/kg)	Min	21	9
	Max	36	35
	Mean	30	25
Chelate Zinc (mg/kg)	Min	0	0
	Max	101	101
	Mean	26	31
ZnSO <sub>4</sub> (mg/kg)	Min	0	0
	Max	104	104
	Mean	32	31
ZnO (mg/kg)	Min	0	0
	Max	47	47
	Mean	27	27

#### 1.3.4.3 Bone Zn results

Bone zinc showed a linear response for those treatments containing Zn up to 80 mg/kg of diet Zn in average, at which the supplementation response stabilizes, probably due of the proximity to the physiological maximum (Figure 6). An optimal concentration can be reached at approximately 90 mg/kg of Zn. Animal Zn status prior to the experiment may also play a role in the response, as a bird with a high Zn level would show a lower response to Zn supplementation.



The source doesn't seem to play an effect on Zn bioavailability (Figure 7), which is also in agreement with a meta-analysis performed by Schlegel *et al.* (2013). In that study, the authors compare chelates and inorganic sources of zinc and don't see an effect of the source in Zn supplementation in broilers.

Seven out the eight experiments aimed to evaluate different types of chelate sources, and only 1 experiment looked at different sources of ZnO. In all studies, Zn sulfate was used as the standard source to be compared. Although studies are focused on the different chelates forms, in practice, ZnO is still widely used. Additionally, data to assess bioavailability of ZnO is rather old and shows great variability within sources. Thus, the need to update the knowledge on supplemented sources and further investigate to explain this variability.

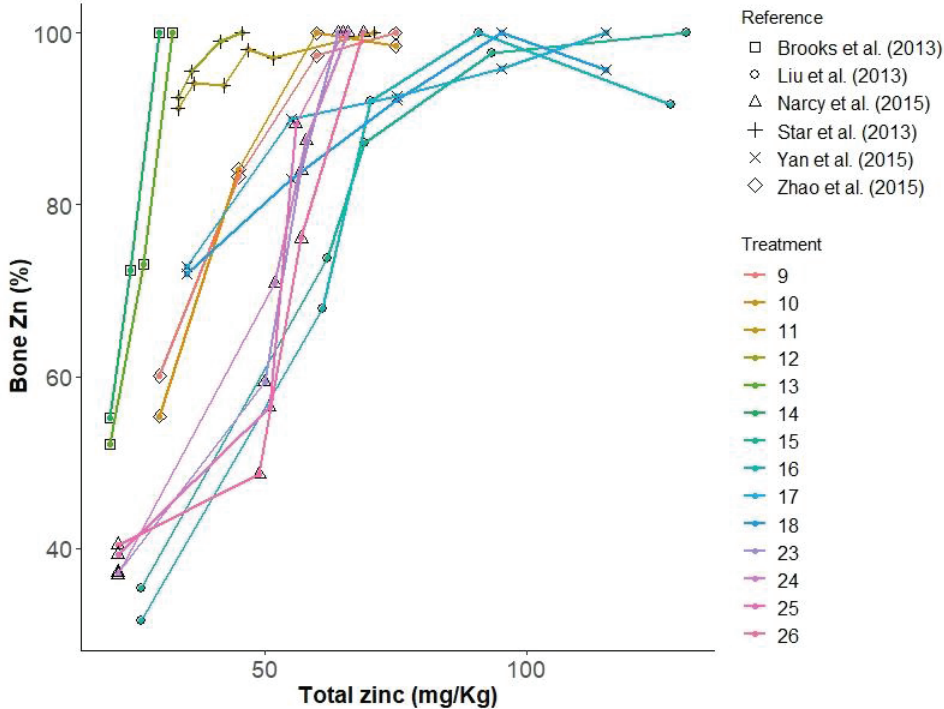


Figure 6: Bone Zn (%), in which 100% corresponds to the maximum Zn concentration in the bone for a given treatment in response to Zn supplementation.

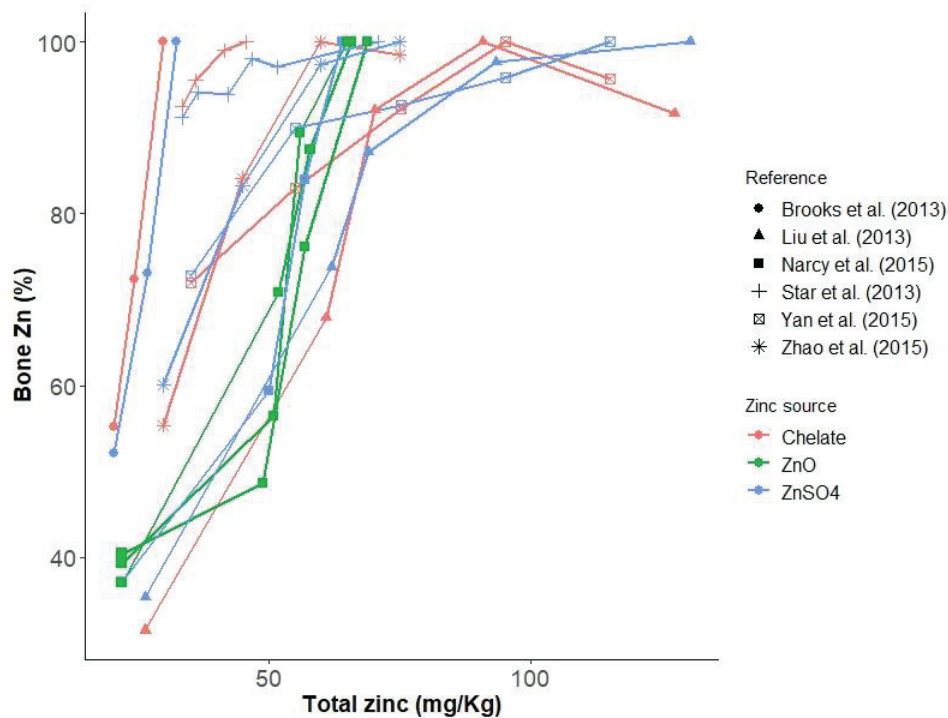


Figure 7: Bone Zn (%), in which 100% corresponds to the maximum Zn concentration in the bone for a given treatment in response to different Zn sources supplementation.

#### 1.3.4.4 ADG results

ADG dose response on Figure 8, showed also a quadratic response, but rather discrete when compared to bone Zn. The effect of Zn supplementation was only observed in one experiment, in which the negative control has a lower ADG when compared to the different supplemented levels. In that experiment, animals were submitted to a 7-days Zn depletion period prior to the experiment (Brooks *et al.*, 2013). The level and source had no great effect on ADG in the other experiments, which was also noted by those authors. This might be due the short period of experiments, as the Zn requirement for growth performance is less sensitive than bone. While bone data showed a response of up to 60% according to the different level of supplementation, ADG represented only up to 15 - 20% of the Zn dose-response. Additionally, the plateau reached by Zn supplementation seem to be different according to the parameter

evaluated: ADG seemed to be reach the plateau at 40 mg Zn/kg, whereas, Zn in the bone achieved homeostatic regulation at 80 – 90 mg Zn/kg. This observation confirms the importance of the criteria considered to establish requirements as previously shown by (Schlegel *et al.*, 2013).

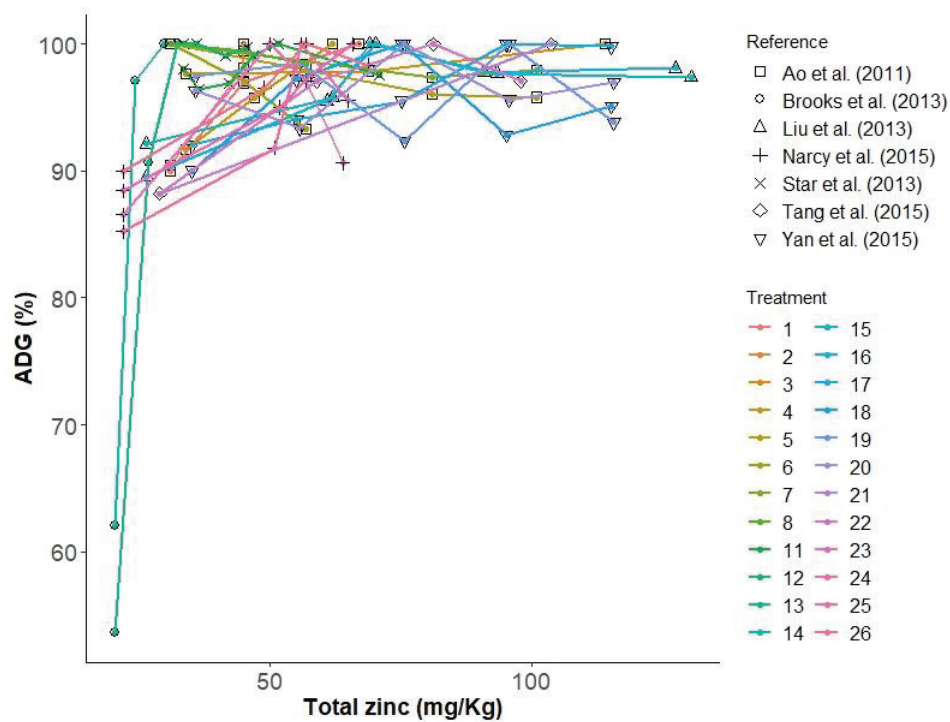


Figure 8: Average Daily Gain (ADG) in % in response to Zinc supplementation.

#### 1.4 CHARACTERIZATION PHYSICOCHEMICAL OF POWDERS

Powders, like ZnO, have a wide applicability in food, chemical and pharmaceutical industries. In Europe, around 60% of what is manufactured in the chemical industry are powders (Leturia *et al.*, 2014). Powder technology science, apart from traditional processes like grinding, mixing and formulating, is also focusing in the manufacturing processes involved in the modification of a powder and particle properties to manipulate solubility and dissolution properties (Khadka *et al.*, 2014). As in animal nutrition, the properties of those powders have an

important impact in the final product quality and the efficiency of processes (Sutton *et al.*, 2017).

#### 1.4.1 DISSOLUTION AND SOLUBILITY OF POWDERS

Solubility and dissolution are different parameters, but related. Solubility is the concentration in a saturated solution at equilibrium, while dissolution is the process by which the compound goes into solution. Solubility is the endpoint of dissolution, this happens when the concentration and the solid form do not change over time (Sugano *et al.*, 2007). Various parameters influence the rate at which a solute reaches its solubility. For a given substance, the amount of solute dissolved at the endpoint is the same at the same conditions;

The solubility of Zinc, described by Reichle *et al.* (1975), shows that the solubility of Zn increases exponentially in a pH below 6 (Figure 9) which, in the gastric condition of broilers would represent a full solubilization of the Zn supplemented in the diet.

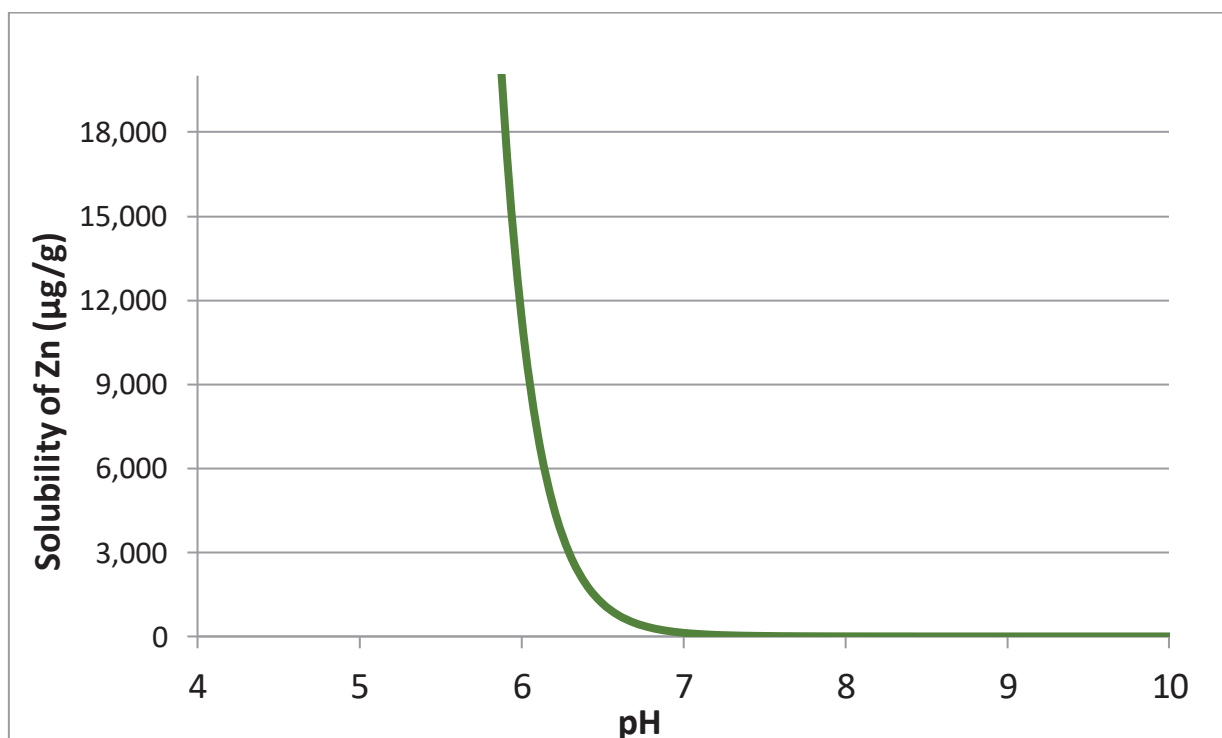


Figure 9: Solubility of Zinc in relation to different pH.

However, the speed to get there, known also as dissolution kinetics, will be influenced by the physicochemical properties of the solid. Solubility does not depend on particle size or other factors; provided enough time is waited for, even large particles will eventually dissolve (Savjani *et al.*, 2012). When it concerns the GIT, the transit time determines the absorbed amount of a given substance and therefore, absorption is a kinetic process influenced by dissolution kinetics rather than by solubility.

This topic is widely explored in the pharmaceutical field in the assessment of drugs profile. Like powders, drug absorption is dependent on the balance between the solubility, dissolution rate and permeability of a drug through biological barriers. Each of those steps can have a limiting factor and therefore is set into categories of oral absorption profile: (1) Dissolution rate limited absorption: the permeation of the intestine is larger than the dissolution rate, so that the dissolved drug will disappear instantly in the intestine as it dissolves. (2) Permeability limited absorption: the permeation is slow and the dissolution is fast, so that drug accumulates in the intestine lumen. (3) Solubility limited oral absorption: when the concentration of the substance reaches its saturation, the drug cannot be further dissolved (Sugano *et al.*, 2007).

The reduction of the particle size, increases the surface area of the solid, which can improve the first scenario, when dissolution is limiting. When the permeability is limiting, the reduction in particle size plays no effect but rather an increase of the dose rises the concentration in the intestinal lumen. The same when solubility is the limiting factor, reducing the particle size might not increase the absorption, because fast dissolution makes the drug to accumulate in the gut and reach its saturation concentration (Sugano *et al.*, 2007).

This example illustrates the importance of physicochemical properties of feed compounds to achieve an ideal absorption. Knowledge in which factor is limiting for absorption, allows the choice for a product with an optimal dissolution to support maximum absorption.

#### 1.4.2 PROPERTIES OF DRY POWDERS

The use of powders in animal feeding involves two main factors: (1) human safety. Dust powders can cause respiratory injuries to the feed premix workers. (2) fate in biological tissues of the animal. Powders with specific properties can have various behavior in the body. Size and specific surface area, are the parameters most explored in powders analysis due their impact on dissolution speed on the compounds.

Powder behavior depends on (1) intrinsic properties, such as particle size, density, shape, roughness and porosity and (2) bulk properties: corresponding to size distribution, bulk density and cohesive and frictional interactions (Leturia *et al.*, 2014). Furthermore, Sutton *et al.* (2017), classifies inorganic powder properties into 3 categories: morphology, chemistry and microstructure. In this study, to simplify, morphology and microstructure were organized in the same category named as structural, that also comprise intrinsic and bulk properties of powders (Figure 10).

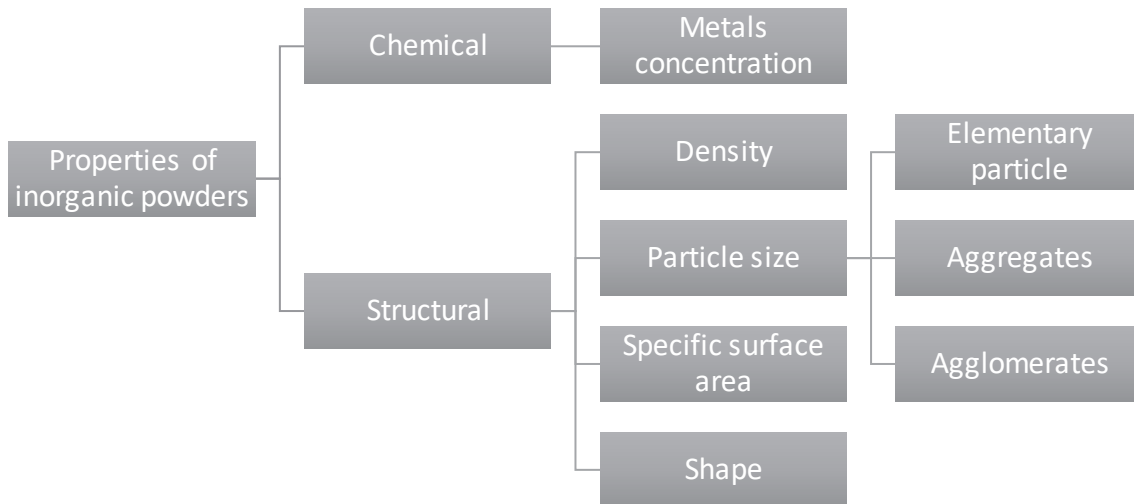


Figure 10: Properties of inorganic powders.

#### 1.4.2.1 Metal Concentration

Inductively coupled plasma optical emission spectrometry (ICP-OES, ICP-AES or ICPS) is a powerful analytical technique for rapid multi elemental analysis. It is the preferred choice to define the total metal concentrations in the powder. For ICP analysis, samples must be solubilized in acid (Sutton *et al.*, 2017). This analysis allows to assess the concentrations of metal and detect contaminations by heavy metals, if they exist.

The raw material used to produce ZnO is usually a source recycled from the industry, that can contain high concentration of other metals (e.g. Iron) and heavy metals (Moezzi *et al.*, 2012). To comply with regulations of feed safety, a control of the quality of raw material is important to guarantee product quality.

#### 1.4.2.2 Density

The density of a powder is the ratio of mass over volume. There is not a fixed value of density to a given powder, as it is influenced by the consolidation state of the granular material: either aerated (bulk density) or tapped (Leturia *et al.*, 2014). The measurements of these properties

can provide information about the powder flowability (Abdullah and Geldart, 1999; Sutton *et al.*, 2017). A low bulk density value characterizes a powder with strong structural strength. This is due to a high friction between particles. Oppositely, low friction between particles from structurally weak powders results in a high density value (Abdullah and Geldart, 1999). The tapping forces the particles to jump and lose contact with each other for a moment. Free-flowing powders have little scope for consolidation and bulk density and tapped density have similar values. Particles with reduced friction will collapse improving packing conditions (Abdullah and Geldart, 1999), but it can affect the product quality and the efficiency of the process due to wastage, machinery maintenance and downtime (Leturia *et al.*, 2014).

The ratio of bulk and tapped density is called Hausner ratio and it is a useful tool to define cohesion of a powder (Abdullah and Geldart, 1999; Leturia *et al.*, 2014). The lower the ratio the less cohesive is the powder, meaning to have a low tendency for further consolidation than a powder with a higher ratio value. Van der Waals forces act between particles in a powder defining adhesive and cohesive forces. These forces decrease as the particle size increases (Aulton and Taylor, 2013).

Adhesion and cohesion of powder have some of its properties controlled by external factors related to particle properties such as size, shape and density. Fine particles with high specific surface area are more cohesive than coarser particles, which are influenced more by gravitational forces (Aulton and Taylor, 2013).

Particles larger than 250  $\mu\text{m}$  are usually relatively free flowing. Particles with size below 100  $\mu\text{m}$  become more cohesive and resist to flow under gravity. However, when these small particles are aggregated/agglomerated as larger particles, the flowability of the powder is then controlled by the bigger particles (Aulton and Taylor, 2013). Nevertheless, the flow



properties of a powder can change according to packing as submitted to different stress levels. Due to the action of higher stresses, the powder structure loses the individualized character of agglomerates, which leads to a formation of a more compact system (caking) (Leturia *et al.*, 2014).

#### 1.4.2.3 Shape

According to synthesis technique ZnO powders can have a wide variety of possible shapes (Moezzi *et al.*, 2012). There is still controversy in the literature about the effect of shape on the dissolution behavior of nanoparticles. Based on theoretical grounds, Borm *et al.* (2006) proposes that convex features a faster dissolution because they have a thinner stagnant diffusion layer extending from the surface toward the shear plane. In opposite, concave structures have a thicker stagnant layer since it is recessed on the surface, leading to a slower dissolution rate. Some nanoparticles powders are inhomogeneous in size and shape, varying from batch-to-batch. In addition, aggregation of particles leads to formation of several irregularly shaped structures that their effects become uncertain.

Shape can have an effect on the Hausner ratio as the further particle shape deviates from spherical, the higher the ratio of the tapped to the aerated density for the bulk powder is observed (Abdullah and Geldart, 1999).

#### 1.4.2.4 Particle size

Primary particles are single crystals or crystal composed of crystallites. Aggregates are strong bounds of primary particle, whereas agglomerates are loose assemblage of particles or aggregates (Nichols *et al.*, 2002; Union, 20 October 2011). Agglomerates can be disassembled into aggregates if a force is applied but the aggregate once formed, cannot return to crystal

size (Nichols *et al.*, 2002). In other words, aggregates are irreversible. Agglomerates can be broken by mechanical stress.

Nanomaterials are defined as natural, incidental or manufactured materials containing particles in unbound state, aggregate or agglomerate form in which 50% or more of the particles have a number size distribution in the size range from 1 nm to 100 nm (Union, 20 October 2011).

According to European Union (20 October 2011), nanomaterials are defined only on the size of the material. However, size and size distributions may not provide same results due to different measurement methods because powders consist of a complex mixture of primary particles and particle assemblages in the form of aggregates and agglomerates (Nichols *et al.*, 2002).

There are several techniques to assess the size of dry powders properties: dynamic light scattering, UV-Vis absorption spectroscopy, selected area electron diffraction (SAED), powder X-ray diffraction (XRD), transmission and scanning electron microscopy (TEM and SEM), zeta potential (Beegam *et al.*, 2016). The most usual techniques to assess particle size are summarized in Table 4.

Table 4: Advantages and drawbacks of the most used techniques to assess particle size of dry powders.

Technique	Particle size	Advantage	Drawback
X-Ray Diffraction	Primary particle	Able to measure the size of nanocrystals. Quantify and qualify the purity of the sample.	Cannot assess sizes larger than 100 nm A prior database is required to identify the nature of crystal
TEM	Primary particle	Image, shape and size	Sampling time consuming Numbered measurements for statistical significance of size Two-dimensional images
SEM	Aggregates and agglomerates	High resolution images	
Laser light scattering	Aggregates and agglomerates		Consider spherical particles only

#### 1.4.2.5 X-ray diffraction

This technique is used to identify crystal structures. These structures are then determined by indexing the peak positions to the same pattern of the powder diffraction library (Sutton *et al.*, 2017).

X-ray diffraction can also be used to predict crystal size, applying the width at half-height of the peaks to the Scherrer formula. However, this measurement is limited for crystals smaller than 100 nm.

#### 1.4.2.6 TEM and SEM

Transmission and Scanning electron microscopy are powerful tools to directly observe the particles. They allow visual observation of the shape and primary particle size of the powder.

The drawback of TEM is the two-dimension approach for calculate particle size may not be very accurate due to possible bias and it can be time consuming. Additionally, the amount of particle needed to obtain statically significant results is still unclear (Powers *et al.*, 2007; Sutton *et al.*, 2017).

#### 1.4.2.7 *Dynamic Light Scattering*

This method is normally preferred because it measures large number of particles in a short period to obtain significant statistical average (Powers *et al.*, 2007; Sutton *et al.*, 2017). The assumption is samples being perfect spheres, which can be misleading in case of irregular particles and those with high aspect ratios (Sutton *et al.*, 2017). Nevertheless many particle systems are in irregular shape, so it is customary to express particle size in terms of equivalent spherical diameter (Powers *et al.*, 2007).

#### 1.4.2.8 *Specific surface area*

The specific surface area corresponds to the total surface area of a material per unit of mass, expressed as the unity:  $m^2/kg$ . It is often stated that dissolution rate is directly proportional to the specific surface area. Accordingly, a decrease in particle size will result in an increase on dissolution rate (Mosharraf and Nyström, 1995). The interfacial reaction between the solid phase and liquid phase, resulting in the liberation of solute molecules from the solid phase. The higher the specific surface area, the faster is the first stage dissolution of the particles (Mosharraf and Nyström, 1995).

## CHAPTER 2

**« Si tu diffères de moi, mon frère, loin de me léser, tu m'enrichis »**

*Antoine de Saint-Exupéry*

## BIOAVAILABILITY OF ZINC OXIDE: RELATIONSHIPS BETWEEN DISSOLUTION KINETICS AND PHYSICOCHEMICAL CHARACTERISTICS OF ZINC OXIDE SOURCES USED IN ANIMAL FEEDING

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### HIGHLIGHTS

- Variability in ZnO dissolution kinetics is accounted for through statistical analysis
- Dissolution kinetics of coarse ZnO powders depends on specific surface area
- Dissolution kinetics of fine ZnO powders depends on aggregation and agglomeration

### ABBREVIATIONS

ZnO: zinc oxide; PCA: principal component analysis; HAC: hierarchical ascendant analysis; SAP: small aggregated particles; SAAP: small aggregates and agglomerated particles; LDP: large dense particles.

## ABSTRACT

The bioavailability of a trace mineral source is related to its *in vivo* dissolution, which in turn is determined by its physicochemical properties. It is still not clear which characteristics are more relevant in affecting solubility and bioavailability of feed compounds. Zinc oxide (ZnO) is a common feed additive used to supplement zinc in the diet of monogastric animals. However, different sources have shown different responses on animal bioavailability. It is hypothesized that various physicochemical properties control dissolution kinetics, which affect the fate in the gastrointestinal tract and zinc bioavailability. Samples of ZnO collected from the feed industry worldwide were analyzed for density, tapped density, particle size, shape and specific surface area. Dissolution kinetics in water was assessed *in vitro* by kinetic measurements of the acid demand for keeping a constant pH with an automatic burette. A principal component analysis (PCA) allowed sorting ZnO grades in three clusters corresponding to families having different physicochemical properties. A correlation statistical analysis was performed to disclose the most relevant physicochemical characteristics affecting dissolution; they are density, agglomerate size and specific surface area. Dissolution kinetics depends on different variables according to ZnO families. A dissolution mechanism in 2 steps is put forwards: the dissolution of ZnO into  $Zn^{2+}$  ions from the surface of ZnO primary particles, followed by diffusion to the bulk aqueous medium through the liquid medium in quiescent state between aggregates of primary particles. The rate-determining step of dissolution kinetics and the physicochemical parameters controlling dissolution are not the same for the three ZnO families.

**KEYWORDS:** zinc oxide, bioavailability, physicochemical characterization, dissolution kinetics

## INTRODUCTION

Zinc (Zn) is an indispensable mineral present in many physiological pathways in biological functions. In cereal based feed, Zn is a limiting ingredient and it is often supplemented in diets of monogastric animals (Schlegel *et al.*, 2010; Brugger and Windisch 2017; Narcy *et al.* 2015). The most common sources of zinc used in the feed industry are: Zinc Oxide (ZnO), Zinc sulfate and Zinc chelates. There is a poor characterization of supplemented sources; they are most often just differentiated by their solubility in water. The solubility of a feed compound is related to its bioavailability. However, studies comparing *in vitro* solubility and *in vivo* digestibility of minerals have shown a negative correlation (Anwar *et al.*, 2016). It is thought that a highly soluble source could overcome the uptake capacity of the gut, what would not happen in case of slow release from a coarse source (Zhang and Coon, 1997). Furthermore, fast solubilized minerals may interact with feed compounds, such as phytate, forming insoluble complexes that cannot be absorbed by the animals (Brugger and Windisch, 2017).

Sulfates and chelates, sources with high solubility in water (Schlegel *et al.*, 2010), and in the ruminal fluid (Spears and Kegley, 2002) are claimed to have a higher bioavailability than oxides (Schlegel *et al.*, 2013). Although ZnO is not soluble in water, this mineral is highly soluble in gastric conditions where pH ranges from 2 to 4 (Reichle *et al.*, 1975). The literature reported a variation on bioavailability of ZnO among the different sources from 30 to 105% compared to the zinc sulfate reference compound (Narcy *et al.*, 2015; Edwards and Baker, 1999).

The different manufacturing processes of ZnO sources, lead to a diversity of colours and powder structure, as observed by Edwards and Baker (1999). ZnO similarly presents differences in structural characteristics, which could justify the large variability of



bioavailability reported by researchers. However, the mechanisms underlying solubility, interactions and bioavailability of ZnO are still not completely elucidated.

It is hypothesized that the properties of ZnO can affect its dissolution kinetics and further bioavailability. The aim of this study was to characterize and categorize samples of ZnO used in animal feeding to further understand their influence on the mechanism of dissolution in the digestive tract of monogastric animals.

34 samples of ZnO were collected from the feed industry in Europe, Asia and Americas. Samples were analyzed for crystal size and shape, particle size, specific surface area (SSA), density and tapped density and then categorized according to their properties. A Principal Component Analysis (PCA) clustered samples into families, according to their characteristics.

An *in vitro* assay was performed to observe the dissolution kinetics of samples and understand the properties influencing dissolution. A mechanism of dissolution is proposed.

## **MATERIALS AND METHODS**

### X-Ray Diffraction

Crystallographic and size information on zinc oxide samples were obtained using a Bruker AXS D8 Advance diffractometer at the 'Centre de Diffractométrie Henri Longchambon' facility of the University Lyon 1 (<http://cdalpha.univ-lyon1.fr/>). operating in the Bragg  $\theta$ - $2\theta$  configuration using Cu K $\alpha$  radiation (0.154 nm wavelength) provided Debye-Scherrer X-ray diffraction powder patterns in an angular domain from 10° to 70° at scanning rates of 0.25°·min<sup>-1</sup>.

### Transmission Electron Microscopy (TEM)

Size and shape of structures were determined by TEM analysis, with images taken at an acceleration voltage of 80 kV on a Philips CM120 instrument at 'Centre Technologique des Microstructures' facility of the University of Lyon 1 (<http://microscopies.univ-lyon1.fr/>). Specimen for all TEM experiments were prepared by dispersing particles in distilled water (0.1%) by sonication for 1 min at 50% amplitude with a Hielscher UP400S ultrasound device. 5  $\mu$ L of the dispersion was spread on Formvar/carbon grids and dried before observation.

### Scanning Electron Microscopy (SEM)

Size and shape were complementary determined by SEM. The images were taken at 'Centre Technologique des Microstructures' – University of Lyon. The instrument used was a FEI Quanta FEG 250 at an acceleration voltage of 10 kV. Samples were prepared with the deposition of a thin layer of ZnO powder on a conductive carbon paste tape, followed by metallization with a 10 nm thick deposit of copper.

### Particle Size

The diameter of ZnO aggregates and agglomerates in water was measured by low-angle laser light scattering using a Malvern Mastersizer 3000 instrument. Agglomerate and aggregate size distributions were calculated to Mie theory using the refractive indices of water (1.33) and ZnO (2.004) and absorption index of ZnO of 0. Agglomerates were accessed by adding directly the powder in the water. To measure aggregate sizes, a dispersion of ZnO at a concentration of 10% was dispersed using an IKA Ultra-Turrax Rotor-Stator device rotating at 15,000 rpm for 15 min to reach smaller particle size.

### Specific Surface area (SSA)

Specific surface area of the dry powders were assessed by nitrogen adsorption experiments using a Tristar 3000 Micromeritics BET instrument. The specific area was determined by the Brunauer-Emmet-Teller (BET) multipoint method. SSA is calculated from nitrogen adsorption measurements by fitting the BET equation to experimental data for relative pressures taken between 0.05 and 0.3. This allows the determination of the amount of nitrogen gas adsorbed at the surface as a dense monomolecular layer of close-packed molecules. The full area of the powder is then calculated from the area of an adsorbed nitrogen molecule ( $16.2 \text{ \AA}^2$ ).

### Density and Tapped density

40 g of dry ZnO powder was poured into a graduated cylinder for its volume to be measured. Bulk density is defined as mass over volume.

Tapped density was measured using a Tapped Density Tester series JV from Copley Scientific according to the ISO 8398 standard (1989). The final volume was noted after  $250 \pm 15$  tapings and tapped density was calculated as mass over tapped volume.

### Dissolution kinetics

Kinetics was assessed with 200 mL of deionized water and 0.15 g of ZnO at  $40^\circ\text{C}$  at pH 4 for 1 h using an automatic burette (877 Titrino plus, Metrohm, Switzerland) that recorded the volume of hydrochloric acid (HCl 1 M) required for keeping pH at the constant value of 4 while ZnO was dissolving. In addition, samples were collected at times 3, 10, 20, 30, 40, 60 and 120 min, centrifuged for 5 min at 14,000 rpm and have the supernatant collected. 100  $\mu\text{L}$  of each sample was diluted in 10 mL of deionized water for ICP-OES analysis of Zn. ICP-OES analysis was performed using a Thermo Scientific™ iCAP™ 7000.

## **MULTIVARIATE STATISTICAL ANALYSIS**

Multivariate data analyses were performed using XLSTAT software (Addinsoft, [www.xlstat.com](http://www.xlstat.com)).

### Principal Component Analysis (PCA)

PCA aims at reducing the dimensionality of the dataset by building a new coordinate space whose axes are called Principal Components (PCs) and correspond to linear combination of the initial variables. PCs are calculated so as to account for most of the variance in the dataset (Gabriel, 1971; Brereton, 2007). PCA calculations were performed using standardized initial variables so as to give them equal weight in the analysis.

### Hierarchical Ascendant Clustering (HAC)

HAC is a clustering method based on an agglomerative (bottom up) approach which initially considers each sample as a cluster. Pairs of clusters are hierarchically put together by using the Ward's linkage criterion based on the Euclidian distance metric that seeks minimizing intra-cluster variance while maximizing inter-cluster one (Ward, 1963; Everitt, 2001). These successive clustering operations allow obtaining a clustering tree called dendrogram.

A Principal Component Analysis (PCA) was performed to group into classes samples according to their physicochemical properties. A correlation between properties analyzed and zinc concentration at 10 min was considered to assess which factors have an influence on the dissolution kinetics of the different grades of ZnO. A *t*-test was done to evaluate if there was a significant difference between the different dissolution curves.

## **RESULTS AND DISCUSSION**

### Physicochemical characterization

Differentiation between feed grade zinc oxide is often based on purity, composition, specific surface area and manufacturing process (Moezzi *et al.*, 2012). Dry powders consist of a complex assembly of crystals, known as primary particles, associated in the form of aggregates and/or agglomerates (Nichols *et al.*, 2002). Aggregates and agglomerates behave as large particles with internal porosity (Borm *et al.*, 2006). Aggregates comprise crystals strongly bound by sintering, usually formed during powder processing. Aggregates cannot be broken by common mechanical means. Agglomerates are loose arrangements of primary particles or aggregates in which bounds are weak, such that they can be broken into aggregates; but aggregates cannot break into primary particles (Balakrishnan *et al.*, 2010; Nichols *et al.*, 2002; Borm *et al.*, 2006). In this framework, the 'size' of particles is defined by the three sizes of primary particles, aggregates and agglomerates. Clear-cut assessment of such complex structures is a difficult task because the various tools for structural investigation do not provide definite measurement of each size. Techniques for measuring sizes are optical and electron microscopy, light scattering, X-ray diffraction. Nitrogen adsorption measurements known as the BET method provide the specific surface area of the powder. Finally, density of the dry powder gives an indirect assessment of the formation of aggregates and agglomerates. The experimental results coming from these techniques are presented in the following.

### **Primary particle size**

There are a few methods that can be used to measure the size of primary particles depending on their size-range such as: X-ray diffraction (XRD), TEM and Optical Microscopy. XRD confirmed the same chemical composition and crystallographic structure of all samples. XRD pattern was compatible with zincite crystal structure of hexagonal Wurtzite according to the standard database (EVA 00-005-0664) (data not shown). The crystal size can be calculated

from the width at half-height of Bragg peaks of XRD by applying the Scherrer equation (Patterson, 1939):

$$D = \frac{0.9 \lambda}{\beta \cos \theta} \quad (\text{Eq. 1})$$

where  $D$  is the mean crystallite size (nm),  $\lambda$  is the wavelength of the X-ray radiation (0.154 nm),  $\theta$  and  $\beta$  are the diffraction angle and the resolution-corrected width at half height of Bragg reflections. The present resolution was  $0.55^\circ$ , a broadening of XRD peaks by 20% of the resolution cannot be measured with enough accuracy. Taking the 100 Bragg reflection at  $\theta = 31.75^\circ$ , a peak broadening by  $0.1^\circ$  corresponds to  $\approx 100$  nm. An accurate determination of crystal size is limited to crystals smaller than 100 nm. Such analysis did not allow an estimate of crystal size for the whole dataset because some of the samples had crystal sizes larger than 100 nm. In addition, primary particles may contain several crystals. So, Scherrer analysis based on XRD was discarded from the dataset aiming at a statistical analysis of relationships between structure and dissolution kinetics. TEM images can be used to assess the mean size of primary particles from size measurements of a large collection of particles., But it was not possible to observe a large enough number of particles for reaching a relevant statistical average when samples made of large primary particles. Furthermore, when particle shapes are platelets or rods (needles), they tend to lay flat on the surface of the grid they are deposited on and only the largest dimension can be visualized on the two-dimensional images; this leads to bias results (Powers *et al.*, 2007). Optical microscopy is the most appropriate method to measure the size of large crystals, but it cannot be applied for small-sized particles. Owing to the large differences of primary particle sizes between samples and the impossibility to use a unique technique to measure such size, this parameter was excluded from the analysis. The specific surface area (SSA) was taken as a better compromise parameter related to the average

primary particle size, as a decrease in particle size results in the increase of SSA (Mosharraf and Nyström, 1995).

### **Primary particle shape**

Morphology of ZnO particles is very sensitive to preparation conditions and methods (Li and Haneda, 2003; Moezzi *et al.*, 2012). Typical SEM and TEM pictures shown in Figure 2 illustrate the different properties among samples. Shape varied from nano to micro-sized platelets, rod-like particles and needles (Figure 2). Some samples contain mixed types of particles. Samples of Figure 2A, C and D showed to be platelet-shaped, whereas those of Figure 2B presented a 'rod-like' shape with some needles presence. Studies of cytotoxicity did not find any effect of the morphology (shape) on the dissolution kinetics of ZnO due to the aggregation effect of nanoparticles (Peng *et al.*, 2011). Nevertheless, Heng *et al.* (2011) found differentially shaped ZnO, with different dissolution rate between spheres and platelets ZnO. It is still controversial the effect of shape on dissolution behaviour of powders. In the present study, no correlations were observed in relation to the shape. Shape could not be included in the statistical analysis because the shape remains a parameter of descriptive better than quantitative nature.

### **Aggregate and agglomerate sizes**

Although TEM pictures allow observation of shapes and sizes of primary particles, their aggregation and agglomeration observed in the pictures can be a bias, because they can be caused by the drying process for sample preparation (Powers *et al.*, 2007). Aggregate and agglomerate sizes were measured by low-angle light scattering. This technique is preferred because the large number of particles measured provides a nice statistical average of the size (Powers *et al.*, 2007). Due to dry powders properties, particle size distribution can be influenced in many ways, therefore setting the materials sampling methodology for measuring

is very important (Marsalek, 2014). The size of aggregates was measured after a powerful dispersion of ZnO powder in water using a rotor-stator disperser rotating at 15,000 rpm for 15 min. It was checked that a faster rotation or a longer dispersion time did not decrease the measured particle size, showing that the measured size was that of the unbreakable aggregates. The size of agglomerates was measured by a mild dispersion of ZnO powder in water that did not break agglomerates. The ratio of agglomerates to aggregates size was called 'agglomeration rate'.

Primary particles sizes that can be roughly estimated from TEM images (Figure 2) were smaller than sizes coming from small-angle light scattering measurements, as was already noticed by Peng *et al.* (2011). Small nanoparticles with a high SSA tend to aggregate and form micro-sized particles in aqueous environment (Heng *et al.*, 2011; David *et al.*, 2012). The present nanoparticles observed in TEM images appeared assembled as sub-micron-size aggregates and agglomerates.

### **Specific surface area (SSA)**

Specific surface area is a measurement that aims to assess the surface exposure of a particle. SSA is associated to primary particle size since aggregation does not decrease SSA. The results are summarized in Table 1. The smallest SSA that can be measured by the BET method was  $0.1 \text{ m}^2\cdot\text{g}^{-1}$ . Sample ZnO-20 that its SSA is marked  $0.0 \text{ m}^2\cdot\text{g}^{-1}$  in Table 1 has actually a SSA lower than  $0.1 \text{ m}^2\cdot\text{g}^{-1}$ . Samples of the ZnO-D type showed the largest SSA values of  $41 \text{ m}^2\cdot\text{g}^{-1}$ . Samples of the type of Figure 1A had the lowest SSA values of less than  $0.1$  to  $0.5 \text{ m}^2\cdot\text{g}^{-1}$ . Those of Figures 1B and C showed variable SSA values. High values of SSA are often associated to a faster dissolution, as there is a greater area of contact between the particle surface and aqueous solution facilitating dissolution.



Table 1: Physicochemical properties of feed grade ZnO samples.

Sample	Shape	Aggregate <i>D</i> (50) (μm)	Agglomerate <i>D</i> (50) (μm)	Agglomeration rate	Density (g·cm <sup>-3</sup> )	Tapped density (g·cm <sup>-3</sup> )	Hausner ratio	SSA (m <sup>2</sup> ·g <sup>-1</sup> )
ZnO-1	Platelet	4.6	117	35.0	0.93	0.98	1.05	37.0
ZnO-2	Platelet	5.5	111	52.3	0.95	1.03	1.08	25.9
ZnO-3	Platelet	5.6	118	46.2	1.00	1.05	1.05	31.6
ZnO-4	Platelet	6.4	158	54.7	1.00	1.08	1.08	38.0
ZnO-5	Platelet	18.0	22.7	12.3	2.01	2.36	1.18	0.5
ZnO-6	Platelet	27.6	43.6	23.4	2.35	2.58	1.10	0.3
ZnO-7	Platelet	16.8	28.2	13.7	2.23	2.51	1.12	0.3
ZnO-8	Sphere	8.3	26.6	14.0	1.00	1.25	1.25	19.4
ZnO-9	Platelet	9.6	12.0	6.3	0.78	1.08	1.38	18.7
ZnO-10	Platelet	14.4	13.5	8.1	0.40	0.49	1.21	28.1
ZnO-11	Platelet	8.7	8.7	5.6	0.76	1.05	1.40	7.2
ZnO-12	Platelet	7.7	14.4	7.9	0.56	0.73	1.29	26.5
ZnO-13	Rod-like	7.0	39.4	19.3	0.60	0.73	1.22	2.6
ZnO-14	Rod-like	5.6	33.8	22.9	0.66	0.76	1.15	4.3
ZnO-15	Rod-like	7.2	129	64.8	0.78	0.87	1.11	2.4
ZnO-16	Rod-like	5.4	45.8	30.0	0.70	0.80	1.14	5.1
ZnO-17	Rod-like	5.9	75.7	45.5	1.00	1.11	1.11	6.3
ZnO-18	Rod-like	3.8	8.8	2.6	0.46	0.58	1.26	4.2
ZnO-19	Rod-like	5.8	5.6	2.5	0.80	0.98	1.22	2.0
ZnO-20	Platelet	17.9	36.6	23.7	2.00	2.22	1.11	0.0
ZnO-21	Rod-like	4.7	7.0	4.6	0.64	0.74	1.17	4.7
ZnO-22	Rod-like	5.8	4.7	2.3	0.51	0.63	1.24	2.9
ZnO-23	Rod-like	3.6	39.4	23.2	0.48	0.59	1.24	13.1
ZnO-24	Rod-like	5.4	4.0	2.7	0.56	0.65	1.14	4.4
ZnO-25	Rod-like	4.0	15.8	6.9	0.68	0.87	1.28	16.8
ZnO-26	Rod-like	4.2	32.7	24.6	0.28	0.34	1.19	12.6
ZnO-27	Platelet	5.5	12.8	6.1	0.46	0.63	1.38	29.0
ZnO-28	Platelet	5.3	25.6	8.2	1.13	1.23	1.09	41.0
ZnO-29	Rod-like	15.0	25.8	13.2	2.35	2.67	1.13	0.2
ZnO-30	Platelet	12.9	23.7	10.6	2.22	2.50	1.13	0.1
ZnO-31	Platelet	6.6	35.1	15.9	2.50	2.67	1.07	0.1
ZnO-32	Platelet	5.4	9.1	5.1	0.69	0.82	1.18	21.2
ZnO-33	Platelet	24.3	44.6	18.0	2.51	2.87	1.14	0.4
ZnO-34	Platelet	4.7	5.8	3.4	0.67	0.80	1.20	35.9

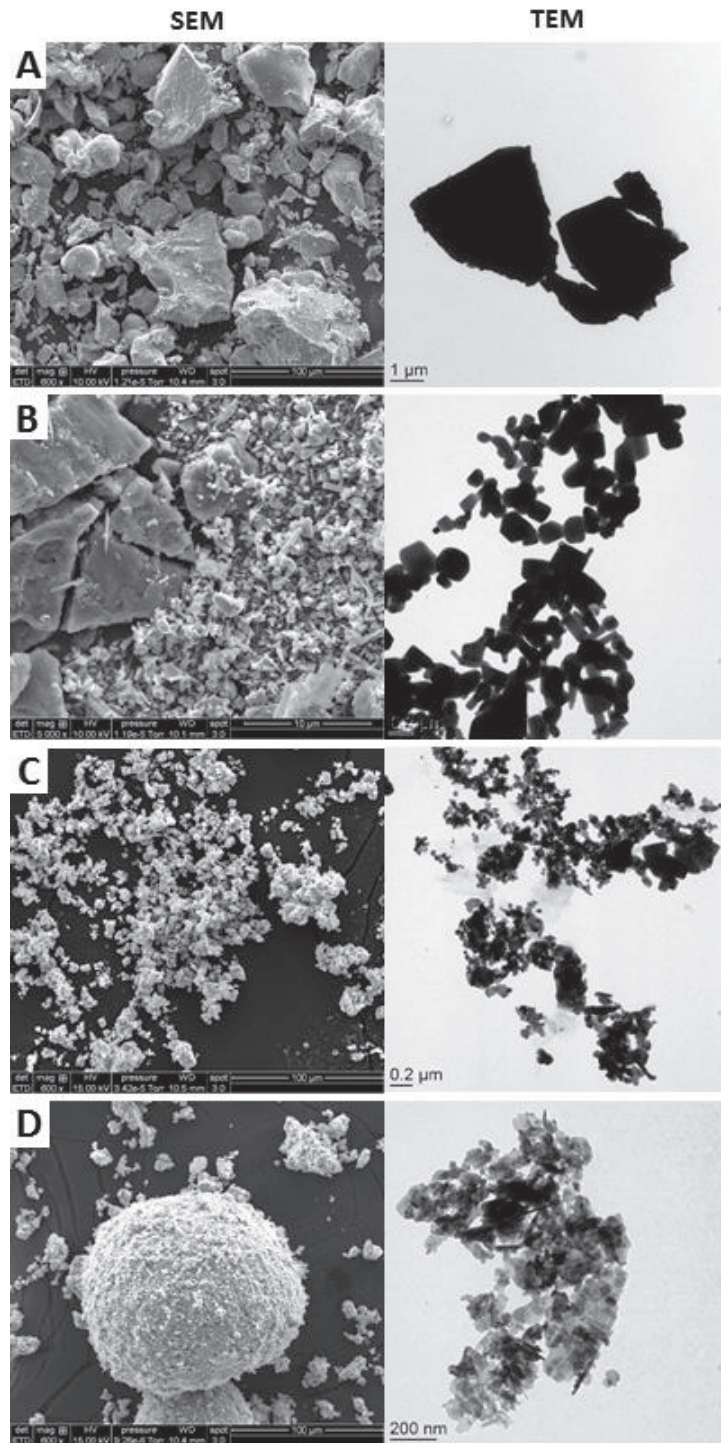


Figure 1: Feed grade ZnO powders. SEM (left) and TEM (right) show the main structures of different powders.

Pictures A, B, C and D correspond to ZnO-6, ZnO-21, ZnO-12 and ZnO-4 in Table 1.

### Density, tapped density and Hausner ratio

Density of a powder is the ratio of mass by the volume occupied by the powder. Low densities are associated to loosen aerated aggregates and/or agglomerates of particles stuck together

during the fabrication or drying process. Sticking by means of sintering yields irreversible bonds that cause formation of unbreakable aggregates. Agglomerates are formed through bonds coming from interparticle forces like van der Waals ones that can be broken by mechanical stirring during dispersion process in aqueous medium. Such bonds are mainly formed between primary particles of small size. With regards to the structure of ZnO particles, density provides an indirect estimate of the extent of aggregation and agglomeration. This relationship is in agreement with present findings. The lowest density values were related to particles with small size of primary particles. Conversely, particles of large size and rounded shape can pile as more compact powder of high density (Abdullah and Geldart, 1999).

Tapped density is obtained by tapping the container holding the aerated sample. Powder particles are forced to rearrange to close contact upon tapping. Tapped density is the densest density the powder can achieve under a moderate compression stress. Tapping erases the memory of the last stages of the manufacture process, mainly drying and final manipulations. The tapped density better characterizes particles packing under the control of interparticle interactions in dry medium. Hausner ratio is tapped density over bulk density; it is a useful tool to define cohesion of a powder (Leturia *et al.*, 2014; Abdullah and Geldart, 1999). The lower this ratio the less cohesive is the powder, meaning the lowest tendency for further consolidation. The forces acting between particles causing cohesion decrease as the primary particle size increases (Aulton and Taylor, 2013).

The lowest variation observed was 5% of volume reduction from density to tapped density. When the powder is given mobility through tapping, large and small particles are able to rearrange. As most of the small particles migrate to the bottom between the largest, the highest volume variation of samples observed was 40% for samples composed of mixed fine

powders and big clusters that, once tapped, adjust to the container when small particles migrate into interstices between large ones (Abdullah and Geldart, 1999).

### **Summary of experimental characterizations**

The analytical results are given in Table 1. The main characteristics of samples allow to sort them into 4 different groups corresponding to types of Figure 2A, B, C and D. This classification relies on electron microscopy pictures that provide rather subjective pieces of information. Physicochemical properties look being correlated with the structural types inferred from electron microscopy. Several samples presented two-size populations as observed on SEM pictures (Figure 1), whereas others presented a homogeneous size distribution of small primary particles, in which the latter presents large agglomerates structure. These 4 groups correspond to the different manufacture processes presented in the next section. However, the aim of the present paper is to reach a classification of ZnO particles in terms of their physicochemical properties that would be free of subjectivity. This will be achieved by means of a rigorous statistical analysis using PCA.

### Manufacturing process of ZnO

Zinc oxide is obtained mainly through three different processes: (1) Direct method, also known as the American process, with the oxidation of zinc metal. This method uses ore as raw materials. Due to the purity of the raw material, the final product is usually of a lower quality than the indirect method and has a wide range of chemical properties and physical characteristics. The SSA is very low, between 1 and 3  $\text{m}^2\cdot\text{g}^{-1}$ . Properties can be correlated to samples shown in Figure 1A. (2) The indirect or French method consists of the reduction of zinc ore followed by re-oxidation. This method generates 'rod-like' shape primary crystals varying in size from 30-2000 nm. SSA is in the range 3-5  $\text{m}^2\cdot\text{g}^{-1}$  up to 12  $\text{m}^2\cdot\text{g}^{-1}$ . Figure 1B shows

a representative sample of this method. (3) Wet method with the precipitation of the oxide from an aqueous solution with subsequent calcination (Moezzi *et al.*, 2012). Also known as 'active zinc oxide', ZnO obtained from the wet method, have an irregular particle morphology and porous with high specific area. (4) Spray pyrolysis also yields powders of high SSA, but with a narrower size distribution. The characteristics of ZnO prepared by these two later processes correspond to Figure 1C and D, even though some integrant showed a low SSA. It is difficult to discriminate these two manufacture processes from electron microscopy pictures. The management of manufacturing process of ZnO can control the SSA (in which a low SSA is associated to high temperatures) (Moezzi *et al.*, 2012).

### Dissolution kinetics

Solubility and rate of dissolution are important parameters that characterize powder particles (Avramescu *et al.*, 2017; David *et al.*, 2012). When assessing the solubility of feed compounds, dissolution seems to give a clear impression of the digestion process rather than solubility. The dissolution kinetics illustrates how fast a given element solubilizes, and this might have an impact on feed interactions, absorption and passage rate of that element.

Solubility is a state of equilibrium of a medium, but when the topic is digestion, solubility does not correctly address the questions, as reaching equilibrium of solubility can take longer time than digestion itself. Dissolution kinetics, though, will show how fast or slow a given feed compound takes to achieve equilibrium solubility.

In a study, Avramescu *et al.* (2017) summarized studies using different conditions to mimic biological fluids such as medium pH and temperature according to the purpose of each study. The authors report the difficulties to assess kinetics in these media due to their complexity. Nevertheless, pH and temperature are key parameters influencing dissolution of metals in *in*

*vitro* assays, and biological medium may have constituents, such as organic and inorganic ligands that may increase or decrease dissolution, and therefore impact understanding the dissolution mechanism of compounds (David *et al.*, 2019; Avramescu *et al.*, 2017). Owing to such issues, the authors suggest a first screening to evaluate dissolution kinetics, in water medium, at a specified pH and temperature according to the purpose of the study.

In this study, the dissolution kinetics was assessed in water at pH 4 at 40°C. Temperature and pH were selected to simulate the gastric pH of monogastric animals. Water medium was selected as to minimize the interactions with medium components and therefore have a better understanding of powders behaviour.

ZnO does not dissolve in water as ZnO species but mainly as Zn<sup>2+</sup> ions. There are actually several soluble zinc species whose respective concentrations depend on the pH. This is well-documented since the work by Reichle *et al.* (1975). Zn<sup>2+</sup> is the predominant species at pH 4; it is 99.99% of dissolved zinc. Only Zn<sup>2+</sup> ions are considered in the following. Dissolution of ZnO is accompanied by hydrolysis of ZnO into Zn<sup>2+</sup> according to the chemical reaction (Eq. 2)



Such reaction releases hydroxide ions (OH<sup>-</sup>), so that, the pH is increasing when ZnO dissolves into water. Maintaining the pH constant during the dissolution process requires addition of acid. Such demand for acid corresponds to the amount of OH<sup>-</sup> ions released according to the dissolution reaction (Eq. 2). As two moles of OH<sup>-</sup> ions are release for each mole of dissolved ZnO, the mole number of H<sup>+</sup> to be supplied is twice the mole number of dissolved Zn<sup>2+</sup>. In that way, the volume of acid supplied as a function of time can be converted into the concentration of dissolved Zn<sup>2+</sup>, that is, into dissolution kinetics of ZnO. An automatic burette was used to

deliver hydrochloric acid (HCl 1 M) in such a way that the pH is regulated at a constant value; the volume of added acid was recorded as a function of time.

$$[\text{Zn}^{2+}] = \frac{\text{Volume of acid supplied} \times [\text{HCl}]}{2 \times \text{Volume of solution}} \quad (\text{Eq. 3})$$

This method designed for easy measurement of dissolution kinetics is indirect and relies on the assumption of the chemical reaction (Eq. 2) being unique. Such a method was validated by comparison to direct measurements of soluble Zn by ICP-OES in samples collected during dissolution experiments. ICP-OES provides the concentration of soluble zinc, whatever its speciation (chemical form). The method using the automatic burette was satisfactorily validated since the concentrations of soluble Zn determined by this method were in very good agreement with those determined by ICP-OES (Figure 2).

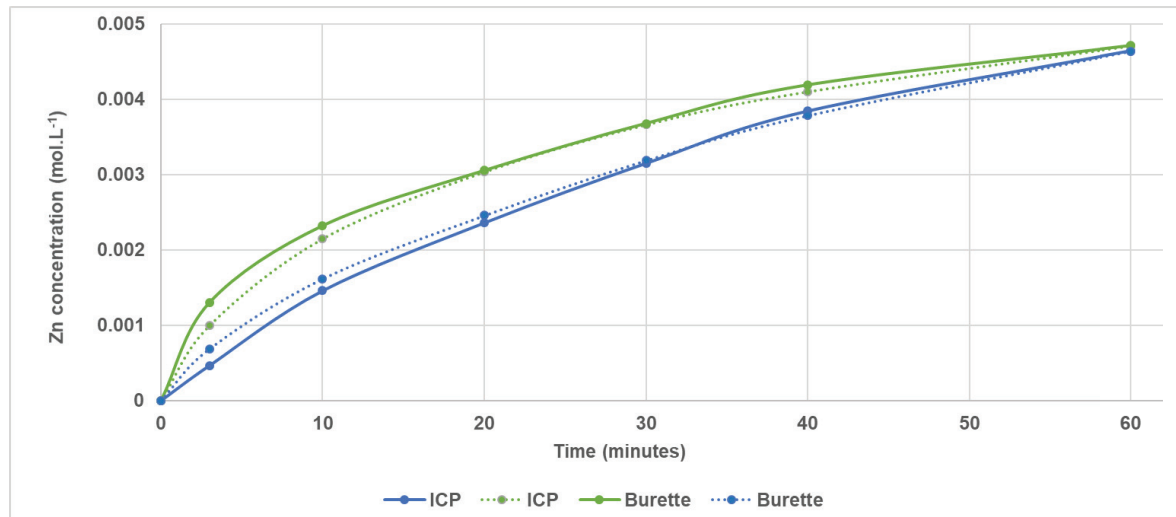


Figure 2: Validation of dissolution kinetics of ZnO using an automatic burette and through ICP-AOS analysis.

The dissolution kinetics curve had 3 main behaviours (Figure 3). The ZnO sources dissolving the fastest reached full dissolution in less than 20 min, while the slowest source did not reach 50% of dissolution during 1 h of experiment. The kinetics curves were characterized by the

concentrations of Zn dissolved after 10 min and after 20 min for their use in statistical analyses.

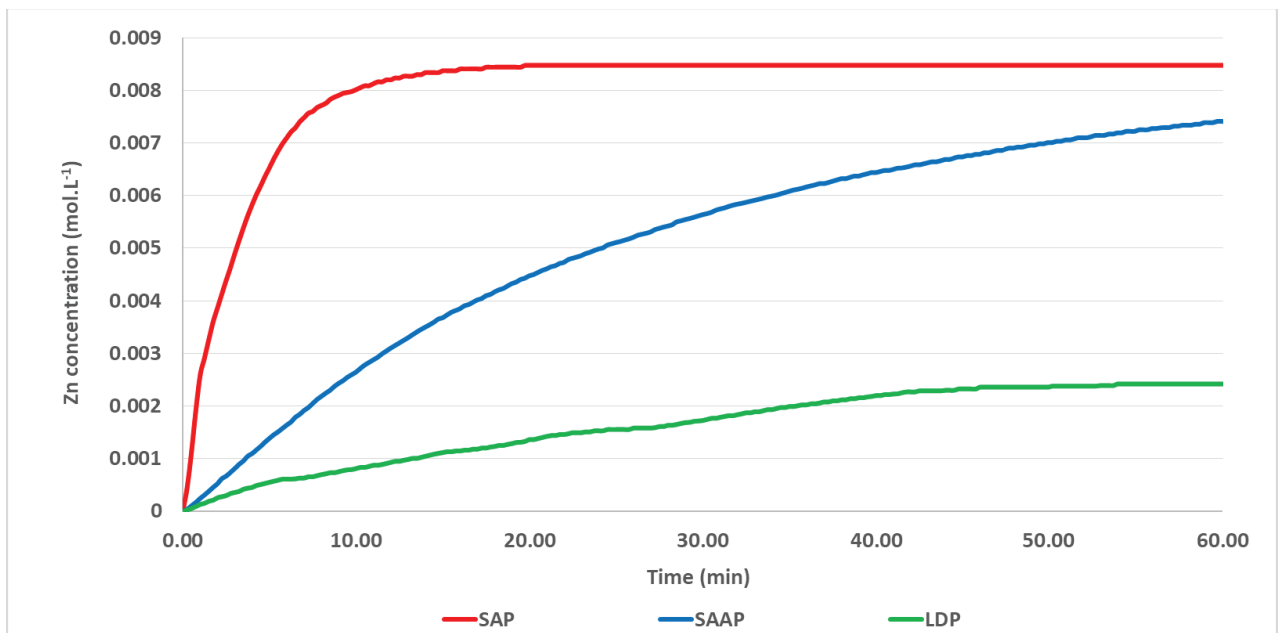


Figure 3: Dissolution kinetics of ZnO in water at pH 4 at 40°C.

### Classification of ZnO grades with respect to their physicochemical properties

PCA was firstly used to represent ZnO samples in a low-dimensional space. The purpose was to categorize ZnO grades in terms of their physicochemical characteristics, to identify which properties are characteristic of each family, and to select one representative sample in each family for further studies.

The raw dataset (Table 1) contains 34 samples in the 7-dimensional space of the 7 physicochemical parameters that are more or less correlated. The physicochemical parameters were reduced in standardized form by rescaling them with both their average value and standard deviation for each parameter so as they contribute with the same weight in the PCA analysis. The number of variables needs being reduced to those of highest



relevance, that is, with the highest contribution to the variance. The samples were placed in a lower-dimensional space by applying a PCA to the dataset.

The projections of physicochemical parameters on the plane defined by the two Principal Components (PCs) contributing the most to the variance (PC1 and PC2) are represented in Figure 4A. The parameters closest to the circle are most represented in the projection and their distances to PC1 and PC2 axes indicate whether they contribute most to PC1 or PC2. PC1 is mainly related to the aggregate  $D(50)$  size, the density values and, to a lesser extent, to the specific surface area. PC2 is essentially related to the Hausner ratio, the agglomerate  $D(50)$  size and the agglomeration rate (size ratio agglomerate/aggregate).

The scores plot in Figure 4B shows three distinct clusters of samples. Samples of Group A highlighted high density and large aggregate diameter as well as low SSA. Most samples were in Group B characterized by relatively low agglomerate and aggregate diameters and by high Hausner ratio. Finally Group C was made of samples characterized by large agglomerate diameter and SSA values. This distribution in clusters relies on a visual observation of the scores plot. This is open to some subjectivity.

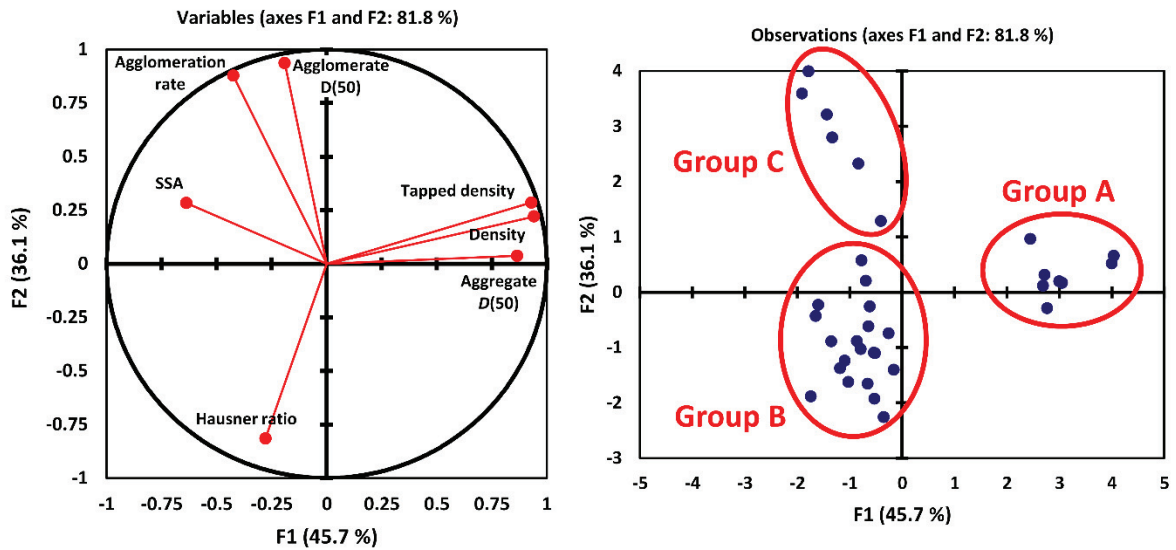


Figure 4: Loadings (A) and scores (B) plots obtained from the PCA analysis of the 34 ZnO samples characterized by 7 physicochemical parameters mapped onto the first two principal components PC1 and PC2 providing a cumulative variance of 81.8%.

A more rigorous analysis of clustering relying on statistics makes use of Hierarchical Ascendant Clustering (HAC). The outcome of HAC is a tree called 'dendrogram' where the population of samples distributes into classes (clusters) according to the Euclidian distance between samples called dissimilarity (Figure 5). The dissimilarity that minimizes the intra-cluster variance and maximizes inter-cluster variance according to Ward's criterion (Ward, 1963) was 53 (the dashed line in Figure 5). The dendrogram from HAC calculations indeed indicates three clusters of samples as it has been inferred by visual observation of PCA results in the PC1-PC2 frame. So HAC analysis yielded three clusters according to a statistical analysis that made the clustering process free of subjectivity.

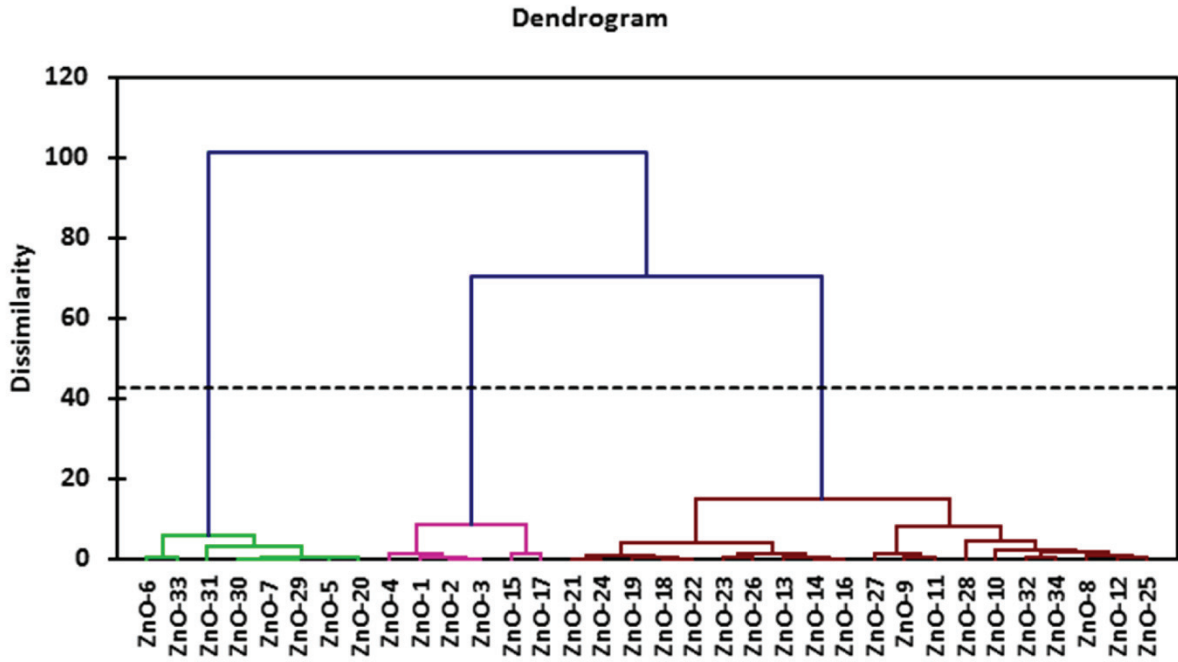


Figure 5: Hierarchical Ascendant Clustering Dendrogram obtained from the analysis of the 34 ZnO samples described by the 7 standardized physicochemical parameters. The dashed line shows the optimum dissimilarity inferred from the Ward's criterion.

The mean profile of each cluster in terms of the 7 initial standardized physicochemical parameters is given in Figure 6. Class 1 including 8 samples corresponded to the Group A of samples as defined by PCA: these samples were mainly characterized by high density, low SSA values and large aggregate median diameter (Figure 6). More precisely, as described by the distribution of the density parameter presented by a histogram, these samples exhibited density larger than  $2 \text{ g}\cdot\text{cm}^{-3}$  while samples belonging to other clusters of the dataset had density lower than  $1.2 \text{ g}\cdot\text{cm}^{-3}$  (Figure 7a). Except for the ZnO-31 sample with  $6.6 \mu\text{m}$  aggregate diameter, samples of Class 1 were made of the largest aggregates with  $D(50)$  ranging from  $12.9$  to  $27.6 \mu\text{m}$  (Figure 7b). Aggregate sizes measured by light scattering corresponded to the size of primary particles estimated from microscopy. Those samples are not aggregated and they are slightly agglomerated. Indeed, the mean value of agglomeration rate took the low

value of 2.2 (Figure 7c). This mean shifted down to 1.7 if the ZnO-31 sample is discarded. These values close to one meant that particles were not agglomerated. In accordance to their large particle size and high density, Class 1 samples had small specific surface areas that corresponded to the smallest values observed in the dataset ( $SSA < 0.5 \text{ m}^2\cdot\text{g}^{-1}$ ) (Figure 7d).

The 22 samples of Class 2 were related to Group B and exhibited low density and both small aggregates and agglomerates (Figure 6). The agglomeration rate was widely scattered, which meant that some samples of Class 2 were agglomerated whereas some others were not. Finally, Class 3 corresponding to Group C includes six samples made of small aggregates and large agglomerates ( $D(50) > 100 \mu\text{m}$ ) leading to the highest values of the agglomeration rate ( $> 20$ , Figure 7c). They also exhibited the highest specific surface areas ( $SSA > 25 \text{ m}^2\cdot\text{g}^{-1}$ , Figure 7d).

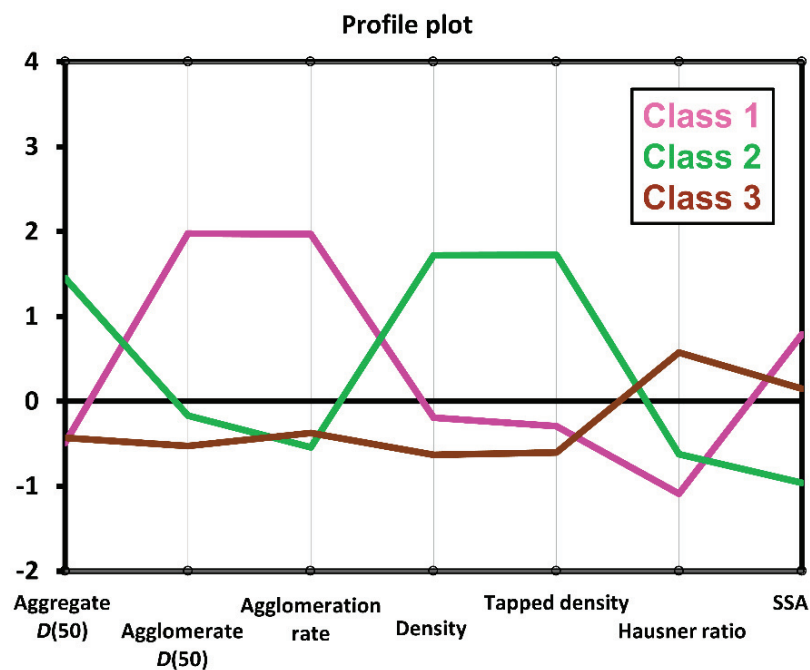


Figure 6: Mean standardized physicochemical profile for each of the three clusters as defined by HAC.

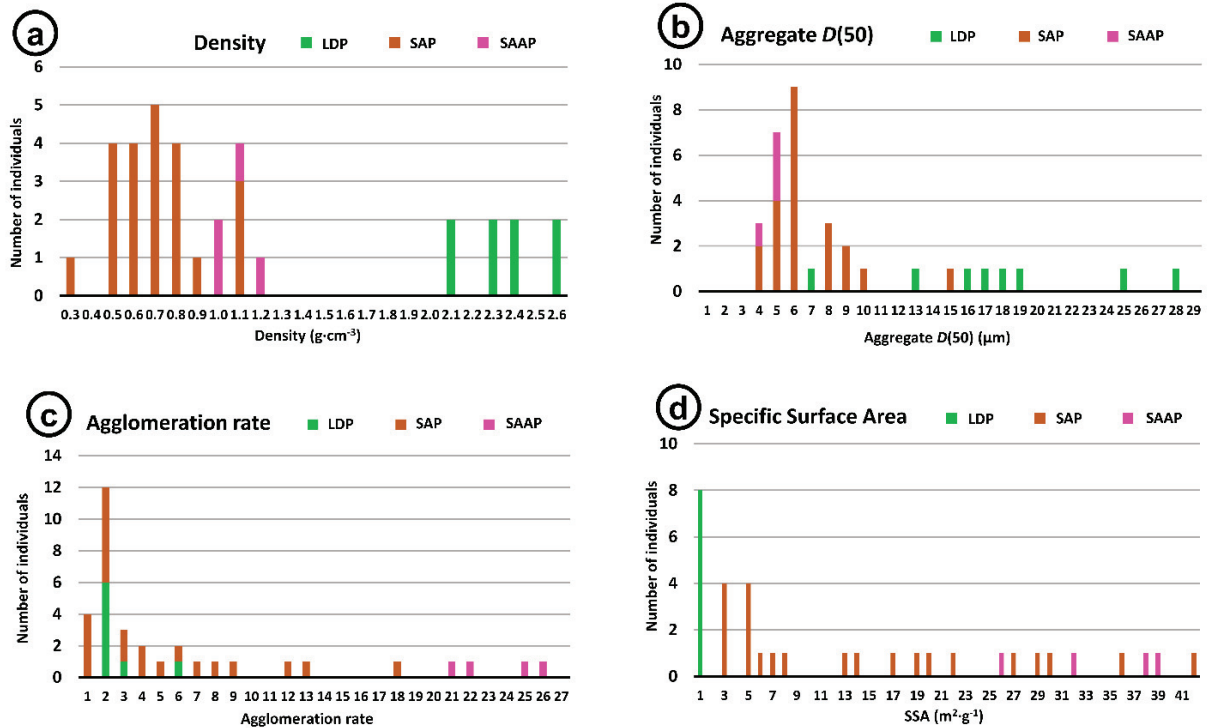


Figure 7: Distribution of physicochemical parameters. (a) density, (b) aggregate  $D(50)$ , (c) agglomeration rate, (d) SSA.

As a summary of these clustering analyses, three different groups of samples were highlighted and they were described by their characteristic physicochemical properties. Names have been allocated to the groups according to their main physicochemical features:

- 'Large dense particles' (LDP): Class 1 is made of large massive particles that are not agglomerated. The high density and electron microscopy pictures show that such particles are massive and not aggregated. The specific surface area is low because the particles are large and dense (not porous). This class probably corresponds to zinc oxide manufactured by the American process and subsequently grinded.
- 'Small aggregated and agglomerated particles' (SAAP): Class 3 is made of very small elementary particles as disclosed by electron microscopy observations. As a consequence, density and tapped density of dry powders are low. Such ZnO grades were probably produced

by the French process or the wet process (precipitation from a solution). Extensive agglomeration may come from a drying process by spray drying.

- 'Small aggregated particles' (SAP): Class 2 samples are also made of very small elementary particles. They are aggregated and only part of them is agglomerated. The dry densities are low (less than  $1 \text{ g}\cdot\text{cm}^{-3}$ ). But their characteristics are spread over the wide range for the other physicochemical parameters. The SAP class is a grey area where aggregates are either not or weakly agglomerated (less than for the SAAP class. Their manufacture process is the same as for the SAAP class.

### Dissolution kinetics

The purpose of this section is to search for correlations between dissolution kinetics and physicochemical properties. Dissolution rate results were full curves showing variation of zinc concentration against time (Figure 4). They were expressed as the zinc concentration after 10 min and 20 min dissolution times. Results are represented in Figure 8. These two concentrations are well-correlated, so that using only one of them would be enough. Such correlation shows that the physicochemical phenomena are the same at these two times; in other words there is no change of dissolution mechanism during the dissolution process. The clusters marked in colours in Figure 8 clearly appear as having different kinetics. Samples from LDP and SAAP classes exhibited the slowest dissolution kinetics. This behaviour may be obviously related to their low specific surface area for LDP class. It is tentatively related to their highly agglomerated state for SAAP class since large size of agglomerates is a specific feature of SAAP class compared to SAP class. Such visual observation of the influence of HAC clusters on dissolution kinetics clearly teaches that physicochemical properties control

dissolution rate and that the clustering made by HAC can provide clues for understanding relationships between dissolution kinetics and physicochemical properties.

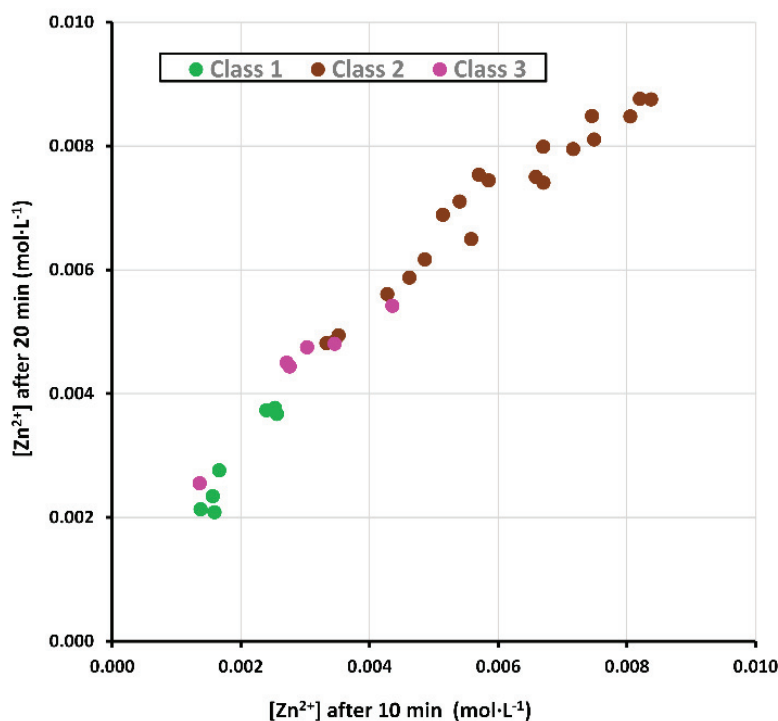


Figure 8: Dissolution kinetics results expressed as the Zn concentration after 10 min and 20 min for the 34 ZnO grades.

A correlation analysis was performed to further investigate such relationships. The Pearson's correlation coefficients ( $R$ ) were determined from the whole dataset. The correlation matrix (Table 2) shows that the dissolution rate was significantly anti-correlated with the density parameters and with both the agglomerate and aggregate median diameters. As expected, this indicates that large aggregates or agglomerates and/or dense ZnO particles make the dissolution process slow. The correlation is stronger with the density than with aggregate or agglomerate size. This appears quite surprising because no significant correlation was obtained with SSA since so many literature reports claim that dissolution is related to the available contact area of the solid and liquid media.

Table 2: Correlation matrix of Pearson's correlation coefficients determined for the 34 ZnO samples. The significant  $R$  values (at 0.05 level of significance) are indicated in bold italics.

	Aggregate $D(50)$ ( $\mu\text{m}$ )	Agglomerate $D(50)$ ( $\mu\text{m}$ )	Agglomeration rate	Density ( $\text{g}\cdot\text{cm}^{-3}$ )	Tapped density ( $\text{g}\cdot\text{cm}^{-3}$ )	Hausner ratio	SSA ( $\text{m}^2\cdot\text{g}^{-1}$ )	$[\text{Zn}^{2+}]$ after 10 min ( $\text{mol}\cdot\text{L}^{-1}$ )	$[\text{Zn}^{2+}]$ after 20 min ( $\text{mol}\cdot\text{L}^{-1}$ )
Aggregate $D(50)$ ( $\mu\text{m}$ )	1								
Agglomerate $D(50)$ ( $\mu\text{m}$ )	-0.073	1							
Agglomeration rate	-0.333	<b>0.945</b>	1						
Density ( $\text{g}\cdot\text{cm}^{-3}$ )	<b>0.764</b>	0.066	-0.150	1					
Tapped density ( $\text{g}\cdot\text{cm}^{-3}$ )	<b>0.783</b>	0.014	-0.204	<b>0.996</b>	1				
Hausner ratio	-0.172	<b>-0.600</b>	<b>-0.523</b>	<b>-0.460</b>	<b>-0.385</b>	1			
SSA ( $\text{m}^2\cdot\text{g}^{-1}$ )	<b>-0.401</b>	0.309	<b>0.437</b>	<b>-0.422</b>	<b>-0.440</b>	0.012	1		
$[\text{Zn}^{2+}]$ after 10 min ( $\text{mol}\cdot\text{L}^{-1}$ )	<b>-0.362</b>	<b>-0.479</b>	<b>-0.375</b>	<b>-0.709</b>	<b>-0.676</b>	<b>0.653</b>	0.171	1	
$[\text{Zn}^{2+}]$ after 20 min ( $\text{mol}\cdot\text{L}^{-1}$ )	<b>-0.417</b>	<b>-0.419</b>	-0.304	<b>-0.777</b>	<b>-0.749</b>	<b>0.621</b>	0.189	<b>0.982</b>	1

To illustrate these results, the zinc concentration after 10 min dissolution time was represented as a function of i) the density, ii) the agglomeration rate, and iii) SSA (respectively in Figures 9a, b and c). The samples of LDP class appeared to strongly impact the correlation coefficients: they clearly induce a strong correlation with density (Figure 9a) while they make it weaker with the size ratio (Figure 9b). No significant linear relationship was observed between SSA and the dilution rate by considering the whole dataset (Figure 9c). But these two parameters are quite related since all of the samples of LDP class characterized by the lowest SSA values ( $\leq 0.5 \text{ m}^2\cdot\text{g}^{-1}$ ) had low dissolution rates. The other samples (SAP and SAAP classes) with SSA ranging from 2.0 to 41.0  $\text{m}^2\cdot\text{g}^{-1}$  may exhibit all types of dissolution kinetics.



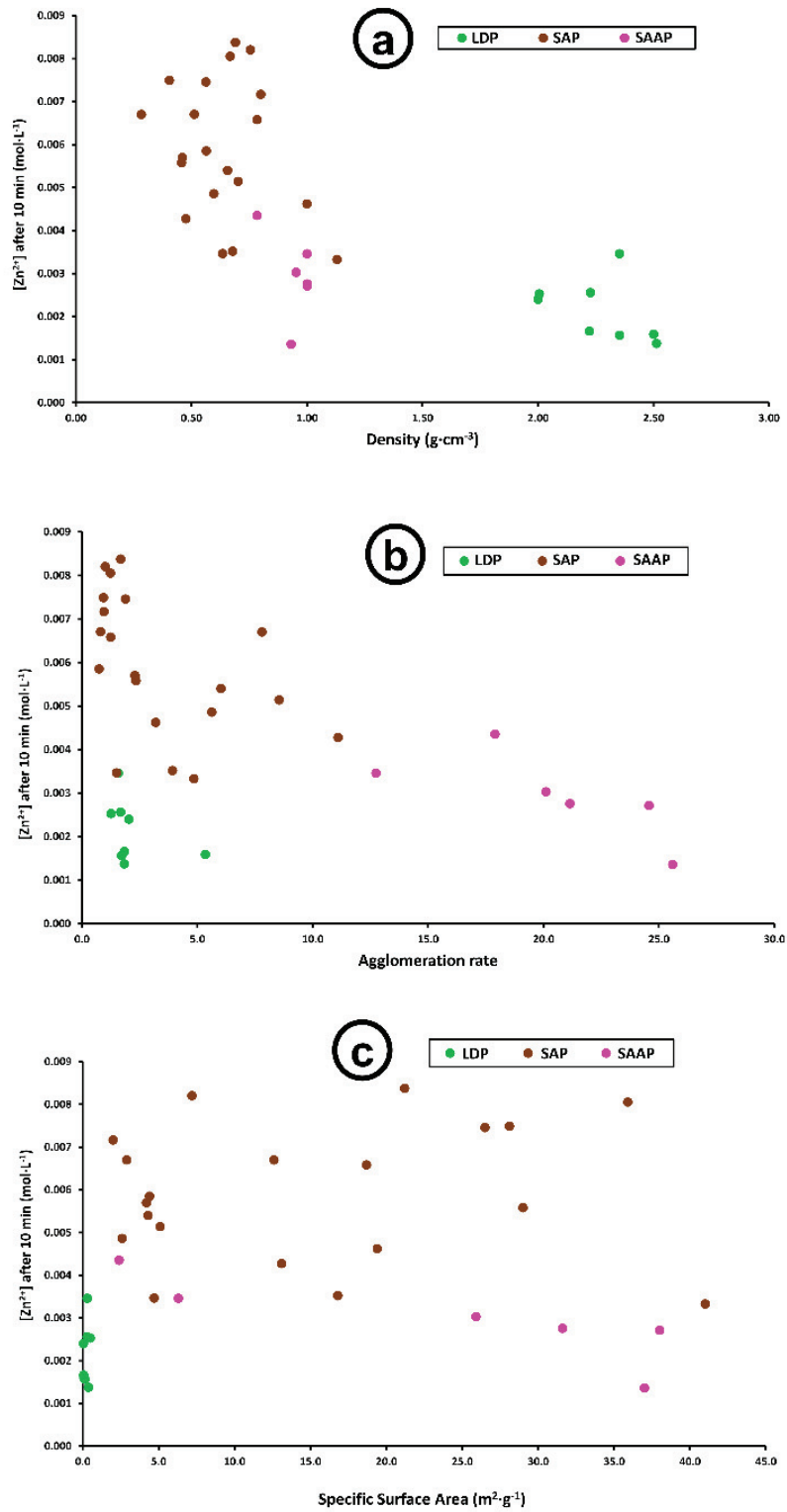


Figure 9: Representation of the zinc concentration after 10 min dissolution time as a function of density (a), agglomeration rate (b) and SSA (c) for the 34 ZnO samples.

A refined analysis was done by considering together samples of both SAP and SAAP classes. The corresponding correlation matrix (Table 3) disclosed that the dissolution kinetics was significantly related to the agglomerate size, the agglomeration rate, and the dry density parameters. For both dissolution measurements, the highest  $R$  values were obtained for the agglomeration rate. The negative correlation coefficient indicated that dissolution was favoured in ZnO samples characterized by low size ratios, i.e. by aggregates that were poorly agglomerated. As in the correlation analysis of the full dataset, the dissolution was improved for samples exhibiting low density revealing large porosity of the aggregates. The striking feature is that dissolution kinetics is not correlated with SSA.

Table 3: Correlation matrix of Pearson's correlation coefficients determined for the 26 samples of merged SAP and SAAP classes. The significant  $R$  values (at 0.05 level of significance) are indicated in bold italics.

	Aggregate $D(50)$ ( $\mu\text{m}$ )	Agglomerate $D(50)$ ( $\mu\text{m}$ )	Agglomeration rate	Density ( $\text{g}\cdot\text{cm}^{-3}$ )	Tapped density ( $\text{g}\cdot\text{cm}^{-3}$ )	Hausner ratio	SSA ( $\text{m}^2\cdot\text{g}^{-1}$ )	$[\text{Zn}^{2+}]$ after 10 min ( $\text{mol}\cdot\text{L}^{-1}$ )	$[\text{Zn}^{2+}]$ after 20 min ( $\text{mol}\cdot\text{L}^{-1}$ )
Aggregate $D(50)$ ( $\mu\text{m}$ )	1								
Agglomerate $D(50)$ ( $\mu\text{m}$ )	-0.080	1							
Agglomeration rate	-0.206	<b>0.972</b>	1						
Density ( $\text{g}\cdot\text{cm}^{-3}$ )	-0.012	<b>0.551</b>	<b>0.513</b>	1					
Tapped density ( $\text{g}\cdot\text{cm}^{-3}$ )	0.086	0.377	0.325	<b>0.957</b>	1				
Hausner ratio	0.264	<b>-0.683</b>	<b>-0.702</b>	<b>-0.483</b>	-0.215	1			
SSA ( $\text{m}^2\cdot\text{g}^{-1}$ )	0.094	0.309	0.345	0.376	0.312	-0.221	1		
$[\text{Zn}^{2+}]$ after 10 min ( $\text{mol}\cdot\text{L}^{-1}$ )	0.347	<b>-0.700</b>	<b>-0.751</b>	<b>-0.558</b>	<b>-0.401</b>	<b>0.617</b>	-0.236	1	
$[\text{Zn}^{2+}]$ after 20 min ( $\text{mol}\cdot\text{L}^{-1}$ )	0.302	<b>-0.701</b>	<b>-0.753</b>	<b>-0.587</b>	<b>-0.440</b>	<b>0.594</b>	-0.295	<b>0.982</b>	1

It remains the LDP class where the small number of individuals compromises the accuracy of its statistical analyses. It is characterized by a low SSA; but SSA does not significantly contribute to the variance of the whole dataset (Figure 9c). Figure 9c shows the origin of such lack of correlation between dissolution rate and SSA: samples with low dissolution rate are those with the lowest SSA (LDP class) and the highest SSA (SAAP class). All samples of the LDP class have a low SSA and a slow dissolution rate, so that a low SSA is indeed associated to a slow dissolution. Figure 9c shows two different regimes of dissolution according to the SSA. Dissolution rate increases with respect of SSA for samples with SSA lower than  $2 \text{ m}^2\cdot\text{g}^{-1}$ , and

stays more or less constant for higher SSA with a large scatter of data points that prevents a statistical correlation. So it can be inferred that the SSA parameter controls dissolution for samples with low SSA, even though this is not an outcome of the correlation analysis.

### Dissolution mechanism

The dissolution rate is often claimed directly proportional to the SSA of the powder, meaning that the smaller the particles, the faster dissolution happens (Mosharraf and Nyström, 1995). However in the current experiment, results showed a greater influence of agglomerates to dissolution than SSA. Agglomeration appears in the parameters of higher statistical relevance: agglomerate size, agglomeration rate, density and tapped density. The analysis showed a rate-limiting step in the kinetics of dissolution given by the structural arrangement of particles.

Thus, the mechanism by which zinc oxide is dissolved can be explained by a two-step process: (1) the dissolution itself in the surface of the particles, which is very fast and (2) the diffusion of the molecules outside the agglomerate (Figure 10). The dissolution happens faster in powders with high specific area; however the diffusion out of the agglomerates is slow. The dissolution kinetics is determined by the rate of the slowest stage (Mosharraf and Nyström, 1995). Diffusion is the rate-limiting step for the SAP and SAAP classes as it has been inferred by David *et al.* (2012) with ZnO samples of the SAP or SAAP class. The relationship between density, agglomerate size and dissolution corresponds to the length of the path from the  $Zn^{+2}$  from the interface of solid and liquid phase into the bulk medium, that is, the diffusion distance to escape the agglomerate.

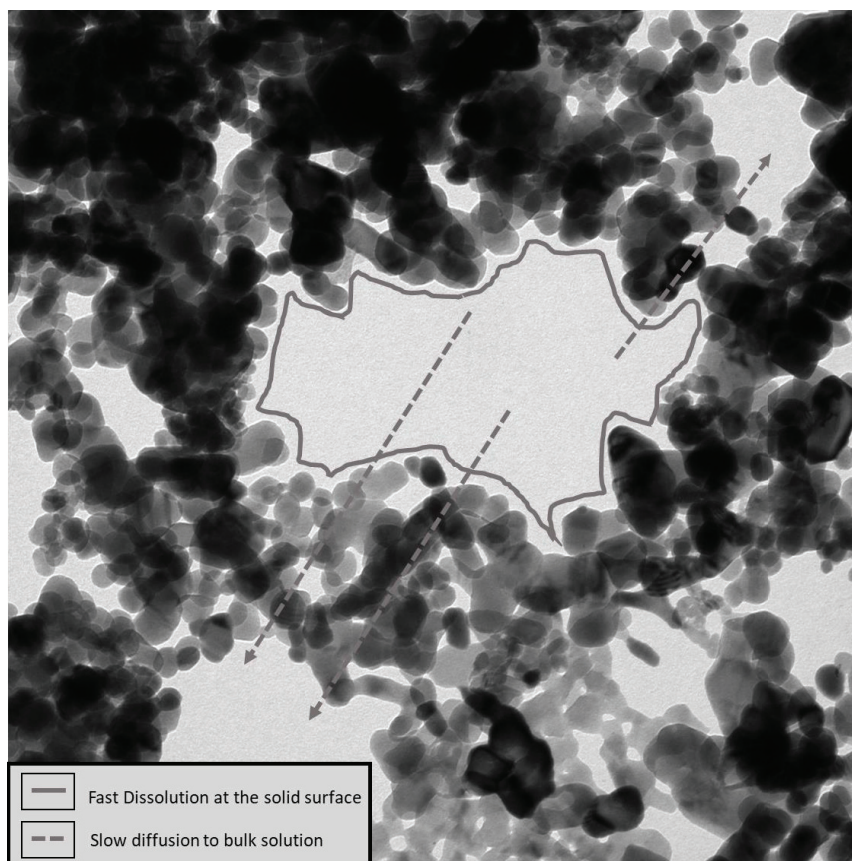


Figure 10: Illustration of proposed dissolution mechanism of ZnO of the SAP and SAAP classes. In the first step, there is fast dissolution of ZnO into  $Zn^{+2}$  at the surface of the solid, followed by ions diffusion through the quiescent medium to the bulk solution.

## CONCLUSION

The present study showed great differences among evaluated sources.

Different physicochemical properties have an impact on the dissolution kinetics which can affect *in vivo* solubility and bioavailability in monogastric animals. Coarse particles of low specific surface area dissolve slowly as it is expected from common sense: small contact area causes slow dissolution. The great majority of feed grade ZnO powders investigated are made of aggregates (and agglomerates) of small primary particles. The SSA is large; but dissolution can be slow. In this case, dissolution kinetics involves a two-step process related to the SSA and the density of the powder. Density is a way to assess aggregation and agglomeration of

small primary nanoparticles. It is easy to measure and strongly correlated with dissolution rate. A systemic study is being developed to assess the *in vitro* dissolution kinetics vs *in vivo* bioavailability relationships.

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## CHAPTER 3

***« Science sans conscience n'est que ruine de l'âme »***

*François Rabelais*

## **EFFECT OF THE PHYSICOCHEMICAL PROPERTIES OF ZINC OXIDE SOURCES ON THEIR BIOAVAILABILITY IN BROILERS**

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### **ABSTRACT**

The bioavailability of a trace mineral source is related to its in vivo solubility, which in turn is determined by its physicochemical properties. It is still not clear which characteristics are more relevant in affecting solubility and bioavailability of mineral sources. Zinc oxide (ZnO) is a common feed additive used to supplement zinc in the diet of monogastric animals. However, different sources have shown different responses on animal bioavailability. This study hypothesized that different sources of feed grade ZnO have various physicochemical features that lead to distinct bioavailability values. Feed grade ZnO samples collected from the feed industry worldwide and characterized for their physicochemical features were tested in broilers and their zinc bioavailability determined. A total of 135 male Cobb broiler chickens were fed a standard starter diet from day 1 after hatching up to day 7. At day 8, animals were allocated in individual cages and fed one of the following dietary treatments during 15 days: a basal diet with 23.5 ppm of zinc and 7 diets with supplemented ZnO or zinc sulphate (ZnSO<sub>4</sub>) at 6 or 12 ppm. Different sources of ZnO showed an effect of Zn solubility in the stomach and Zn supplementation influenced total Zn in the ileum. The bioavailability of the different



sources varied from 49 to 160% in relation to ZnSO<sub>4</sub>. Aggregate size of particles seems to explain a large variability in the bioavailability of the different sources tested in broilers. In conclusion, physicochemical properties of Zn oxide can partly explain the variability observed in terms of Zn biological value. Further work is needed to define precisely the most accurate set of predictors of Zn bioavailability.

## **INTRODUCTION**

Zinc (Zn) is a trace element essential for a proper body function. Present in over 300 metalloenzymes and 2000 transcription factors, Zn is mainly involved in gene expression, immune function and oxidative stress (Underwood, 2010). Poultry diets often do not have the enough amount of Zn to support optimal animal growth and supplementation is necessary (Schlegel *et al.*, 2013). Zn requirement, defined by old studies (NRC, 1994), is 40 ppm. However, genetic companies recommend 110 ppm of Zn in the total diet (Aviagen, 2014). This gap between requirements and recommendations demands for a further understanding of the mechanism underlying Zn digestion and absorption. Additionally, there is a pressure to reduce the safety margins of this mineral in animal feed to diminish the environmental footprint left by the livestock production, by a better adjustment of dietary supply to the animal needs and a better characterization of supplemented sources to understand the mechanisms affecting Zn absorption in the body.

The intestinal uptake of Zn is mainly influenced by the source and interaction with feed components, such as phytate (Schlegel *et al.*, 2013). Zinc sulphate (ZnSO<sub>4</sub>) and oxide (ZnO) are the most common Zn sources used to supplement diets. Zinc sulphate is a water-soluble source and therefore, considered to have a bioavailability of 100%. Zinc oxide, a non-water-soluble source, showed a bioavailability varying from 37% to 105 % in relation to the sulphate

counterpart (Edwards and Baker, 1999; Narcy et al., 2015). The manufacturing process of ZnO produces powder with different physicochemical properties (Moezzi *et al.*, 2012) which lead to variable speed of dissolution in vitro. We hypothesized that these different characteristics could explain the variability observed on Zn bioavailability values (Cardoso *et al.*, 2019). Therefore, the objective of the present study was to relate the physicochemical properties of different sources of ZnO used in the feed industry to their in vivo bioavailability.

## **MATERIAL AND METHODS**

### Birds and housing

The experimental protocol was approved by the Regional Ethical committee on animal experiment (N°19) and by the French ministry of higher education and research.

A total of 135 one-day-old male Cobb broiler chicken were used in a dose-response experiment to assess the bioavailability of 7 zinc sources. Each treatment consisted of 9 replicates. At day 1, birds arrived at the poultry facility of INRA Val de Loire (Nouzilly, France), were weighted and housed in floor pens and fed a standard diet until day 7. At day 8, broilers were allocated according to body weight (BW) in individual cages resulting in similar mean BW per treatment. Birds were housed in these individual cages until the end of the experiment at day 22 or 23. Ambient temperature was gradually decreased in average 0.5°C per day from 31°C at day 1 to 22°C at the end of the trial. Feed and water were supplied for ad libitum intake throughout the entire experiment. Birds were weighed at day 1, 8 (to standardize mean BW per treatment) and 22 or 23 of the experiment. Individual feed intake was recorded from day 8 to day 22 or 23. Feed conversion ratio (FCR) was calculated from day 8 to day 22 or 23. Mortality was registered daily throughout the experiment. At the day of sampling, the feed was removed in the morning and animals were weighted. Feed was re-introduced individually

3 hours prior to slaughter to synchronize with bird digestive physiology. Birds were euthanized by pentobarbital injection (1mL/kg of BW; Laboratoire Vetoquinol SA, Lure, France). A sample of blood was withdrawn and centrifuged to collect the plasma before storage at -20°C. The gastrointestinal tract of each bird was removed and separated in the different segments: (1) proventriculus and gizzard, (2) duodenum, (3) jejunum and (4) ileum. The content from each segment was collected as well as the right tibia and stored at -20°C until further analysis.

### Experimental Diets

Birds were fed a standard diet from day 1 upon to 7 days of age that covers all nutrients requirements. A basal diet (Table 1) containing corn (62%), potato protein (17%) and starch (13%) was formulated in a way that Zn originated exclusively from plant feedstuffs using a zinc-free premix. Seven different sources of feed grade zinc were thus tested according to a dose-response experiment: (1) Zinc sulfate ( $ZnSO_4$ ) used as a standard zinc source, (2) SAAP-1; ZnO source characterized as small aggregates and agglomerates particles, (3) SAAP-2, (4) SAAP-3, (5) LDP-1; ZnO source presenting large dense particles, (6) LDP-2 and (7) SAP, ZnO characterized as small aggregates and large agglomerates (HiZox<sup>®</sup>, Animine, France). The study included 15 dietary treatments consisting of a basal diet, with no zinc added, and zinc sulfate or one of ZnO sources providing at 6 and 12 mg/kg of zinc.

Experimental diets were manufactured by adding the respective zinc source to the basal diet. Prior to this in vivo experiment, different feed grade ZnO samples were collected and characterized according to their physicochemical properties (Table 1). Samples were assessed for particle size, specific surface area, and in vitro dissolution kinetics. The different ZnO sources were then gathered into families and representative samples of each family was selected for this bioavailability study.

Table 1: Characteristics and dissolution behaviour of ZnO used in the present study.

	Zinc source	Properties <sup>1</sup>	Speed of dissolution <sup>2</sup>	Origin
1	ZnSO <sub>4</sub>	*	+++	Europe
2	SAAP-1	Small aggregates and agglomerates; platelet shaped	+++	Asia
3	SAAP-2	Small aggregates and agglomerates; platelet shaped	+++	Europe
4	SAAP-3	Small aggregates, large agglomerates; 'rod-like' shaped	+++	Asia
5	LDP-1	Large dense particles	+	Europe
6	LDP-2	Large dense particles	+	North America
7	SAP	Small aggregates, large agglomerates; platelet shaped	++	Europe

\* Characterization not applicable for ZnSO<sub>4</sub>. <sup>1</sup>Properties are described by Cardoso *et al.* (2019). <sup>2</sup>The speed of dissolution described in vitro as: (1) +++: fast dissolution, (2) ++ intermediate dissolution and (3) +: slow dissolution rate.

All diets met or exceeded nutrient requirements of broilers according to French standards, except for dietary zinc. From day 8 onward, the birds were fed their respective pelleted experimental diets until the end of the experiment.

Prior to experimental feed manufacturing, Zn was analyzed in critical feedstuff (e.g. maize, potato, starch, casein) and supplemented sources. Based on the analyzed values, experimental diets were optimized according to experimental setup (Table 2). Each zinc source was added to the basal diets at the highest level, and then basal diet were mixed to high level treatment to create the intermediate level of treatments. The Zn concentration of each experimental diet was checked before the start of the trial.

### Zinc Analysis

Dry matter was determined for tibia diets and digestive contents (103°C for 4 h). To assess the soluble and insoluble fraction, the rest of the digestive content was centrifuged and the liquid

phase separated from the solid. This step was repeated, with the addition of 5mL of distilled water prior the second centrifugation. Samples were then ashed at 550°C and was then solubilized with nitric acid (16 M) and Zn content determined using ICP-OES (Thermo Scientific™ iCAP™ 7000).

Table 2: Composition of the basal diet without added zinc sources\*

Item	Basal diet
Ingredient (%)	
Corn	62.4
Potato protein	17.2
Starch	13.0
Dicalcium phosphate	2.36
Limestone	0.70
Soybean oil	2.00
Salt	0.35
Premix† (Zn-free)	0.50
Casein	1.00
Lysine HCL	0.14
Methionin DL	0.20
Threonin	0.08
L-tryptophan	0.02
Anticoccidiostatic	0.05
Nutrient (g/kg)	
DM	915
CP	201
CF	44.3
AMEn (kcal/kg)	3213
Phosphorus (mg/kg)	6.1 (6.6)*
dig Lys	12.1
dig SAA	8.3
Calcium (mg/kg)	9.5 (10.4)*
Zinc	22 (23.5)*

\*Analyzed values in brackets

†Supplied per kilogram of diet: 12,500 IU vitamin A (retinyl acetate); 4300 IU vitamin D<sub>3</sub> (cholecalciferol); 100 IU vitamin E (DL-alpha-tocopherol); 5 mg vitamin K<sub>3</sub> (menadione); 5 mg vitamin B<sub>1</sub> (thiamine); 8 mg vitamin B<sub>2</sub> (riboflavin); 100 mg vitamin B<sub>3</sub> (PP, niacin); 25 mg vitamin B<sub>5</sub> (Ca panthotenate); 7 mg vitamin B<sub>6</sub> (pyridoxine); 0.3 mg vitamin B<sub>8</sub> (biotin, H); 3 mg vitamin B<sub>9</sub> (folic acid); 0.02 mg vitamin B<sub>12</sub> (cyanocobalamin); 550 mg choline; 50 mg Fe (FeSO<sub>4</sub>); 16 mg Cu (CuSO<sub>4</sub>); 80 mg Mn (MnO); 2 mg I (Ca(IO<sub>3</sub>)<sub>2</sub>); 0.2 mg Se (Na<sub>2</sub>SeO<sub>3</sub>).

## Statistical Analysis

Observations identified as outliers and excluded from the data set before statistical analysis if an observation exceeding the parameter mean by more than two times the standard deviation (SD). The experimental data were analysed by ANOVA (GLM procedure) using R software (3.5.1 edition). The following model was used:

$$Y_{ij} = \mu + \text{Block}_i + \text{Treatment}_j + e_{ij}$$

Where Y is the Zinc concentration in tibia ash,  $\mu$  is the overall mean, Block is the effect of replicated block ( $i = 1$  to 9), Treatment is the effect of zinc source and dose level ( $j = 1$  to 15) and e is the random error. Treatment means were compared by least significant differences. A P-value  $< 0.05$  was considered statistically significant and  $0.05 < P < 0.10$  was considered a trend. The dose-response relationship between Zn intake and tibia Zn was tested according to the method of Littell et al. (1997). The slope of the Zn sulphate source was considered as the reference to estimate the relative bioavailability of ZnO sources. The correlation between physicochemical properties and zinc bioavailability were then determined using the correlation procedure of R software.

## **RESULTS**

### Dietary Zinc Levels

The Zn level was determined in each dietary treatment as shown in Table 3. The analyzed zinc level in basal diet was 23.5 mg/kg, as expected. Supplementation of the Zn sources was also in accordance with the expected dose levels. Results described below will refer to supplemental Zn intake of birds throughout the experiment.

Table 3: Analyzed zinc levels in each of the experimental diets.

Treatment	Zinc source	Analyzed zinc level in the diet (mg/kg)	Expected zinc supplementation (mg/kg)	Realized zinc supplementation <sup>1</sup> (mg/kg)
1	Control	23.5	0	0
2	ZnSO <sub>4</sub>	28.4	6	4.9
3		34.5	12	11
4	SAAP-1	28.5	6	5
5		35.4	12	11.9
6	SAAP-2	28	6	4.5
7		34.4	12	10.9
8	SAAP-3	29.7	6	6.2
9		36.6	12	13.1
10	LDP-1	29.3	6	5.8
11		33.6	12	10.1
12	LDP-2	29.3	6	5.8
13		33.1	12	9.6
14	SAP	28.9	6	5.4
15		34.5	12	11

<sup>1</sup>Calculated as the analysed zinc content of experimental diet minus zinc content of the control diet.

### Production Performance

Results in feed intake, BW gain and FCR are given on Table 4. Daily feed intake, body weight gain per day from 8 to 22 or 23 days and FCR were not significantly affected by the dietary zinc source and supplemented level among the different treatments. The feed intake varied from 27.5 to 36.8 g per day per bird. The BW gain varied from 37.5 to 42.8 g per bird per day. FCR showed a variation from 1.19 to 1.49.

### Tibia zinc content

Results on Zn on tibia dry matter content are described on Table 4. Zinc in the tibia in the control diet was 85.7 mg of Zn /kg of DM. In the supplemented diets, Zn in tibia varied from 91.16 in the LPD-2 treatment to 120.7 mg of Zn /kg of DM in the treatment supplemented with SAP. The supplementation of either 6 or 12 mg/kg of Zn increased significantly the Zn in the tibia for all treatments ( $P < 0.001$ ) compared with the unsupplemented diet.

Table 4: Daily weight gain and FCR from day 8 to 22/23 days of treatments. a–d Means with different superscript within a column differ significantly ( $P < 0.001$ ).

Treatment	Zinc source	Diet Zn (mg)	Feed intake (g/day)	Weight gain (g/day)	FCR (g/g)	Tibia Zn (mg/kg DM)
1	Control	23.5	34.36	40.25	1.38	85.69 <sup>d</sup>
2	ZnSO <sub>4</sub>	28.4	32.98	42.33	1.36	100.38 <sup>abcd</sup>
3		34.5	29.23	39.56	1.44	107.55 <sup>abc</sup>
4	SAAP-1	28.5	31.23	37.46	1.20	101.54 <sup>abcd</sup>
5		35.4	33.13	42.39	1.32	115.95 <sup>ab</sup>
6	SAAP-2	28	30.46	42.80	1.41	103.14 <sup>abcd</sup>
7		34.4	27.50	37.57	1.39	113.45 <sup>ab</sup>
8	SAAP-3	29.7	31.40	40.26	1.30	102.25 <sup>abcd</sup>
9		36.6	29.52	42.60	1.49	116.66 <sup>ab</sup>
10	LDP-1	29.3	30.99	40.21	1.30	102.34 <sup>abcd</sup>
11		33.6	34.86	43.81	1.28	113.83 <sup>ab</sup>
12	LDP-2	29.3	30.38	42.64	1.41	91.16 <sup>cd</sup>
13		33.1	36.76	42.82	1.19	99.94 <sup>bcd</sup>
14	SAP	28.9	30.51	39.08	1.34	100.81 <sup>abcd</sup>
15		34.5	33.14	42.50	1.32	120.68 <sup>a</sup>
	p-value		0.4060	0.158	0.207	< 0.001

### Bioavailability of zinc sources

The zinc content in tibia dry matter and the supplemental zinc intake throughout the experiment were used to calculate the relative bioavailability of the respective sources. All sources showed a linear response for both levels of supplementation. The linear slope ratio of sources is shown on Figure 1. A significant difference in bone zinc was observed according to the source and to the supplemental zinc intake ( $p < 0.001$ ). The bioavailability is represented by the slope of the different linear dose-response of each source. The higher the slope, the higher is the bioavailability. At the opposite, a lower slope indicates a lower response to the supplementation. The slopes and bone relative bioavailability values (RBV) are described on Table 5. The coefficients of relative bioavailability of zinc were estimated considering ZnSO<sub>4</sub> as 100%. Sources bioavailability had a significant variability, from 49% for a ZnO source made



of large dense particles (LDP) to 160% for SAP, a ZnO source made of small aggregates and large agglomerates, in relation to ZnSO<sub>4</sub>.

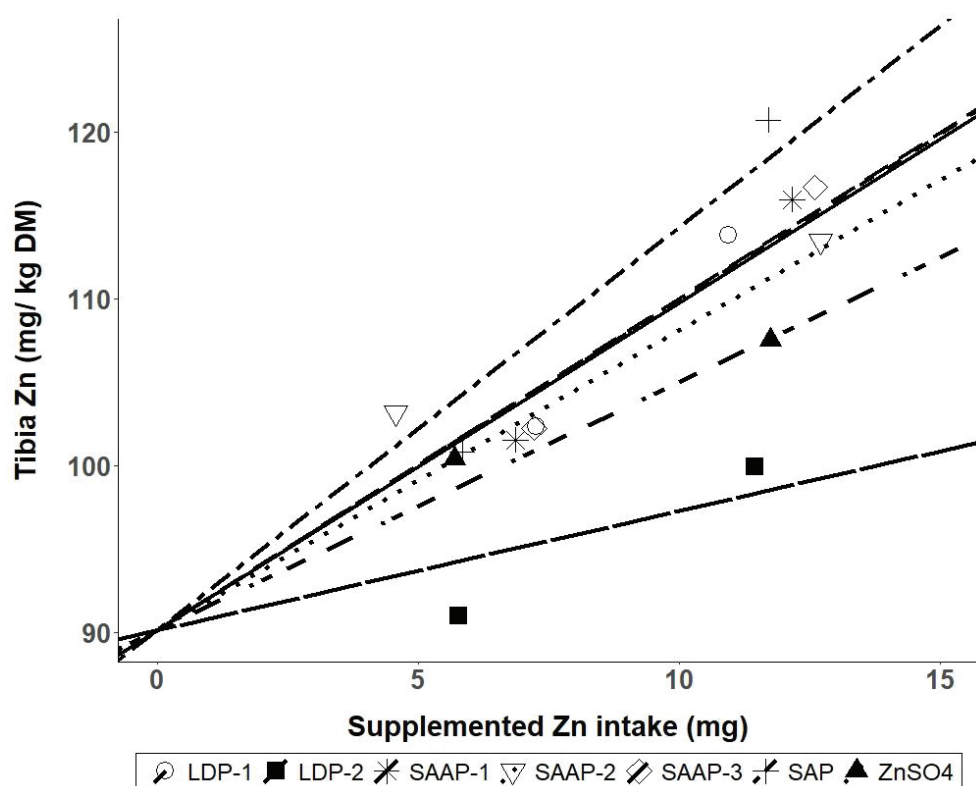


Figure 1: Linear dose response relationship between supplemental zinc intake (mg) and tibia zinc content (mg/kg of DM).

Table 5: Relative biological value (RBV) of different ZnO sources in comparison to Zn sulfate, slope and standard error of different source of ZnO.

Source	Slope	SE	RBV Zn % Bone
ZnSO <sub>4</sub>	1.49	0.42	100 <sup>ab</sup>
SAAP-1	1.96	0.39	133 <sup>ab</sup>
SAAP-2	1.80	0.38	120 <sup>ab</sup>
SAAP-3	1.98	0.36	132 <sup>ab</sup>
LDP-1	1.98	0.40	131 <sup>ab</sup>
LDP-2	0.71	0.40	49 <sup>b</sup>
SAP	2.41	0.40	160 <sup>a</sup>

### Gastrointestinal contents

No differences were observed regarding the supplemented Zn intake in the 3 hours before sampling (Table 6). The Zn intake in the 3 hours prior to euthanasia was not affected by the source of Zn supplemented, but was significantly higher than Zn in the control diet as expected. The supplemented Zn intake varied from 0.31  $\mu\text{g}$  in the SAP treatments to 0.47  $\mu\text{g}$  in the SAAP-3 treatment. The gastric content showed no differences in the total Zn and soluble content for any of the treatments, but a significant difference ( $p = 0.011$ ) in the insoluble part of the gastric content. LDP-2 had the highest content of insoluble Zn in the stomach with 5.9  $\mu\text{g/g DM}$ , as  $\text{ZnSO}_4$  the lowest showing 1.5  $\mu\text{g/g DM}$ . Interestingly, LDP-2 showed to have the highest percentage of soluble Zn in the stomach with 29% of Zn soluble while birds fed the SAAP-3 showed 17% of soluble Zn. In the ileum, no differences were observed within sources in the different analyzed contents. The soluble part showed to be numerically higher for  $\text{ZnSO}_4$  source with 6.8  $\mu\text{g/g DM}$ , which represented 4.7 % of soluble Zn, while birds fed LDP-2 source showed only 4.3  $\mu\text{g/g DM}$  to be soluble in the ileum that characterized 3.0% of soluble Zn. The control diet, however, showed a significant lower concentration of total Zn ( $p < 0.001$ ) in the ileum, which is expected as the Zn content was lower than the other treatments. The highest Zn content in the ileum was for birds in the SAAP-2 treatments with 146  $\mu\text{g/g DM}$ , while birds in the control diets presented 101  $\mu\text{g/g DM}$ .

Table 6: Zn content in the soluble and insoluble part of gastrointestinal content

	Proventriculus and Gizzard content					Ileum content			
	Supplemented Zn intake 3h (µg)	Insoluble Zn (µg/g DM)	Soluble Zn (µg/g DM)	Soluble Zn (%)	Total Zn (µg/g DM)	Insoluble (µg/g DM)	Soluble Zn (µg/g DM)	Soluble Zn (%)	Total Zn (µg/g DM)
Control	0.00 <sup>b</sup>	1.9 <sup>b</sup>	1.3	17.4	8.56	23.4	4.6	4.41	101 <sup>b</sup>
ZnSO <sub>4</sub>	0.43 <sup>a</sup>	1.5 <sup>b</sup>	2.4	20.2	9.75	29.8	6.8	4.69	140 <sup>a</sup>
SAAP-1	0.45 <sup>a</sup>	1.9 <sup>b</sup>	1.3	18.9	9.78	31.2	6.1	4.33	140 <sup>a</sup>
SAAP-2	0.34 <sup>a</sup>	2.8 <sup>ab</sup>	3.0	21.3	14.78	31.1	5.6	3.96	146 <sup>a</sup>
SAAP-3	0.47 <sup>a</sup>	2.3 <sup>ab</sup>	1.8	17.1	12.21	28.0	5.5	3.92	145 <sup>a</sup>
LDP-1	0.40 <sup>a</sup>	2.2 <sup>ab</sup>	2.0	19.7	12.46	29.0	5.3	3.63	145 <sup>a</sup>
LDP-2	0.36 <sup>a</sup>	5.9 <sup>a</sup>	4.2	29.2	13.93	26.7	4.3	3.03	145 <sup>a</sup>
SAP	0.31 <sup>a</sup>	2.7 <sup>ab</sup>	2.8	23.9	10.87	28.9	6.4	4.64	143 <sup>a</sup>
<b>P-value</b>	< 0.001	0.011	0.135	0.5608	0.361	0.3081	0.1898	0.251	< 0.001

### Physicochemical correlation

The correlation between physicochemical properties and Zn bioavailability, described on Figure 2, showed a significant impact of aggregate size, density and specific surface area ( $P < 0.05$ ) in Tibia Zn. In vitro dissolution kinetics and agglomerate size did not significantly correlate with bone Zn in the present experiment.

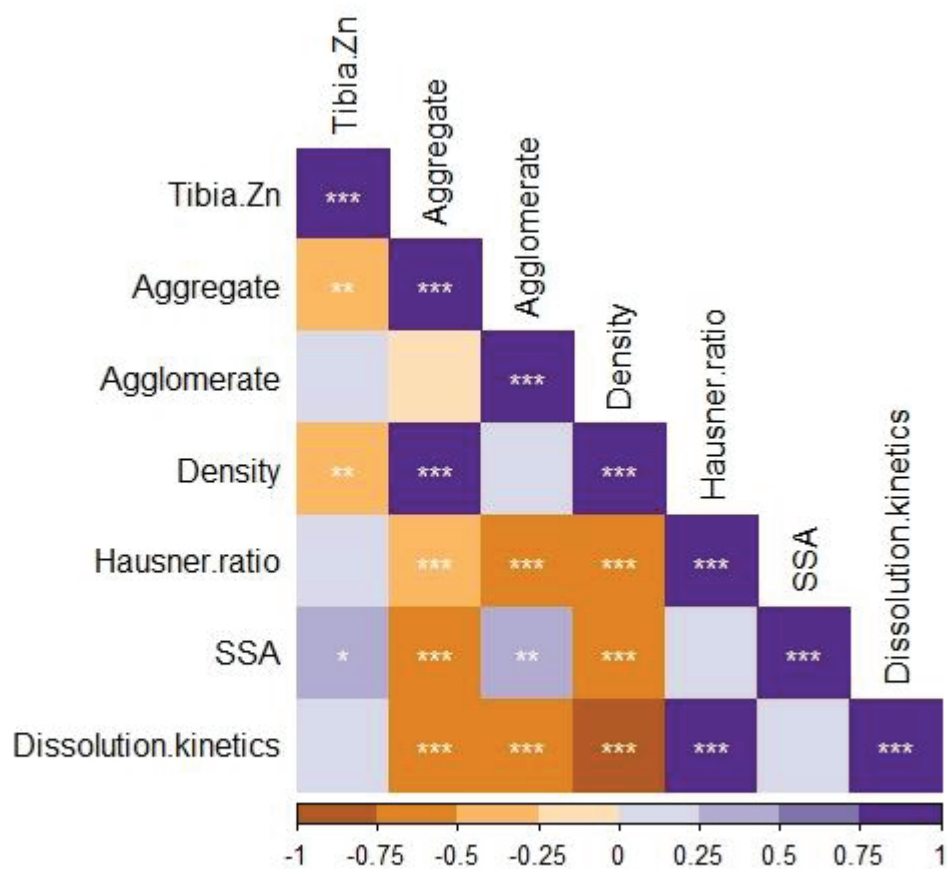


Figure 2: Correlations between tibia Zn and physicochemical properties of ZnO sources Significance levels: (\*\*\*) 0.001, (\*\*) 0.01 and (\*) 0.05.

## DISCUSSION

Zn is an essential micronutrient to ensure a proper body function. Broiler zinc requirement is estimated to be 40 mg /kg (NRC, 1994), however innumerable studies have reported improvement in animal performance when Zn was supplemented (Brooks *et al.*, 2013; Zhao *et al.*, 2016). Therefore, a supplementation in the feed is a current practice in poultry production. It is reported in the literature that zinc deficiency can reduce feed intake and limit growth of animals (Salgueiro *et al.*, 2000). However, this was not observed in the present experiment, which is consistent with previous studies from Edwards and Baker (1999); Mohanna *et al.* (1999); Narcy *et al.* (2015); Star *et al.* (2012); Yan *et al.* (2016). The short period of the present trial might explain the lack of response to zinc supplementation. Brooks *et al.* (2013) found a difference between the supplemented sources and the control diet in birds submitted to a Zn depletion over 7 days prior to the beginning of the trial, which was not the case in the present study.

ZnO is a source widely used to supplement the feed of broilers. However, this source is poorly investigated and mainly tested in old studies (Edwards and Baker, 1999). As a non-water soluble source, ZnO is considered to have a low bioavailability when compared to ZnSO<sub>4</sub>, a water-soluble compound. The great variability in bone Zn concentrations from the different sources of ZnO observed in this study are in agreement with previous works. Edwards and Baker (1999) evaluated the bioavailability of different sources of ZnO from various manufacturing processes. The study showed a variation from 34% to 97% in comparison to ZnSO<sub>4</sub> in which the manufacturing process showed a great effect on relative bioavailability (RBV) measured. The LDP-2 originating from the US, obtained from the Waelz-process,

resulting in greenish-brown powder showed the lowest RBV (34%) (Edwards and Baker, 1999), which was confirmed in the present study with the lowest RBV of 49%.

To be absorbed in the body, Zn needs to be solubilized in its ionic form to be taken up by the protein transporters in the enterocytes membrane (Cousins, 2010). Nevertheless, in its ionic form Zn is more vulnerable to interact with other feed compounds, forming complexes that unable its absorption (Brugger and Windisch, 2017; Schlegel and Windisch, 2006). Phytate (myo-inositol phosphate), widely present in cereal based diet of poultry, is an important source of phosphorus and the most important feed component affecting the bioavailability of Zn. Phytate is a negative charged molecule that has a great affinity for divalent cations, such as  $Zn^{+2}$ , forming an insoluble complex precluding the uptake of this mineral (Brugger and Windisch, 2017; Schlegel *et al.*, 2013; Schlegel and Windisch, 2006). The stomach is a dynamic environment, where reactions occur permanently. The liquid phase is where these reactions take place: molecules are broken into minor structures, and complexing again with other compounds. Bioaccessibility, which represents the soluble fraction of the total amount of a substance, is necessary for potential intestinal absorption. The duodenum is the main site of absorption of minerals. This short segment of the small intestine, comprise the end of the stomach up to the pancreatic duct, where enzymes and bicarbonate are released, increasing the pH. In the duodenum, the pH remains low, favouring the solubilization of minerals and therefore their absorption (Vieira, 2008).

The physicochemical properties of powders showed to influence their *in vitro* dissolution kinetics, which could explain their different *in vivo* bioavailability. The previous article Cardoso *et al.* (2019) related three main criteria according to the physicochemical of ZnO sources: (1) Small aggregates and agglomerates particles (SAAP), showing a fast dissolution kinetics,

related to the small particle size of the powders. (2) Large dense particles (LDP), characterized by ZnO sources with low specific area and large size, resulting in a slow dissolution kinetics and (3) Small aggregate particles (SAP), a ZnO source with large agglomerates and large specific surface area that results in an intermediate dissolution kinetics, in which the mechanism is explain in two steps: the fast dissolution in the surface of the powders, followed by the slow diffusion of the ion from the quiescent to the solution media.

Oral absorption is a kinetic process, the transit time in the gastrointestinal tract determines the amount that can be absorbed. A fast dissolution can cause a saturation limiting absorption when the dissolution overcomes the capacity of the gut to absorb nutrient (Sugano *et al.*, 2007), as the absorption of Zn in the duodenum and ileum suggests a saturable carrier-mediated pathway (Liu *et al.*, 2008). Additionally, it is believed that sources with fast dissolution, such as ZnSO<sub>4</sub>, release Zn ions in the stomach in a very short time after feed ingestion, interact with feed compounds before they are broken down, forming insoluble complexes which are unable to dissociate. In vitro studies failed to assess a relationship between in vitro solubility and *in vivo* digestibility when attempting to predict bioavailability of minerals (Anwar *et al.*, 2016). The study performed with calcium, comparing coarse and fine particles, showed that fine particles where more soluble in vitro, but with lower digestibility values in vivo, while the opposite was observed for coarse particles. Mineral interaction and speed of dissolution and capacity of absorption might be one of the reasons to explain these results. Additionally, the lack of techniques to identify the state of the minerals in the soluble content, might blur the understanding of the mechanisms affecting Zn bioavailability. In the present study, gastric and intestine Zn content seem to partially explain the relation between in vitro and in vivo dissolution kinetics. The high concentration of LDP-2

in the insoluble part of the gastric content agrees with the in vitro dissolution, showing an inert source, as it does not dissolve, hence, stays longer in the gastric environment. On the contrary, the low content of Zn in the insoluble gastric part of ZnSO<sub>4</sub> and SAAP-1 agrees with a fast dissolution of these sources. Additionally, no differences were observed in the total Zn in the stomach, which suggest as the sources dissolves the solubilized portion will leave the faster the gastric compartment, towards the intestine.

A source of Zn that dissociates at a slower rate, is less likely to complex and therefore more ions will be available for absorption in the small intestine. Nevertheless, a source that does not dissociate will remain inert and not available for absorption in the small intestine, which is most likely to explain the low bioavailability of LDP-2. In this case, the absorption is limited by its dissolution rate, whereas the ability of absorption is higher than the capacity of the powder of dissolve. In this scenario, an increase in the amount of powder increases proportionally the absorption, because the total solid surface area is proportional to dose amount (Sugano *et al.*, 2007). As many factors can influence the observation of in vivo dissolution kinetics, such as: time and amount ingested by each bird, water ingestion, transit time of the feed, further studies are necessary to better relate in vitro and in vivo dissolution kinetics of sources.

A correlation analysis, relating Zn bioavailability in bone and physicochemical properties, suggests that aggregates size, density and specific surface area are the most relevant properties influencing the bioavailability of ZnO powders. Aggregate size showed a negative correlation of 43%, meaning the smaller the aggregate size, the larger the Zn content in the bone. Specific surface area, demonstrated a correlation of 29% to tibia Zn. As density and



aggregate size are largely correlated, most likely the strong relationship between density and tibia Zn is due to the aggregate size of powders that relates to the density values.

The small size of aggregates and large specific surface area favors the dissolution of ZnO in the gastric media, whereas large particles take a longer time to dissolve, or remains in the insoluble content of the digestive content, being unavailable to absorption, which it showed to be the case for LDP-2. Dissolution kinetics did not show any direct relationship with tibia Zn, but it seemed to explain at least partially the Zn content in the gastrointestinal tract, in which LDP summed up to the major insoluble content of gastric Zn.

## **CONCLUSION**

The bioavailability of ZnO is variable according to the physicochemical properties of the sources with aggregate size and specific surface area as the main parameters. In vitro dissolution kinetics influenced by the properties of the sources partially explains the fate of ZnO sources in the gastrointestinal tract. However, in vitro dissolution property cannot be considered as a unique predictor of Zn bioavailability in broilers. The development of techniques that allow to identify the species of Zn and its complexes in the soluble content in the digestive tract and the description of the different ZnO used in animal feeding studies will help to compare and elucidate how the physicochemical properties of ZnO powders can affect bioavailability in broilers.

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## CHAPTER 4

## 4. GENERAL DISCUSSION

### 4.1 Techniques to assess physicochemical properties of powders

Inorganic powders have a wide application in the industry, as such: cosmetics, food, construction, engineering, medicine, electronics. The development of nanotechnology has increased the interest for these materials, so as the debate associated to their potential risks to environment and human health (Hristozov and Malsch, 2009; Leturia *et al.*, 2014).

Regulations and development of techniques to assess the properties of these materials, have been discussed to define physicochemical properties, to evaluate their hazard for human health and the environment. There is a variety of methodologies available to define those characteristics. The discussion around this topic is still controversial, and shows that it is not possible to reach a definite conclusion from a single analysis.

As an example, many samples of feed grade ZnO analyzed showed a lack of homogeneity within the sample. As exemplified on Figure 1, in the same sample it is observed two populations of particles of very different sizes. This characteristic was observed on samples belonging to group A and B from samples analyzed, which may be the manufacturing process does not result in a homogenous final product. The two-sized population sources of ZnO can distort the understanding of the physicochemistry in relation to its behavior *in vivo*. When sampled and measured, the results might seem biased within different batches, which can influence the interpretations.

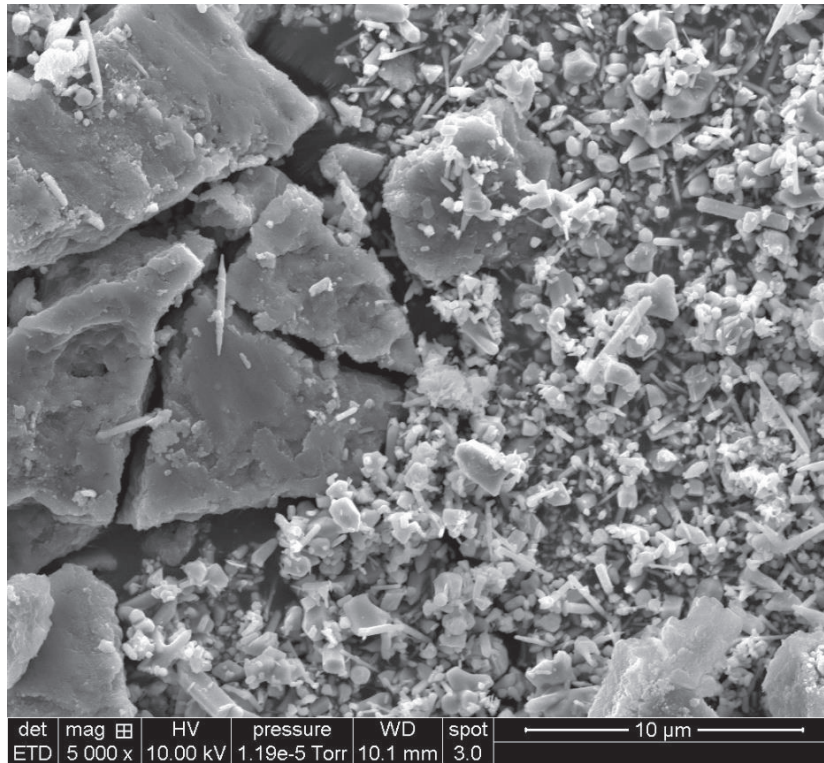


Figure 1: Feed grade ZnO sample observed in a SEM. Two-sized population is clearly observed.

The presence of a large number of samples presenting this two-population characteristic may explain the lack of a significant relationship between SSA and particle size. The SSA of a particle increases as the size decreases, however some samples showed a small particle size and low SSA values. As measurements of particle size by light scattering and specific surface area by nitrogen adsorption are estimated on average values, large and small particles have a weight in the final values which can mislead interpretation of values. Therefore, selecting techniques that can provide reliable information can be challenging with heterogeneous samples and only a single technique may not provide all necessary information.

In the counterpart, the innumerable techniques available can provide redundant and sometimes unnecessary results. It is, then, imperative to consider what information is desirable in order to select which technique to use to narrow down to those that offer the

most information. Accessibility and price should also be considered as relevant criteria to define the most important.

For powders, like ZnO, it is also necessary to consider not only the fate in the digestive tract but: (1) the safety of the workers, for example, dusty products can affect the health of employees due to inhalation of hazardous compounds and (2) the form in which this product reaches the environment and the impact on it.

#### **4.2 Dissolution kinetics**

Before starting to discuss the dissolution kinetics process of the different ZnO powders, it is important to differentiate solubility and dissolution kinetics. For an animal nutritionist, when one talks about the solubility it is often misunderstood as a 'picture' of the dissolution kinetics process. The solubility is the end point and dissolution kinetics is the process how to get there. This process will be influenced by the properties of each powder source. The dissolution kinetics process can take up to weeks, which is not necessarily relevant for the digestion process as the transit in gastrointestinal tract is faster. In the gastric compartment, when the feed is solubilized, it will flow to the intestine and continue the digestion. Yet, after some time, if a given compound is not solubilized, this material will move to the next compartment anyway. As the transit time in the stomach of a broiler is between half an hour to an hour (Svihus, 2014), the kinetics reflects better the 'solubility' of a certain compound.

There are many factors influencing dissolution kinetics in biological fluids. In the gastric compartment, the presence of feed compounds, pH, temperature, transit time, etc. To predict this multifaceted environment *in vitro* is difficult due to the complexity of the media. Therefore, to attempt at understanding the mechanism underlying the dissolution of ZnO

powder, as a primary categorization, assays were performed in water at different pH – as pH is one of the key parameters affecting the dissolution of metals (Avramescu *et al.*, 2017).

The gastric juice secreted in the stomach of broilers has a pH of 2, but due to the feed ingested, retention time and other factors, it is often observed in pellet feed of broiler a pH between 3 and 4 (Svihus, 2014). In the present study, the *in vivo* study showed an average pH of 3.0 among treatments, showing to be in agreement with the literature.

Out of the 34 ZnO sources analyzed in this study, 3 main behaviors were observed. Representative samples from each category had their dissolution kinetics behavior analyzed at pH 2, 3 and 4. The results for all samples showed a faster dissolution as the pH decreased ( $P < 0.05$ ). Although there is a significant change in the speed of dissolution with the pH variation for all samples, they seem to be more relevant as the speed of dissolution decreases, when comparing the behavior of these 3 representative samples of ZnO (slow, intermediate and fast dissolution).

The different speed on dissolution kinetics is explained by the different physicochemical properties as reported on chapter 2. In short: small aggregates of particles showed a faster dissolution than larger aggregates. An intermediate step was observed in samples with small aggregates but large agglomerates, in which a fast dissolution happened in the particle surface but the time by which the  $Zn^{2+}$  ions took to reach the bulk solution, outside the agglomerate slows down the dissolution process.

As observed in Figure 2, a sample characterized with a fast dissolution behavior, had more than 80% of the particles dissolved within 10 minutes at all pH's tested. In the counterpart, a powder with a slow dissolution kinetics at pH 4 within the 1-hour duration of the experiment, had only approximately 30% of the powder dissolved (Figure 3). At pH 3, 50% of the powder



was dissolved within 30 minutes and at pH 2 this was observed at the minute 10. A sample with an intermediate dissolution performance, showed 50% of the particles dissolved in the pH 4, 3 and 2 at the minutes: 17, 7 and 4 respectively, as it can be observed on Figure 4.

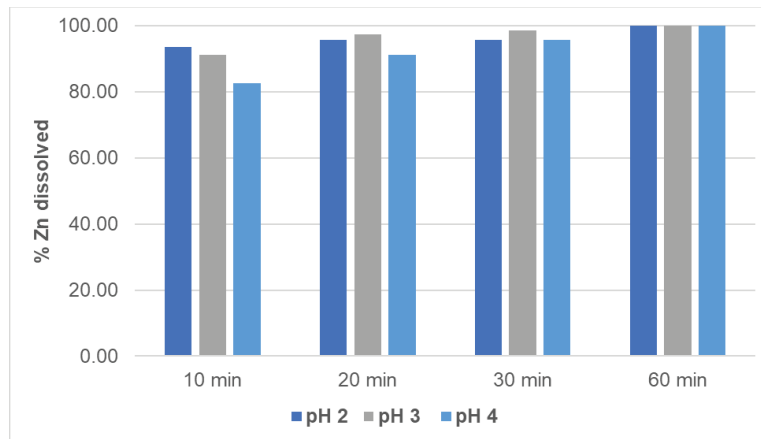


Figure 2: Percentage of Zn dissolved over time at different pH. ZnO sample with a fast dissolution kinetics behavior.

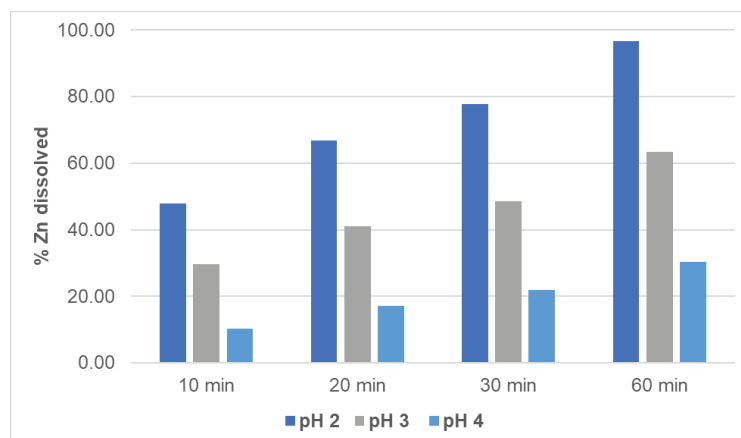


Figure 3: Percentage of Zn dissolved over time at different pH. ZnO sample with a slow dissolution kinetics behavior.

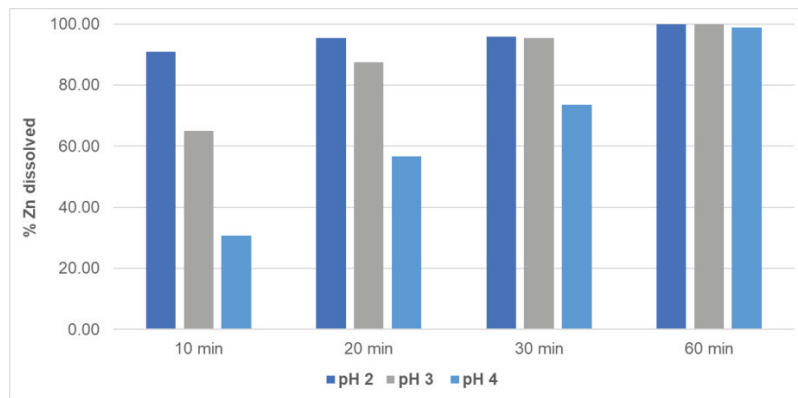


Figure 4: Percentage of Zn dissolved over time at different pH. ZnO sample with an intermediate dissolution kinetics behavior.

When comparing the behavior of these 3 samples and the dynamic environment of the stomach of birds it is possible to assume that: (1) The slower the rate of dissolution of a sample, the more important is the pH of the stomach to guarantee its dissolution. (2) Even though a fast dissolution ZnO sample showed a quicker dissolution as the pH decreased, it was less significant than sources with a very slow dissolution behavior, in which the decrease of pH, decreased the time to dissolve, being more compatible with the digestion time.

The relationship between the *in vitro* analysis of ZnO sources and the *in vivo* bioavailability study have shown that large particles with low specific surface area values had the slowest dissolution rate correlated with the lowest bioavailability values when compared to zinc sulfate (< 50%). This result might indicate the need for a balance, to avoid interaction and the need to solubilize so it can be taken up in the enterocytes in the small intestine. In the counterpart, the source with an intermediate dissolution value *in vitro*, presenting small aggregates and large agglomerates, had the highest bioavailability value, which may agree with the hypothesis that slowing down the dissolution may improve the bioavailability of Zn in broilers, due to decrease of interaction with antinutritional factors. The two-step dissolution takes place for ZnO powders having large agglomerates: fast dissolution of Zn from the particle

surface allows Zn to be solubilized inside large agglomerates; it is followed by slow diffusion from the inside to the outside of the agglomerate that slows down the time of exposure of Zn to antinutritional factors and avoids a 'flush' of Zn at the same time in the intestine, which can saturate the transporters responsible for Zn absorption.

In the stomach, feed compounds are being digested into smaller molecules, solubilizing, complexing with each other. As a nutrient is solubilized, it will flow to the small intestine where the digestion continues and nutrients are absorbed. In the literature, the main site of absorption of minerals like Zn is still debatable. Some authors claim the duodenum to be the preferable site, as the pH is still low from the stomach, favoring the solubilization of minerals. On the other hand, Liu *et al.* (2008) suggested that Zn absorption in the duodenum and jejunum is a saturable carrier-mediated pathway, but the ileum is the preferential site for Zn ion transport in the small intestine of chickens, regulated by a non-saturable diffusive process.

One can speculate that, a fast dissolution source would lead to a high amount of Zn flowing at the same time to the small intestine, and as it is a saturable process, would have its absorption limited, at least in the first segments of the small intestine. Lack of absorption of these ions, will allow the complexation with other feed compounds, such as phytate, in the small intestine.

Interestingly, the *in vivo* data on Zn content in gastric and intestinal content are not so clear, regarding the dissolution kinetics. It is important to keep in mind that the digestion is a complex process, involving many physiological processes that can affect the fate of ZnO powders in the gastrointestinal tract of broiler chickens and not only the physicochemical properties. Additionally, the physicochemical properties investigated in these experiments

might not be enough to clarify all mechanisms behind the dissolution kinetics of powders and further analysis may help to better understand the digestion process of ZnO.

The *in vivo* data showed one of the two slow-dissolution sample to be in the highest amount in the insoluble content of the stomach (Figure 5) and the highest percentage of zinc soluble in the stomach as well (Figure 6). This is in agreement with the hypothesis that as ZnO solubilizes, it flows to the next segment of the digestive tract, so less Zn is remaining in the total content of the stomach. As the dissolution slows down, it stays for longer time retained in the stomach. Nevertheless, the other slow-dissolution sample, didn't show the same behavior trend. Samples in this category showed to have two-population size may have led to different outcomes. Intermediate dissolution also showed a higher Zn in the insoluble part and higher percentage of soluble Zn in gastric compartment, when compared to fast dissolution sources. Additionally, fast dissolution sources presented mainly the same behavior among each other as could be expected from the *in vitro* dissolution, except for one of the sources that showed a higher concentration in the gastric compartment, similar to slow dissolution samples.

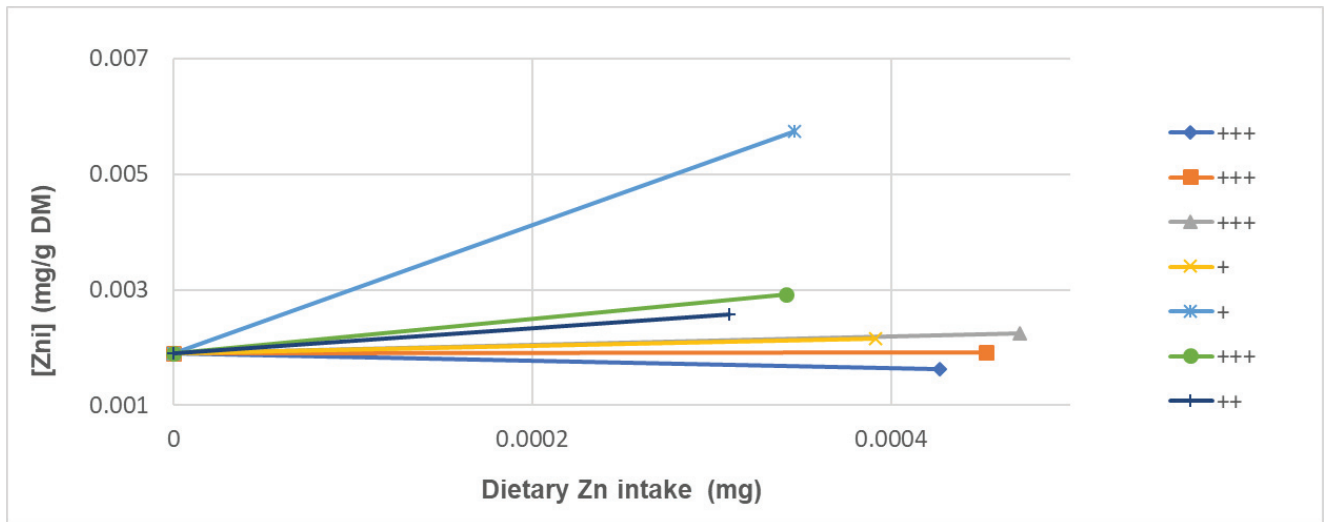


Figure 5: Zinc concentration in the insoluble content in the proventriculus and gizzard of broiler. Sources are classified as their dissolution speed (+++: fast dissolution, ++: intermediate dissolution speed and +: slow dissolution).

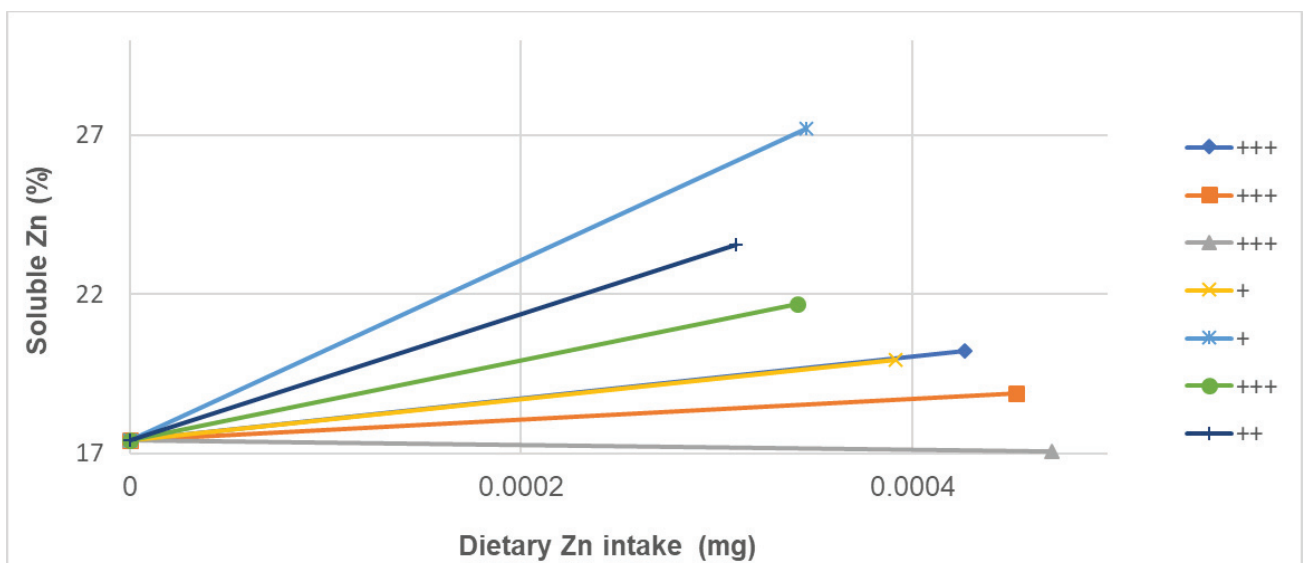


Figure 6: Percentage of Zn in the soluble content in the proventriculus and gizzard of broilers. Sources are classified as their dissolution speed (+++: fast dissolution, ++: intermediate dissolution speed and +: slow dissolution).

In the ileum, total Zn showed to be higher for those sources that stayed longer in the stomach (Figure 7), suggesting that their digestion process was slower during the whole length of the digestive tract. Figure 8 illustrates the Zn content in the soluble part of the ileum, in which the slow-dissolution source showed to remain insoluble, explaining its low bioavailability values.

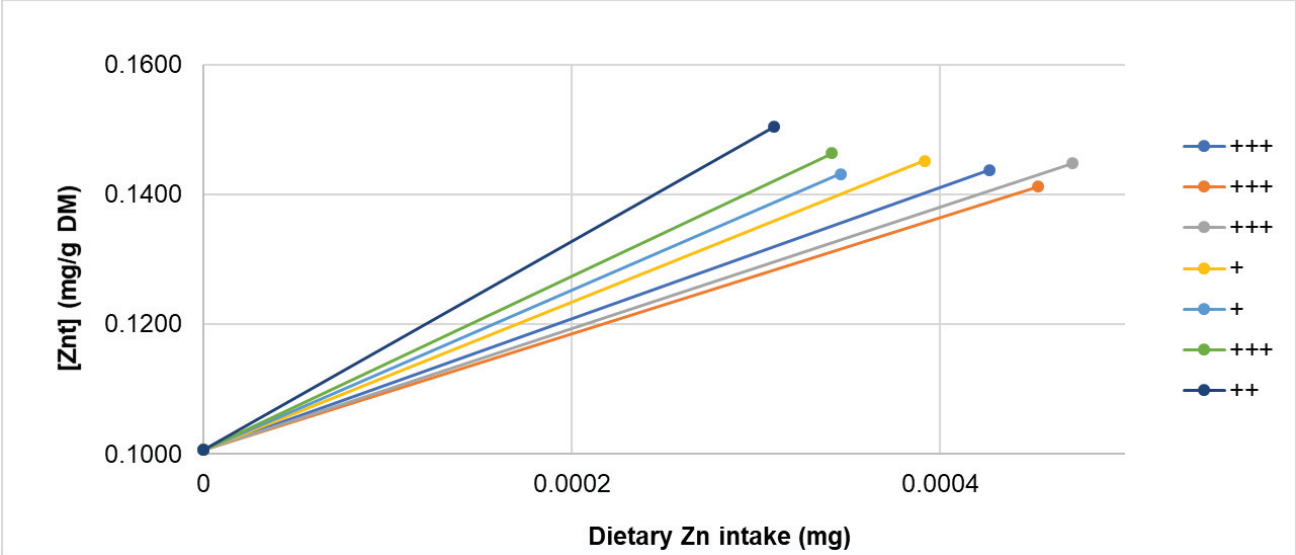


Figure 7: Total Zn concentration in the ileum content of broilers. Sources are classified as their dissolution speed (+++: fast dissolution, ++: intermediate dissolution speed and +: slow dissolution).

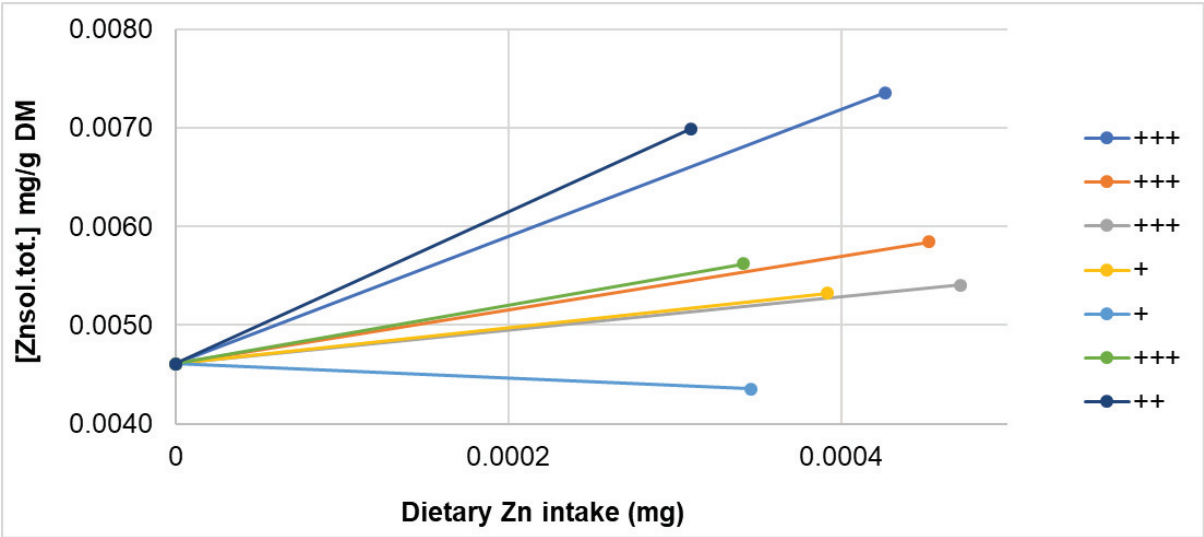


Figure 8: Soluble Zn concentration in the ileum content of broilers. Sources are classified as their dissolution speed (+++: fast dissolution, ++: intermediate dissolution speed and +: slow dissolution).

### 4.3 Phytate

Phytic acid, as it has been discussed before, is widely present in the feed and it is an important antinutritional factor when related to Zn bioavailability.

Phytate is a negative charged molecule that serves as a source of phosphorus (P) for the plants. The animals however, are not able to break this molecule, and when solubilized it feels great attraction for divalent cations, such as Zn, Ca, Mg, etc. Phytate has a great affinity for Zn but its relevance is obfuscated by Ca, due to the higher concentration (100 fold) of this mineral compared to Zn.

The solubility and complexation of phytate with the different feed compounds is pH dependent and it can be briefly summarized as:

The solubility of phytic acid decreases as the pH increases, forming insoluble salts at neutral pH. Under the gastric condition phytic acid is likely to: (1) solubilize as phytic acid, (2) form binary complexes with proteins, that happens at pH values below the isoelectric point of proteins (optimal pH at 2.5) and (3) associate as ternary complexes, between phytic acid, proteins and divalent cations. In the stomach, binary complexes are more likely to happen, but as the pH increases and the proteins reach their isoelectric point, these complexes are released and likely to form ternary complexes. The small intestine conditions increase the affinity of phytic acid for minerals. These complexes are soluble in the gastric conditions and precipitate when the pH is above 4-5, resulting in less phytate available for phytase action (Humer *et al.*, 2015). The fast dissolution of a Zn source will release Zn ions that are susceptible to bind to phytic acid. The flow of a high amount of Zn to the small intestine, where the pH is optimal to this complexation, will cause binding to phytic acid and precipitation as the pH

increases. It is hypothesized, that a source with a slow release will be more uptaken in the first segment of the small intestine, as it is a saturable process and it would give some time to phytase to act before the Zn binds to phytic acid. So, quick dissolution is more likely for the Zn present in the stomach to bind to phytate. In the case presented here that a source with a fast dissolution have 50% of its particles dissolved in 1 or 4 minutes, the impact on interacting with feed compounds is greater, no matter the pH, whereas a slower release source can reduce those interactions due to an extra time provided for other feed compounds to act, such as phytase.

Phytases are the enzymes often added to the diet to break phytate. Anyhow, most of the phytases used in the market today, have an optimal pH around 4-5.5 with a main action in the small intestine. *In vitro* tests have shown that those phytase take 10 – 20 minutes to break the phytate. A phytase source with an optimal activity in the stomach at a pH of 3.5 has been reported, but, even though, this source is claimed to have a fast action, it still takes approximately 10 minutes to break IP-6 into IP-5.

If Zn<sup>2+</sup> ions reach phytate before it could be degraded by phytase, the enzyme cannot act on breaking phytate down. Therefore, a supplemented Zn source that slows down its dissolution process can optimize the use of phytase, resulting in less interaction between Zn and phytate and a higher availability of Zn and Phosphorus for the animal.

#### **4.4 Speciation**

When a Zn compound is solubilized, it is not necessarily in its ionic form, Zn<sup>+2</sup>, like mentioned before. The gastrointestinal tract is a dynamic environment where reactions are happening all the time.



When the solubility of Zn is analyzed, it is often assumed that it is in its ionic form and ready to be absorbed. Nonetheless, this Zn can be attached to other molecules and become unavailable to absorption. For example, the complex Zn-phytate, is soluble under the stomach conditions, where the pH is below 4, but as this complex flow to the small intestine it precipitates and becomes unavailable for uptake by the enterocytes.

Additionally, increasing environmental concerns raise the attention to the mineral species present in the feces of the animals, and how they affect the soil in terms of reactivity and bioavailability for the plants.

The development of techniques that allow the identification of species of Zn present in the different segment of the gastrointestinal tract and in the excreta of animals can help to elucidate the fate of different zinc sources in the gastrointestinal tract and in the environment.

## CONCLUSION AND PROSPECTS

*« I'm astounded whenever I finish something. Astounded and distressed. My perfectionist instinct should inhibit me from finishing: it should inhibit me from even beginning. But I get distracted and start doing something. What I achieve is not the product of an act of my will but of my will's surrender. I begin because I don't have the strength to think; I finish because I don't have the courage to quit. This book is my cowardice »*

*Fernando Pessoa*

This thesis had the objective to assess the effect of physicochemical properties of different sources of ZnO used in animal feeding on their bioavailability in broilers. To bring together chemistry and nutrition expertise allows a different look into the behavior of powders in the gastrointestinal tract and how it affects the digestion of birds.

The manufacturing process indeed affects the rate of dissolution of the sources as assessed *in vitro*. These characteristics seem to play a role in the bone bioavailability of broilers. The study also showed that aggregate size and specific surface area are the most relevant properties influencing the bioavailability, although the complexity of the digestive tract requires further studies to clarify the mechanisms involved in the bioavailability of Zn. Slow dissolution kinetics may come either from a low specific surface area of ZnO powder, or from the formation of large aggregates or agglomerates that prevent fast release of Zn<sup>2+</sup> ions into the bulk digestive medium.

This thesis achieved an important goal to clarify differences within ZnO sources, and that not necessarily they have an inferior bioavailability compared to Zn sulfate. It is important, for further studies to properly characterize ZnO samples, so studies can be compared and better understood.

Additionally, a limited number but robust tests that can characterize powders and properly predict *in vivo* bioavailability is necessary. Methodologies that are accessible, affordable and standardized, to avoid different results among the techniques.

The physicochemical characterization along with the development of *in vitro* techniques that are able to predict bioavailability, will reduce costs and avoid the use of animal trials. This combination, can bring in the future an easy way for feed companies to better select their mineral compounds.

The study of Zn speciation when solubilized in digestive media will allow to further understand the mechanism underlying what happens in the gastrointestinal tract of the birds. The identification of Zn species will also help at a better understanding of the interactions with other feed compounds.

## ANNEXES

## **ANNEX 1**

### **Methodologies and validation methods of particle size and dissolution kinetics analyses**

#### **Particle Size**

The diameter of ZnO aggregates and agglomerates in water was measured by low-angle laser light scattering using a Malvern Mastersizer 3000 instrument. Agglomerate and aggregate size distributions were calculated to Mie theory using the refractive indices of water (1.33) and ZnO (2.004) and absorption index of ZnO of 0. Agglomerates were accessed adding directly the powder in the water. To measure aggregates, a solution of Zinc Oxide at a concentration of 10% was submitted to dispersion using a Rotor-Stator at 15,000 RPM for 15 minutes to reach the smaller particle size.

In order to define the dispersion process and duration to obtain the smallest size, samples were analyzed after dispersion using Ultrasound (US) and a Rotor-Stator (RT). The objective was to select the process and duration in which samples reached the smallest size in each technique.

Two samples of ZnO were used to assess the size over time. Samples were prepared at a concentration of 10% and analyzed for the following times: 5, 10, 15 and 20 minutes, as shown on Figure 1. Both samples achieved the smallest size after 15 minutes submitted to the Rotor-Stator.

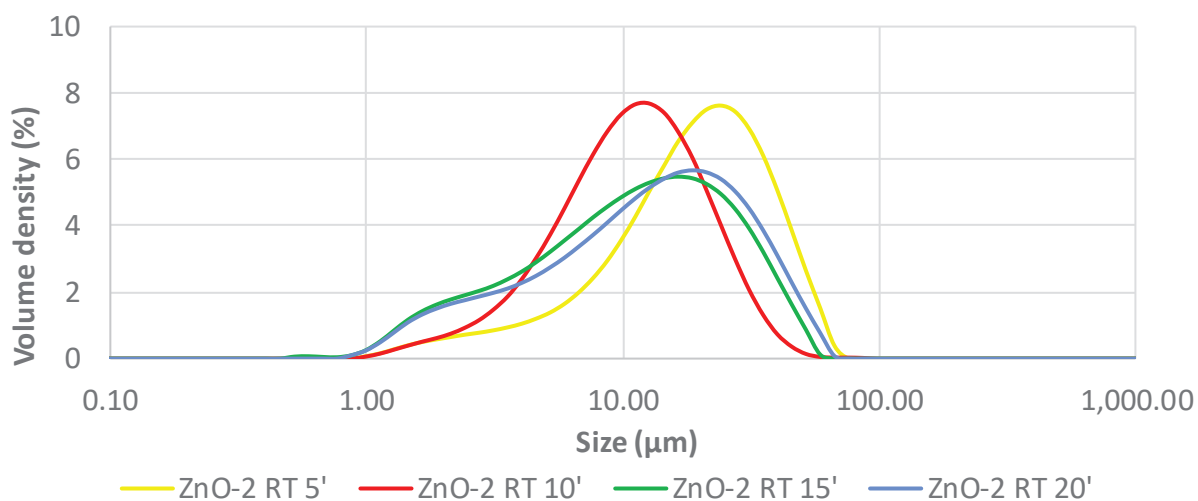
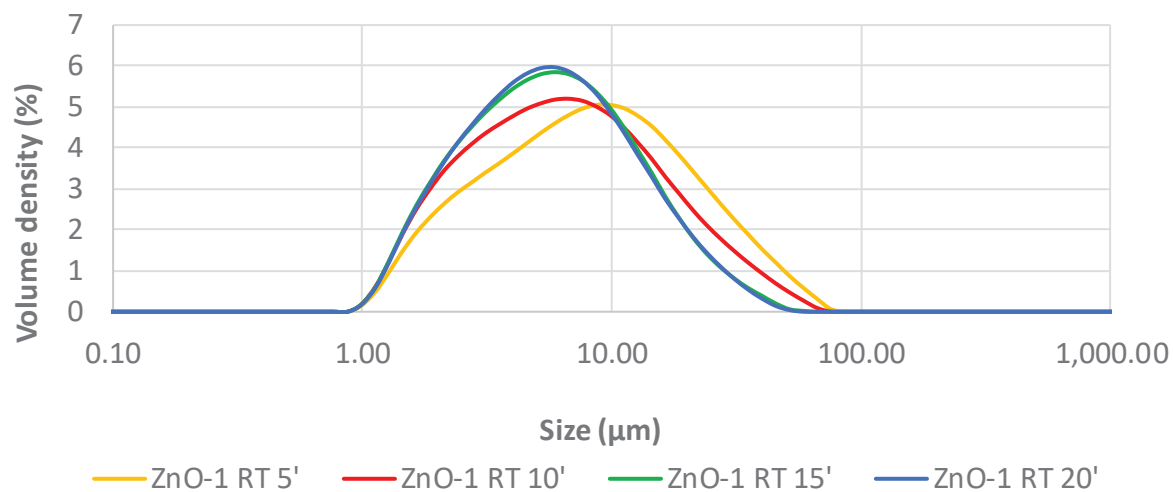


Figure 1 : Particle size of 2 samples of ZnO over time using a Rotor-Stator at 14,000 RPM.

To evaluate the efficacy of the Rotor-Stator, an Ultrasound technique was used to measure the particle size over time. As shown on Figure 2, the smallest size seemed to be achieved at 4 minutes.

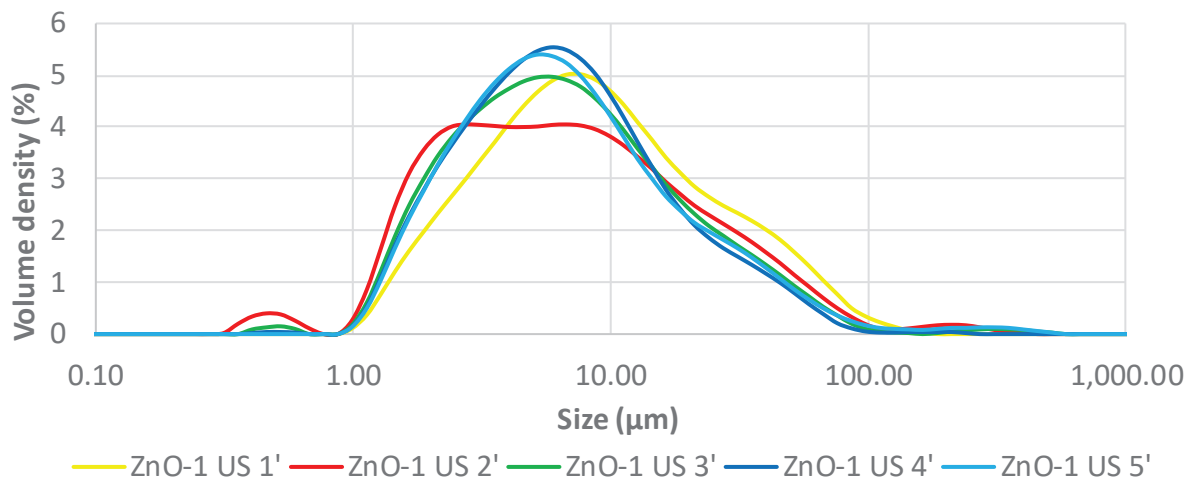


Figure 2: Ultrasound technique used to measure particle size

The plot of both techniques at the maximum time, showed no differences between the techniques (Figure 3). Hence, the Rotor-Stator was selected as the main technique, due to laboratorial practicalities.

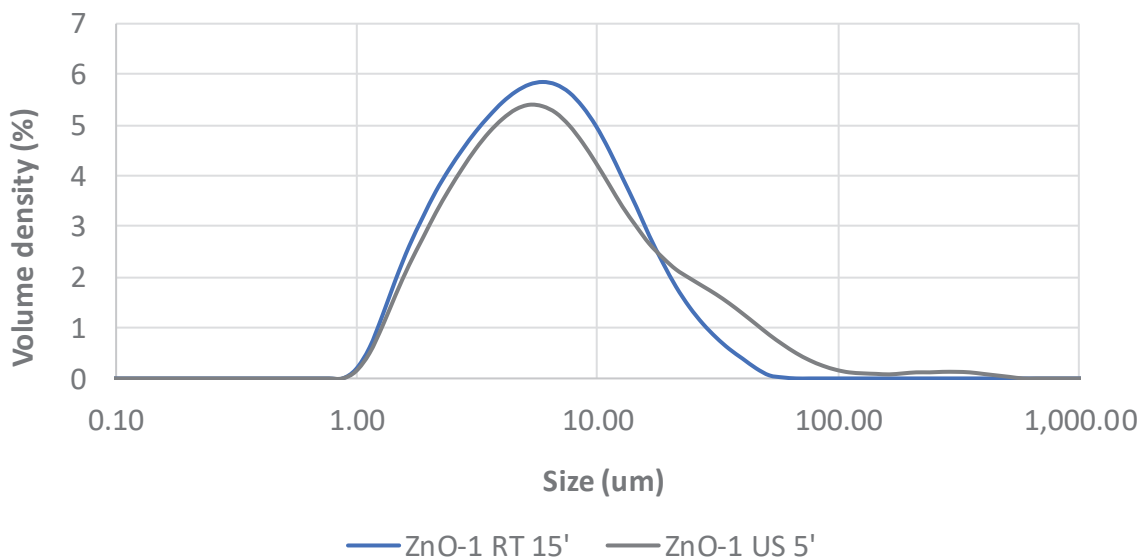


Figure 3: Particle size of a designated ZnO sample using either Rotor-Stator or Ultrasound technique after 15 and 5 minutes respectively.



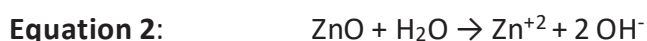
## Dissolution kinetics

Dissolution kinetics was assessed from the estimation of acid released during dissolution of ZnO powder in water. 200 mL of deionized water and 0.15g of ZnO at pH 3 for 1 hour using an automatic burette (877 Titrimo plus, Metrohm, Switzerland).

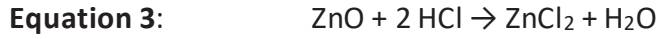
To validate this methodology, the kinetics of 2 samples of Zn was measured using ICP-AOS. 200 mL of distilled water had the pH adjusted to 3 using the automatic burette containing HCl at a concentration of 1 mol/L, then a sample (2 mL) was collected as a blank. The volume of acid added to adjust the pH was noted, to be adjusted for the estimation of Zn dissolution in relation to the supply of HCl. Samples (mL) were collected at times 3, 10, 20, 30 and 40, and 60 minutes. Samples were set in 2 Eppendorf tubes (1.5mL) and centrifuged for 5 minutes at 14,000 RPM and the supernatant collected. Samples were stored for further ICP-AOS analysis. Concentration of Zinc from ICP-AOS given in mg/L, were converted in mol/L through the equation 1:

$$\text{Equation 1:} \quad [\text{Zn}^{+2}] \text{ mol/L} = [\text{Zn}^{+2}] \text{ g/L} / (1000 \times \text{molar mass of Zn})$$

The dissolution of ZnO is accompanied by hydrolysis of ZnO into  $\text{Zn}^{+2}$  according to the chemical reaction:



The Equation 4 refers to the estimation of  $[\text{Zn}^{+2}]$  from the release of  $\text{OH}^-$  that is compensated by the supply of acid, based on the theoretical concentration of ZnO (Equation 3):



**Equation 4:** 
$$[\text{Zn}^{+2}] \text{ mol/L} = \frac{\text{volume HCl added (mL)} \times [\text{HCl}]}{(2 \times \text{Volume of solution (mL)})}$$

A Student test was performed to evaluate the difference between the concentration of Zn assessed from HCl supply and the direct measurement of Zn by ICP. No difference was observed between samples ( $p > 0.05$ ) as showed on Figure . The burette method of measuring the Zn dissolution was validated.

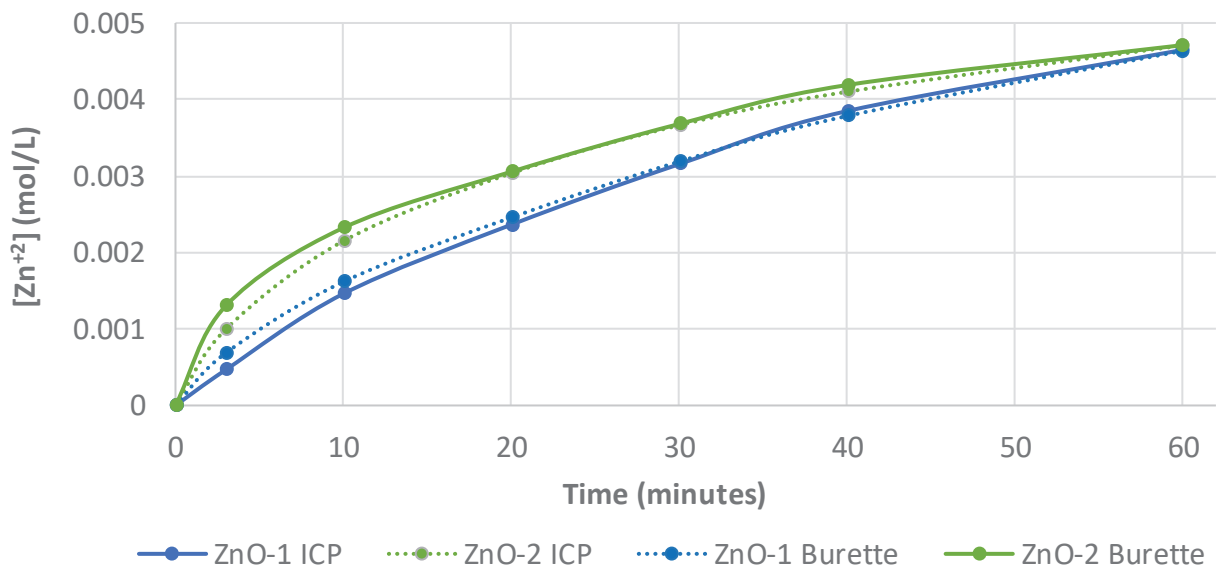


Figure 4: Validation for dissolution kinetics method comparing ICP-AOS and automatic burette ( $p > 0.05$ ).

## **Annex 2**

### **Physicochemical characterization of feed-grade zinc oxide sources**

Denise Cardoso, Yves Chevalier, Agathe Romeo and Stéphane Durosoy

*16<sup>th</sup> International Symposium on Trace Elements in Man and Animals*

*Saint Petersburg, Russia, June 26-29, 2017*

### Objectives of the study

Characterize and categorize different sources of feed grade zinc oxide (ZnO).

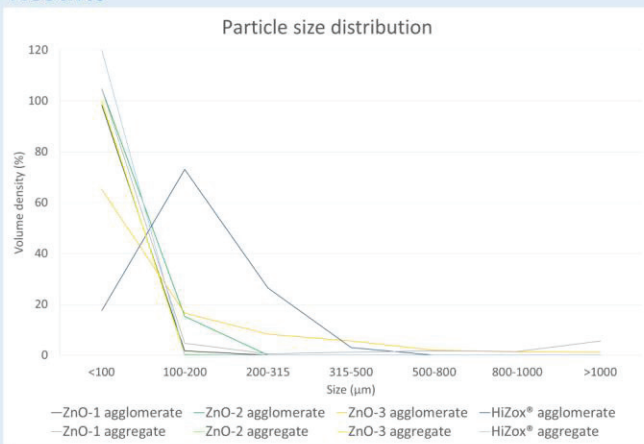
### Outcome

Better understanding ZnO fate in the digestive tract and differences in bioavailability in animals.

### Material and methods

From a total of over 30 samples of ZnO collected from the feed industry worldwide, 4 representative samples were selected and classified according to shape, particle size aggregation and specific surface area using electron microscopy (SEM and TEM), low-angle light scattering, and BET nitrogen adsorption isotherms.

### Results

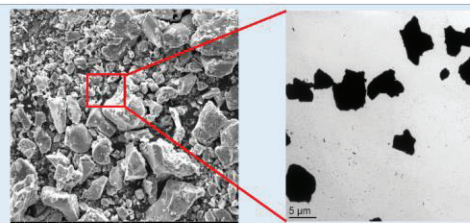


**Particle size distribution for agglomerates and aggregates.** HiZox®: large agglomerates (100 - 200 µm) and small aggregates (<100 µm). ZnO-1, -2, -3: agglomerates and aggregates smaller than 100µm.

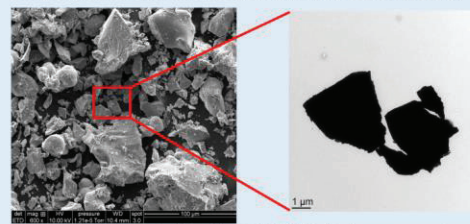
Agglomerate is a loose arrangement of aggregates, readily dispersible. Aggregate consists of primary particles strongly bound, not readily dispersible.

Sample	BET (m <sup>2</sup> /g)
ZnO-1	0.5
ZnO-2	0.3
ZnO-3	2.4
HiZox®	42

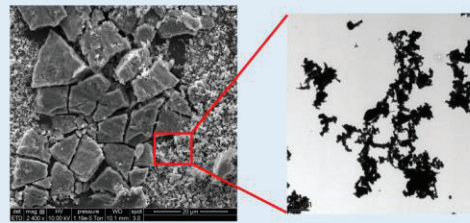
Specific area of the powders by nitrogen adsorption applied using BET theory



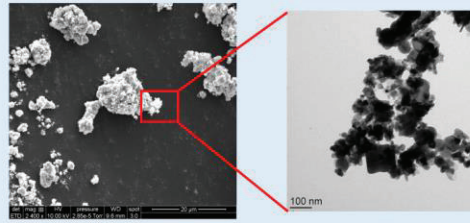
ZnO-1: SEM (left) dense, grinded particles. TEM (right) big platelets not aggregating



ZnO-2: SEM (left) dense, grinded particles. TEM (right) big platelets not aggregating



ZnO-3: SEM (left) dense, grinded particles, broad size distribution. TEM (right) medium size rod-shape particles forming aggregates



HiZox®: SEM (left) agglomerates with a porous internal structure. TEM (right) small platelets forming aggregates

### Conclusion

ZnO sources have different characteristics which can affect their *in vivo* solubility and bioavailability. Further studies are being developed to understand this relationship.

## **Annex 3**

### **The effect of physicochemical properties of feed grade zinc oxide sources in dissolution kinetics**

Denise Cardoso, Yves Chevalier, Agnès Narcy, Agathe Romeo and Stéphane Durosoy

*11<sup>th</sup> Asia Pacific Poultry Conference  
Bangkok, Thailand, March 25-29, 2018*

*Digestive Physiology of Pigs  
Brisbane, Australia, August 21-24, 2018*

### Objectives of the study

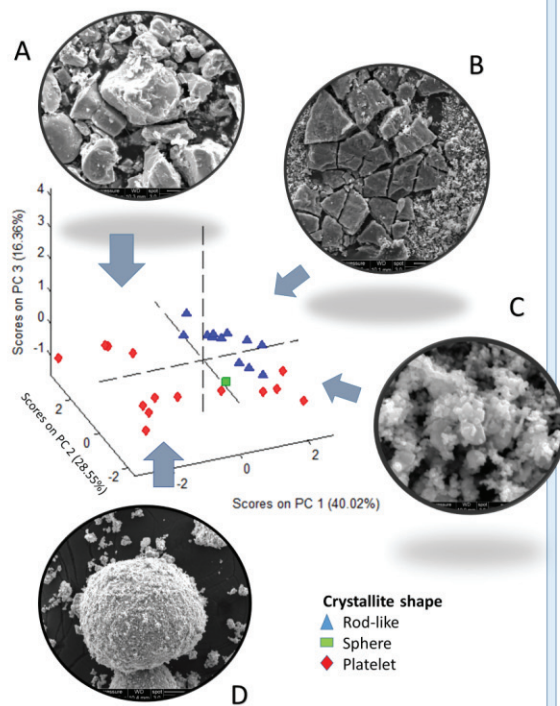
To group different ZnO in terms of their physicochemical characteristics. Relate dissolution rates to those characteristics.

### Material and methods

27 samples of ZnO samples collected from the feed industry worldwide were analyzed for density, tapped density particle size, shape, specific area and porosity of powder. Dissolution kinetics was assessed in vitro from a pH range of 2 to 4 in water with an automatic burette and zinc concentration measured.

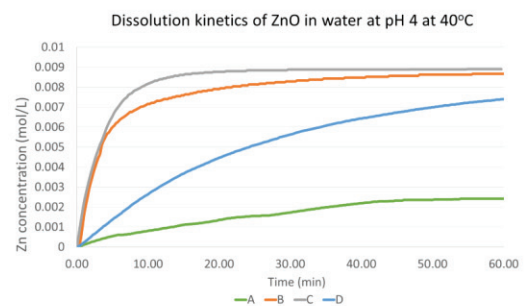
## Results and discussion

### Characterization



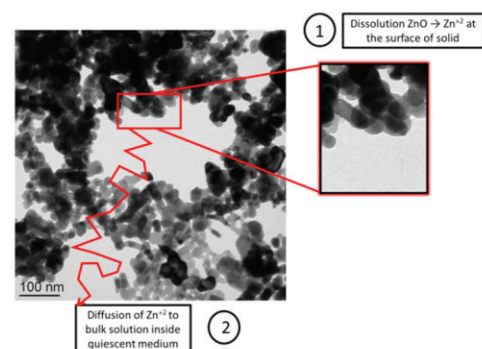
Main contributions to the principal components:  
 PC1 – Density and tapped density  
 PC2 – Agglomerate size and BET  
 PC3 – Crystal size

### Dissolution



Most relevant parameters are agglomerate size and density.

Proposed mechanism with 2 steps:  
 Fast dissolution at the surface of ZnO  
 and slow diffusion to the bulk solution



### Conclusion

Different ZnO show various characteristics which reflect in their dissolution kinetics. The fate of ZnO in the upper part of the digestive tract will influence intestinal health and zinc bioavailability.

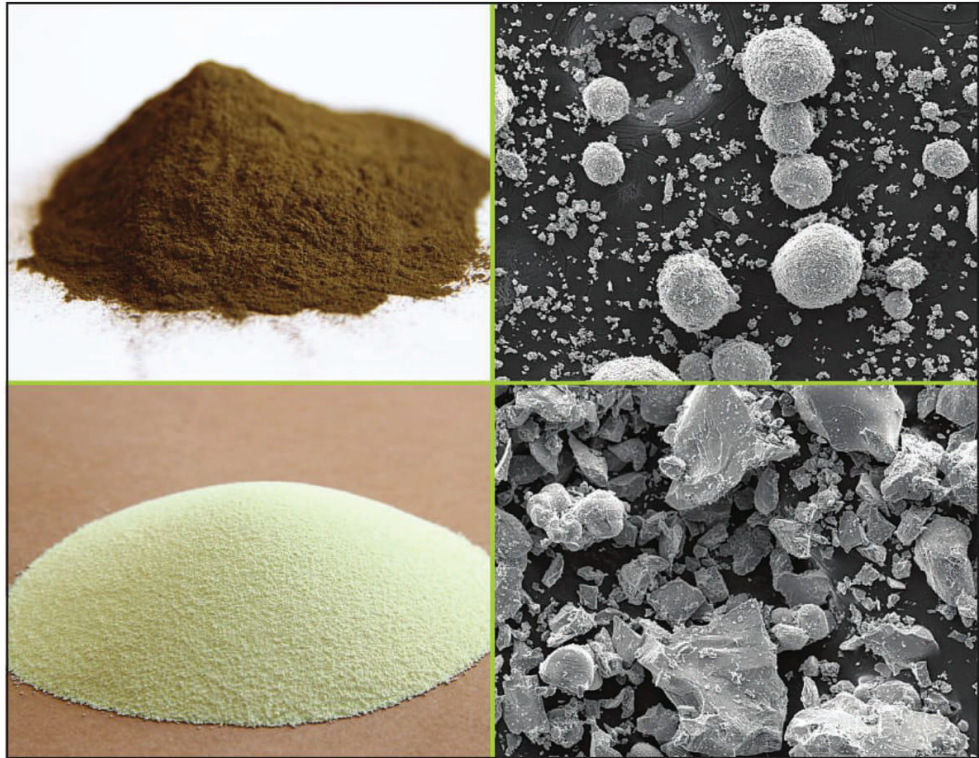
## **Annex 4**

### **Risks of high iron in zinc oxide in animal diets**

Denise Cardoso and Agathe Romeo

*Feed Strategy*

*November 2018*



Different sources of feed-grade zinc oxide from diverse manufacturing processes and raw materials generate products with different physicochemical properties. *Animine*

## Risks of high iron in zinc oxide in animal diets

Analysis examines the effects of excess, unintended iron levels in zinc oxide

DENISE CARDOSO AND AGATHE ROMEO

**M**icro- and macrominerals are essential to the positive function of organisms.

In swine production, for example, iron and zinc are often supplied in piglets' early life. To combat post-weaning diarrhea, which presents production challenges, zinc oxide (ZnO) is usually supplied at a pharmacological dose of 3,000 ppm to prevent this

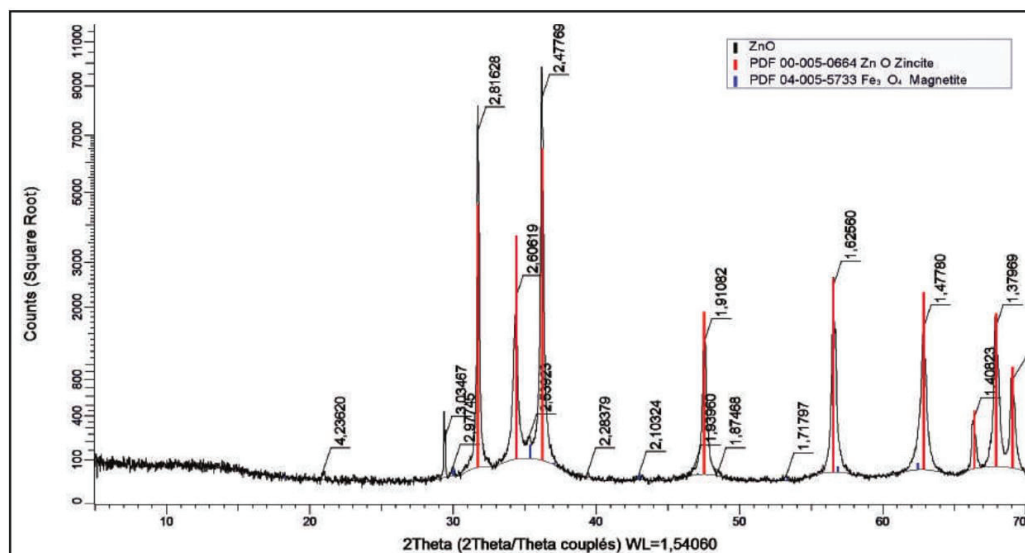
disease, reducing morbidity and mortality. Iron is also fed at high levels to combat anemia in piglets.

However, little is known about the possible negative effects of high iron levels or its bioavailability from the different iron sources supplied. The following article reviews available data on zinc, zinc oxide and the impact of excess iron in animal diets.

[www.WATTAgNet.com](http://www.WATTAgNet.com) | November 2018



## RISKS OF HIGH IRON IN ZINC OXIDE



X-ray diffraction showing peaks corresponding to ZnO and iron oxide (magnetite –  $\text{Fe}_3\text{O}_4$ ). Axis (x) represents the position recorded as the angle 2Theta; (y) record the x-ray intensity as counts.

### Study of zinc oxide sources

Depending on the manufacturing process and raw material sourcing, feed-grade zinc oxide (ZnO) materials can differ greatly from one to another. Macroscopically, different sources of ZnO vary in color, granulometry and density and they show diverse structural characteristics, such as particle size and shape, metal contamination, porosity, etc.



Improving piglet gut health with nutrition: [www.WATTAgNet.com/articles/9906](http://www.WATTAgNet.com/articles/9906)

To better understand the properties of the different ZnO sources, one study analyzed more than 40 samples collected from feed industries worldwide. The samples were characterized and categorized according to their physicochemical properties, and their metal contamination was assessed according to the European Standard NF EN 15510 and analyzed by ICP-OES.

The presence of iron in ZnO sources can vary greatly according to the source of raw materials. Analyses have

confirmed that some samples of ZnO have very high concentrations of iron, corresponding up to 2 percent of total zinc oxide.

For example, 2 percent iron content in zinc oxide sources fed to piglets at 3,000 ppm would correspond to 60 ppm of iron coming from zinc.

### Iron bioavailability

To gain better understanding of the bioavailability of the iron present in ZnO, samples were characterized by X-ray diffraction to establish in which form iron is present.

Iron is mainly present in the form of iron oxide — magnetite ( $\text{Fe}_3\text{O}_4$ ). EFSA considers iron oxide a source of iron that should not be used to meet animal requirements. Furthermore, EFSA does not state data regarding the biological fate of microparticles of iron oxide ( $\text{Fe}_3\text{O}_4$ ).

Oxides normally are soluble at low pH; however, the dissolution rate depends on its physicochemical properties. If the iron present in zinc oxide cannot be dissolved in the stomach, it won't be absorbed by the animals' body and it will flow on the gastrointestinal tract where it can interact with other feed compounds, microbiota and intestinal

Mass fraction of metals in ZnO (ppm)	
SAMPLE	Fe (mg/kg)
ZnO 1	6.2
ZnO 2	7.3
ZnO 3	19.9
ZnO 4	29.9
ZnO 5	31.2
ZnO 6	32.3
ZnO 7	81.9
ZnO 8	189.2
ZnO 9	411.6
ZnO 10	488.2
ZnO 11	1,991.1
ZnO 12	2,264
ZnO 13	2,289.3
ZnO 14	5,688.9
ZnO 15	6,910.3
ZnO 16	24,451.7
ZnO 17	26,647.3

Source: Animine

**The source of the raw materials greatly influences variations in the presence of iron in zinc oxide (ZnO) sources.** | ICP-OES

epithelia. There is still little knowledge about the effect of excess iron in piglet feed; however, there is an increasing concern about this matter.

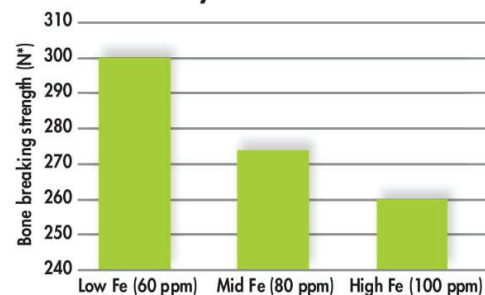
### Effect of excess iron in animal feed

The following review of literature examines the health effects of excess iron in animal diets:

#### ■ Weaning piglets

Interactions between trace minerals are extensively reviewed in literature. In a recent study conducted in Canada, the effects of high doses of ZnO on iron status of weaning piglets were observed. Depending on the farm, piglets were fed with nutritional dosage (< 500 ppm), high

### Broiler bone breaking strength fed different dietary Fe doses



\*Newton  
Source: Akter, 2017

**When dietary iron (Fe) increases, the tibia breaking strength decreases.**

dosage (2,000-3,000 ppm) or very high dosage (> 3,000 ppm) of ZnO during the post-weaning period.

In conclusion, the odds of nursery pigs being anemic was three times greater for pigs consuming high doses of dietary zinc, compared with pigs consuming zinc at nutritional dosage, and four times greater for those fed starter diet containing very high dosage of zinc.

The mechanisms of this antagonism are not fully understood: the current hypotheses include a competition for the transporter DMT1 and an interference of zinc with the conversion of iron into ferritin.

Despite the high iron concentration in some ZnO sources analyzed, the use of these sources at pharmacological level can affect the iron status of piglets and consequently their performances.

#### ■ Negative side effects of iron

During the past decade, some studies conducted with mammals suggest that high levels of dietary iron can have negative effects on gut health. For example, a deleterious effect of 750 ppm of dietary iron on gut integrity was recorded in calves.

Similarly, a high dose of dietary iron (> 500 ppm) could increase the intestinal permeability of weaning piglets and alter the gut morphology, with villous atrophy.

## RISKS OF HIGH IRON IN ZINC OXIDE

**Association of dietary ZnO level and piglet anemia**

	Odds ratio	Standard error	95% confidence interval
High dose	3.4	1.97	1.114 - 10.595
Very high dose	4.1	2.55	1.206 - 13.889

Source: Perri, 2016

The odds of piglets being anemic is greater for pigs consuming high doses of dietary zinc oxide (ZnO).

This effect on barrier function could be related to oxidative damages and lipid peroxidation in mucosa. These oxidative damages can harm not only the epithelia but also cause stress to local microbiota.

#### ■ Interaction with phytase

High dosages of iron could inhibit phytase efficacy. A recent study conducted in broilers showed a negative effect of high dietary iron (100 ppm) on nutrient utiliza-

## HIGH DOSAGES OF IRON could inhibit phytase efficacy.

#### ■ Gut microbiota

Human studies have suggested that unabsorbed iron can stimulate growth and virulence of pathogens in the intestinal environment. Bacteria may solubilize iron oxide by lowering the external pH, reducing the iron to the more soluble ferrous forms or binding to siderophores.

Iron can bind to phytate and become unavailable to the host. However, studies in rats have shown that gut microbiota can degrade iron bound to phytate and use it for itself.

tion and on tibia breaking strength. Indeed, high iron levels in the gastrointestinal tract could lead to the formation of iron-phytate complex and reduce the phytate hydrolysis by phytase.

The literature implies that excess iron can comprise gut health and, therefore, animal performance. Hence, it can be suggested that iron present in ZnO is rather a contamination than a supplementation. ■

*References available upon request.*

*Denise Cardoso and Agathe Romeo work in Animine's research and development department.*

## FS PRODUCTS



### SPIRAX SARCO SPIRA-TROL MODULAR CONTROL VALVES

Spirax Sarco Spira-trol are modular control valves. Their modular design with a quick-change "clamp-in-place" seat means their duty can be altered to match changing plant conditions, rather than replacing all of the valves. The self-aligning "clamp-in-place" seat also simplifies maintenance, so no special tools are required, and there's no need to take the valve out of the pipeline. There are different versions available: electrically and pneumatically actuated Spira-trol valves from 0.5 inches to 4 inches.

[www.spiraxsarco.com](http://www.spiraxsarco.com)

### FEED ENERGY R<sup>2</sup> PREMIUM BRAND OF NUTRACEUTICALS

The Feed Energy R<sup>2</sup> Premium Brand of Nutraceuticals provides a solution in the detection and control of foodborne hazards in poultry and hog production. It has natural antipathogenic properties that help to mitigate the three major pathogens that threaten all poultry and swine producers. Those pathogens include *E. coli*, *Salmonella* and *Campylobacter*.

[www.feedenergy.com](http://www.feedenergy.com)

## **Annex 5**

### **Zinc and Copper oxides: The best combination**

Denise Cardoso and Stéphane Durosoy

*Pig Progress*  
*December 2018*

# Zinc and copper oxides: The best combination

It is difficult to find molecules which are more popular than zinc and copper in pig production. Zinc oxide, named 'vitamin Z' by veterinarians, has strongly established anti-diarrhoea effects after weaning. A potentiated form of zinc oxide and a new source of copper are now available for the optimal supply of these nutrients which are essential for the animal and for the gut.



BY DENISE CARDOSO AND STÉPHANE DUROSOY, ANIMINE

It has been decades since the pharmacological dosage of zinc oxide, 20 times more than physiological requirements, has been routinely added to piglet feeds after weaning. Field experience proved the beneficial effects on diarrhoea, and scientific meta-analysis confirmed improvement of feed intake and animal growth. However, the downsides are known too: excess usage due to too high supplementation duration or level, nutritional interactions,

risks of contaminants, risk of accumulation in the environment, risk of increased microbial resistance. In some areas like the European Union, such practices have been regulated through veterinary prescription of medicated feeds. Permanent usage of high dosed ZnO in piglet feeds is more and more restricted. This is why feed manufacturers have looked for more sustainable practices, either with other zinc sources, or with improved forms of zinc oxide. Different zinc compounds, like sulphate and chelated sources, have been tested in the 2000's, without convincing results. Coated forms of zinc oxide failed to achieve the same results as high dosed ZnO. As

Figure 1 - Effect of ZnO on bacterial populations in the stomach and ileum of weaned piglets.

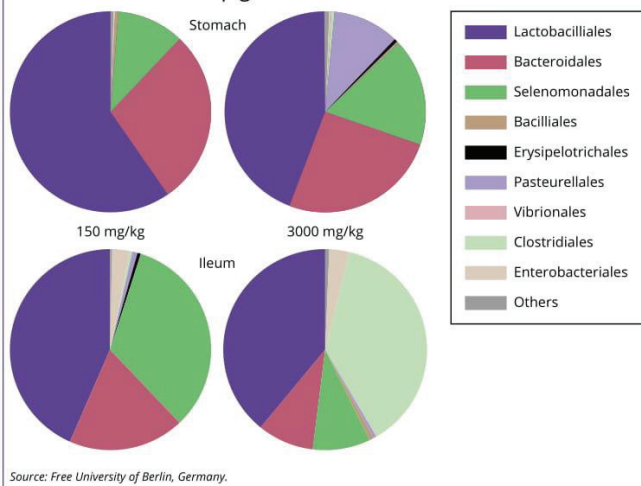
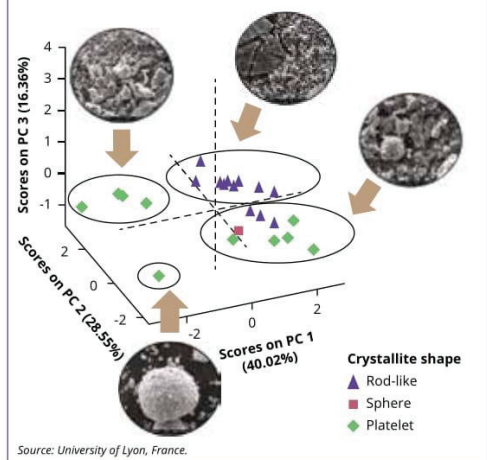
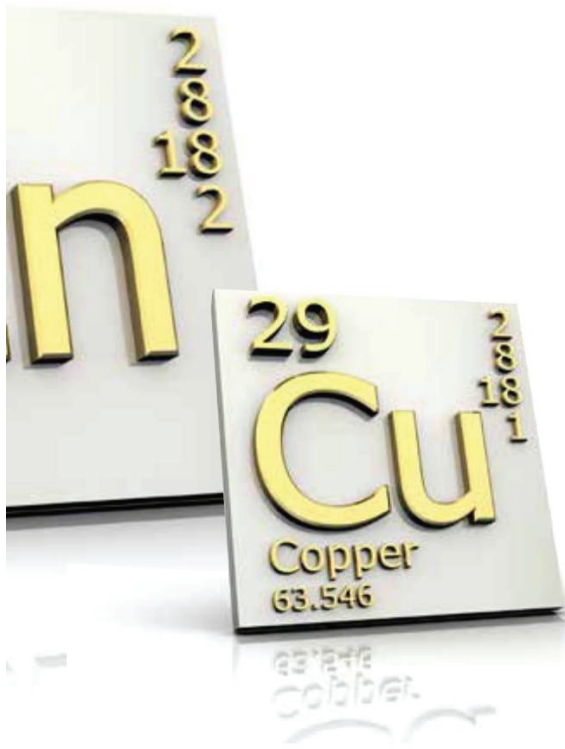


Figure 2 - Principal component analysis of zinc oxide sources.





encapsulation only releases ZnO in the small intestine, it can not act as early as in the stomach (Figure 1). Only zinc oxide works, but there is a wide heterogeneity of feed grade zinc oxides used by pig producers or feed manufacturers. Feed safety and animal health may be degraded by contaminants (heavy metals, dioxins, etc). Some products may have a very high concentration in iron, thus leading to possible counteraction on *E.coli* populations. Differences in physio-chemical features will affect dissolution kinetics of ZnO in the gastrointestinal tract, which will impact the beneficial effect on intestinal health and the bioavailability at absorption sites. From more than 40 samples of feed grade ZnO collected worldwide, a potentiated form of zinc oxide (HiZox) showed significant differences with other products (Figure 2). This explains how the product, when supplied at low dosages, can improve intestinal health through reduction of pathogenic bacteria and improvement of gut integrity, as recently shown by Ghent University in Belgium (*J. Anim. Physiol. Anim. Nutr.*, 2018 ;1-11).

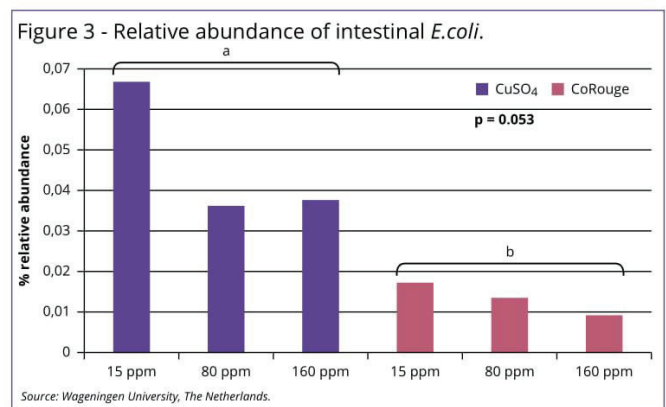
### Copper oxide: Same properties as zinc oxide

Copper compounds face the same challenge as zinc when feeding livestock and poultry: their biosolubility is required in typical conditions of the digestive tract of animals. Even if the gastric pH of the weaned piglet is not so acidic due to the immature digestive system and buffer capacity of the feed. When solubility is too high, copper ions are liberated too fast, thus binding to antagonistic factors in the upper part of the gastrointestinal tract. On the other hand, some copper compounds will be insufficiently dissociated, as is the case with copper(II) oxide. This black coloured copper oxide is not

utilised in animal nutrition because of very poor bioavailability. Copper(I) oxide is another chemical form: it is the monovalent state of copper oxide, recently authorised in the European Union. CoRouge is a highly concentrated (75% Cu) source of copper(I) oxide, in a non hygroscopic, free-flowing and dust-free powder form. With high antibacterial activity due to the monovalent state of copper, the product improves the gut health of monogastric animals. This has been shown in an experiment at Wageningen University, the Netherlands, involving 600 piglets. Two sources (copper sulphate; CoRouge) at three dosages (15 ppm; 80 ppm; 160 ppm) were tested for five weeks after weaning. Effects on animal growth, intestinal microbiota and mineral metabolism were measured. There was a significant improvement of weight gain from increased Cu supplementation levels. The highest difference in BW at 35 days was 3.6 kg, comparing 160 ppm from the copper(I) oxide solution to 15 ppm from copper sulphate. The improved growth performance from CoRouge could be explained by modification of intestinal populations profile. Treatments affected *Lactobacillaceae* and *Streptococcaceae* which were the most abundant in ileum and colon. There was a source effect when focusing on intestinal *E.coli* populations (Figure 3).

### Toolbox of digestive security

Zinc and copper supplementation in pig feeds are highly popular worldwide because they perform well and are cost effective. Eliminating pharmacological dosages of zinc oxide from piglet diets may be more challenging for diarrhoea control than banning some antibiotics. It will be highly difficult for any dietary intervention or feed additive supplementation to achieve the same level of growth improvement as with copper. As mineral tools in the toolbox of digestive security, zinc oxide and copper(I) oxide can offer the best combination. As dosages decrease due to regulatory constraints, nutritionists look for well identified trace mineral sources which offer the best guarantees of safety, and most robust proofs for optimal performance on intestinal health and bioavailability. HiZox and CoRouge deliver this.



## **Annex 6**

### **Impact Des Caractéristiques Physico-Chimiques De L'oxyde De Zinc Sur La Biodisponibilité Du Zinc En Poulet De Chair**

Denise Cardoso, Agathe Romeo, Stéphane Durosoy, Nathalie Meme,  
Yves Chevalier, Agnès Narcy

*Treizièmes Journées de la Recherche Avicole et Palmipèdes à Foie Gras  
Tours, March 20-21 2019*

## IMPACT DES CARACTERISTIQUES PHYSICO-CHIMIQUES DE L'OXYDE DE ZINC SUR LA BIODISPONIBILITE DU ZINC EN POULET DE CHAIR

Cardoso Denise<sup>1,2,3</sup>, Roméo Agathe<sup>3</sup>, Durosoy Stéphane<sup>3</sup>, Meme Nathalie<sup>2</sup>,  
Chevalier Yves<sup>1</sup>, Narcy Agnès<sup>2</sup>

<sup>1</sup>Université Lyon 1, LAGEP, CNRS UMR 507, 69622 VILLEURBANNE

<sup>2</sup>UMR BOA, INRA, Université de Tours, F-37380 NOUZILLY

<sup>3</sup>Animine – 335 chemin du Noyer – 74330 SILLINGY

[dcardoso@animine.eu](mailto:dcardoso@animine.eu)

### RÉSUMÉ

La biodisponibilité d'un oligoélément peut être liée à sa solubilité *in vivo*, qui dépend elle-même des caractéristiques physico-chimiques du produit. Néanmoins, le lien exact entre ces caractéristiques, la solubilité et la biodisponibilité des minéraux n'est pas encore pleinement élucidé. L'objectif de l'étude était d'évaluer la biodisponibilité de plusieurs sources d'oxyde de zinc (ZnO), qui présentaient des propriétés physico-chimiques différentes. Environ 40 échantillons de ZnO ont été analysés pour connaître leurs caractéristiques physico-chimiques. Une analyse en composantes principales a été réalisée pour déterminer les critères les plus pertinents et diviser les échantillons en plusieurs groupes. Des échantillons représentatifs de chaque famille ont été choisis pour un essai *in vivo* sur poulets de chair. 117 animaux étaient nourris avec un aliment standard de 1 à 7 jours. Le 8<sup>ème</sup> jour, les poulets étaient placés en cages individuelles et recevaient l'un des traitements expérimentaux : contrôle négatif (23 ppm de Zn natif sans Zn ajouté) ou un aliment complété à raison de 6 ou 12 ppm de ZnO ou de sulfate de zinc (ZnSO<sub>4</sub>). Les animaux étaient abattus les jours 22 et 23. La concentration de Zn dans l'os était retenue comme critère de biodisponibilité. Les biodisponibilités des sources testées variaient de 56 à 152% comparées à la biodisponibilité de ZnSO<sub>4</sub>. La taille des agrégats semblait expliquer la grande variabilité des valeurs obtenues pour la biodisponibilité du Zn en poulet de chair. En conclusion, les propriétés physico-chimiques des sources de ZnO peuvent expliquer en partie la variabilité observée pour la biodisponibilité. D'autres recherches sont en cours pour déterminer des critères plus précis visant à prédire la biodisponibilité des sources de ZnO.

### ABSTRACT

#### Physicochemical properties of ZnO correlated to their bioavailability in broilers

The bioavailability of a trace mineral source is related to its *in vivo* solubility, which in turn is determined by its physicochemical properties. It is still not clear which characteristics are more relevant in affecting solubility and bioavailability of feed compounds. Zinc oxide (ZnO) is a common feed additive used to supplement zinc in the diet of monogastric animals. However, different sources have shown different responses on animal bioavailability. This study hypothesized that different sources of feed grade ZnO have various physicochemical features that lead to distinct bioavailability values. Over 40 samples of ZnO have been collected from the feed industry worldwide. Samples were analyzed for density, tapped density, particle size, shape, specific surface area and dissolution kinetics. A principal component analysis (PCA) was performed to define the most relevant physicochemical characteristics and categorize the samples into groups. Representative products from each family were selected for *in vivo* trial to measure the effect of their characteristics on the zinc bioavailability in broilers. 117 animals were fed a standard starter diet from day 1 after hatching up to day 7. At day 8, animals were allocated in individual cages and fed one of each treatment. Treatments consisted of a basal diet with 23ppm of Zinc and diets with supplement zinc oxide or sulfate at 6 or 12 ppm. Animals were slaughtered at day 22 and 23. Bone zinc was used to analyze zinc availability. The bioavailability of the different sources varied from 56 to 152% from zinc sulfate. Aggregate size seems to explain a large variability in the bioavailability of the different sources tested in broilers. In conclusion, physicochemical properties of Zn oxide can partly explain the variability observed in terms of Zn biological value. Further work is needed to define precisely the most accurate set of predictors of Zn bioavailability.



## INTRODUCTION

Le zinc (Zn) est un oligo-élément essentiel supplémenté à un maximum de 120 mg/kg dans les aliments complets destinés au poulet de chair. Le sulfate de Zn ( $ZnSO_4$ ) et l'oxyde de Zn (ZnO) sont deux sources de Zn communément utilisées dans l'alimentation des monogastriques, mais leurs valeurs de biodisponibilité sont très variables (Edwards & Baker, 1999 ; Narcy, 2015) et aucune corrélation n'a pu être établie entre leur solubilité *in vitro* et leur biodisponibilité *in vivo* (Anwar, 2016). Une caractérisation plus fine de ces sources est donc requise pour permettre de prédire plus précisément leur biodisponibilité.

Dans ce but, des échantillons de ZnO ont été collectés sur le marché international de l'alimentation animale et leurs caractéristiques physico-chimiques ont été mesurées ; plusieurs de ces échantillons ont ensuite été testés sur le poulet de chair. L'objectif de l'étude était d'établir un lien entre les caractéristiques physico-chimiques des sources de ZnO et leur biodisponibilité pour les volailles.

## 1. MATERIELS ET METHODES

### 1.1. Mesure des caractéristiques physico-chimiques

Les critères retenus pour caractériser les produits sont la granulométrie, l'aire de la surface spécifique, la densité apparente, la densité après tassement.

La taille des particules (agrégats et agglomérats) est mesurée par granulométrie laser par voie humide, en utilisant un instrument Malvern Mastersizer 3000. La détermination de l'aire de la surface spécifique (ASS) des poudres sèches est basée sur l'adsorption de l'azote, avec un instrument Tristar 3000 Micrometrics BET.

La densité apparente correspond à la masse par unité de volume. La densité après tassement est mesurée en utilisant un Tapped Density Tester ; le nombre de tassements est fixé en suivant les recommandations de l'Organisation Internationale de la Standardisation (ISO) :  $250 \pm 15$  chutes. Le ratio Hausner (ratio entre densité apparente et densité après tassement) est ensuite calculé.

La cinétique de dissolution est réalisée à l'aide d'une burette automatique (877 Titrino plus, Metrohm, Suisse) ; la dissolution du Zn est estimée en fonction de la quantité d'acide délivrée par la burette

### 1.2. Animaux et design expérimental

L'essai *in vivo* est réalisé sur 117 poussins mâles (Cobb) âgés d'un jour. Les animaux sont pesés, répartis dans des cases et nourris avec un aliment standard jusqu'au 7<sup>e</sup> jour. Le 8<sup>e</sup> jour, les poulets sont pesés à nouveau et répartis en cages individuelles à raison de 9 blocs. Ils reçoivent l'un des aliments expérimentaux pendant 14 ou 15 jours ; ces aliments

sont composés d'une base pauvre en zinc (23 ppm de Zn natif), supplémentée avec 6 ou 12 mg/kg de  $ZnSO_4$  ou de ZnO (Tableau 1).

**Tableau 1** : Aliments expérimentaux

Source de Zn supplémenté	Dose de Zn ajouté (mg/kg)	Zn analysé (mg/kg)
Aucune (base)	0	24,5
ZnSO <sub>4</sub>	6	28,4
	12	34,5
HZ (HiZox®)	6	28,9
	12	34,5
ZnO-1	6	29,7
	12	36,6
ZnO-2	6	29,3
	12	33,6
ZnO-3	6	29,3
	12	33,1
ZnO-4	6	28,5
	12	35,4

Les niveaux de supplémentation ont été choisis en-deçà des besoins des poulets de chair, pour évaluer plus finement la biodisponibilité des différentes sources testées.

### 1.3. Analyses sur l'os

A J22 et J23, en fin d'essai, tous les animaux ont été abattus (9 par traitement) et leur tibia droit a été prélevé. Une partie des échantillons est immédiatement stockée dans de l'azote liquide (-80°C) en attendant des analyses complémentaires. L'autre partie est pesée puis placée au four à 103°C pendant 12h pour la mesure de matière sèche, puis à 500°C pour 6h pour la détermination du contenu en cendres. La teneur en Zn est ensuite analysée par ICP-AOS.

### 1.4. Contenus intestinaux

Les contenus du proventricule-gésier et de l'intestin grêle ont été collectés. Les matières sèches des contenus digestifs ont été mesurées après 4h à 104°C avant d'être incinérés (550°C, 8h). Les cendres des contenus ont ensuite été solubilisées dans l'acide nitrique 16N et le peroxyde d'hydrogène 30% sur un bain à sec jusqu'à évaporation, et finalement diluées dans l'acide nitrique 0,4N. La teneur en Zn est finalement analysée par ICP optique.

### 1.5. Analyses statistiques

L'analyse en composantes principales (ACP) est utilisée pour déterminer les caractéristiques physico-chimiques liées à la cinétique de dissolution.

La concentration de Zn osseux est analysée par la procédure ANOVA du logiciel de traitement statistique R. Une régression linéaire est effectuée entre la concentration de Zn dans le tibia et la consommation de Zn des poulets (Littel, 1997). Les

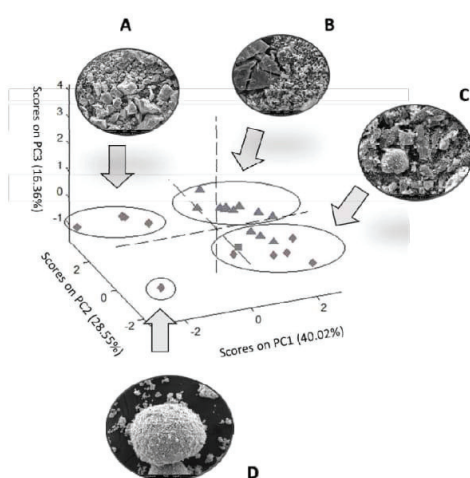
sources ont été discriminées à partir de l'estimation des intervalles de confiance.

## 2. RESULTATS ET DISCUSSION

### 2.1. Caractéristiques physico-chimiques

La caractérisation des échantillons de ZnO nous a permis de les répartir dans 4 groupes distincts (Figure 1).

**Figure 1 :** Division des échantillons en 4 groupes par ACP



Les familles A et B présentaient une grande amplitude pour la taille de leurs particules, alors que les familles C et D avaient une granulométrie plutôt homogène. Des agglomérats assez larges (100-300  $\mu\text{m}$ ) ont été observés dans le groupe D.

Les poudres comportent des particules primaires libres et des assemblages constituant des agglomérats et/ou des agrégats (Nichols et al., 2002). Les particules primaires peuvent correspondre à des cristaux simples ou à des cristaux composés de cristallites. Les agrégats et les agglomérats agissent comme des particules de grande taille avec une porosité interne (Borm et al., 2016). Les agrégats sont constitués de particules primaires liées solidement entre elles, en général au cours du processus de fabrication du proI amduit. Les agglomérats sont des assemblages plus lâches de particules primaires ou d'agrégats, qui peuvent être assez facilement brisés, contrairement aux agrégats (Balakrishnan et al., 2010 ; Borm et al., 2006 ; Nichols et al., 2002).

Le ZnO est majoritairement obtenu via trois procédés de fabrication. Les caractéristiques des ZnO produits par ces procédés peuvent être reliées aux 4 groupes de notre étude (Tableau 2).

**Tableau 2 :** Caractéristiques des sources de ZnO en fonction des procédés de fabrication

Méthode	Description de la méthode	Description du produit	Groupe
Directe	Oxydation du Zn métal	ASS entre 1 et 3 $\text{m}^2/\text{g}$	A
Indirecte	Réduction du minerai de Zn puis réoxydation	Particules primaires de 30 à 2000 nm, ASS de 3 à 12 $\text{m}^2/\text{g}$	B
Humide	Précipitation de l'oxyde d'une solution aqueuse suivie d'une calcination	Poreux, ASS très élevée	C, D

La cinétique de dissolution a montré une dissolution lente pour les échantillons du groupe A (moins de 50% du Zn dissolu après 60 minutes). Pour les groupes B et C, le même schéma de dissolution était observé, avec une dissolution complète en 20 minutes. Les échantillons du groupe D présentaient un comportement unique, avec une dissolution lente (50% de Zn dissolu après 30 minutes).

### 2.2. Essai *in vivo*

Des échantillons de chaque famille ont été retenus pour l'essai *in vivo* : ZnO-1 et ZnO-3 pour le groupe A, ZnO-4 pour le groupe B, ZnO-2 pour le groupe C et HZ pour le groupe D.

Les niveaux de Zn analysés dans les aliments correspondaient aux valeurs attendues. Aucune différence n'a été observée sur le poids vif. L'effet de la dose sur l'IC dépendait de la source.

Les valeurs de biodisponibilité calculées sont présentées dans le tableau 3, sachant que la biodisponibilité de  $\text{ZnSO}_4$  était fixée à 100%. Les régressions linéaires entre le niveau de Zn dans l'os et la consommation de Zn présentaient des pentes différentes. Des différences significatives ont été observées pour la concentration de Zn dans l'os, dépendant de la source ( $P < 0,05$ ) (Tableau 4).

**Tableau 3 :** Biodisponibilité relative de Zn en fonction de la source

Source	Biodisponibilité relative dans l'os (%)
ZnSO4	100 <sup>ab</sup>
HZ	152 <sup>a</sup>
ZnO-1	124 <sup>ab</sup>
ZnO-2	126 <sup>ab</sup>
ZnO-3	56 <sup>b</sup>
ZnO-4	126 <sup>ab</sup>

Les valeurs obtenues variaient de 56 à 152%. Cette grande variabilité est en accord avec les résultats mesurés dans des études antérieures (Edwards & Baker, 1999 ; Narcy, 2015). Les résultats suggèrent que les caractéristiques physico-chimiques pourraient avoir un impact non négligeable sur la biodisponibilité des produits et, la cinétique de dissolution pourrait jouer un rôle sur la biodisponibilité.

Concernant la concentration en Zn dans le contenu du gésier et le contenu de l'intestin, aucune différence significative n'a été observée. La concentration de Zn dans l'iléum des oiseaux ayant consommé du ZnO-3 était la plus élevée :  $0,189 \pm 0,027$  mg Zn/g contenu intestinal sec pour la dose la plus élevée, tandis que les autres sources se situaient entre  $0,157 \pm 0,005$  mg/g et  $0,164 \pm 0,008$  mg/g à même dose. Ce résultat est cohérent avec sa biodisponibilité très basse et la faible concentration de Zn dans l'os. L'étude *in vitro* avait déjà montré que cette source de ZnO est presque inerte ; elle pourrait passer dans le tractus gastro-intestinal sans être dissoute, et donc sans être absorbée par l'animal.

La corrélation entre la biodisponibilité mesurée dans l'os et les caractéristiques physico-chimiques a été calculée ; cette analyse suggère que la taille des agrégats et l'aire de la surface spécifique sont les critères les plus pertinents pour prédire la biodisponibilité des poudres de ZnO. Des études

complémentaires sont néanmoins nécessaires pour définir les critères les plus pertinents de prédiction de la biodisponibilité des sources.

## CONCLUSION

La biodisponibilité des sources de ZnO varie avec leurs propriétés physico-chimiques. La cinétique de dissolution est influencée par ces propriétés *in vitro*. Il est notamment possible que les caractéristiques physico-chimiques puissent avoir un effet sur la vitesse de dissolution. Une corrélation entre les valeurs de biodisponibilité et les propriétés physico-chimiques a montré que 43% de la biodisponibilité pouvait être expliquée par la taille des agrégats, et 27% par l'aire de la surface spécifique des échantillons.

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**Tableau 4** : Performances de croissance et Zn osseux

Source	Dose	Poids J22/23 (g)		IC J8-J22/23			Zn osseux (mg/Kg MS)		
Basal	23,5	744	± 43,73	1,38	± 0,14	85,69	± 4,22		
ZnSO <sub>4</sub>	28,4	765,78	± 51,82	1,36	± 0,07	100,38	± 4,81		
	34,5	771	± 43,77	1,44	± 0,1	107,55	± 2,92		
HZ	28,9	761,78	± 21,68	1,47	± 0,14	100,81	± 4,10		
	34,5	816,11	± 24,91	1,32	± 0,07	120,68	± 3,40		
ZnO-1	29,7	783,44	± 37,4	1,3	± 0,05	99,92	± 2,70		
	36,6	800,33	± 17,72	1,49	± 0,07	113,83	± 3,05		
ZnO-2	29,3	780,63	± 37,98	1,3	± 0,05	101,54	± 4,58		
	33,6	840,11	± 24,14	1,28	± 0,07	115,95	± 4,05		
ZnO-3	29,3	819	± 21,33	1,41	± 0,06	91,16	± 4,12		
	33,1	821,11	± 17,94	1,19	± 0,07	99,94	± 5,77		
ZnO-4	28,5	840,11	± 24,14	1,28	± 0,07	102,25	± 3,07		
	35,4	806,33	± 30,79	1,37	± 0,06	116,66	± 3,28		
	Source	NS		NS			< 0,001		
P-value	Dose	NS		< 0,01			< 0,001		
	Source*Dose	NS		< 0,01			NS		

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