An *in vitro* assessment of the effects of feed-grade trace minerals on commercial phytase activity

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Declaration of Authorship

This thesis has not been submitted in whole or in part, to this or any other university, for any degree and is, except where otherwise stated the original work of the author.

Signed: _____

Rachel O'Rourke

Date: _____

Dedication

For my Mum.

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Abstract

Exogenous phytases have the capacity to increase the nutritional value of animal feeds through the hydrolysis of phytate-bound phosphorus. Inorganic trace minerals (ITM) routinely added to feed can negatively impact phytase activity. Recent research has shown that replacement of ITMs with organic trace minerals (OTMs) may overcome these inhibitory effects due to their greater levels of stability.

The objective of the present study was to assess the effects of ITMs and OTMs on the enzyme activities of five different commercial phytases. Exposure to copper sulphate (ITM) greatly inhibited phytase activity compared to OTMs, at reflective inclusion levels. Similarly, exposure to iron sulphate (ITM) had a greater effect on phytase activity than OTMs, at reflective inclusion rates. The observed responses were instigated by trace mineral source, elemental concentration and phytase mode of action.

Additional studies assessed the effects of simulated mineral mixes containing sequentially added copper, iron, zinc, and manganese. Similar to previous findings, exposure to ITM mixes resulted in high losses of phytase activity (85 – 95 %). Comparatively, considerably more phytase activity was retained with OTM mixes (55 – 80 %). Interestingly, diverse activity retentions were observed after the exposure of phytase to commercial organic premixes (20 – 80 %), further indicating that the classification of OTM was a key determinant of phytase function.

The effect of pH changes, reflective of the poultry gastrointestinal tract (GIT), on the activity of a 3- and 6-phytase in the presence of mineral mixes was assessed. Once again, OTMs had greater enzyme retention for both phytases. Unlike the 3-phytase, 6-phytase was dependent on pH, again emphasising that phytase mode of action was integral for activity retention.

Findings from the present study indicated that the retention of exogenous phytase function was dependent on trace mineral source. These responses differed between individual minerals and phytases, an important formulation consideration for maximising feed quality.

Abbreviations

ANF	Antinutritional Factor
AAFCO	Association of American Feed Control Officials
BPPhy	Beta-propeller phytase
CPhy	Cysteine phytase
DM	Dry Matter
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESFA	European Food Safety Authority
GIT	Gastrointestinal tract
HAPhy	Histidine acid phytase
IP6	Inositol hexaphosphate
ITM	Inorganic trace mineral
NRC	National Research Council
OTM	Organic trace mineral
P	Phosphorus
PAP	Purple acid phosphatase
PU/g	Phytase units per gram
RA	Relative activity
TRT	Total Replacement Technology
SDS	Sodium dodecyl sulphate
SmF	Submerged fermentation
SSF	Solid state fermentation

1 Introduction

By 2050, the global population is projected to increase by 2.3 billion people, representing over a third of today's population. As the world's population grows and life expectancy increases, the agricultural industry faces a number of challenges, in particular food security, environmental concerns and the use of feedstocks for biofuel production (FAO, 2009). Moreover, as consumer health and environmental awareness grows, the demands for high quality animal goods produced following safe and sustainable practices is being matched with stricter levels of regulation and legislation. Health promotion and disease prevention are important considerations for the future, of which food chain integrity and safety are of primary concern (Kumar and Preetha, 2012).

Animal feed and associated enzymes play a significant role in the production of safe, high quality and affordable food. It is well established that specific enzymes can increase the nutritional value of animal feeds, potentially improving feed and cost efficiencies (Brufau *et al.,* 2006). In the past 20 – 30 years, the supplementation of these enzymes has improved the efficiency of meat and egg production, particularly for monogastric animals (Barletta, 2011). Improved feed efficiencies can lead to higher levels of production, as well as reduced costs due to reduced need for additional supplements (Ravindran, 2013). This can also allow for the use of lower grade raw materials, of which the nutritional value is increased with the use of feed enzymes (Paloheimo *et al.*, 2011). Moreover, feed enzymes can help to reduce the excretion of feed components (such as trace minerals and phosphorus) into the environment (Singh, 2008).

The following sections look to assess how feed-grade commercial phytases, routinely used in monogastric nutrition, interact with other feed components. It also describes the effects of phytic acid on the nutritional quality of feed. Finally, considerations for the supplementation of trace minerals are discussed, with regards to mineral source, the importance for animal health, and the potential interactions with other dietary components which may affect overall feed value.

1.1 Overview of phytic acid

1.1.1 Origin, structure, and occurrence in plant sources

Phytic acid, also referred to as myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate or IP6, is a naturally occurring compound present in many plant sources. It consists of an inositol ring linked to six phosphates and has a molecular weight of 660.04 g/mol (Figure 1.1). The occurrence of phytic acid in foods can have a significant effect on functional and nutritional properties (Singh, 2008).

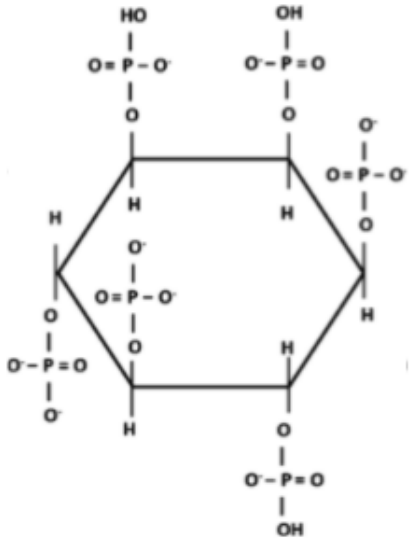

Figure 1.1 Structure of phytic acid.
Sourced from Humer *et al.*, 2015.

Phytic acid can be present as a free acid, phytate, or phytin depending on the environmental pH and presence of metal ions. These terms are often interchangeably used in literature to describe the substrate of phytases. Phytate refers to the chelated salt form of phytic acid, a chelation which typically occurs with divalent metal ions such as zinc and iron. Phytin refers to a salt form of phytic acid, involving calcium and magnesium (Singh, 2008).

Phytic acid is the principle storage unit of phosphorus in plant sources and is predominantly found in cereal seeds, pulses, and oleaginous plants. It can account for up to 80 % of the total phosphorus in animal feed ingredients, as depicted in Table 1.1. Although often abundant in nature, phosphorus retained in phytic acid is largely unavailable to monogastric animals due to low levels of intrinsic phytase activity.

Table 1.1 Phytate content of various feed ingredients commonly used in animal nutrition.

Feed Ingredients	Phytate - P* (g/100g DM**)	Phytate - P* (% of Total P*)
Cereals		
Rice (unpolished)	0.27	77
Corn/Maize	0.24	72
Wheat	0.27	69
Oats	0.29	67
Sorghum	0.24	66
Barley	0.27	64
Rice (polished)	0.09	51
Cereal by products		
Rice bran	1.03	80
Wheat bran	0.81	73
Oil seed meals		
Sunflower	0.89	77
Soya bean	0.39	60
Oil Seed Rape	0.7	59
Pulses		
Field peas	0.24	50
Roots and Tubers		
Potato	0.24	21

Adapted from Singh (2008).

*Phosphorus

**Dry matter

In general, greater amounts of phytate are found in cereal by-products and oleaginous seeds than in cereals and pulses. Concentration is dependent on the part of the plant from which they are derived (Table 1.1). In cereals, phytate is predominantly found in the outermost part of the endosperm (aleurone layer), whereas in pulses and oil seeds, phytate is dispersed in protein globoids in the kernel (Singh, 2008). In germinating seeds, phytate is broken down to release inorganic phosphate, providing energy for the emerging seedling (Azeke *et al.*, 2011). A number of different factors affect the phytic acid content of crops, including fertiliser application, climate and soil conditions, as well as the level of plant maturity (Reddy, 2001).

1.1.2 Antinutritional characteristics of phytic acid

Phytic acid is widely regarded as an antinutritional factor (ANF) in diets due to its ability to readily interact (directly or indirectly) with various feed ingredients, such as feed enzymes, macro- and micro-minerals, proteins, starch, and lipids (Oatway *et al.,* 2001). Phytic acid has a propensity to interfere with mineral availability due to its strong chelating ability. The chemical composition of phytic acid is reflective of its chelating potential. As depicted in Figure 1.1, phytic acid is a highly negatively charged molecule with 12 protons that can be easily displaced. Half of these protons are highly acidic, while the remaining six have decreasing levels of acidity (Pang and Applegate, 2006). These characteristics make phytic acid a strong chelator for multivalent cations. It also retains its negative charge across a broad pH range, enhancing the potential to chelate positively charged agents, such as macrominerals, trace minerals, and proteins with acidic, neutral and basic states (Greiner *et al.,* 2006). Such interactions can occur within the plant, as well as in the gastrointestinal tract (GIT) of animals and humans (Yu *et al.,* 2012).

1.1.2.1 Phytate-mineral interactions

It had been demonstrated in the literature that phytic acid can readily bind to divalent minerals, some of which include Zn^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, and Cu^{2+} (Greiner *et al.,* 2006; Yu *et al.,* 2012). Binding of cations to phytate reduces bioavailability and absorption of these minerals by plants and animals; potentially leading to mineral deficiencies. Various factors determine the solubility and stability of mineral-bound phytate, including the metal ion, environmental pH, presence of potential competing compounds, and the molar ratio of metal ion to phytate (Greiner *et al.,* 2006). The solubility of phytate is highly dependent on pH. In mildly acidic conditions (~ pH 4 upwards), phytate and minerals tend to interact to form insoluble complexes, whereas more soluble complexes are formed at lower pH values (Tangkongchitr *et al.,* 1982; Bohn *et al.,* 2008). Although phytate may be soluble in the more acidic sections of the GIT, it can become insoluble in the more neutral environment of the small intestine (Vieira, 2008). Different ratios of metal ion to phytic acid will also affect the solubility of phytate, with excess metal ions forming insoluble phytate complexes and vice versa (Bohn *et al.,* 2008). This poses a problem when formulating diets with respect to predicting phosphorus release and trace mineral absorption.

1.1.2.2 Phytate-protein interactions

Phytate is also known to interact with proteins, forming binary or ternary protein-phytate structures. Phytate can interact with positively charged proteins to form binary structures at pH values less than their isoelectric point (Cosgrove, 1966). Conversely, ternary structures are likely to form if a protein is negatively charged and the pH is above its isoelectric point. Binary structures arise from phytate interacting with basic amino acids, whereas ternary structures are stabilised by metal ion bridges between proteins and phytates. Ca^{2+} usually forms the cationic bridge in this instance (Selle *et al.*, 2012). These interactions can have a negative impact on the digestibility of amino acids and peptides.

1.1.2.3 Phytate-starch interactions

Phytic acid has also been shown to negatively affect starch digestibility; however, this relationship hasn't been fully elucidated due to the different potential interactions. One way in which phytic acid may reduce starch digestibility is through interactions with the starch-degrading enzyme, amylase. Knuckles and Betschart (1987) reported that the presence of phytate reduced starch digestion by 91.5 % using human α-amylase. In an alternative approach, dephytinisation of navy beans was shown to increase starch digestibility by 25 % (Thompson, 1986). Another potential way in which phytic acid can affect starch digestibility is through chelation of metal ions associated with the activation of amylases (Rickard and Thomspon, 1997). Phytic acid may also interact with starch through phosphate linkages or by binding to proteins associated with the carbohydrate molecule (Yoon *et al.*, 1983).

Inhibition of these intrinsic enzymes can greatly impact digestibility and absorption of nutrients by the animal, leading to decreased feed efficiencies and lower production outputs, often resulting in financial losses. The implementation of enzyme-enriched feed formulations in monogastric nutrition has the ability to overcome these antinutritional effects. Exogenous feed enzymes, like carbohydrases, proteases and phytases, are routinely added to monogastric diets as a cost-effective method of increasing the nutritional value of feed, thereby improving feed efficiencies and maximising production (Barletta, 2011).

1.1.3 Phytic acid in animal nutrition

Phosphorus is an essential macromineral, fundamental to all life forms and holds great importance in animal nutrition. After calcium, phosphorus is the most abundant mineral within an animal, with 80 % of it deposited in the skeleton and the remainder dispersed throughout the body, incorporated within proteins and fats (Suttle, 2010). Phosphorus is required for overall animal health and function, playing a vital role in numerous metabolic processes. The largest proportion of absorbed phosphorus is involved in the growth and maintenance of a healthy skeleton. It also has key roles in energy utilisation and transfer, blood buffering, protein synthesis, reproduction, and feed efficiency (Suttle, 2010; Oster *et al,* 2016). Low levels or unavailable forms of phosphorus can quickly result in deficiency of the mineral, manifesting itself in a number of ways in the animal. The initial sign of a phosphorus deficiency presents as decreased blood plasma phosphate levels, which can lead to calcium and phosphorus withdrawal from skeletal deposits, potentially resulting in bone weakness and breakages. Compromised immunity, loss of appetite, and poor feed efficiencies are typically associated with phosphorus deficiency and can have performance and financial implications (López-Alonso, 2012).

1.1.3.1 Environmental pollution

Phytic acid is the primary storage form of phosphorus in plant sources, such as corn, oil seed rape and potatoes. The availability of this form of phosphorus to different animal species varies greatly. Ruminants have the ability to dephosphorylate phytic acid to release inorganic phosphate, which is mediated by microbial enzymes within the rumen. Conversely, phosphorus release by monogastric animals, such as poultry and swine, differs greatly from that of ruminants as they possess much lower levels of intrinsic phytase activity (Yank *et al.,* 1998). Consequently, monogastric feeds are often supplemented with an additional source of inorganic phosphorus to help meet nutritional needs. Monogastric meals are typically cereal-based with phosphorus predominantly in the form of phytic acid. The combination of unusable phytate-phosphorus and high levels of supplemented inorganic free phosphorus often results in diets that exceed actual dietary requirements. High levels of phosphorus, in either organic or inorganic form, will inevitably result in its excretion via manure, and quickly become a pollution concern (Oster *et al.,* 2016). Eutrophication (from excessive

phosphorus levels) of aquatic systems stimulates the prolific growth of different microorganisms such algae and cyanobacteria, leading to hypoxia, and subsequently harming and/or killing fish and other forms of aquatic life (Singh, 2008).

1.1.3.2 Improving phosphorus availability with exogenous phytases

Reducing the excessive levels of phosphorus present in poultry feed (and other monogastric animals) is of utmost importance to protect the environment. This can be achieved by the formulation of specialist diets containing highly digestible feed ingredients and supplements to aid the degradation of phytic acid, meeting the nutritional needs of poultry while minimising the need for supplemental phosphorus. The benefits of exogenous microbial phytases in monogastric feeds has been reviewed extensively (Singh, 2008; Humer *et al.*, 2015). Amongst numerous studies in this area, Manangi and Coon (2008) demonstrated that phytate hydrolysis and phytate retention levels increased with higher applications of a commercial 6-phytase. Similarly, Truong *et al.* (2017) reported phytate degradation levels of 95.5 %, as well as increased starch and protein digestibility in phytase-supplemented diets. Another study illustrated that the addition of a fungal phytase to broiler diets significantly reduced phosphorus excretion, as well as significantly improving weight gain in the chicks (Abdel-Megeed and Tahir, 2015).

The degradation of phytic acid through exogenous phytases offers many potential benefits such as the overall improvement of phosphorus availability, reduced need to supplement inorganic phosphorus sources; the absorption of trace minerals, amino acids, and proteins, and the reduction of phosphorus excretion into the environment. Phytase supplementation may also be superior to alternative methods (such as phosphorus extraction and precipitation) as it doesn't have a negative effect on the nutritional quality of the product and has the potential to incur less cost through reduced inorganic phosphorus supplementation and better mineral uptake (Pandey *et al.*, 2001).

1.2 Overview of phytases

Enzymes are proteins produced by all living organisms that catalyse biochemical reactions within cells. Under normal conditions, they can initiate and regulate catalytic reactions without any permanent changes to themselves. Enzymes catalyse reactions by

binding to specific substrates and lowering the activation energy required for hydrolysis. Products of the reaction are released, and the enzyme is then free to catalyse more reactions. For example, xylanases are glycosidic enzymes that are involved in the degradation of complex xylan chains present in plant cell walls, to smaller subunits through the hydrolysis of 1,4-β-D-xylosidic linkages (Collins *et al.*, 2005)

Phytases (myo-inositol hexakisphosphate phosphohydrolase) are hydrolytic enzymes which are members of the phosphatase family. They are responsible for catalysing the step-wise dephosphorylation of phytic acid (myo-inositol hexakisphosphate) and its salt (phytate) resulting in the release of inorganic orthophosphate and the generation of myo-inositol after complete hydrolysis. In 1907, four years after the discovery of phytic acid (Posternak, 1903), the first report of phytases in literature was documented (Suzuki *et al.*, 1907). The nutritional benefits of phytic acid are often unrealised with monogastric species unless the compound is degraded to release the inorganic phosphate locked within. Consequently, phytases are of great commercial importance for monogastric diets, as they have the potential to liberate bound phosphate, helping to meet the nutritional requirements of the animal while reducing costs associated with inorganic phosphate supplementation.

Natuphos® was the first commercial phytase produced for use in poultry diets and was introduced to the EU market in 1991 by BASF. Natuphos® is a 3-phytase produced from the fermentation of a genetically modified *A. niger* strain. Since its advent, many other phytase preparations have come to the market, including Phyzyme®, Quantum Blue®, Ronozyme®, and Optiphos®.

1.2.1 Classification of phytases

Phytases are a broad group of enzymes, varying in size, function and structure. They are typically classified based on their catalytic function; histidine acid phytases (HAPhys), β-propeller phytases (BPPhys), cysteine phytases (CPhys), and purple acid phosphatases (PAPs). Phytases can be sub classified as acid or alkaline phytases depending on their pH optimum. They can also be separated based on their mode of action, to either 3-phytases, 4/6-phytases and 5-phytases. 3-phytases (E.C.3.1.3.8) initiate dephosphorylation at the C3 position, and are typically produced by fungi (Wyss *et al.*, 1999). 4/6-phytases (E.C.3.1.3.26) initiate hydrolysis at the C4 or C6 position and are typically found in bacteria and plants (Greiner and Konietzny, 2011). 5-phytases

(E.C.3.1.3.72) initiate hydrolysis at the C5 position and tend to be less common, having previously been identified in lily pollen and *Selenomonas ruminantium* (Puhl *et al.*, 2008).

1.2.1.1 Histidine acid phytases

Most known phytases belong to the histidine acid phosphatase group of phytases and have been found in microorganisms, plants and animal tissues. Histidine acid phytases (HAPhys) are a diverse group of phytases that share a unique active site motif, RHGXRXP and a dipeptide (HD), both of which are responsible for hydrolysis (Greiner and Konietzny, 2011). Dephosphorylation of phytic acid to release orthophosphate and myo-inositol is catalysed by a two-step reaction involving an initial nucleophilic attack by histidine to the scissile phosphoester bond, followed by protonation of the leaving group by the HD motif. It has been noted in the literature that not all HAPhys have specificity for phytic acid. Wyss *et al*. (1999) identified two classes of phytases within the HAP family; the first was a group of fungal HAPhys that had broad substrate specificity but a low affinity for phytic acid, the second group was one that had narrow substrate specificity but a high affinity for phytate. To date, all commercial phytases qualified for use in animal feeds are HAPhys belonging to 3- and 6-classes.

1.2.1.2 Beta-propeller phytases

Beta-propeller phytases (BPPhys) are a distinct class of phytases that do not share amino acid sequence homology with any other known phosphatases (Mullaney and Ullah, 2003). They also do not contain the conserved active site motif, RHGXRXP, or the HD dipeptide of HAPhys (Kerovuo *et al.*, 2000), making them a distinct class of phytases. BPPhys were initially purified from the Gram positive bacterial species, *Bacillus* sp. (Kumar *et al.*, 2017). They have a propeller-like structure containing six blades. It has been reported that the catalytic activity of these phytases is dependent on the binding of calcium ions (Oh *et al*., 2001; Shin *et al.*, 2001). It has also been suggested that calcium ions are associated with the enhanced thermostability of BPPhys (Mullaney and Ullah, 2003).

1.2.1.3 Purple acid phosphatases

Purple Acid Phosphatases (PAPs) are another group of enzymes that display phytase activity in some instances (Hegeman and Grabau, 2001). PAPs are responsible for the hydrolysis of phosphorylated molecules. They are also distinct from HAPhys as they do not contain the conserved active site motif. PAPs have been identified across a range of sources, including animals, plants, and fungi. To date, they have not been identified in bacterial sources; however, homology with known gene sequences suggests a possible presence in cyano- and mycobacteria (Schenk *et al.*, 2000). The mass and structure of PAPs can differ depending on their source. Mammalian PAPs tend to be smaller (~ 35 kDa), monomeric enzymes, whereas plant sources are typically larger (~ 110 kDa), homodimeric enzymes. Each subunit of their active site contains Fe^{3+} linked to a divalent metal. PAPs purified from red kidney beans were shown to have active sites containing Fe^{3+} linked to Zn^{2+} (Truong *et al.*, 2005), whereas a PAP purified from sweet potato contains a $Fe^{3+} – Mn^{2+}$ unit (Schenk *et al.*, 2005). However, in the case of mammalian PAPs, the active site contains a ferric – ferrous/ferric unit (McGeary *et al.*, 2009). Few PAPs display specificity for phytic acid or its salt, phytate. The first PAP displaying phytase activity was identified in the germinating seedling of soybeans (Hegeman and Grabau, 2001). To date, most PAPs exhibiting phytase activity have been reported in plant sources (Zhang *et al.*, 2008; Dionisio *et al.*, 2011).

1.2.1.4 Cysteine phytases

Cysteine Phytases (CPhys), also referred to as Protein Tyrosine Phosphatase-like Phytases (PTPLPs), are the last group of enzymes that exhibit phytate degrading abilities. They are classified as PTPLPs due to their similarities to protein tyrosine phosphatases in catalytic mechanism and protein folding (Chen *et al.*, 2015). Many of the reported CPhys have been purified from *Selenomonas ruminatium*, a bacterium present in the ruminant GIT (Gruninger, 2012; Puhl *et al.*, 2008; Chu *et al.*, 2004). Their active site contains the HCXXGXXR(T/S) motif, sharing homology with protein tyrosine phosphatases (Chen *et al.*, 2015).

1.2.2 Use of phytase in monogastric animal nutrition

Various feeding studies have demonstrated the advantages of incorporating phytase into the diets of monogastric animals to improve the availability of inorganic phosphorus, as well as improving mineral bioavailability through the degradation of phytate complexes. One of the early studies by Nelson *et al.* (1971) reported that the addition of exogenous phytase to broiler feeds helped to improve the availability of phosphorus within corn and soybean meals. Similarly, Cromwell *et al.* (1995) reported the improved bioavailability of phosphorus when Allzyme®, a fungal-derived phytase, was included in pig diets. Although earlier research showed the potential benefits of phytase supplementation in monogastric diets, it was not until the early 1990s that a phytase was developed for commercial feed inclusion (Selle and Ravindran, 2007).

The application of microbial phytases in monogastric diets was also prompted by the need to reduce phosphorus pollution caused by the accumulation and leaching of excreted phosphorus into the soil and water sources (Selle and Ravindran, 2007). Simons *et al.* (1990) reported that the supplementation of 1500 FTU/kg phytase reduced the phosphorus load of faeces by 50 % in broilers and by 35 % in pigs. More recently, Htoo *et al.* (2007) reported that the supplementation of Natuphos® to pig feeds improved the digestibility and uptake of phosphorus, resulting in lower levels of phosphorus excretion.

The supplementation of exogenous phytases has also been associated with other potential health benefits for monogastric species, such as enhanced egg shell quality in laying hens (Zyla *et al.*, 2012), improved growth performance of poultry and swine (Dilger *et al.*, 2004; Onyango *et al.*, 2005; Olukosi *et al.*, 2007), increased weight gain and feed intake in poultry and swine (Lei *et al.*, 1993; Pirgozliev *et al.*, 2007).

1.3 Mineral nutrition

There are approximately thirty different known elements required to sustain animal and plant life. These can be classified into four major groups; bulk elements, macrominerals, microminerals, and trace minerals (including metals and non-metals) (Roat-Malone, 2007). Macrominerals, including Ca, K, Mg, and Na, are elements that are required at relatively high levels (above 100 ppm), whereas trace elements, including Cu, Fe, and Zn, are microminerals typically required at levels below 100 ppm. Although

microminerals are required in trace amounts, even as low as ppb, it does not mitigate their importance in the animal diet (Lukić *et al.*, 2009).

Trace mineral requirements in animal nutrition is of particular interest to producers, premix manufacturers, veterinarians, and scientists, as they are of great importance to the overall health and productivity of the animal; with vital roles in immunity, growth, and reproduction (Yatoo *et al.*, 2013). Adequate dosing rates and bioavailability of trace minerals is crucial in preventing potential mineral deficiencies. Antagonism of minerals with other feed components can lead to issues with uptake efficiency resulting in deficiencies of certain minerals. Animal diets are routinely supplemented with trace minerals in an attempt to meet nutritional demand, due to low levels of minerals or lack of availability within the basal feed source (Nollet *et al.*, 2007).

1.3.1 Importance of trace minerals in poultry nutrition

There are a number of factors that make a trace mineral an essential requirement; if physiological deficiency occurs upon removal or reduction of a mineral, if a deficiency is remedied by the addition of a mineral, and if the mineral is specifically responsible for a biological function (Roat-Malone, 2007). Their functions can be described by four broad categories, encompassing structural, physiological, catalytic, and regulatory considerations. Structural function refers to minerals integral for forming structural components, such as the role of copper in the synthesis and maintenance of collagen (Richards *et al.*, 2010). Physiological roles refer to when trace minerals maintain osmotic pressure, acid-base balance and membrane permeability. Catalytic function refers to trace minerals which act as co-factors to metalloenzymes and hormone systems, such as manganese acting as an activator for enzymes involved in the production of polysaccharides and glycoproteins. Regulatory function refers to the influential role of trace minerals in cell replication and differentiation (Suttle, 2010). Given all of these key functions, it is imperative to ensure adequate mineral status of the animal.

Basal poultry diets are considered nutritionally insufficient; thus, the supplementation of trace minerals is required to meet or exceed dietary requirements. Deficiencies are often due to intensive production practices or a lack of bioavailable nutrients within animal feed ingredients. Although appreciation for the necessity of

trace minerals in animal diets has increased in the last number of years, the importance of mineral source is often overlooked. An inadequate supply of trace minerals often results in subclinical deficiencies which can be difficult to identify as they do not present with specific symptoms. As depicted in Figure 1.2, even slight deficiencies in trace mineral status can greatly impact the overall health of an animal (López-Alonso, 2012). The initial sign of a subclinical deficiency is reduced immunity and enzyme function. As trace mineral status of the animal declines, maximum growth rates, poor feed efficiencies and reproduction frequencies decrease, resulting in reduced production and output.

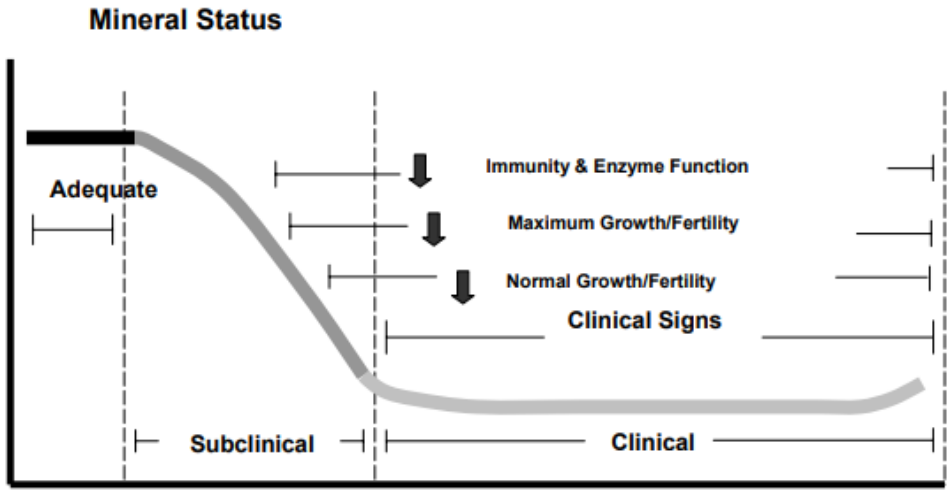

Figure 1.2 Effect of trace mineral intake on animal performance.

General symptoms are often difficult to detect, leading to major issues for animal health and performance when this transitions into diagnosable clinical conditions
Sourced from Olson (2007).

Trace minerals, such as zinc (Zn), copper (Cu), iron (Fe), and manganese (Mn), are crucial for a wide number of metabolic processes in animals and humans alike (Richards *et al.*, 2010). Although trace minerals have highly specific individual functions, they are also important across multiple roles, often acting in synergistic fashion along various biochemical pathways. Table 1.2 provides a general overview of the role of trace minerals in animal nutrition, as well as some of the potential symptoms arising from deficiencies, demonstrating the importance of trace minerals animal diets.

Table 1.2 Role of trace minerals and potential deficiency symptoms in agricultural animals.

Trace mineral	Role	Deficiency symptoms
Copper	Co-factor to a number of enzymes involved in structural strength, elasticity of connective tissues and blood cells.	Reduced growth rates, delayed feathering, weak bones, abnormal skeletal disorders, lack of keratinisation.
Iron	Forms haemoglobin and myoglobin which are necessary for oxygen transport and cellular use. Co-factor for certain enzymes.	Anaemia, poor growth, compromised immunity, de-pigmentation of feathers, diarrhoea.
Zinc	Co-factor to several hundred enzymes, comprising all six classes. Essential for immunity, reproduction and sexual maturity.	Reduced growth rates, loss of appetite, compromised immunity, weak bones, poor skeletal development.
Manganese	Co-factor to enzymes involved in metabolism of fats, carbohydrates, and proteins. Plays a role in structure and growth.	Reduced growth rates, impaired metabolism of nutrients, reduction in hatchability.

Adapted from Ewing and Charlton (2007), Richards (2010) and Suttle (2010).

1.3.2 Trace mineral source

Traditionally, the supplementation of trace minerals to animal feeds has been done through the means of inorganic sulphates, chlorides, oxides, and carbonates. These trace minerals are often added at excessive levels to meet the nutritional requirements of the animal, largely due to poor bioavailability and lower solubility within the GIT. Although there is a shifting trend in animal production to utilise organic mineral sources, inorganic minerals are still often employed (Nollet *et al.,* 2007)

Upon entering the GIT, inorganic trace minerals are solubilised by digestive fluids and metal ion dissociation occurs. Some metal ions can be absorbed via normal metal ion channels in the small intestine; however, this process can be inefficient (Rutz *et al.,* 2004). Divalent metal ions readily attach to certain feed components, having

positive or negative effects on mineral absorption. Certain ligands, such as amino acids and organic acids, have the capacity to improve mineral absorption (Lönnerdal, 2000). More often than not, dissociated metal ions associate with other dietary components, forming complexes which prevent uptake of the mineral and contribute to excessive waste pollution. Phytic acid has a strong chelating potential and can bond with dissociated metal ions, forming soluble and insoluble mineral-phytate complexes, which limit mineral uptake and absorption (Akter *et al.*, 2015). Trace minerals, particularly in their inorganic form, have been shown to influence phytase function, both directly and indirectly. Inorganic sulphates of Cu, Fe and Zn are known to be potent inhibitors of HAPhy activity (Greiner and Konietzny, 2011). Numerous studies have demonstrated the negative effects of these minerals on the phytase function of fungal and bacterial sources of HAPhys (Igamnazarov *et al.*, 1999; Maenz *et al.*, 1999; Quan *et al.*, 2004). Commercial phytases in the form of HAPhys are routinely added to monogastric feeds, thus there is an inherent risk of antagonistic relationships developing between trace minerals and exogenous phytases. Due to their perceived stability, the use of organic sources of trace minerals may help to overcome these potential antagonistic relationships. With production efficiencies typically gained through feed composition, these factors should be taken into consideration when formulating feeds.

Minerals can also interact with each other, leading to reduced mineral absorption and metabolism (Vieira, 2008). These factors should also be considered when formulating animal feeds, to eliminate potential antagonisms. Figure 1.3 illustrates the broad range of mineral-mineral interactions that can occur. Calcium has been shown to negatively affect the absorption of iron (Hallberg *et al.*, 1991). Zinc has also been reported to affect iron and copper absorption (Sandström, 2001).

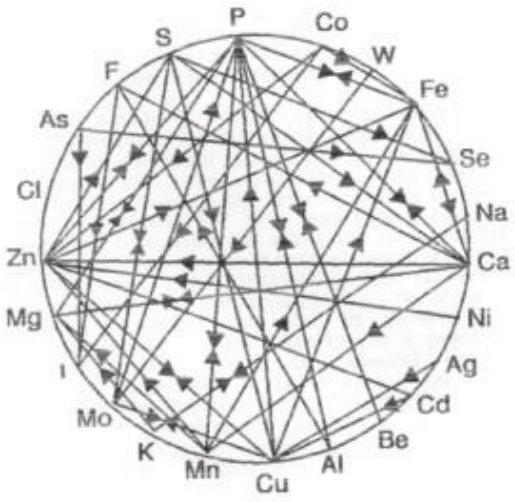

Figure 1.3 Potential interactions between minerals.

Antagonistic relationships can arise between different minerals potentially affecting their metabolism and absorption.
Sourced from Vieira (2008).

Various forms of organic trace minerals (OTM) have been available since the 1980s. OTMs have the potential to offer greater stability and bioavailability to animals than their inorganic counterparts due to their ligand bonding properties. Greater stability of these complexes may also help to reduce mineral-mineral interactions. A number of different types of organic trace mineral products have been developed since their advent, including metal amino acid complexes, metal amino acid chelates, metal proteinates, metal polysaccharide complexes, metal propionates, and metal methionine hydroxy analogue chelates. While all are classed as OTMs, they have very distinct and individual definitions as laid out by AAFCO (1998) (Table 1.3).

Table 1.3 Definitions of organic trace mineral products.

Organic trace mineral	**Definition**
Metal amino acid complex	Product resulting from complexing a soluble salt with an amino acid (≤300 Da).
Metal (specific amino acid) complex	Product resulting from complexing a specific soluble metal salt with an amino acid.
Amino acid chelate	Product resulting from the reaction between a cation from a soluble metal salt with amino acids in a 1:1 – 3 (preferably 2) of metal to amino acids to form coordinate covalent bonds. The average weight should of the amino acids should be ~ 150 Da, and the end product should not exceed 800 Da.
Metal proteinate	Product resulting from the chelation of a soluble metal salt with amino acids and/or partially hydrolysed protein.
Metal polysaccharide complex	Product resulting from the complexing of a specific soluble metal salt with a polysaccharide solution.
Metal propionate	Product resulting from the reaction of a soluble metal salt with propionic acid.
Metal methionine hydroxy analogue chelate	Product resulting from the reaction of a metal salt with 2-hydroxy-4-methylthiobutanoic acid (HMTBa), having a chelated molar ratio of one mole of metal to two moles of HMTBa to form coordinate covalent bonds.

Adapted from AAFCO (1998).

Although the trace minerals presented in Table 1.3 are defined as organic sources, they do not share identical chemical properties, primarily due to their ligand and bond type. Organic trace minerals are formed by complexing or chelating mineral salts with organic compounds (ligands). A metal complex consists of a central metal ion electrostatically or covalently linked to atom(s) within a ligand. Monodentate ligands contain only one interactive atom, whereas polydentate ligands contain two or more, inferring enhanced stability. Chelation arises from polydentate ligands (or chelating agents) complexing to a metal ion to form a heterocyclic ring structure (Vieira, 2008). It is important to note that while all chelates are complexes, not all complexes are chelates. Chelated trace mineral sources, such as proteinates and amino acid chelates, have greater stability due to the bond strength between the mineral and ligand. Stability constants of OTMs define bond strength, with a high stability constant indicating greater bond strength, which also infers that the mineral is less likely to interact with other feed components. Furthermore, it has been suggested that OTMs can be effective at reduced inclusion rates due to their enhanced integrity within the GIT. Manangi *et al.* (2012) reported that reduced rates of chelated sources of copper, manganese and zinc were effective as alternative mineral sources to inorganic counterparts in poultry, resulting in improved foot pad health and decreased levels of trace minerals within excreta.

Comparatively, inorganic trace minerals have limitations in animal nutrition, primarily due to their low bioavailability, high supplementation levels and potential toxicity at higher levels (NRC, 1994; Yenice *et al.*, 2015). It has been proposed that chelated minerals are superior alternatives to inorganic minerals as they can overcome these limitations (Nollet *et al.*, 2007). Organic trace minerals are more bioavailable due to their stability, which helps to protect the metal from dietary antagonists that can prevent the absorption of the mineral. In general, chelated minerals are soluble across a broad range of pHs and are free to pass through the GIT without interacting with other potentially limiting agents, such as phytic acid. It should be noted that some organic trace mineral sources are more bioavailable than others, which is due to the characteristics of the organic ligand and differences in their dissociation rates within the GIT (Richards *et al.*, 2010).

1.3.3 Potential mineral interactions with feed enzymes

Trace minerals have been shown to interact with a number of different feed enzymes, including xylanases, glucanases and phytases. In the case of phytase, it is difficult to ascertain whether this is through direct interaction with the enzyme or indirectly with phytic acid (Konietzny and Greiner, 2002). Relevant *in vitro* studies have typically been conducted using inorganic sources of trace minerals, in order to determine potential modulators of phytase activity (Wyss *et al.,* 1999; Tai *et al.,* 2013; Monteiro *et al.,* 2015). The effects of inorganic trace minerals on phytase function have been reported across numerous sources. Ca^{2+} was shown to enhance the activity of a phytase from *Pantoea* sp. 2-fold, whereas Cu^{2+} slightly inhibited the activity (Suleimanova *et al.,* 2015). Casey and Walsh (2003) reported that $CaCl_2$ moderately inhibited (32 %) the activity of a phytase from *A. niger*. $CuSO_4$ and $FeSO_4$ were reported to inhibit the enzyme activity of a phytase from *A. niger* by ~ 96 % and ~ 40 %, respectively (Sariskya *et al.,* 2005). Previous studies have demonstrated that organic trace mineral sources may have less of an impact on the phytate degrading activity of phytases. Santos *et al.* (2015) reported higher phytase retention in the presence of Fe and Zn proteinate sources for phytases from *P. lycii* and *E. coli* sources. Additionally, the authors reported that Cu proteinate sources caused significantly less inhibition to *E. coli* and *A. niger* derived phytases in comparison to other trace mineral sources. Furthermore, a varying degree of phytase activity was observed in the presence of different OTM sources. Pang and Applegate (2006) reported higher levels of phytate degrading activity in the presence of organic copper lysinate in comparison to inorganic copper sulphate. These collective studies emphasise the potential use of OTMs in animal nutrition with respect to minimising antagonisms between exogenous phytases and trace minerals within feeds.

1.4 Future perspectives

It is imperative that the animal production industry continues to evolve to meet consumer demands, the needs of animals and the growing population. By 2050, the population is predicted to rise by one-third of the current population, correlating to a 70 % increase in food production demand (FAO, 2009). Feed enzymes have an important role to play in the efficient production of safe, high quality and affordable food to meet these demands. With their capacity to increase the nutritional value of feed sources, alternative, lower grade food substances can be utilised, allowing higher value cereals, like wheat and corn, to be used directly in human nutrition.

Supplementation of exogenous phytases to monogastric diets is required to enhance the nutritional value of feeds, leading to increased productivities and efficiencies. Likewise, it is of utmost importance to add bioavailable trace minerals to monogastric diets to satisfy dietary requirements and minimise potential antagonistic relationships which may occur between dietary components. Organic trace minerals may help to achieve this, with the potential to result in improved overall health, as well as feed and production efficiencies.

1.5 Objectives of the project

The overall objective of the study was to determine the effects of different inorganic and organic trace mineral sources on the activity of commercially-available phytases. Within this, the following aims were investigated:

1. To determine the phytate-degrading capacities of commercial phytases at pH values associated with the digestive tract of poultry.

2. To elucidate the effects of potential modulators, such as trace minerals, protein disrupting agents and chelating agents, on the activity of a commercial phytase, as a means to determine what substances could potentially alter phytase function.

3. Given the ability of phytase activity to be compromised, the effects of individual feed-grade trace mineral sources on enzyme function of commercial phytases.

4. To determine the effects of simulated mineral mixes on enzyme function of commercial phytases. Additional research was conducted to look at the combined effect of trace minerals.

5. Following the effects of simulated mineral mixes, the effects of commercially obtained premixes were assessed.

6. To examine the effect of gastric pH on phytase function in the presence of feed-grade mineral mixes, as a way of modelling potential activity *in vivo*.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals, solvents, and other reagents

All materials used were of ACS grade or higher where appropriate. 2-mercaptoethanol, acetic acid, acetone, ammonium molybdate, calcium chloride, dithiothreitol, ethylenediaminetetraacetic acid (EDTA), magnesium sulphate, phytic acid, potassium chloride, sodium acetate, sodium chloride, sodium dodecyl sulphate (SDS), and sulphuric acid were obtained from Sigma Aldrich, Arklow, Ireland.

Five commercially available phytases from different bacterial and fungal sources were obtained for the purpose of this study. Phy 1 and Phy 5 were from bacterial sources and were characterised as 6-phytases. Phy 2, 3, and 4 were from fungal sources and were characterised as 3-phytases. Phytases were supplied by industrial sources.

Trace minerals came in the form of copper, iron, manganese, and zinc. Organic proteinates and inorganic sulphates were kindly supplied by Alltech. All other organic minerals were obtained from independent distributors. Commercial premixes were also supplied by Alltech and other independent distributors.

2.2 Methods

2.2.1 Mineral analysis

Elemental concentrations of Cu, Fe, Mn, and Zn from organic and inorganic trace mineral sources were analysed using inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent Technologies, Waldbronn, Germany). Briefly, 0.1 g of each mineral source was weighed in triplicate and digested with 10mL of HNO_3 for 35 minutes at 180 °C in a CEM Discover microwave (CEM Corporation, Matthews, NC). After digestion, the samples were diluted with >18 MΩcm water to the expected mineral concentration. Samples and standards were matrix-matched to 2 % HNO_3 prior to analysis.

2.2.2 Phytase activity assay

Commercial phytases were examined for phytase activity using a modified version of the assay described by Engelen *et al.* (1994). The assay was based on the hydrolysis of 0.5 mL aliquots of 2.5 mM phytic acid (from rice) in 0.2 M acetate buffer pH 5.0, by 0.5 mL of appropriately diluted phytase sample in 5 mM acetate buffer. After 10 minutes, the reaction was terminated by the addition of 2 mL of colour/stop solution. The colour/stop solution was composed of a 2:1:1 ratio of acetone, 10 mM ammonium molybdate, and 5 N sulphuric acid, respectively. Excess molybdate was bound by the addition of 0.1mL of 1 M citric acid. Blanks were prepared by adding colour/stop solution before the addition of phytic acid. Sample absorbance was then assessed at λ_{380nm} to determine the level of orthophosphorus release from the hydrolysis of phytic acid. Absorbance readings of the samples were converted to inorganic phosphorus (KH_2PO4) concentrations using a standard curve (ranging from 0.1 µmol/mL to 0.5 µmol/mL of KH_2PO4). Relative phytase activity was calculated thereafter.

Phytase activity was defined as the amount of enzyme that can liberate 1 µmol of inorganic phosphate per minute and is calculated as outlined below:

$$\frac{PU}{g} = \left(\frac{\Delta A_{380}\ x\ F\ x\ D}{10}\right)$$

ΔA_{380} = the difference in absorbance between the sample and the blank
F = the phosphate concentration (µmol/mL) corresponding to the absorbance ($\lambda_{380\ nm}$)
2 = multiplication factor to a standard of 1 mL
10 = the time of the reaction
D = the required dilution to be within the limits range of the standard curve

2.2.3 Commercial phytase preparation

Stock solutions of phytases were prepared in 5 mM acetate buffer (pH 5) and extracted for 1 hour at room temperature. Supernatant was collected by centrifugation (4000 rpm for 1 min9ute) and phytase was diluted appropriately to fall within assay absorbance parameters. Phytases were prepared fresh each day.

2.2.4 Optimal pH of commercial phytases

The effect of simulated gastric pH on phytase activity was assessed by incubating extracted phytase preparations for 15 minutes in different buffers. The pH range assessed was between 2.0 – 6.0, with increasing increments of 0.5. pH values were reflective of poultry gastric conditions, ranging from the highly acidic environment of the stomach (~ pH 2.5 – 3.5), to the mildly acidic environment of the crop (~ pH 4 – 5), to the neutral environment of the small intestine (~ pH 6). Buffers included glycine-HCl buffer (pH 2 and 2.5) and 50 mM acetate buffer (pH 3 – 6). Residual phytase activity was assessed in triplicate as detailed in Section 2.2.2.

2.2.5 Effect of potential modulators of phytase activity

The effect of metal ions and other potential modulators (as detailed in Section 2.1.1) on phytase activity was assessed by incubating phytase preparations in 1 mM and 10 mM treatments for 15 minutes. Treatments are fully detailed in Table 3.1. Residual phytase activity was assessed as detailed in Section 2.2.2.

2.2.6 Commercial mineral preparation

Stock solutions of minerals were prepared in 5 mM acetate buffer (pH 5). Minerals were extracted for 20 minutes at room temperature and supernatant was collected by centrifugation (4000 rpm for 1 minute). Further concentrations were prepared to reflect the levels of elemental mineral that would be employed at higher levels in industrial settings and relatively lower levels for Total Replacement Technology (TRT). Minerals were prepared fresh each day.

2.2.7 Effect of trace minerals on activity of commercial phytases

Phytases and minerals were prepared as detailed in Sections 2.2.3 and 2.2.6, respectively. The effects of individual inorganic and organic copper and iron mineral sources were assessed by incubating phytase preparations in mineral extracts for 15 minutes, followed by testing for residual phytase activity.

The effect of inorganic and organic mineral combinations on enzyme activity was assessed thereafter. Phytase preparations were incubated in the presence of a 2-way,

3-way and 4-way mineral mix composed of copper, iron, zinc, and manganese. The same experimental parameters were applied as previously described.

Sulphates were employed to represent inorganic trace minerals. Organic equivalents included proteinates, amino acid complexes, and chelates. Both inorganic and organic trace minerals were applied at rates indicative of those commonly used in the poultry industry and under TRT programmes.

2.2.8 Commercial premix preparation

Stock solutions of premixes were prepared in 5 mM acetate buffer (pH 5). Premixes were extracted for 20 minutes at room temperature and supernatant was collected by centrifugation (4000 rpm for 1 minute).

Premixes were normalised to one elemental mineral, i.e.: copper or iron. Further concentrations were prepared to reflect the levels of elemental mineral that would be applied in industrial settings and for TRT (as detailed in Table 2.1). Premixes were prepared fresh daily for each experiment.

Table 2.1 Recommended trace mineral levels for poultry premixes.

Level	Cu (ppm)	Fe (ppm)	Zn (ppm)	Mn (ppm)
Commercial	16	80	100	100
TRT	4	11	30	40

2.2.9 Effect of gastric pH on mineral-phytase interactions

Stock solutions of phytases and mineral were prepared as detailed in Sections 2.2.3 and 2.2.6. Phytase stock (9 mL) was then dispensed into sterilins in triplicate. Mineral mix (1 mL) was then added to the phytase. Phytase-mineral mixes were then incubated at 40 ºC for 15 minutes. After 15 minutes, the pH of the phytase-mineral mixes was adjusted to pH 2.5 using 1 M hydrochloric acid. They were then incubated at 40 ºC for 15 minutes. After each incubation step, residual phytase activity was measured as detailed in Section 2.2.2.

2.2.10 Statistical analysis

All data was tested for significance by a one-way analysis of variance (ANOVA) based on a confidence interval of 95 % unless otherwise stated. Data were analysed using the Minitab statistical software package, version 17.0 (Coventry, UK).

3 Results and Discussion

The supplementation of exogenous phytases to monogastric animal feeds has been described as an effective method of increasing the availability of phosphorus within feed as well as reducing its presence within faeces, subsequently minimising excretion into the environment (Selle and Ravindran, 2007; Dersjant-Li *et al.,* 2015). Trace minerals are also added to monogastric premixes, often at excessive levels, in an attempt to maximise production and avoid potential deficiencies (Leeson, 2003). Published literature has demonstrated that certain inorganic mineral salts can have an antagonistic effect on phytase function (Chantasartrasamee *et al,* 2005; Shao *et al.,* 2008; Greiner *et al.,* 2009; Rani and Ghosh, 2011; Yao *et al.,* 2014). Recent research conducted by Santos *et al.* (2015) reported that certain organic trace mineral sources had less of a negative impact on phytase function than inorganic sources.

Following initial biochemical assessments of commercially-available phytases (Section 3.1), an overall objective was established to determine the effects of inorganic and organic trace mineral sources on the activity of five phytases, the results of which are presented in Sections 3.2 – 3.4.

3.1 Influence of extrinsic factors on commercial phytases

The extent that an enzyme retains its function is dependent on a number of factors, such as pH, metal ions, salinity, and temperature. An enzyme can become reversibly or irreversibly denatured depending on the extent of damage caused by these external factors. Denaturing can be induced by disruption of disulfide bridging or other internal bonds that may stabilise the enzyme.

pH has a strong influence on enzyme function. Changes in pH can induce alteration of the catalytic site and affect the overall stability of enzymes. These catalytic groups are composed of amino acids, and are subject to protonation or deprotonation with changing pH. While minor changes in pH can have a negative impact on the overall function of an enzyme, major changes in acidity or alkalinity can cause complete denaturation (Bisswanger, 2014).

Metal ions have also been shown to have both positive and negative effects on enzyme function. They can reduce or enhance enzyme activity by binding to non-specific sites of the enzyme, as well as the active site, altering the enzyme's shape. They

can also bind directly to substrates thereby preventing enzyme-substrate binding (Berg and Jain, 2002). Metal ions have been shown to affect a broad range of enzymes commonly used in animal feeds. These effects have been previously reported on phytases (Yao *et al.*, 2014; Santos *et al.*, 2015), xylanases (Isil and Nilufer, 2005), and cellulases (Zeng *et al.*, 2016). A 10 mM treatment of Ca^{2+} was shown to have inhibitory effects on a xylanase from *Thermomyces* sp. (Gaffney *et al.* 2009). Conversely, a treatment of 1 mM Ca^{2+} was shown to enhance the activity of a xylanase produced from *Trichoderma* sp. (Isil and Nilufer, 2005). Furthermore, it is important to note that these metal ions, often in the form of inorganic salts, are supplemented to animal feeds at levels typically higher than recommended rates. This can lead to unwanted enzyme-mineral interactions, potentially leading to reduced enzyme function, which was the focus of the present study. This section focuses on the effect of pH and modulators on the activity of commercial phytases.

3.1.1 Effect of pH on commercial phytase activity

The optimum pH range of a phytase can vary greatly depending on its biological source. Phytases, often found in plants, bacteria, fungi, and yeasts, can be classed into either acid or alkaline phytases depending on their optimum pH range (Pandey *et al.*, 2001). In general, phytases have one pH optimum; however, some fungal phytases have been shown to have a secondary peak of activity (Han *et al.*, 1999). As previously mentioned in Section 1.2, phytases are classified based on their catalytic function. The majority of phytases used in animal nutrition are histidine acid phytases (HAPhys) which typically have optimal activity under acidic conditions. This makes them suitable in poultry nutrition, as well as in swine and fish diets, given the broad acidic pH range associated with the gastrointestinal tract of these animals (Konietzny and Greiner, 2002). pH is also an important consideration with regards to storage of an enzyme, as storage outside of its optimum pH can negatively impact performance in the long term (Bisswanger, 2014).

The effect of pH on phytase activity was assessed by incubating phytase preparations in glycine-HCl buffers (pH 2 and 2.5) and 50 mM sodium acetate buffers (pH 3 – 6). pH values were chosen based on poultry gastric conditions, ranging from the highly acidic environment of the stomach (~ pH 2.5 – 3.5), to the mildly acidic environment of the crop (~ pH 4 – 5), to the neutral environment of the small intestine

(~ pH 6). Commercial phytases (Phy 1 – 5) from different microbial sources were used for the purpose of this study. The relative activities of Phy 1 – 5 were presented in Figure 3.1 and were based on the highest phytase activity measured for each respective phytase.

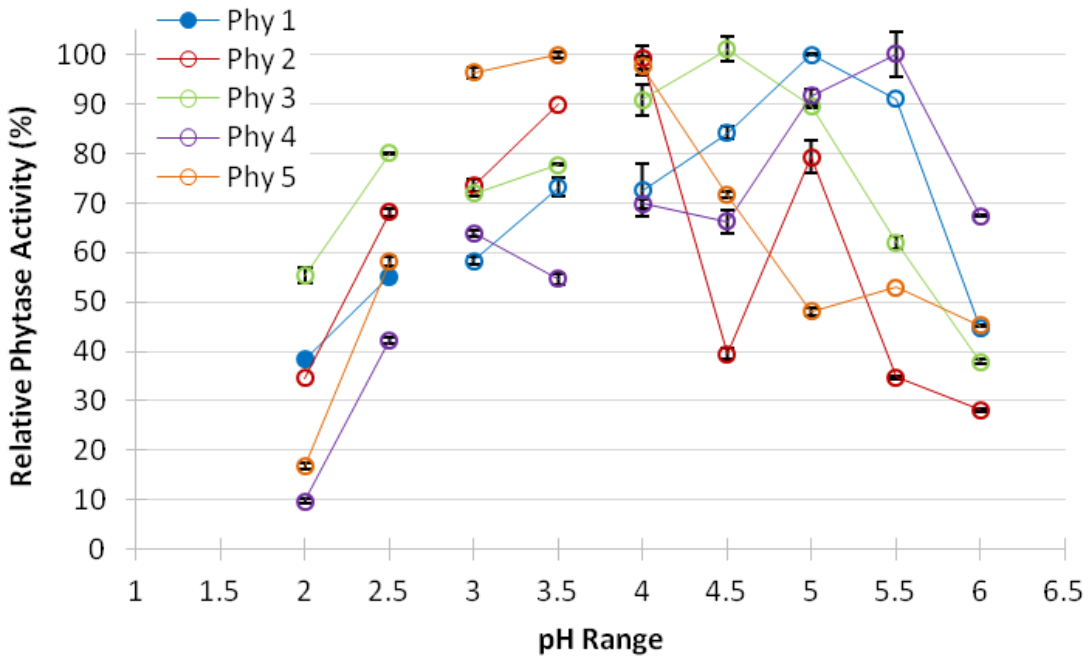

Figure 3.1 pH profiles of commercial phytases, Phy1 – 5.

Data was presented as means of triplicate values with standard deviation.

Phy 1, 3, and 5 were commercially-available bacterial phytases. Phy 2 and 4 were commercially-available fungal phytases. Phytases were subjected to pH values reflective of the poultry GIT, i.e.: pH 2 – 6 (increments of 0.5) to determine the effect on relative activity. Buffers included glycine-HCl buffer (pH 2 and 2.5) and 50 mM acetate buffer (pH 3 – 6).

Activities of all the commercial phytases studied varied over the gastric pH range assessed (Figure 3.1). Highest relative activities were observed from pH 3 – 5.5. Although Phy 1 and Phy 4 were from different microbial sources, both had optimal activities around pH 5 to 5.5, demonstrating similarities between biochemical characteristics of HAPhy enzymes. Phy 2 and Phy 4 were both from *A. niger* sources but had slightly different pH profiles which may have been due to the strain of *A. niger* used and the different fermentation techniques, Solid State Fermentation (SSF) and Submerged Fermentation (SmF).

The optimum pH range of Phy 1 was between pH 5 and 5.5, with relative activities of 100 % and 91.1 %, respectively. However, Phy 1 was considerably stable across a broad pH range (pH 3.5 – 5.5), only losing ~ 17.8 – 27.4 % of its activity when incubated at pH 3.5 – 4.5. This agrees with the literature as the pH optima of phytases produced from *E. coli* typically falls around pH 4.5 – 5.5 (Naves *et al.*, 2012; Shao *et al.*, 2008). In the present study, activity decreased outside of the optimum pH range; however, comparatively good levels were still retained. At pH 2.5 and 3, phytase activity was 55.1 % and 58.4 %, respectively; demonstrating that Phy 1 had moderate tolerance at more acidic pHs. The lowest levels of activity were observed at pH 2 (38.3 %) and pH 6 (44.7 %). These low levels of activity demonstrated the phytase's inability to dephosphorylate phytic acid/phytate at pH 2 and pH 6 which may an indication of lower activity in the stomach and small intestine (*in vivo*).

Phy 2 had optimal levels of activity from pH 3.5 – 4, with a second peak in activity at pH 5. The pH maximum of 3.5 – 4 was slightly lower than expected for a phytase produced and expressed by *A. niger* (Wyss *et al.*, 1999; Casey & Walsh, 2003); however, pH optima as low as 2.5 and 3.5 have been observed for certain fungal phytases, including *A. niger* (Wyss *et al.*, 1999; Quan *et al.*, 2004). At pH 5, a peak in activity (~ 80 %) was observed in Phy 2. Conversely, the second peak in activity has been typically observed below pH 3 (Greiner *et al.*, 2009). High levels of activity were also observed at pH 2.5 and 3 (68.1 % and 73.6 %, respectively), demonstrating that Phy 2 had a broad range of considerably high activity. Overall, the high levels of activity maintained at pH 2.5 – 3.5 demonstrated that Phy 2 may perform well in the acidic regions of the GIT *in vivo*. However, the lower levels of activity observed at mildly acidic to neutral pH values may indicate impacted ability of Phy 2 to dephosphorylate phytic acid/phytate in the small intestine. Igbasan *et al.* (2000) reported that phytases produced by *Aspergillus* sp. had a broad range of activity from pH 2 – 6,

retaining at least 50 % of their maximal activity in this range. In the present study, at least 30 % of activity was retained across the gastric pH range, with retentions of at least 70 % from pH 2.5 – 4 and at pH 5.

Phy 3 had highest levels of activity (89.1 – 100 %) from pH 4 – 5, with maximum activity at pH 4.5. This was similar to relevant literature with regards to phytases originating from *Buttiauxella* sp. (Shi *et al.*, 2008; Dersjant-Li *et al*., 2015). High levels of activity still remained at pH 2.5 – 3.5 (72 – 80 %); demonstrating the phytase's stability over a wide range of pH values associated with gastric pHs. Of all the commercial phytases assessed, Phy 3 exhibited the highest level of activity at pH 2 (55.4 %), showing the phytase's pH stability and potential for phytate hydrolysis in the stomach.

Phy 4 had a relatively narrow range of optimal activity; with highest activity levels observed at pH 5 and 5.5 (91.7 % and 100.1 %, respectively). Decreases in activity were observed outside this optimal pH range; however, activity was consistent between pH 3 and 4.5 (~ 54.7 – 69.9 % of maximum activity). The lowest level of activity (9.2 %) was observed at pH 2; showing the phytase's sensitivity to highly acidic environments. A comparatively high level of activity remained at pH 6 (67.4 %).

Phy 5 had a maximum activity range around pH 3 – 4, with the highest activity reported at pH 3.5. Kim *et al.* (2003) reported similar results for a phytase produced by *Citrobacter* sp. Outside of the optimal pH range, changing pHs negatively impacted activity of Phy 5. Similar to Phy 4, a reduction in activity of ~ 75 % was observed between pH 2 and 2.5, demonstrating the phytase's sensitivity to acidic environments. From pH 5 – 6, reductions in activity were observed (45.2 – 53.0 %); however, levels were relatively stable within this range. Overall, Phy 5 had a very narrow range of activity; pH had a detrimental impact on the phytase's activity outside of its optimal range (pH 3 – 4). These results indicated that the efficacy of Phy 5 may be affected at different areas of the poultry GIT, especially in the stomach.

All commercial phytases displayed a broad range of activity across the gastric pH range employed for this study. Although the optimum pH values of these enzymes fell between pH 3.5 – 5.5, differences in activity were observed outside of this range. All phytases retained at least 40 % activity when exposed to a pH range representing that of the stomach (pH 2.5 – 3.5), with Phy 5 displaying maximum activity at pH 3.5. Within this pH range, Phy 1 – 5 were subject to reductions in activity of ~ 15 – 40 % when acidity was increased from pH 2.5 to 2, indicating varying degrees of sensitivity

to increased acidity. In the majority of cases, at least 70 % activity was observed from pH 4 – 5, with the exceptions of Phy 2 at pH 4.5 and Phy 5 at pH 5. These results may be an indication of potential activity levels in the crop section of the GIT, which is of importance as the main area for exogenous phytase activity in poultry is within the crop and upper part of the GIT (Dersjant-Li *et al.,* 2015). Activity retentions of ~ 30 – 70 % were observed when Phy 1 – 5 were exposed to pH levels similar to that of the small intestine (pH 6). Phy 1 – 3 and Phy 5 displayed relative activities of 30 – 45 % at pH 6, demonstrating their similar catalytic responses at this pH, whereas Phy 4 retained ~ 70 % activity showing its higher tolerance level at pH 6.

As the optimum pH of the phytase enzymes studied were within the range of pH 3.5 – 5, an assay condition of pH 5 was employed for the study.

3.1.2 Effect of potential modulators of commercial phytases

Commercial phytases are typically produced via microbial fermentation, either through SSF or SmF. Mineral salts are one of the key components of the growth media responsible for sustaining microbial growth (Basu *et al.,* 2015). Similarly, mineral salts are often supplemented to animal feed to fulfill nutritional requirements. They can be added as macronutrients, such as calcium and phosphorus, or micronutrients, such as copper and iron (Lukić *et al.,* 2009). Traditionally, trace minerals, such as copper and iron, have been added to feed in their inorganic salt form (e.g.: $CuSO_4.5H_2O$, $FeSO_4.H_2O$). These compounds have the potential to interact with different high-value animal feed components, particularly enzymes like phytases, xylanases, and glucanases. Enzyme function can be influenced through interactions with amino acid residues at active catalytic sites or through alterations to enzyme structure (Ullah and Mullaney, 1996; Igamnazarov *et al.,* 1999). These interactions can result in agonistic and antagonistic modulation of enzyme activity (Tran *et al.,* 2011).

Herein, the effects of metal ions (typically found in animal feed) on phytase activity were assessed, as well as other potential modulators. A commercial phytase of bacterial origin was used for the purpose of this study. Phy 1 was exposed to 1 mM and 10 mM treatments of metal ions and other potential modulators. Residual phytase activity was measured after 15 minutes by means of a colorimetric assay as detailed in Section 2.2.2. Relative activities were expressed as a percentage of activity observed

after treatment with deionised water in Table 3.1. Statistical analysis (ANOVA) was completed to determine any significant differences across the group.

Phytase activity was significantly affected by a number of compounds at both 1 mM and 10 mM treatment concentrations ($p \leq 0.05$) (Table 3.1). Most notable was the significant inhibition of activity observed when the phytase was exposed to metallic coumpounds commonly added to animal feeds. Table 3.2 provides the elemental levels of each trace mineral per modulator treatment.

Table 3.1 Effect of metal ions and potential modulators of phytase activity from a bacterial source.

Relative activities (RA) are represented by mean triplicate readings with standard deviations compared to a control sample (water). Means lacking the superscript letter [(a)] in the same line differ significantly ($p \leq 0.05$) by way of the Dunnett's test.

Modulator	Chemical Formula	Treatment (1 mM)		Treatment (10 mM)	
		RA (%)	SD	RA (%)	SD
Water	H_2O	100.0 [a]	1.9	100.0 [a]	1.9
Calcium Chloride	$CaCl_2$	108.9	0.2	105.6	1.6
Copper Sulphate	$CuSO_4$	11.7	0.5	5.2	0.1
Ferrous Sulphate	$FeSO_4$	10	0.5	-	-
Magnesium Sulphate	$MgSO_4$	103.2 [a]	2.1	111.5	0.6
Manganese Sulphate	$MnSO_4$	89.1	1.7	82.4	1.3
Potassium Chloride	KCl	82.9	1.4	94.8	0.5
Sodium Chloride	NaCl	102.8 [a]	4.8	97.2 [a]	1.2
Zinc Sulphate	$ZnSO_4$	8.5	0.3	-	-
2-Mercaptoethanol	C_2H_6OS	95	1.8	90.1	2.7
Dithiothreitol	$C_4H_{10}O_2S_2$	89.2	1.1	89.3	0.6
SDS	$NaC_{12}H_{25}SO_4$	-	-	-	-
EDTA	$C_{10}H_{16}N_2O_8$	103.4 [a]	2.5	104.7	0.8

Table 3.2 Elemental levels of trace minerals per modulator treatment.

		Elemental concentration (ppm) per modulator treatment	
Trace Mineral	Metal ion	1 mM	10 mM
$CuSO_4.5H_20$	Cu	60	600
$FeSO_4.H_2O$	Fe	54	540
$MnSO_4.H_2O$	Mn	55	550
$ZnSO_4.H_2O$	Zn	64	640

As demonstrated in Table 3.1, treatment with 1 mM and 10 mM copper, ferrous, and zinc sulphate strongly inhibited phytase activity ($p \leq 0.05$). Enzyme activity was not detected after exposure to 10 mM ferrous and zinc sulphate, and little activity remained after 1 mM treatments (10 ± 0.5 % and 8.5 ± 0.3 %, respectively). As depicted in Table 3.2, 1 mM treatments of ferrous and zinc sulphates equated to 54 ppm Fe^{2+} and 64 ppm Zn^{2+}; 1.5 – 2-fold lower than application rates recommended for use in poultry nutrition. Similarly, exposure to cupric sulphate at levels higher than 1 mM resulted in < 10 %

phytase activity. It is widely documented that these metal ions can have a highly inhibitory effect on phytase activity (Igamnazarov *et al.,* 1999; Konietzny & Greiner, 2002; Tai *et al.,* 2013; Monteiro *et al.*, 2015). It has been suggested that inhibitory effects can arise from enzyme-mineral interactions, as well as phytate-mineral interactions, particularly in the case of Fe^{2+} (Greiner & Farouk, 2007). Although cupric, ferrous and zinc sulphates had highly inhibitory effects on phytase activity in the present study, it is important to note that not all phytases may be affected in the same fashion. Monteiro *et al.* (2015) demonstrated that a phytase from *A. niger* UFV-1 retained 81.2 % and 87.7 % activity after exposure to 5 mM treatments of $CuSO_4$ and $ZnSO_4$, respectively. Conversely, Quan *et al.* (2004) observed that a 1 mM treatment of Zn^{2+} reduced activity of a phytase from *Cladosporium* sp. FP-1 by 46 %. These findings demonstrate the different responses of phytases to mineral interference, highlighting the different properties of microbial phytases.

In the present study, exposure of a bacterial phytase (Phy 1) to Ca^{2+} ions slightly improved activity ($p \leq 0.05$). Higher Ca^{2+} stimulations of phytase activity in BPPhy-producing strains of bacteria have been described in the literature (Igamnazarov, 1999; Sariyska *et al.,* 2005; Suleimanova *et al.,* 2015). However, the slight increase in phytase activity observed in the present study does not strongly imply that Ca^{2+} is required for optimal phytase activity. Furthermore, Ca^{2+} activation is not a common biochemical characteristic of acid phytases (Oh *et al.,* 2004). In addition to this, EDTA did not have an inhibitory effect on phytase activity, further suggesting that the phytase in question did not require metal ions, like Ca^{2+}, for maximal activity (Tai *et al.,* 2013). Exposure to 10 mM treatments of magnesium sulphate also slightly improved phytase activity (+ 11.5 ± 0.6 %). Similar responses have been seen in phytases produced by *E. coli* (Tai et al., 2013).

It is also important to note that treatment with protein denaturing agents (2-mercaptoethanol and dithiothreitol) only slightly affected phytase activity, resulting in reductions of 5 – 11 % at both treatment levels. These denaturing agents disrupt disulfide bridging within the enzyme, altering its structure and ultimately its activity (Ullah *et al.,* 2005). These results may have indicated that strict rigidity was not a prerequisite of enzyme function.

It can be concluded that various types of compounds can have an impact on phytase function. The trace minerals copper, ferrous and zinc sulphate, had the most inhibitory effect on phytase activity, which is highly significant as they are the mineral

form traditionally used in monogastric feeds. From these findings, the influence of copper and iron sources and their effective concentrations were further investigated in Section 3.2.

3.2 Effect of individual feed-grade mineral sources on activity of commercial phytases

As previously mentioned in Section 1.3, minerals are classified as either macro or micro, depending on their required concentrations and function. They play a central role in many metabolic systems within animals, and are required for overall good health. Minerals have been utilised in the poultry industry since the 1950s, largely in the form of inorganic sulphates, oxides, and carbonates. These trace minerals are often added at excessive levels, in an attempt to avoid potential deficiencies by exceeding dietary needs, as well as trying to maximise production and performance (Nollet *et al*., 2007). However, research has demonstrated that minerals in their inorganic form may not fulfil poultry nutritional requirements, with efficacy compromised by their lack of bioavailability. Although sulphates are considered more bioavailable than oxides, they are still not as readily absorbed by the animal (Nollet *et al.,* 2007). This lack of bioavailability and excess concentration can lead to mineral excretion which can accumulate in the environment, potentially leading to land pollution and eutrophication of water sources (Jungbloed *et al.,* 1999; Grana *et al,* 2013).

In recent years, interest in organic trace mineral sources has garnered more attention due to their increased bioavailability and overall stability (Carvahlo *et al.,* 2015). Furthermore, it is suggested that it is not necessary to add organic trace minerals to animal feeds at the high levels proposed by the industry for inorganics because of the enhanced characteristics of organic minerals in the GIT and their reduced propensity to interact with other dietary components (Asku *et al.,* 2010; Manangi *et al.,* 2012).

3.2.1 Effect of copper-based mineral sources on the activity of commercial phytases

Copper plays a key role in many metabolic processes, including immune functions, energy production, and acting as a co-factor for certain enzymes, such as lysyl oxidase which is responsible for the maturation of collagen subunits into more stable protein

structures (Richards *et al.,* 2010). The EU feed industry recommends the supplementation of copper to poultry feed at an inclusion rate of 16 ppm (EFSA, 2016).

As previously mentioned in Section 3.1.2, a number of compounds had a significant effect on phytase activity. Most notable of these results was the inhibitory effect of feed-grade inorganic trace minerals on phytase activity. Following on from these results, the effect of individual feed-grade mineral sources (at levels consistent with feed inclusion) on the activity of commercial phytases, Phy 1 – 5, was investigated. Commercial phytases were incubated in the presence of inorganic and organic sources of copper as per Section 2.2.7. Copper sulphate ($CuSO_4.5H_2O$) was used as an inorganic source of copper as it is typically used in poultry nutrition. Organic sources of copper employed were in the form of proteinates, chelates, and amino acid complexes. As previously illustrated in Table 2.1, inorganic minerals are typically added at high levels to animal feeds as guided by the EU feed industry (NRC, 1994; ESFA, 2016), whereas organic minerals tend to be added at lower levels. Certain practises involve total replacement technology (TRT), i.e.: the complete replacement of an inorganic mineral source with an alternative organic one based on greater bioavailability (Nollet *et al.,* 2007). The effects of both inorganic and organic minerals on phytase activity were assessed at TRT (4 ppm copper) and commercial (16 ppm copper) levels. Residual phytase activity was assessed as detailed in Section 2.2.2.

The effect of copper-based minerals, at both TRT and commercial levels, on the activity of commercial phytases (Phy 1 – 5) is presented in Table 3.3 (a) and (b). Copper sources assessed included inorganic feed-grade copper sulphate (ITM) and organic feed-grade proteinates (OTM 1 and 4), a chelate (OTM 3) and an amino acid complex (OTM 2). All copper sources had an inhibitory effect on activity of Phy 1 – 5; however, the level of inhibition appeared to be dependent on the copper source and concentration, as well as individual phytase source.

Table 3.3 Effect of copper-based mineral sources at (a) TRT and (b) commercial levels on activity of commercial phytases.

Relative activities are presented as means of triplicate values from triplicate experiments, with corresponding deviations. Statistical analysis is presented as a one-way ANOVA with Tukey's test. Trace minerals included: copper sulphate (ITM), a proteinate (OTM 1), an amino acid complex (OTM 2), an amino acid chelate (OTM 3), and alternative proteinate (OTM 4).

(a)

Mineral Source		**Phy 1**		**Phy 2**		**Phy 3**		**Phy 4**		**Phy 5**	
		RA (%)	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*
ITM	*TRT*	75.45 ^{a,b}	2.6	35.2 ^{a}	3.5	75.7 ^{a,b}	9.8	66.2 ^{a}	2.6	45.7 ^{a}	2.6
OTM 1	*TRT*	78.5 ^{a}	1.7	37.4 ^{a}	2.5	81.7 ^{a,c}	3.2	70.3 ^{b}	1.8	66.0 ^{b}	2.3
OTM 2	*TRT*	73.5 ^{b}	0.9	52.4 ^{b}	2.2	73.0 ^{b}	1.1	83.8 ^{c}	1.2	55.9 ^{c}	3.9
OTM 3	*TRT*	77.0 ^{a}	2.9	53.0 ^{b}	8.2	84.4 ^{c}	1.1	71.4 ^{b}	2.9	75.8 ^{d}	3.9
OTM 4	*TRT*	73.2 ^{b}	3.1	34.7 ^{a}	1.7	75.7 ^{a,b}	1.8	76.0 ^{d}	1.8	57.8 ^{c}	3.5

(b)

Mineral Source		**Phy 1**		**Phy 2**		**Phy 3**		**Phy 4**		**Phy 5**	
		RA (%)	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*
ITM	*Commercial*	42.6 ^{a}	4.1	12.9 ^{a}	2.1	42.8 ^{a}	3.0	34.3 ^{a}	1.5	16.1 ^{a}	1.1
OTM 1	*Commercial*	50.9 ^{b}	2.1	15.6 ^{a}	4.4	57.4 ^{b,c}	2.8	62.1 ^{b}	1.8	28.5 ^{b}	1.2
OTM 2	*Commercial*	46.4 ^{c}	1.7	28.5 ^{b}	1.3	39.7 ^{d}	1.4	59.8 ^{c}	1.2	17.7 ^{a}	1.4
OTM 3	*Commercial*	57.4 ^{d}	2.0	24.3 ^{c}	2.8	58.8 ^{b}	1.1	61.4 ^{b,c}	1.2	36.2 ^{c}	0.7
OTM 4	*Commercial*	45.3 ^{a,c}	0.9	19.8 ^{d}	1.3	55.5 ^{c}	2.2	67.4 ^{d}	1.2	27.1 ^{b}	1.3

Inorganic trace minerals are added to animal feeds with large safety margins to help prevent potential mineral deficiencies and maximise potential production outputs (López-Alonso, 2012). Relevant literature has shown organic trace minerals to be more bioavailable to the animal and consequently, are typically added at lower inclusion rates (Bao *et al.*, 2007). Taking this into account, the impact of inorganic and organic sources of copper on phytase activity was compared at both TRT and commercial levels to gain perspective on an *in vitro* level.

Varying degrees of inhibition were observed when Phy 1 – 5 were exposed to inorganic copper (ITM) at TRT and commercial levels. At TRT levels (4 ppm copper), decreases of 25 – 65 % were observed after exposure to ITM across the range of phytases (Table 3.3 (a)). Higher mineral levels, reflective of inclusion in a commercial setting (16 ppm copper), had a greater inhibitory effect of activity of Phy 1 – 5, resulting in reductions of ~ 57 – 87 % (Table 3.3 (b)). Similarly, different levels of inhibition were observed when Phy 1 – 5 were exposed to OTM 1 – 4 at TRT and commercial levels. In the majority of cases, the least inhibition was observed when Phy 1 – 5 were exposed to OTM 1 – 4 at TRT levels. A greater level of inhibition was seen when commercial phytases were exposed to organic copper sources at higher commercial levels; however, in most cases, relative phytase activity was at least 40 %. The phytase source also appeared to have an effect on the outcome of mineral interactions. For example, exposure of Phy 2 and Phy 3 to TRT levels of ITM resulted in reductions of 65 % and 25 %, respectively. Phy 2 was from a fungal source, whereas Phy 3 originated from a bacterial source and was expressed in a fungal host, which may be indicative of the different responses observed. Overall, these results indicated copper source and concentration, as well as the phytase source influenced activity of Phy 1 – 5.

In the case of Phy 1, a bacterial phytase, ITM significantly reduced activity by 57.4 % at commercial levels ($p \leq 0.05$), whereas OTM 1 – 4 has less of an inhibitory effect at TRT levels, reducing activity by only 21.5 – 26.8 %. The reduction in activity after exposure to ITM was not unexpected due to the high levels of inhibition caused by 1 mM treatments of copper sulphate in Section 3.1.2. It was also interesting to observe that the increase in ITM concentration (from TRT to commercial levels) caused a further decrease in activity of approximately 43 %, whereas increasing the concentration of OTM 1 – 4 reduced activity by ~ 25 – 36 %; potentially indicating greater stability of organic copper sources in certain instances. Exposure to OTM 1 and OTM 3 resulted in significantly higher retentions of phytase activity in comparison to exposure to OTM 2

and OTM 4 ($p \leq 0.05$). Organic copper sources included two proteinates (OTM 1 and OTM 4), an amino acid complex (OTM 2), and an amino acid chelate (OTM 3). These results demonstrated that copper source influenced phytase efficacy and that the amino acid complex (OTM 2) may have had different properties to the other chelated mineral sources. Although Pang and Applegate (2006) determined that copper concentration and source had an effect on the ability of a bacterial phytase to hydrolyse phytic acid, they reported that copper lysinate, an amino acid complex, didn't impact activity as much as inorganic copper sources. At pH 5.5, the presence of inorganic copper sulphate and copper chloride significantly reduced phosphorus release by approximately 20 – 80 % (Pang and Applegate, 2006).

The efficacy of Phy 2, a fungal phytase, was negatively affected by ITM at commercial levels. Activity was reduced by 87.1 % after exposure to the inorganic copper sulphate (Table 3.3 (b)). It has been demonstrated in the literature that phytases produced by *A. niger* can be strongly inhibited by 1 mM $CuSO_4$. Soni *et al.* (2010) reported that copper sulphate negatively impacted the activity of *A. niger* NCIM 563 by 89 %. Similarly, relative activity of just 4.6 % was observed after a phytase produced from *A. niger* 306 was exposed to copper sulphate at levels as low as 1 nM. Higher levels of phytase activity remained after exposure to OTM 1 – 4; however, organic mineral source still had quite a negative effect on phytase function, with only 34.7 – 53.0 % of total activity retained. Exposure to OTM 2 and OTM 3 had the lowest level of impact on relative activity of Phy 2, which was also reflected when the concentration of these OTMs was increased to commercial levels. It was evident that copper, in both its inorganic or organic form, had a negative impact on the ability of Phy 2 to hydrolyse phytic acid.

The ability of Phy 3 to dephosphorylate phytic acid was hampered by the presence of ITM. Activity was significantly reduced to 75.7 % after exposure to the inorganic copper source at TRT levels. After increasing the concentration of ITM to commercial levels, relative phytase activity dropped to 42.8 %, representing a decrease of ~ 43 %. Conversely, this retention was higher than results reported in published literature whereby a phytase produced by *Buttiauxella* sp. was highly susceptible to Cu^{2+} ions (Shi *et al.*, 2008). OTM 1 – 4 had less of an inhibitory effect on activity, with reductions ranging from 15.6 – 27.0 %. Some significant differences were observed between organic copper mineral sources at lower TRT levels; however, OTM 2 induced

a significantly higher level of inhibition in comparison to ITM and the other OTMs at higher commercial levels ($p \leq 0.05$), in a similar trend to Phy 1.

A significant reduction in activity (65.7 %) was detected when Phy 4 was exposed to ITM at commercial levels ($p \leq 0.05$), which represented a 48 % decrease in activity following exposure to TRT levels. Similar to the other phytases previously mentioned, OTM 1 - 4 had less of a negative effect, reducing activity by only 16.4 – 29.7 % at TRT levels (Table 3.3 (a)). In addition to this, significant differences were observed between the organic copper sources ($p \leq 0.05$), with OTM 2 causing the lowest levels of inhibition. This again demonstrated that each organic copper source affected the ability of Phy 4 to degrade phytic acid. In this instance, the amino acid complex caused significantly less inhibition of phytase activity in comparison to the other organic sources. Similar results were seen when commercial phytases were exposed to copper lysinate; they concluded that this may have been due to copper lysinate forming less insoluble copper-phytate structures compared to copper sulphate and copper chloride (Pang and Applegate, 2006). Interestingly, the increased concentration of organic copper at commercial levels inhibited the activity of Phy 4 to a lesser extent than the previous phytases mentioned (Table 3.3 (b)). This was represented by ~ 10 – 30 % reduction of activity seen after exposure to TRT levels of OTMs. This demonstrated that the activity of Phy 4 may not have been dependent on the concentration of organic copper source (within the limits typically used in poultry feed).

Similar to Phy 4, a significant reduction in activity (45.7 %) was observed when Phy 5 was exposed to ITM at TRT levels ($p \leq 0.05$). A further reduction of 29.6 % was seen when Phy 5 was exposed to higher commercial levels of ITM, representing a 65 % loss in activity between the two concentration levels. Similar results were observed in a phytase produced by *Citrobacter* sp., whereby low concentrations of Cu^{2+} strongly inhibited activity (Kim *et al.,* 2003). Exposure to organic copper sources resulted in significantly higher retentions in phytase activity. Relative activity ranged from 55.9 % to 75.8 % after exposure to OTM 1 – 4. Highest phytase activity was observed after exposure to proteinate sources of copper (OTM 1 and 4); however, OTM 1 had more of a negative effect than OTM 4. Increasing the concentration of OTMs to commercial levels (16 ppm) caused further reductions in phytase activity of up to ~ 68 % of those observed after exposure to OTMs at TRT levels. Phy 5 was similar to Phy 2 as both inorganic and individual copper sources had a considerable impact on their efficacies.

Overall, the results in the present study demonstrated that copper was influential to the ability of phytases to dephosphorylate phytic acid and consequently, its ability to release inorganic phosphate. In the case of Phy 2 (a 3-phytase from a fungal source) copper source and concentration had a highly negative impact on phytase activity ($p \leq 0.05$). These effects were observed with both inorganic and organic Cu sources at TRT and commercial rates, and may have been due to its mode of action and biological source. Similarly, both organic and inorganic copper sources (at the higher commercial inclusion levels) caused high inhibition in the phytate-degrading ability of Phy 5 which was a 6-phytase from a bacterial source. Some significant differences were observed between organic copper sources at TRT levels in the cases of Phy 1, Phy 3, and Phy 4, indicating dependence on organic copper source in certain instances. The phytase function of Phy 4 did not appear to be highly dependent on the concentration of organic copper sources; however, its activity was dependent on the concentration of inorganic copper source. These results demonstrated the diverse range of phytase activities in response to copper sources and concentrations, indicating individual mode of action is an important factor when predicting retained phytase function in feed.

3.2.2 Effect of iron-based mineral sources on the activity of commercial phytases

Iron is another trace mineral that plays a vital role in animal health and performance. It forms part of many proteins, such as haemoproteins like haemoglobin and myoblobin, and non-haemoproteins like ferritin. It also acts as a co-factor for many iron-dependent enzyme systems (Shinde *et al.,* 2011). Like copper (and other trace minerals), iron is traditionally supplemented to animal feeds in an inorganic form and in excess amounts in an attempt to maximise production at low cost (Yang *et al.,* 2011). The National Research Council had previously recommended iron supplementation levels of 80 ppm for poultry (NRC, 1994); however, iron is often reported to be used at much higher levels than these recommendations. Phytic acid is a known chelator of many divalent metal ions, such as calcium, copper, iron, and zinc. Mineral deficiencies can occur in animals due to mineral-phytate complexes forming from the interaction between phytic acid and minerals. Phytate complexes can be soluble or insoluble depending on the pH at which they form, which affects mineral uptake (Akter *et al.,* 2015). Ferrous sulphate

has been shown to be a potent inhibitor of phytate hydrolysis (Maenz *et al.,* 1999; Kalsi *et al.,* 2016).

As demonstrated in Section 3.1.2, exposure of Phy 1 to 1 mM ferrous sulphate resulted in a reduction in phytase activity of ~ 90 %, demonstrating the highly inhibitory nature of ferrous sulphate. It was also important to note that 1 mM of ferrous sulphate had an elemental iron concentration of 54 ppm, which was 40 % lower than the levels often used in the poultry industry (in excess of 80 ppm). Following on from these results, the effect of individual feed-grade mineral sources on phytase activity was investigated. Commercial phytases were incubated in the presence of inorganic and organic sources of iron. Ferrous sulphate was utilised as an inorganic source of iron as it is commonly used in poultry nutrition. Organic sources of iron included two proteinates (OTM 1 and 3) and an amino acid complex (OTM 2). The effects of both inorganic and organic minerals on phytase activity were assessed at TRT and commercial levels. As depicted in Table 2.1, inorganic minerals are typically added at high levels to animal feeds as guided by the EU feed industry, whereas organic minerals are typically added at lower levels, in this case at TRT levels. In the case of iron, inorganic minerals are typically added at 80 ppm, whereas organic minerals are added at 11 ppm. In the present study, a reflective value for each organic and inorganic level was used; 2 ppm and 15 ppm for TRT and commercial levels, respectively, due to the highly inhibitory nature of iron. Residual phytase activity was assessed as detailed in Section 2.2.2.

The effect of iron-based minerals, at both TRT and commercial levels, on the activity of commercial phytases (Phy 1 – 5) is presented in Table 3.4 (a) and (b). All iron sources had an inhibitory effect on activity of Phy 1 – 5; however similar to copper sulphate, the level of inhibition appeared to be dependent on the iron source and concentration, as well as the phytase source.

Table 3.4 Effect of iron-based mineral sources at (a) TRT and (b) commercial application rates on activity of commercial phytases.

Relative activities are presented as means of triplicate values from triplicate experiments, with corresponding deviations. Statistical analysis is presented as a one-way ANOVA with Tukey's test. Trace minerals included: iron sulphate (ITM), a proteinate (OTM 1), an amino acid complex (OTM 2), an alternative proteinate (OTM 3).

(a)

Mineral Source		**Phy1**		**Phy2**		**Phy3**		**Phy4**		**Phy5**	
		RA (%)	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*
ITM	*TRT*	81.4 [a]	1.5	76.8 [a]	1.7	73.0 [a]	2.1	88.2 [a]	1.7	82.5 [a]	1.9
OTM 1	*TRT*	66.1 [b]	1.0	72.8 [a,b]	4.2	54.6 [b]	1.3	50.6 [b]	1.3	37.7 [b,c]	1.7
OTM 2	*TRT*	75.2 [c]	2.7	72.2 [b]	4.1	17.3 [c]	1.9	39.0 [c]	2.4	27.2 [b]	1.4
OTM 3	*TRT*	65.3 [b]	3.0	82.1 [c]	2.6	48.3 [d]	2.8	77.3 [d]	1.8	43.2 [c]	4.7

(b)

Mineral Source		**Phy1**		**Phy2**		**Phy3**		**Phy4**		**Phy5**	
		RA (%)	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*	*RA (%)*	*SD (±)*
ITM	*Commercial*	28.3 [a]	1.7	23.3 [a]	1.2	26.4 [a]	0.6	35.9 [a]	1.0	23.4 [a]	0.7
OTM 1	*Commercial*	39.3 [b]	1.1	49.9 [b]	3.2	16.4 [b]	0.1	15.7 [b]	2.6	23.4 [a]	0.6
OTM 2	*Commercial*	43.6 [c]	0.6	35.3 [c]	2.5	8.6 [c]	0.6	40.9 [c]	0.7	13.5 [b]	0.4
OTM 3	*Commercial*	61.5 [d]	1.3	67.7 [d]	0.6	32.0 [d]	0.6	72.5 [d]	1.1	46.3 [c]	1.5

A range of relative phytase activities were observed after the exposure of Phy 1 – 5 to organic and inorganic sources of iron (Table 3.4). Inorganic ferrous sulphate (ITM) at TRT levels caused the least amount of phytase inhibition in all cases ($p \leq 0.05$). Although ferrous sulphate appeared less inhibitory to Phy 1 – 5, only reducing activity by a maximum of ~ 27 %, it is important to note that it is not as readily available to animals in comparison to organic sources, thus this supplementation level wouldn't be effective *in vivo*. Iron supplementation of poultry feed to be effective *(in vivo)* from 25 – 150 ppm (Arnaudova-Matey *et al.*, 2013), thus it is unlikely that $FeSO_4$ would be applied at lower levels in normal circumstances. Reductions of up to ~ 70 % were observed when higher levels (15 ppm) of ITM were applied; demonstrating the influence of inorganic iron concentration on phytase activity. It is possible that low levels of ITM (2 ppm) were not high enough to influence the phytase or phytate present; however, this concentration was used to keep the concentrations relative to each other. In the majority of cases, exposure to OTMs resulted in higher levels of phytic acid degradation at both reflective TRT and commercial levels.

Results in Table 3.4 (a) illustrated that relative activity of Phy 1 was reduced by 18.6 % when TRT levels of ITM were applied. A further reduction (53.1 %) in activity was observed with commercial levels of ITM ($p \leq 0.05$) (Table 3.4 (b)). ITM at commercial levels had a more inhibitory impact on the activity of Phy 1 compared to ITM at TRT levels. These results demonstrated that the concentration of ITM used had a significant effect on the activity of Phy 1 ($p \leq 0.05$). These results were in agreement with published literature; Akter *et al.* (2015) demonstrated that iron significantly reduced the ability of Quantum Blue to hydrolyse phytate-iron complexes. Similarly, Maenz *et al.* (1999) observed that Fe^{2+} was a potent inhibitor of phytase activity.

In comparison to ITM, exposure to organic iron sources, OTM 1 – 3, resulted in significantly higher levels of phytase activity (65.3 – 75.2 %) when reflective TRT levels were applied. This was possibly due to the higher level of stability often observed in organic mineral sources (Arnaudova-Matey *et al.*, 2013; Manangi *et al.*, 2012). Phy 1 was least affected by OTM 2, followed by OTM 1 and 3. OTM 2 was an amino acid complex, whereas, OTMs 1 and 3 were proteinate sources of iron. Significant differences were seen between relative activities after exposure to OTM 2 and OTMs 1 and 3 ($p \leq 0.05$), which indicate that the source of organic iron had an influence on activity. The most probable reason that less inhibition occurred with the organic trace

minerals was enhanced stability (Tamin and Angel, 2003). The higher application rates of OTM 1 and OTM 2 resulted in further decreases in phytase activity; however, this was not observed with OTM 3. An increased concentration of OTM 3 did not cause further inhibition, demonstrating that the activity of Phy 1 was independent to iron concentration for this particular organic proteinate.

ITM at higher commercial levels had the most inhibitory effect on activity (retention of 23.3 %) of Phy 2. The increase from reflective TRT levels to commercial levels caused a 69 % reduction in activity, demonstrating that Phy 2 activity was dependent of ITM concentration. Organic mineral sources, OTM 1 – 3, at lower TRT levels were significantly less inhibitory to Phy 2 ($p \leq 0.05$). OTM 1 – 3 at higher commercial levels also had less of an effect on phytase activity (retentions of ~ 35.3 – 67.7 %) than ITM at commercial levels. Exposure to OTM 3 resulted in significantly higher activity retentions in comparison to OTM 1 and 2 ($p \leq 0.05$). Exposure of Phy 2 to a higher concentration of OTM 3 resulted in a reduction of 18 %, whereas reductions of 31 % and 51 % were observed in the cases of OTM 1 and OTM 2, respectively. This illustrated that individual organic Fe sources also had the potential to impact phytase activity differently.

Results in Table 3.4 (a) showed that ITM at TRT levels had the least effect on activity of Phy 3 (retention of 73 %). Considerable decreases in activity were observed when ITM was increased to reflective commercial levels, representing a 63 % reduction in activity from levels seen after exposure to TRT levels (Table 3.4 (b)). It has been reported in the literature that inorganic sources of iron can have a highly inhibitory effect on activity of bacterial phytases. Iron chloride was found to be highly inhibitory to an *E. coli* phytase over-expressed in *P. pastoris,* resulting in levels as low as 13.3 % (1 mM) and 19.3 % (5 mM) (Tai *et al.,* 2013).

In the present study, both organic and inorganic sources of Fe were quite inhibitory to phytate hydrolysis ability of Phy 3. Furthermore, the effects of organic minerals on the activity of Phy 3 were more detrimental than on Phy 1 and Phy 2. Table 3.4 (a) illustrated activity retentions of 17.3 – 54.6 % after exposure to OTM 1 – 3, with OTM 2 causing the highest level of inhibition. Exposure to OTM 1 and OTM 3 (at TRT levels) resulted in significantly higher levels of activity in comparison to ITM (at commercial levels) ($p \leq 0.05$). Higher levels of activity may have been observed due to the type of chelation associated with proteinates, which enhances their stability (Santos

et al., 2015). OTM 2 had the most negative impact on activity of Phy 3, resulting in an activity retention of 17.3 %, which may have happened due to greater levels of dissociation, as OTM 2 was an amino acid complex with different degrees of binding strength compared to proteinates..

ITM at TRT levels had the least inhibitory effect on activity of Phy 4 (Table 3.4 (a)). A further reduction of 52.3 % was observed when commercial levels of inorganic Fe were applied ($p \leq 0.05$), demonstrating that the concentration of inorganic Fe used had an effect on the ability of Phy 4 to hydrolyse phytate. Akter *et al.* (2015) also demonstrated that Fe^{2+} levels of 70 – 90 ppm negatively impacted phytate hydrolysis.

Organic Fe sources had less of a negative impact on phytase activity at TRT levels in comparison to ITM at commercial levels (Table 3.4). Exposure to OTM 1 – 3 resulted in reductions of activity levels of 22.7 – 61 %, with OTM 2 again having the most negative impact. As previously mentioned, the stabilities of OTM 1 and 3 might be higher than that of OTM 2 due to the different bond strengths, i.e.: OTM 1 and 3 were chelated organic sources, whereas OTM 2 was an amino acid complex. This may have accounted for the higher levels of activity observed after incubation with OTM 1 and 3. Significant differences were observed between organic iron sources, which may have indicated that phytase activity was dependent on organic iron source used. As with Phy 1, the activity of Phy 4 did not decrease further with increased levels of OTM 3, implying that the activity of this phytase did not depend on the concentration of this organic mineral. Increasing the level of OTM 2 also did not affect the activity of Phy 4, showing that phytase activity was independent of OTM 2's concentration.

Overall, incubation of phytase with iron mineral sources had an inhibitory effect on the activity of Phy 5 (Table 3.4). As observed with Phy 1 – 4, increasing the concentration of ITM further reduced the activity of Phy 5; representing the greatest reduction in activity over all of the phytases (~ 79 %). This result implied that Phy 5 was most susceptible to ITM at commercial levels. Exposure to organic iron at lower TRT levels resulted in reductions in activity of 56.8 – 72.8 % (Table 3.4 (a)). Commercial levels of OTM 1 and OTM 2 had an additional negative impact on Phy 5, resulting in relative activities of 23.4 % and 13.5 %. No significant differences were observed between ITM and OTM 2 at commercial levels. These results indicated that Phy 5 was highly susceptible to negative interactions from both inorganic and organic mineral sources. Tamim and Angel (2003) reported similar reductions in phytate

hydrolysis after exposure to both inorganic and organic mixes of minerals (including iron). Another potential reason may have been due to the pH properties of Phy 5. Phytate-mineral destruction has been shown to be pH dependent owing to different solubilities (Akter *et al.*, 2015; Pang and Applegate, 2006). In addition, the pH optimum of phytases may also influence this action; Phy 5 was operating above its optimal pH (4), thus may not have been capable of efficient hydrolysis. Moreover, it is possible that there were insoluble phytate complexes present in the mixture that may have also led to decreased inorganic phosphate release, hence lower relative activity was observed. Similar to Phy 1 and Phy 4, increased levels of OTM 3 did not further impact phytase activity suggesting that concentration of this mineral may not hinder activity of Phy 5.

Overall, the results in the present study demonstrated that iron was influential to the ability of Phy 1 – 5 to break down phytic acid. Negative impacts of ITM were observed across all phytases at TRT levels, with further reductions in activity of 59 – 72 % when the ITM concentration was increased to reflective commercial levels. This suggested that phytase activities of Phy 1 – 5 were dependent on the concentration of inorganic iron applied. Various responses were seen when Phy 1 – 5 were exposed to TRT and commercial levels of OTMs. In most cases, a high degree of phytase inhibition was observed after exposure to commercial rates of OTMs due to the higher concentration of iron. Higher levels of OTM 1 affected Phy 1 – 5 to different degrees; with further reductions of 31 – 70 % detected. Similar levels of reduction were observed when all phytases (excluding Phy 4) were exposed to OTM 2 at commercial levels (42 – 51 %). Higher commercial levels of OTM 3 only negatively impacted Phy 2 and Phy 3. These results demonstrated that iron source and concentration, in addition phytase source can all have an influence on enzyme activity.

3.3 Effect of feed-grade mineral mixes on activity of commercial phytases

In general, trace minerals are not added to poultry diets individually, but rather incorporated as a premix containing a number of different trace minerals, as well as vitamins. These premixes are designed to optimise health and performance of poultry. It is important for nutritionists to identify optimal concentrations of these dietary components to minimise any potential negative interactions between them, while fulfilling the nutrient requirements of the animal (Henry & Miles, 2000). Common trace

minerals added to poultry diets include copper, iron, zinc, and manganese. As demonstrated in Sections 3.1 and 3.2, these minerals, although present in trace amounts, can have a huge effect on phytate hydrolysis. Additionally, agonistic or antagonistic interactions may occur between individual trace minerals due to their stability or lack thereof (Henry and Miles, 2000). These interactions between inorganic trace minerals can occur in the GIT due to their readiness to dissociate at low pHs (Richards *et al.*, 2010). For example, high levels of zinc can impair copper absorption in the intestine, resulting in copper-based deficiencies. Similarly, high levels of copper can reduce zinc absorption. An antagonistic relationship has been proposed between these two minerals due to similar uptake channels (Ao *et al.*, 2009.)

3.3.1 Effect of feed-grade mineral mixes on phytase activity

The individual effects of copper and iron on the activity of Phy 1 – 5 were assessed in Sections 3.2.1 and 3.2.2. In both cases, it was demonstrated that copper and iron sources, as well as concentration, were influential on phytase function. However, as premixes contain multiple trace mineral components, the effect of feed-grade mineral mixes on the activity of commercial phytases was assessed. Trace minerals commonly added to poultry feeds include copper, iron, zinc, and manganese, thus the additive effects of these minerals on phytase activity were determined as outlined in Section 2.2.7. Inorganic (sulphates) and organic (proteinates; OTM 1) mineral combinations were assessed at commercial and TRT levels. Inorganic and organic copper sources were included at rates typically used in feed. Inorganic and organic sources of iron, zinc and manganese were applied at lower relative rates to allow for detection within the range of the *in vitro* assay. Inorganic and organic sources of iron, zinc and manganese were applied at rates of 1 ppm and 7.5 ppm, 5 ppm and 20 ppm, and 5 ppm and 20 ppm, respectively. The results of these experiments were summarised in Figures 3.2 – 3.6.

As previously seen in Sections 3.2.1 and 3.2.2, mineral source influenced the activities of Phy 1 – 5. In the majority of cases, high levels of inhibition were observed when phytases were exposed to inorganic copper and iron at commercial levels.

Figure 3.2 demonstrates the combinatorial effects of inorganic and organic mineral mixes on phytase activity *in vitro*. As with previous findings, the source of mineral mix had a significant effect on activity of Phy 1 ($p \leq 0.05$).

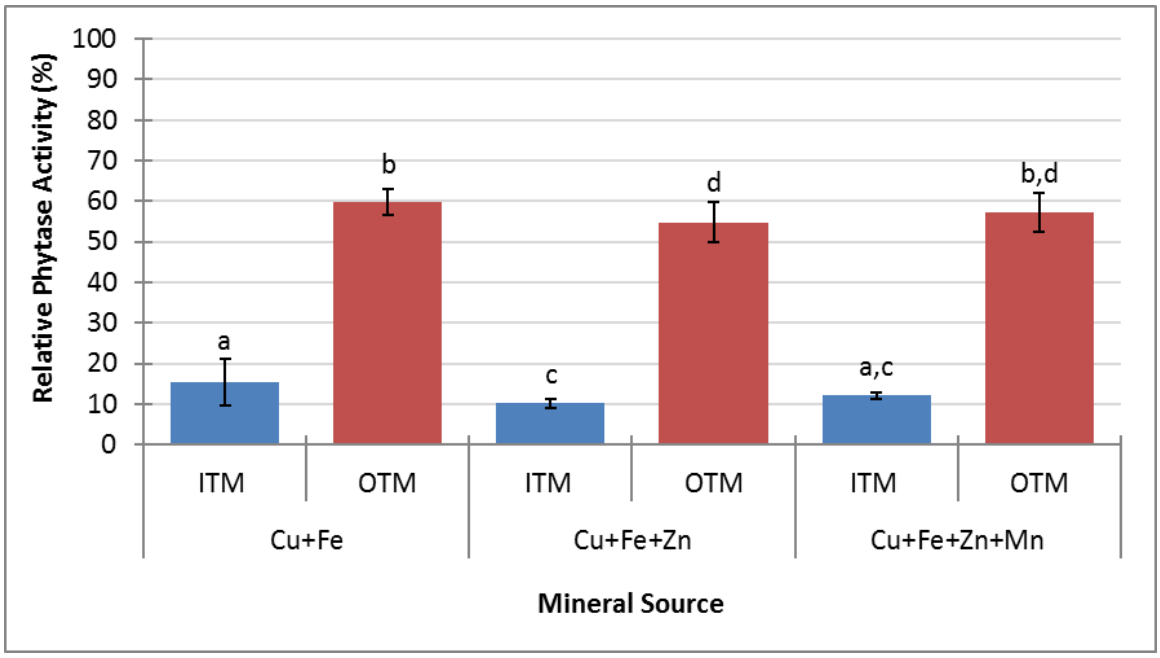

Figure 3.2 Effect of inorganic sulphate and organic proteinate mineral mixes on the activity of a commercial bacterial phytase, Phy 1.

The mineral mixes are presented as ITM (inorganic sulphate) and OTM (organic proteinate). Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Both inorganic and organic sources had a significant effect on activity of Phy 1 ($p \leq 0.05$); however, organic mineral combinations caused considerably less inhibition than inorganic counterparts. Relative activity decreased by 85 % when Phy 1 was exposed to inorganic mixes of copper and iron. This result was in agreement with the individual mineral assessments in Section 3.2, with copper and ferrous sulphates reducing activity by 42.6 % and 28.3 %, respectively. It is likely that the combination of ITM copper and iron caused additional inhibition on the activity of Phy 1 in comparison to the individual ITM sources. This may have been due to non-specific binding of copper and/or iron to the phytase causing a change in its conformation (Kerovuo *et al.*, 2000). It may have also been due to one or both of the ITMs binding to the phytic acid causing it to form mineral-phytate complexes. Phytic acid has been shown to chelate Cu^{2+} and Fe^{2+} which can block phytate hydrolysis due to the formation of insoluble complexes at higher pHs (4 – 7) (Tang *et al.*, 2006; Maenz *et al.*, 1998). These potential interactions may have caused additional antagonism to Phy 1. Numerical differences were observed with the additions of zinc and manganese sulphate. Individually, 1 mM of $ZnSO_4$ (equating to 64 ppm Zn) was found to have a highly inhibitory effect on the activity of Phy 1 in Section 3.1.2, resulting in an activity reduction of 91.5 %. Mineral

saturation of the phytase or phytic acid may account for the lack of further inhibition, due to blocking of respective binding sites, or structure conformation. Another potential reason could be that copper and/or ferrous sulphate may have bound to the phytase with greater affinity.

The presence of organic mineral mixes (OTM) also negatively impacted activity of Phy 1 but to a far lesser extent than ITM mixes (Figure 3.2), with copper and ferrous sulphates resulting in a 40 % reduction in phytase activity. Phy 1 significantly retained ~ 45 % more activity after exposure to the OTM mix of copper and iron in comparison to the ITM mix of copper and iron ($p \leq 0.05$). Similar to the ITM mix, numerical differences were observed when OTM zinc and manganese were added to the mix; however, the observed reductions were minimal (± 5 %). Higher retention levels of Phy 1 after exposure to OTM mixes may have occurred due to higher stability of the organic proteinates over the inorganic sulphates. Inorganic sulphates are known to dissociate readily in mildly acidic environments, whereas it has been suggested that organic trace minerals can offer greater stability in acidic conditions due to their bonding to an organic ligand (Pang and Applegate, 2006; Richards *et al.,* 2010).

Figure 3.3 demonstrates the effect of inorganic and organic mineral mixes on the activity of Phy 2. Greater inhibitory effects were observed with the addition of ITM mixes (a further decrease of ~ 60 %). It was evident that the source of mineral mix had a significant effect on phytase activity ($p \leq 0.05$).

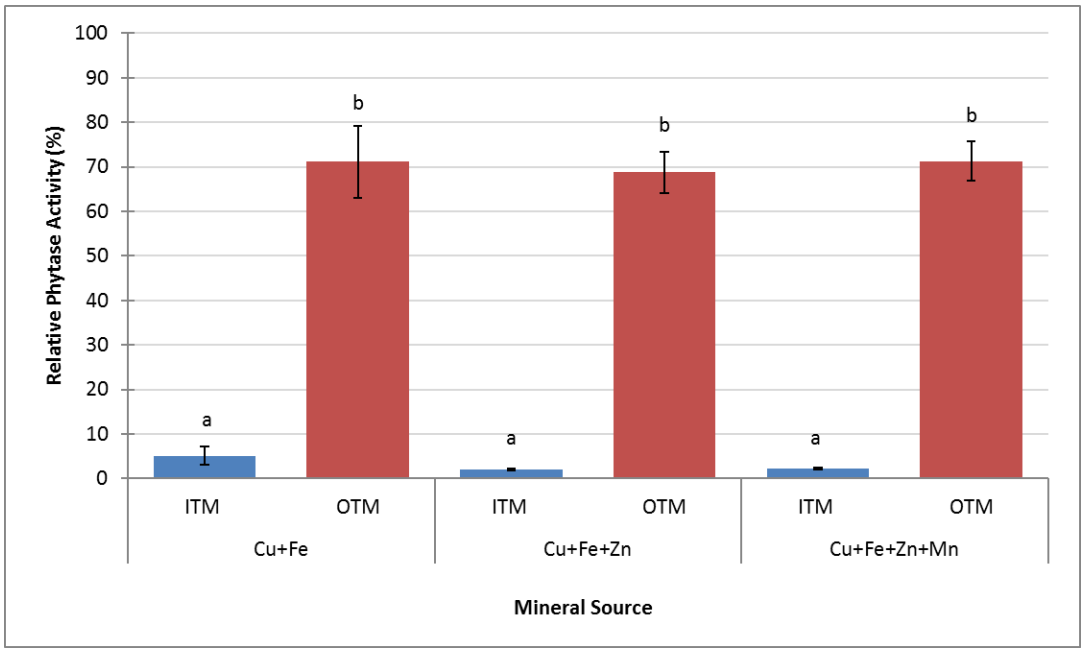

Figure 3.3 Effect of inorganic sulphate and organic proteinate mineral mixes on the activity of a fungal phytase, Phy 2.

The mineral mixes are presented as ITM (inorganic sulphate) and OTM (organic proteinate). Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Both ITM and OTM mixes had a significant impact on the activity of Phy 2; however, the inhibitory effect of OTM mixes was considerably less than that of ITM mixes ($p \leq 0.05$) (Figure 3.3). An activity retention of just ~ 5% was observed after exposure to a combination of ITM copper and iron, which was less than the retentions observed after exposure to individual inorganic sources of copper (12.9 %) and iron (23.3 %) in sections 3.2.1 and 3.2.2, demonstrating the potential negative interaction of combining minerals. Significant differences were not observed with the additions of ITM zinc or manganese; indicating that maximum inhibition occurred with the combined exposure to copper and ferrous sulphates. As previously mentioned, cations of inorganic minerals can be detrimental to the enzymatic activity of phytases. Inhibitory effects can occur due to non-competitive interactions between cations and the phytase or from phytic acid chelating metal ions and forming phytate complexes (Tang *et al.*, 2006; Bekalu *et al.*, 2017). These phytate complexes can be highly resistant to the action of phytase; reducing phytase efficacy. It has been reported in the literature that metal ions can cause varying degrees of inhibition to the phytase function of *Aspergillus* sp.. Tang *et al.* (2006) reported that a phytase from *A. ficuum* was incapable of breaking down phytate in the presence of Fe^{2+}, Cu^{2+}, and Zn^{2+}, due to the stability of these

phytate complexes. Similarly, Maenz *et al.* (1999) demonstrated that Zn^{2+} and Fe^{2+} were potent inhibitors of phytate hydrolysis by a phytase produced by *A. niger*, which again was due to the stability of the mineral-phytate complex. Conversely, Casey and Walsh (2003) reported that $CuSO_4$ and $ZnSO_4$ enhanced the activity of a phytase from *A. niger*. It was also reported that $FeSO_4$ had no effect on the efficacy of that specific phytase (Casey & Walsh, 2003). The results reported in the literature demonstrate that phytases produced from the same genus (or similar strains) may not always have identical responses, indicating that biological source has an effect on biochemical traits.

It was evident that the addition of organic minerals and subsequent incubation had far less of an inhibitory effect than inorganic minerals on activity of Phy 2 (Figure 3.7). Significantly higher phytase activity retentions of ~ 70 % were observed across all OTM combinations, representing ~ 65 % more activity when compared to ITM mixes ($p \leq 0.05$). Significant differences were not observed between OTM mixes suggesting that maximum inhibition occurred with organically bound copper and iron. Overall proteinate stability may have accounted for this. Phy 2 may have been more susceptible to organic copper and iron in comparison to organic zinc and manganese, hence no further inhibition occurred when these minerals were added to the mix. Interestingly, phytase activity after exposure to organic copper and iron was similar to the activity retention observed in the presence of organic iron in Section 3.2.2, suggesting that organic iron impacted phytase function of Phy 2 the most.

Figure 3.4 demonstrates the effects of inorganic and organic mineral mixes on the activity of Phy 3. The source of the mineral mix had a significant effect on phytase activity, as seen previously ($p \leq 0.05$).

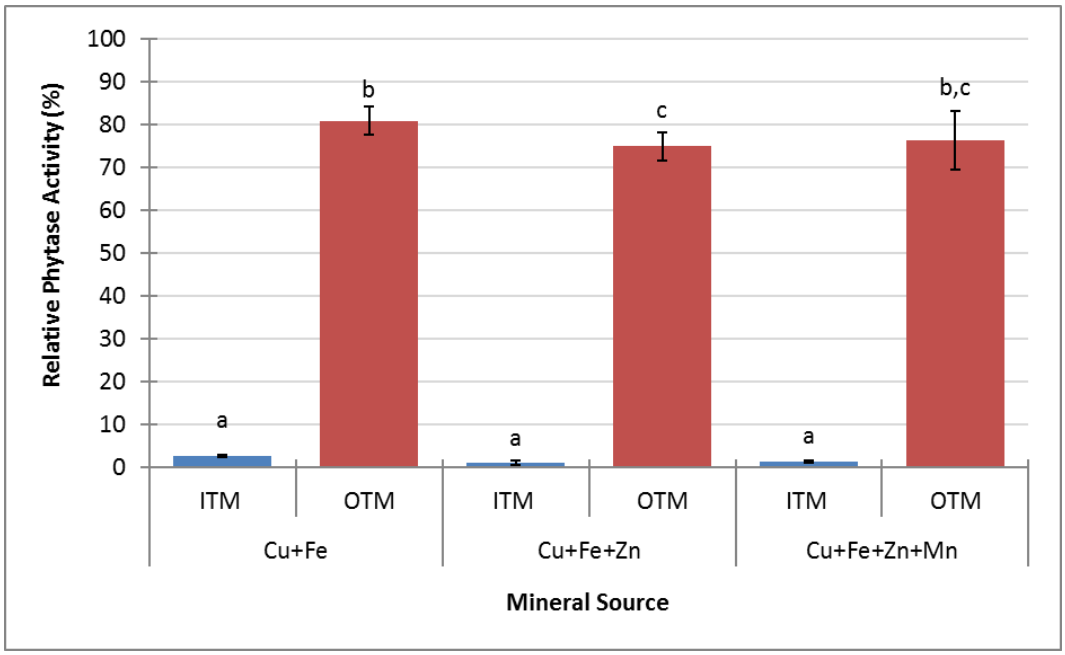

Figure 3.4 Effect of inorganic sulphate and organic proteinate mineral mixes on the activity of a bacterial phytase, Phy 3.

The mineral mixes are presented as ITM (inorganic sulphate) and OTM (organic proteinate). Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Both ITM and OTM mixes had an inhibitory effect on the activity of Phy 3; however, ITM mixes caused significantly greater losses in activity ($p \leq 0.05$) (Figure 3.4). Moreover, Figure 3.4 depicts the highest levels of inhibition after exposure to ITM mixes in comparison to Phy 1 and Phy 2, as phytase activity of Phy 3 was not detectable thereafter. Less than 5 % was detected after incubation with inorganic copper and iron, with no significant differences between ITM mixes ($p \geq 0.05$). It is likely that the inorganic mineral combination of copper and iron caused the most inhibition due to lack of significant differences with the addition of inorganic zinc or manganese. In the case of exposure to individual mineral sources of copper and iron, Phy 3 retained at least 25 – 40 % of its activity (Section 3.2). This may indicate that there was a combined effect of the two minerals which caused the near depletion of phytase activity in this instance.

Significantly higher levels of phytase activity (~ 75 – 80 %) were retained after exposure of Phy 3 to mixes of OTM sources ($p \leq 0.05$). In Section 3.2, individual organic sources of copper and iron reduced phytase activity of Phy 3 by approximately 19 – 45 % (Tables 3.3 and 3.4). In this instance, retention levels were similar to those observed after exposure to organic copper, suggesting that within the organic mix, organically bound copper may have caused the most inhibition. Phytase activity varied minimally

with the addition of chelated zinc and manganese. Overall, phytase activity appeared to be more stable after exposure to OTM mixes in comparison to ITM mixes.

Figure 3.5 illustrates the effects of inorganic and organic mineral mixes on the activity of Phy 4. As in previous instances, mineral mix source had a significant effect on phytase activity ($p \leq 0.05$).

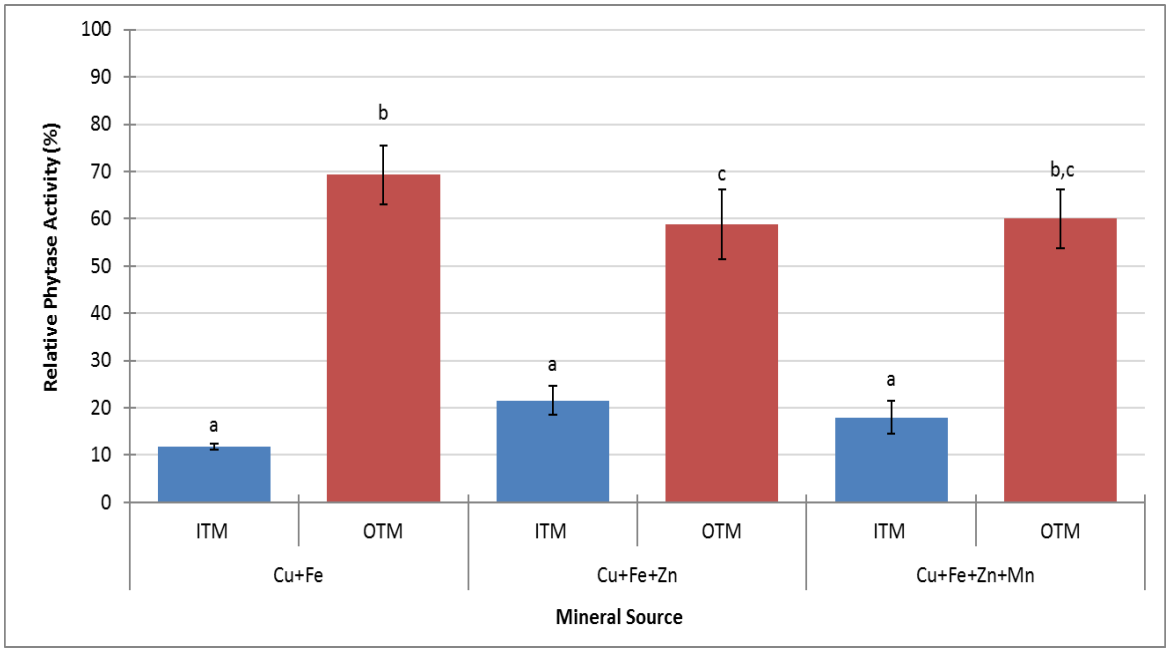

Figure 3.5 Effect of inorganic sulphate and organic proteinate mineral mixes on the activity of a fungal phytase, Phy 4.

The mineral mixes are presented as ITM (inorganic sulphate) and OTM (organic proteinate). Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Both inorganic and organic mineral mixes significantly affected the activity of Phy 4 ($p \leq 0.05$). ITM mixes caused the highest level of phytase inhibition, with low activity retentions of ~ 10 – 20 %; however, these retentions were the highest out of commercial phytases previously discussed (Figure 3.5). In Section 3.2, individual sources of inorganic copper and iron reduced activity to ~ 35 %, with the combination of both reducing activity to ~ 10 %, suggesting that inorganic minerals had combined inhibitory effects on Phy 3 (Figure 3.5). A numerical increase in activity was observed with the further addition of inorganic zinc, which may have indicated a slight stimulatory affect; however, the effect was not significant ($p \geq 0.05$). It was likely that inorganic copper and iron caused the most inhibition due to lack of significant differences with the additions of ITM zinc or manganese.

Similar to the other phytases, significantly less inhibition was caused by OTM mixes to the efficacy of Phy 4 ($p \leq 0.05$). Phytase activity retentions ranged from ~ 60 – 70 %, which was considerably higher (~ 50 – 60 %) in comparison to the retentions seen after exposure to ITM mixes (Figure 3.5). Activity further decreased by ~ 10 % with the addition of organic zinc and manganese, demonstrating that the addition of extra trace minerals to the organic mix did not have a detrimental effect on activity of Phy 4. Similar activity retentions were observed when Phy 4 was exposed to individual organic sources of copper and iron, suggesting that an organic mix of minerals may not further hinder phytase activity. As before, this may have been down to greater stability of the OTMs.

Similar to the previous commercial phytases assessed, mineral mix source had a significant effect on activity of Phy 5 (Figure 3.6). Phy 5 displayed significantly higher levels of activity after exposure to OTM mixes compared to ITM mixes ($p \leq 0.05$).

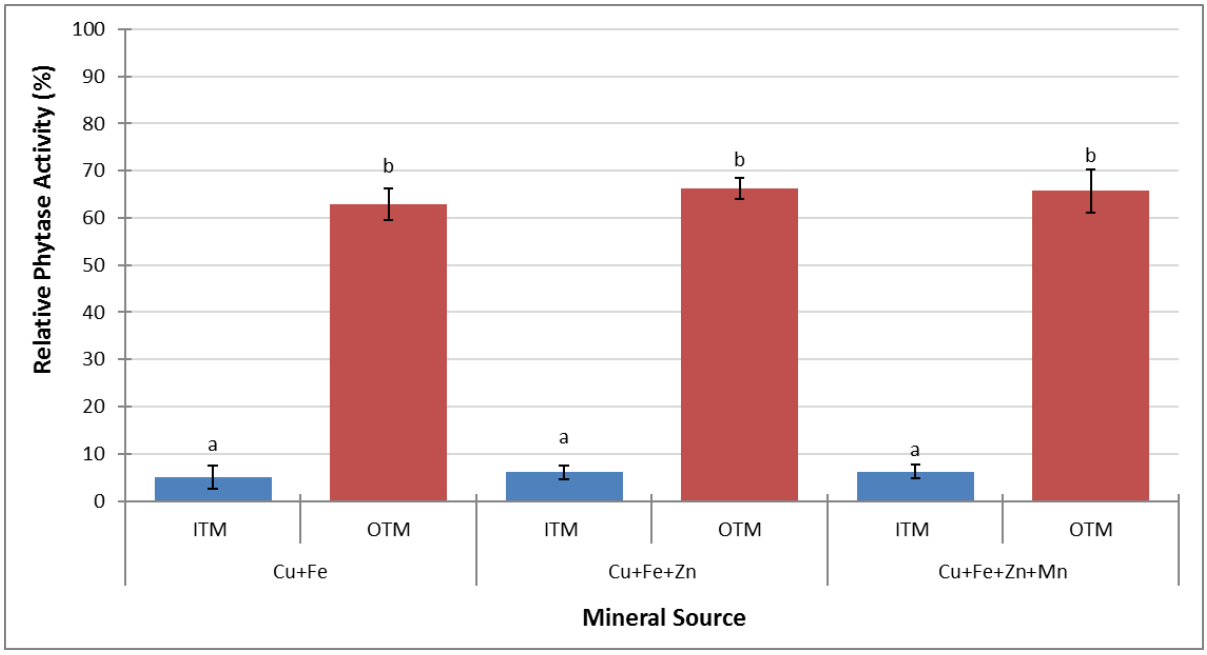

Figure 3.6 Effect of inorganic sulphate and organic proteinate mineral mixes on the activity of Phy 5.

The mineral mixes are presented as ITM (inorganic sulphate) and OTM (organic proteinate). Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Exposure to inorganic and organic mineral sources had adverse effects on phytase activity; however, with ~ 5 % activity remaining, ITM mixes had a much greater inhibitory effect on the activity of Phy 5 (Figure 3.6). Individually, inorganic copper and iron both had detrimental effects on the activity of Phy 5; reducing phytase activity by ~ 78 – 84 %. As with the previous phytases, the combined effect of inorganic copper and iron resulted in a large reduction in the activity of Phy 5. The addition of inorganic zinc and manganese did not have any additional significant effects on phytase activity, suggesting that maximum inhibition had occurred. Again, this may have been due to saturation of the phytase or phytic acid thereby reducing function of the phytase or access to the phytic acid molecule, respectively.

Conversely, OTM mixes had much less of an impact that their ITM counterparts; approximately 55 % more activity was retained when Phy 5 was exposed to organic mineral mixes. No significant differences were observed following the addition of organic zinc or manganese, indicating that organic zinc and manganese had negligible impact on the activity of Phy 5. Activity levels after exposure to OTM mixes were higher than those observed after exposure to individual organic iron minerals (Section 3.2.2). However, activity levels were similar to those observed after exposure to individual organic copper sources (Section 3.2.1), suggesting that most of the impact was due to OTM Cu in comparison to the other OTM sources. This may have been due to a greater reactivity with Phy 5.

The results of this study demonstrated that the source of mineral mix had an impact on the phytase activity of Phy 1 – 5. Overall, the effects of ITM mineral mixes were highly inhibitory, with most phytases only retaining 10 % of their initial activity. Conversely, significantly higher levels of activity were observed after exposure to OTM mineral mixes; with retained activity ranging from 50 – 70 % higher than activity observed after ITM counterparts ($p \leq 0.05$). In general, further inhibition of phytase function was not observed with the addition of inorganic zinc and manganese, suggesting that copper and iron had the most inhibitory effect. However, a numerical increase (~ 10 %) in phytase activity was observed when Phy 4 was exposed to inorganic zinc, indicating that the mineral may have had a slight stimulatory effect on activity but this result was not significant. In the majority of cases, the addition of OTM Zn caused a significant decrease in activity (~ 5 – 10 %); however, this effect was minimal as all the affected phytases still retained activity levels of ~ 55 – 75 %. As

previously mentioned, proteinates possess a higher stability due to their chelated structure and lack of charge; these characteristics may account for the higher levels of retained activity observed in Phy 1 – 5, these characteristics may account for the higher levels of retained activity observed across the commercial phytases tested.

3.3.2 The effect of commercial premixes on phytase activity

While it is important to assess the individual and combined effects of trace minerals on phytase function *in vitro*, it is also useful to look at the effects of commercially formulated premixes which may be formulated with a mixture of different OTM and ITM sources. The current section looked to assess the effects of commercial premixes (containing similar levels of copper, iron, zinc, and manganese) on the phytase function of Phy 1. The commercial premixes chosen were marketed as organic sources of trace minerals, which included a proteinate (A), an amino acid chelate (B), a metal propionate (C), and a methionine hydroxy analogue chelate (D). All premix samples were normalised to their iron content and applied at a reflective TRT level of 2 ppm (as was utilised in Section 3.2.2 and 3.3.1). Figure 3.7 summarises the effects of organic premixes on the activity of Phy 1. As demonstrated with individual organic sources of copper and iron in Sections 3.2.1 and 3.2.2, the source of organic premix had a significant effect on the activity of Phy 1 ($p \leq 0.05$).

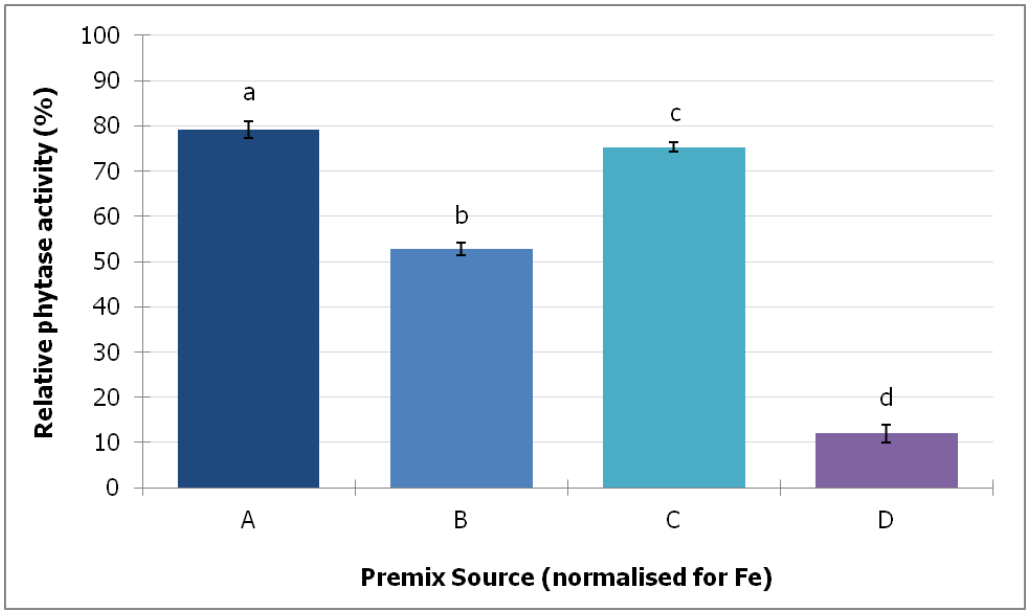

Figure 3.7 Effect organic premixes on the activity of a bacterial phytase, Phy 1. Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$). OTM premixes were as follows: proteinate (A), amino acid chelate (B), metal propionate (C), and methionine hydroxy analogue chelate (D).

A varied response was observed when Phy 1 was exposed to organic premixes, with relative activity ranging from ~ 10 – 80 %. Exposure to Premix A resulted in the highest retention of phytase activity (~ 80 %). As mentioned previously, proteinates are highly stable due to their degree of chelation, which may have accounted for higher levels of phytate hydrolysis in this instance. High levels of activity were also observed after Phy 1 was incubated with Premix C (~ 75 %). Premix C contained trace minerals defined as metal propionates. These organic minerals are formed by the reaction of a metal salt with excess propionic acid and may be more tightly bound than other OTMs, inferring greater stability and less reactivity. Significantly lower levels of phytase activity were observed when Phy 1 was incubated with Premix B (~ 53 %). Although Premix B was composed of chelated trace minerals (inferring greater stability), phytase function was considerably lower in its presence. This may have occurred due to components of the premix binding to the phytase inducing conformation changes, or it may have been due to a greater affinity between phytate and the metal portion of the chelate than their organic ligands. Exposure to Premix D resulted in the lowest phytase activity (~ 10 %). This premix consisted of trace minerals in the form of chelated methionine hydroxy analogues (MHA). The MHA within the premix may have had a

lower stability constant due to its chelation process, inferring less stability, which may have led to greater reactivity between phytic acid and/or the phytase, reducing phytate degradation. Another potential reason for the low activity exhibited in the presence of Premix D may have been due to the higher Cu content of the premix, Cu was 1.7- to 2.5-fold higher than the other premix sources. Additionally, Zn was 1.5- to 1.8-fold higher in Premix D than the other premixes.

These results demonstrated the difference between commercial organic premix sources on the phytase function of Phy 1. The results illustrated the potential interactions between organic premixes a bacterial phytase, showing that different forms of organic trace minerals elicit different phytase responses, indicating that not all organic premix sources react in the same manner.

3.4 Effect of feed-grade mineral mixes on phytase activity in simulated gastric pHs

The poultry gastrointestinal tract (GIT) is comprised of a number of different compartments, with the main areas of importance for phytase activity being the crop, gizzard and small intestine. The pH of these different compartments varies greatly; ranging from pH 4.5 – 5.5 in the crop, pH 2.5 – 3.5 in the gizzard, and pH 5 – 7.5 in the small intestine. Ideally, an exogenous phytase should be able to maintain a moderate to high level of activity across the GIT, but especially in the gizzard and crop, as was demonstrated in Section 3.1.1. As previously illustrated, trace minerals can have an inhibitory effect on phytase activity through non-competitive interactions. Phytic acid can also interact with trace minerals through chelation of their metal ions forming phytate-mineral complexes. At different pHs, soluble or insoluble complexes can arise from these interactions which can potentially reduce or block the action of phytase, compromising dephosphorylation of phytic acid.

The effect of gastric pH on the ability of phytase to dephosphorylate phytic acid in the presence of trace minerals was assessed herein. Two phytases (Phy 1 and Phy 4) were selected based on their catalytic differences; Phy 1, a 6-phytase, from a bacterial source and Phy 4, a 3-phytase, from a fungal source, both with pH optima between pH 5 and 5.5. Both phytases displayed similar responses to individual copper sources and mineral mix sources (Sections 3.2.1 and 3.3.1, respectively), however, slight differences were observed when exposed to individual iron sources (Section 3.2.2). Figure 3.8 and

3.9 demonstrate the effect of pH on phytase-mineral interactions of Phy 1 and Phy 4, respectively.

Figure 3.8 demonstrates the effect of simulated gastric pH and temperature on the catalytic function of Phy 1 in the presence of trace mineral mixes. The temperature of the simulated gastric incubation was 40 ºC, reflective of the internal temperature of poultry. The acidity of the reaction was increased from pH 5 to pH 2.5, reflecting the change of pH conditions in the crop to the gizzard. ITM (sulphate) and OTM (proteinate; OTM 1) mixes used were identical to those used in Section 3.3.1. As seen previously, the relative activity of Phy 1 was negatively impacted after exposure to ITM and OTM mixes. Increasing the pH of incubation significantly affected the phytase activity of Phy 1 when exposed to OTM at TRT levels and ITM at commercial levels ($p \leq 0.05$).

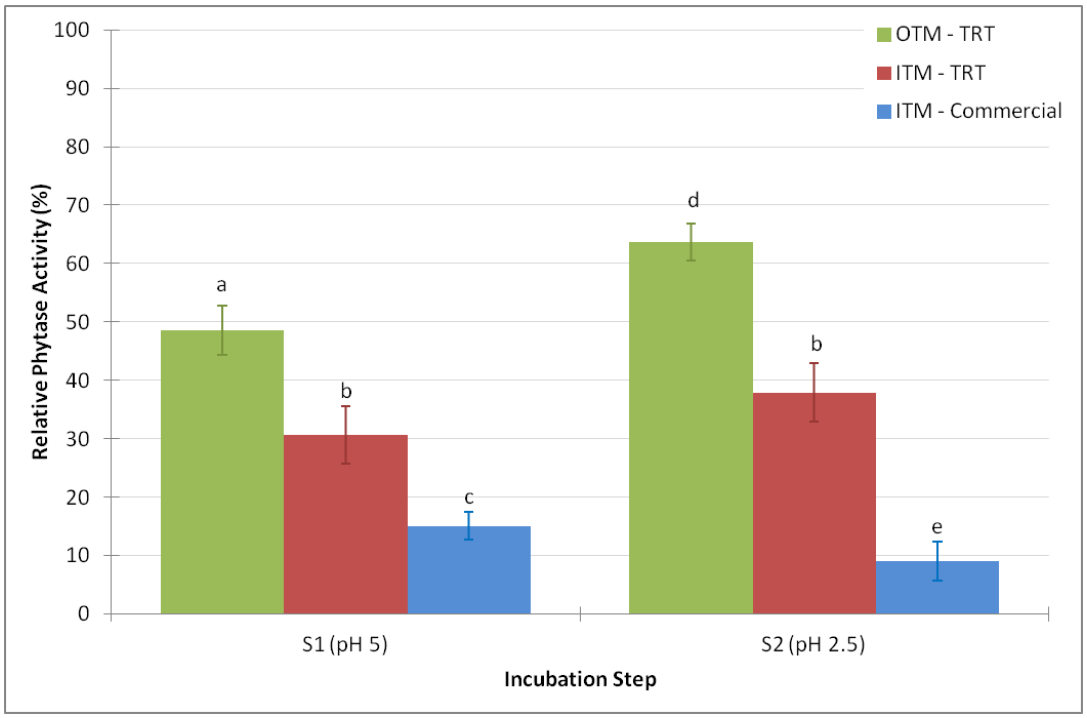

Figure 3.8 Relative activity of a bacterial phytase, Phy 1, after exposure to OTM and ITM mixes at gastric pHs.

Relative activities are presented as n = 9. Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Exposure to ITM and OTM mixes negatively impacted the activity of Phy 1 (Figure 3.8); however, the ITM mix had more of an adverse effect than the OTM mix. Exposure to ITM at pH 5 significantly impacted the activity of Phy 1 at both mineral inclusion levels; 30 % of activity remained at TRT levels, whereas a significant decrease of ~ 15 % was observed when ITM was applied at commercial levels ($p \leq 0.05$). These results were as expected given the relative activities observed in Section 3.3.1. A slight numerical increase in activity of ~ 8 % was observed when Phy 1 was exposed to TRT levels of ITM mixes at pH 2.5 (representing the environment of the gizzard). However, as this increase wasn't significant, it suggested that increasing the acidity did not have a definitive impact on the efficacy of Phy 1 in the presence of ITM mixes at this lower concentration. In Figure 3.8, a significant decrease in phytase activity when Phy 1 was exposed to the ITM mix at higher commercial levels at pH 2.5 ($p \leq 0.05$). This may have occurred due to increased dissociation of mineral salts and subsequent higher cation concentration. Similar results have also been reported in the literature whereby higher pHs hindered phytate hydrolysis in the presence of inorganic minerals (at typical application rates) commonly added to animal feeds (Pang & Applegate; 2006; Akter *et al.,* 2015).

OTM mixes negatively affected phytase activity but to a lesser extent (Figure 3.8). At pH 5, ~ 50 % activity was retained, which was in agreement with results observed previously (Section 3.3.1). At pH 2.5, the relative activity of Phy 1 increased to ~ 63 % in the presence of the OTM mix at TRT levels. In Section 3.1.1, the relative activity of Phy 1 was found to be ~ 55 % at pH 2.5, which may have indicated that the OTM mix did not have an adverse effect on activity in this instance. This may have been due to increased stability of the organic minerals, leading to lower levels of mineral dissociation, resulting in higher levels of phytate hydrolysis. Organic chelated minerals tend to be more chemically inert in comparison to their inorganic sulphate counterparts thus they are less susceptible to dissociation, which may have contributed to increased phytate hydrolysis (Vieira, 2008). Pang & Applegate (2006) reported that pH and copper mineral source and concentration affected phytase activity. They demonstrated that organic Cu sources were less detrimental to phosphorus release at pH 5.5 and 6.5, in comparison to various inorganic Cu sources. They also reported higher phytic acid hydrolysis due to lower levels of dissociation of phytic acid protons at pH 2.5. The authors also noted that phytic acid solubility was higher at more acidic

conditions which would have contributed to higher levels of phytic acid hydrolysis. This may also be an explanation as to higher phytase activities in the current study (Figure 3.8).

Figure 3.9 demonstrates the effects of pH on activity of Phy 4 in the presence of inorganic and organic mineral mixes. The lack of significant differences between simulated gastric environments suggested that pH did not influence the activity of Phy 4 in the presence of mineral mixes ($p \geq 0.05$); however, the source of mineral mix did have a significant effect on phytase activity as previously demonstrated ($p \leq 0.05$).

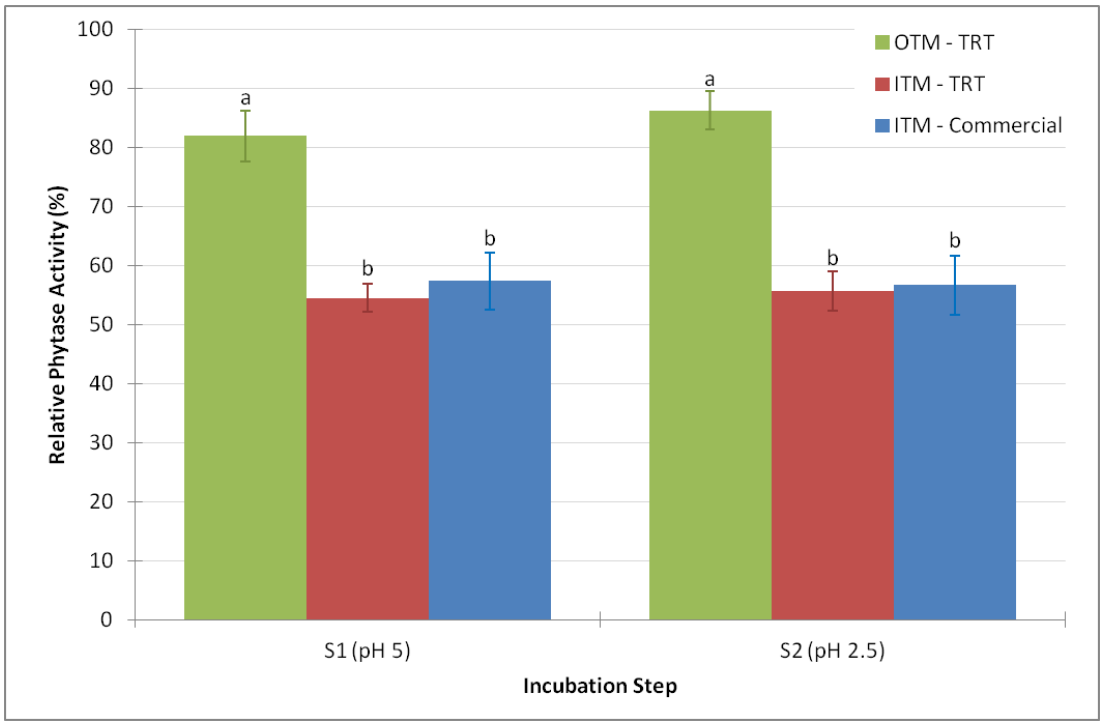

Figure 3.9 Relative activity of a fungal phytase, Phy 4, after exposure to OTM and ITM mixes at gastric pHs.

Relative activities are presented as n = 9. Average values that lack a common superscript differ in statistical significance ($p \leq 0.05$).

Figure 3.9 illustrates that mineral mix source influenced phytase activity of Phy 4. Although both mineral mix sources affected the efficacy of Phy 4, the ITM mix was significantly more inhibitory than the OTM mix, resulting in reduced activity of ~ 55 % ($p \leq 0.05$). Interestingly, the activities observed at pH 5 in the presence of the ITM mix were ~ 30 % higher than those observed in Section 3.3.1. A potential reason for this may have been the temperature of the incubation; 40 ºC was selected to represent the physiological temperature of poultry. It has been reported in the literature that phytases

have optimal activity at temperatures ranging from 30 – 80 ºC (Singh and Satyanarayana, 2013). Naves *et al.* (2012) reported an optimum temperature range of 40 – 45 ºC for two different phytases produced by *Aspergillus* sp.; with decreasing levels of activity from 50 – 60 ºC. This may be indicative as to why higher levels of activity were observed in the current study; the catalytic activity of Phy 4 may have been more effective at 40 ºC than at 50 ºC (temperature employed for Sections 3.1 – 3.3). Increasing the pH of the reaction did not have an effect on phytase activity in the presence of the ITM mix. Increasing the concentration of ITM mix also did not influence the phytase activity.

Phytase activity of Phy 4 was significantly higher in the presence of the OTM mix at both pH values ($p \leq 0.05$). As with the ITM mixes, phytase activity observed was higher (~ 20 %) than that reported in Section 3.3.1, again potentially due to the reduced reaction temperature. As with the ITM mixes, significant differences were not observed when the acidity of the reaction was increased, implying that the interaction was pH independent. Overall, these results were unexpected with regard to pH influence, and also differed to those demonstrated in the literature. Tamim & Angel (2003) reported that inorganic and organic mineral mixes had a negative impact on phytase activity at both pH 2.5 and pH 6.5; with lower levels of phosphorus release at pH 6.5. However, it was also noted that organic mineral mixes in the form of amino acid complexes did not improve phosphorus release (Tamin & Angel, 2003), whereas the results of the present study demonstrated that Phy 4 performed better in the presence of OTM mixes (in the form of proteinates), demonstrating that organic mineral mix source can have an influence on phytase function.

Under simulated gastric conditions, there were clear differences in the activity profiles of Phy 1 and Phy 4 in the presence of trace mineral mixes. Mineral mixes negatively impacted the activity of both phytases; with ITM mixes (at both TRT and commercial application levels) having more of an inhibitory effect than OTM mixes. ITM had more of a negative impact on the activity of Phy 1 than that of Phy 4, with Phy 4 retaining ~ 25 – 45 % more activity. The concentration of ITM had a significant effect on phytase function of Phy 1 but not for that of Phy 4. The activity of Phy 1 decreased from ~ 30 % to 15 % at pH 5, and from ~ 38 % to 9 % at pH 2.5, whereas activity of Phy 4 remained at ~ 55 % when exposed to ITM mixes at both pH levels. pH had an influence on the activity of Phy 1 but not on Phy 4; greater levels of activity were

observed when Phy 1 was exposed to TRT levels of the OTM mix at pH 2.5 than at pH 5. The activity of Phy 4 did not change when the acidity was increased, demonstrating that its activity (in the presence of mineral mixes) was independent of pH changes. As previously mentioned, Phy 4 may have been more effective at 40 ºC, hence the higher levels of activity, firstly in the presence of mineral mixes, and secondly under pH changes.

Overall, it was evident that OTM mixes had less of an inhibitory effect on enzyme activity of both phytases in comparison to ITM counterparts. This was likely due to the enhanced stability of the organic minerals, inferred by their bond strength and orientation. Higher phytase activity in the presence of OTM mixes was also observed at pH 2.5, which was likely due to lower levels of metal ion dissociation at acidic pHs. In addition, phytic acid is more soluble at acidic pHs, thus it was possible that there was a complementary effect of greater phytate solubility and OTM stability. These results demonstrated the vast difference in catalytic activity under simulated gastric conditions, providing a model for testing numerous feed formulations, after which a selection can be validated *in vivo*.

4 General conclusions

Phytases are naturally occurring enzymes that facilitate the dephosphorylation of phytic acid or its salt, phytate, to liberate inorganic phosphorus. They are ubiquitous in nature and are found in an array of microorganisms, plants and animals. In general, monogastric animals have low intrinsic levels of phytases, requiring exogenous microbial phytases to be supplemented to their feedstuffs as a way of increasing the availability of naturally occurring phosphorus. Degradation of phytate also has the capacity to improve digestibility and nutritional value of feed, as phytate has a tendency to chelate nutrients, such as minerals, amino acids and proteins. The addition of phytase to monogastric animal feeds also has the potential to reduce the levels of excreted phosphorus within their faeces, subsequently reducing sources of pollution into the environment (Lei *et al.*, 2013).

The initial objective of the present study was to assess the biochemical properties of five commercial phytases (designated Phy 1 – 5) and how activity is influenced by pH and potential modulators. Enzyme activity profiles were determined for all commercial phytases over a broad pH range *in vitro*, as an indicator of potential activity *in vivo*. Although each of the commercial phytases had diverse pH profiles, maximum activity was observed between pH 3.5 – 5 in all cases. This maximum pH range was expected, as most common feed phytases are classified as HAPhys, which display optimal activity within this range (Greiner and Konietzny, 2011). The pH range investigated broadly covered that of the upper intestinal tract of poultry (encompassing the crop, gizzard and proventriculous), which generally transitions between ~ pH 2.5 and ~ pH 5 (Menezes-Blackburn *et al.*, 2015), demonstrating the potential application and functionality of these commercial phytases in poultry nutrition. The second biochemical property assessed was phytase activity in the presence of different chemicals and minerals (including those typically used in the diets of monogastrics). Inorganic trace minerals (ITM), in the form of cupric sulphate, ferrous sulphate and zinc sulphate, caused the highest levels of phytase inhibition. These findings subsequently formed the main basis for the wider study; that, when added to the diet, trace mineral source could negatively affect the activity of exogenous commercial phytases.

Initially, the effect of individual feed-grade trace mineral sources on phytase function was assessed. Individual sources of copper and iron were utilised for this part of the study, as they had the most inhibitory effect on phytase function in Section 3.1.

Inorganic mineral salts have traditionally been added to feed as sulphates, and as such sulphates were utilised as the source of inorganic trace mineral (ITM) for the present study. The use of organic trace mineral sources (OTM) in feeds is increasing; however, it is important to note that OTM is a generic term and that not all OTMs are identical as illustrated in Table 1.3. Consequently, a range of OTMs were employed for use in the present study, including proteinates, chelates and amino acid complexes. Trace mineral concentrations typically applied in animal nutrition were also assessed, i.e.: the higher commercial rates often reported when ITMs are used in commercial settings and the lower levels often recommended for OTM inclusion. Application rates of OTMs are typically lower than those of ITMs due to their enhanced bioavailability. Some manufacturers recommend full replacement of ITMs with OTMs at reduced inclusion levels. In general, copper source and concentration were influential for retaining phytase function in the majority of cases. Copper sulphate had the most inhibitory effect on phytase activity in each case when assessed at commercial inclusion rates. Greater phytase retention was observed when OTMs were assessed at lower recommended levels. The activity of Phy 4 was not highly affected by increased commercial concentrations of OTMs; however, its activity was reduced by ~ 45 % in the presence of equivalent levels of ITM, demonstrating the benefits of enhanced stability associated with OTMs over ITMs. Additionally, biological source was a key determinant to how the enzyme responded in the presence of copper. Exposure of 6-phytases (Phy 1, 3 and 5) to OTM 2 (an amino acid complex of copper) resulted in lowest levels of activity even at reduced mineral levels. Conversely, exposure of the remaining 3-phytases (Phy 2 and 4) to OTM 2 resulted in the highest levels of activity retention for those respective phytases. This is an important consideration for feed formulation, given that the mineral source, even within organic classifications, may not be compatible with the exogenous phytase being used in the feed.

The source and concentration of iron also had a similar effect on phytase function in the majority of cases. Greater reductions in activity were observed when commercial levels of inorganic iron sulphate were applied in comparison to organic sources. OTM 2 (an amino acid complex of iron) at reduced mineral levels had a considerable impact on enzyme function for Phy 3 – 5, resulting in the greatest reduction of phytase activity. Further decreases in activity were observed when higher levels of OTM 2 were applied to all commercial phytases, except in the case of Phy 4,

suggesting that phytase activity can be independent of iron concentration depending on its biological source (3-phytase).

Following on from assessments of individual minerals, the effects of simulated inorganic and organic mineral mixes (at reduced levels and higher commercial levels) on phytase function were investigated. Simulated mineral mixes consisted of sequentially added copper, iron, zinc, and manganese, with sulphates employed as the ITM source and proteinates as the OTM source. All commercial phytases were negatively impacted by the mineral mixes; however, the ITM mix source caused the highest levels of inhibition, resulting in activity reductions of ~ 80 – 95 % for each of the commercial phytases assessed. Further reductions in activity were not observed with the additions of $ZnSO_4$ and $MnSO_4$, suggesting that $CuSO_4$ and $FeSO_4$ had the most detrimental impact on the activity of Phy 1 – 5, particularly when acting in conjunction with each other. OTM mixes had a far less negative impact on phytase activity than ITM mixes, corresponding to enzyme retention levels of ~ 55 – 80 %. The addition of organic zinc and manganese proteinates did not cause further inhibition of Phy 2 and Phy 5, demonstrating that phytases with different modes of action (i.e.: 3- vs 6-phytases) can react similarly to specific minerals. It was evident that the commercial phytases were less affected by the organic proteinate mixes than their inorganic counterparts, which was likely due to the greater ligand stability associated with these OTMs.

In order to validate the findings from simulated mineral mixes, a number of commercially available premixes were assessed to quantify the effect on phytase function. Phy 1 was exposed to various commercial premixes marketed as organic sources. Premix sources included proteinates, chelates, propionates, and chelated methionine hydroxy analogues. Commercial premixes impacted the phytase function of Phy 1 to varying degrees. The proteinate source had the least inhibitory effect of the premixes tested, resulting in an activity retention of 80 %, which was likely due to higher stability. The amino acid chelated premix source reduced phytase activity by 45 % which was unexpected as these trace mineral sources generally infer greater stability. The findings suggested that not all organic chelated mineral sources interacted with the phytase in the same fashion, which was most likely due to the stability of the binding between ligand and mineral salt.

The final aim of the present study was to determine the effects of simulated gastric conditions (pH 5 to pH 2.5 at 40 °C) on phytase function in the presence of OTM and ITM mixes. Phy 1 and Phy 4 were utilised for the purpose of this experiment and selected based on their biochemical differences. Overall, it was determined that both phytases were negatively impacted by the presence of mineral mixes; however, similar to previous findings, ITM mixes caused greater inhibition in both cases. Higher levels of activity were observed when Phy 1 was exposed to the OTM mix at pH 2.5 than at pH 5, which was likely due to greater stability of the organic proteinate and the increased solubility of phytic acid at lower pHs. Although Phy 4 was also negatively impacted by mineral mixes, it was not affected by changes in acidity, which may be reflective of the different modes of action the two commercial phytases, that is 6- vs 3-phytase.

In conclusion, findings from the present study demonstrated that the activity of commercial phytases can be greatly affected by the trace minerals routinely added to animal feeds. While the use of OTMs resulted in the retention of greater levels of phytase function overall, a number of different factors contributed to this, such as the individual OTM source, the type of organic ligand bound to the mineral, the concentration of OTM and the biological source of the phytase. In the majority of cases, proteinate sources of copper and iron had the least inhibitory effects on phytase function and ITMs had the greatest negative effect on enzyme activity. It is important to note that although these results are not directly reflective of activity *in vivo,* they are a good indicator of the potential interactions which may occur. Overall, the use of OTM sources at lower application levels may help to maximise phytase activity and minimise potential antagonisms between feed components. This is an important consideration for feed formulation due to the costs associated with supplemental feed additives and ensuring feed quality.

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