Research Article

The effect of copper source on the stability and activity of α-tocopherol acetate, butylated hydroxytoulene and phytase

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Abstract

The supplementation of Copper (Cu) is essential for the optimum performance of physiological functions, including growth performance and immune function. Cu is usually formulated into animal premixes in the form of inorganic salts, such as sulphates, or organic minerals. Organic minerals are mineral salts that are either complexed or chelated to organic ligands such as proteins, amino acids, and polysaccharides. Cu is often formulated into premixes alongside other essential components such as vitamins, enzymes and synthetic antioxidants, all of which are susceptible to negative interactions with Cu which can detrimentally effect both their stability and activity. The aim of this study was to determine the effect of five different commercially available Cu sources in relation to their effect on the stability of α-tocopherol acetate and on the activity of Butylated Hydroxytoluene (BHT) and three commercially available phytases in vitro. The results determined that Cu source played a significant role in relation to limiting the interactions between Cu and each of the other components in vitro. There were significant differences ($p \leq 0.05$), not only, between the inorganic and organic Cu sources but also between some of the individual organic Cu sources in relation to their effect on α-tocopherol acetate, BHT and phytase.

Keywords Copper · α-tocopherol acetate · BHT · Phytase · Stability · Premix

1 Introduction

The essential role of Cu in relation to the proper physiological functioning of both poultry and livestock has been well documented [1–7]. Cu and other trace minerals are often formulated into premixes alongside other essential ingredients such as antioxidants, vitamins, amino acids and enzymes, such as phytases. The trace mineral components of premixes can be either inorganic mineral salts such as carbonates, sulphates, and oxides [8] or mineral salts bound to organic ligands such as proteins or individual amino acids. These organic minerals are referred to as organic trace minerals.

The advantages of organic trace minerals over their inorganic counterparts in relation to dietary and environmental antagonisms and bioavailability are well documented [9–17]. However, many of these studies have focused on comparing just one source of organic mineral to a corresponding inorganic mineral salt. In general, organic trace minerals are grouped together under this blanket term even though there are multiple sources of organic trace minerals whose structures and characteristics are highly varied.

The organic trace minerals used within the animal feed industry include a wide range of both complexes and chelates and the ligands can vary from single amino acids to hydrolysed proteins to polysaccharide preparations. The Association of American Feed Control Officials (AAFCO) definitions for the most commonly used organic trace minerals within agricultural practice are shown in Table 1 [19].

Table 1 AAFCO Organic trace mineral definitions

Metal Amino Acid Complex	The product resulting from complexing a soluble metal salt with one or more amino acid
Metal Amino Acid Chelate	The product resulting from the reaction of a metal ion from a soluble metal salt with amino acids having a mole ratio of one mole of metal to one to three (preferably 2) moles of amino acids to form coordinate-covalent bonds. The molecular size of the hydrolysed amino acids should be approximately 150 Dalton while the molecular size of such chelates should not exceed 800 Dalton
Metal Proteinates	The product resulting from the chelation of a soluble mineral salt with amino acids and/or hydrolysed proteins
Metal Polysaccharide Complex	The product resulting from the complexing of a soluble metal salt with a polysaccharide solution

The differences in stability between the organic trace minerals can be attributed, not only, to the type of bonding group used, but also, the production process by which they are produced [18]. The type of ligand and the production process has a direct relationship on the bond strength between the ligand and the mineral salts. The bond strength may, not only, affect the stability of the mineral during the digestive process but may also affect the rate at which the minerals, such as Cu, negatively interact with the other components present within the premixes such as the various enzymes, vitamins, and synthetic antioxidants.

Phosphorous is an essential macromineral required for overall animal health and function. Phytic acid is the natural storage form of phosphorus and, when present in this form, is largely unavailable to monogastric animals due to the low levels of intrinsic phytase activity. To help overcome this issue, exogenous phytases are often added to monogastric animal feeds [21]. Phytases (myo-inositol hexakisphosphate phosphohydrolase) are hydrolytic enzymes which are members of the phosphatase family. They are responsible for catalysing the dephosphorylation of phytic acid into inorganic orthophosphate and myo-inositol which can then be readily utilised by the animal. There are several different classes of phytases, namely histidine acid phytases (HAPhy), beta-propeller phytases (BPPhy), purple acid phosphatases (PAPhy), and cysteine phytases (CPhy). In animal nutrition, HAPhys from fungal or bacterial sources are primarily used [22].

Vitamins are essential organic elements which animals must obtain from the environment as they are unable to synthesise sufficient quantities for normal function of essential physiological processes [9]. As biologically active biochemicals vitamins are, as such, quite sensitive to the physical and chemical environment and can undergo significant degradation under normal storage conditions [10, 23]. The presence of pro-oxidative trace minerals within animal premixes can lead to Fenton type oxidising reactions and the subsequent destruction of vitamins such as retinol, cholecalciferol and tocopherol. The degradation and destruction of these vitamins in storage could ultimately attribute to the presence of vitamin deficiency diseases amongst the animals.

Retinol acetate, the most commonly used source of vitamin A formulated into animal premixes, contains five double bonds within its structure which are highly susceptible to oxidation [9]. α-tocopherol contains a free phenolic hydroxyl group while cholecalciferol contains a hydroxyl group, both of which can be easily oxidised. However, the hydroxyl group present in α-tocopherol is usually replaced with an acetate group when formulated into animal premixes. As α-tocopherol acetate contains no double bonds and no free hydroxyl groups it is significantly more stable in feeds with neutral or slightly acidic pH [9]. However even under slightly alkaline conditions the protective acetate can be cleaved, and free tocopherol is formed, which can be easily and rapidly oxidized [9].

In order to limit the effect of oxidation on the vitamins and other vulnerable ingredients, synthetic antioxidants are usually formulated into the premix. Butylated Hydroxytoluene (BHT) is the most commonly used antioxidant within the food, pharmaceutical and oil industries [24]. BHT, like other synthetic phenolic antioxidants, derives its antioxidant properties from its molecular configuration. BHT contains a labile hydrogen atom within a hydroxyl group that can be easily donated. The donation of this hydrogen atom can reduce the amount free radicals present within the environment which can protect the the stability of other high-cost ingredients present within the premix [30]. The presence of antioxidants such as BHT, not only allow for increased shelf life as it reduces the overall oxidative potential of the premix but, can also prevent rancidity of the fat content and maintain palatability [25]. BHT has been found to be safe within poultry diets up to an inclusion rate of 200 ppm of the total diet [25].

The aim of this study was to analyse the differences between four different commercially available organic Cu sources and inorganic Cu sulphate in relation to their effect on the stability of α-tocopherol acetate and the activity of BHT and three commercially available phytases in vitro.

2 Materials and methods

2.1 Materials

All materials used were of ACS grade or higher where appropriate.

Acetic Acid, acetone, ammonium molybdate, beta-carotene, BHT (≥ 99.0% (GC)), copper (II) sulphate pentahydrate, dimethyl sulfoxide, linoleic acid, methanol, phytic acid, potassium chloride, sodium acetate, sulphuric acid, tetrahydrofuran, and Tween 20 were obtained from Sigma Aldrich, Arklow, Ireland.

The α-tocopherol acetate (50%) was sourced by Masterfeeds, Winnipeg, Canada.

The three phytases used in this study; two 6-phytases (Phy 1 and Phy 2) and one 3-phytase (Phy 3) were obtained from independent distributors, rather than the manufacturers of the products.

Proteinate 1, Proteinate 2, Cu Glycinate and the Cu amino acid complex were also obtained from independent distributors, rather than the manufacturers of the products.

Prior to the experiments the Cu sources were analysed using inductively coupled plasma-mass spectrometry (ICP-MS) to ensure that the mineral concentrations of each was as expected.

2.2 Methods

2.2.1 Beta-Carotene linoleic acid bleaching assay

The five Cu sources were weighed out so that, when extracted with 50 ml of distilled water, each would contain the recommended National Research Council (NRC) level of Cu [26] suggested for broilers. The extracted Cu samples were then filtered and analysed using ICP-MS to ensure that each Cu sample had the same amount of Cu in solution.

BHT inhibition was analysed using a slightly adapted version of the beta-carotene bleaching assay described by Dapkevicius et al. [27]. The analysis was performed by spectrophotometry using a PG Instruments Ltd T70 UV/Vis spectrometer and UV win 5 software.

A stock solution of β-carotene/linoleic acid was prepared by adding 35 µl of linoleic acid and 252 µl of tween 40 to 1.4 ml of β-carotene dissolved in chloroform (0.5 mg/ml). The chloroform was subsequently evaporated using a rotary evaporator. 70 ml of oxygenated distilled water was added with vigorous shaking. 2.5 ml aliquots of this reaction were transferred into cuvettes along with 400 µl of distilled water, 50 µl of a BHT-methanol solution (35 µg/ml) and 50 µl of the trace mineral solution. A positive control was made up by replacing the trace mineral solution with water, while a negative control was made up by replacing the trace mineral solution with water and replacing the BHT-methanol solution with methanol. Each sample was incubated in a waterbath at 50 °C. After an incubation period of 0, 30, 60 and 90 min, the sample absorbance was accessed at λ470nm to determine BHT inhibition.

2.2.2 Quantification of α-tocopherol acetate

Six simulated premixes were formulated so that they would contain 8,000 ppm of Cu and 15,000 ppm of α-tocopherol acetate with limestone as a carrier. Five of the premixes contained a different Cu source while one contained no Cu and was used as a vitamin control. The amount of α-tocopherol acetate present within these premixes was immediately determined by UPLC analysis. The samples were then placed in storage in a dark room with uncontrolled humidity and temperature to mimic the conditions that would be expected within commercial settings. The premixes were again tested after nine weeks to determine the amount of α-tocopherol acetate still present.

The α-tocopherol acetate was extracted by adding 10 ml of mobile phase B (75:25:5 Acetonitrile: Methanol: THF with 0.035% Acetic Acid) to 1 g of each premix. The samples were then placed on a plate shaker for 30 min at 500 rpm. The samples were then centrifuged for 5 min at 4000 rpm and filtered using 3 ml syringes with a leur lock tip and Chromafil RC-45/25 filters. The samples were then diluted by adding 80 µl of the extracted samples to 1.92 ml of mobile phase B and then further filtered into amber glass vials and capped.

The quantitative analysis of the vitamins was performed using an adapted version of the reverse phase high performance liquid chromatographic method for simultaneous determination of fat-soluble vitamins developed by Joseph et al. [28]. The UPLC analysis of the samples was performed on an Agilent 1290 Infinity LC System consisting of a binary pump with integrated vacuum degasser, an autosampler, a thermostatted column compartment containing a Zorbax RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm column and a Diode array detector. The chromatographic parameters were adapted from Joseph et al. [28] with a few alterations (Table 2).

2.2.3 Determination of phytase activity

Three phytases, Phy 1, Phy 2 and Phy 3, were prepared in 5 mM acetate buffer and extracted for 1 h at room

Table 2 Chromatographic parameters used for the Agilent 1290 Infinity LC system

Parameter	
Column temperature	45 ℃
Acquisition rate	10 Hz
Data acquisition	216 nm, 266 nm, 326 nm
Flow cell	10 mm path
Injection volume	1 µl
Sample thermostat	20 ℃
Mobile phase A	95:5; Water:THF with 0.05% acetic acid
Mobile phase B	75:25:5; Acetonitrile:methanol:THF with 0.035% acetic acid
Gradient	0 min → 30% B
	2 min → 75% B
	5.34 min → 100% B
	10 min → 100% B
	10.06 min → 30% B
Post run time	1 min
Flow rate	.49 ml/min

temperature. The supernatant was collected by centrifugation at 4000 rpm.

The Cu sources were prepared in 5 mM acetate buffer and extracted for 20 min. The supernatant was collected by centrifugation at 4000 rpm. The Cu stocks were prepared and subsequently diluted to 16 ppm as this is the inclusion rate of Cu recommended by the EU feed industry for poultry [38].

Residual phytase activity was measured using a modified version of the assay described by Engelen et al. [29]. The assay was based on the hydrolysis of 0.5 mL aliquots of 2.5 mM phytic acid in 0.2 M acetate buffer by 0.5 mL of appropriately diluted phytase sample in 5 mM acetate buffer. After 10 min, 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 0.1 mL 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 λ380nm to determine the level of orthophosphorus release from the hydrolysis of phytic acid. Absorbance readings of the samples were converted to inorganic phosphorus (KH_2PO_4) concentrations using a standard curve (ranging from 0.1 µmol/mL to 0.5 µmol/mL of KH_2PO_4). Relative phytase activity and the subsequent loss in phytase activity was calculated thereafter.

2.2.4 Statistical analysis

To ensure the reproducibility of the results, all the experiments were repeated in triplicate and a mean value with standard deviation was considered. The samples were evaluated for significant differences with Minitab 19.2020.1. The samples were subjected to a one-way analysis of variance (ANOVA) test with a post hoc Tukey honestly significant difference (HSD) test. The significance level was calculated at $p < 0.05$. The data was presented as a mean of triplicate experiments. Average values lacking a common superscript differ in statistical significance.

3 Results

3.1 The effect of Cu source on the antioxidant activity of BHT

The effect of the various sources of Cu on the antioxidant activity of BHT was assessed by subjecting the five different sources of Cu to the beta-carotene linoleic acid bleaching assay in the presence of BHT. The results demonstrated that Cu had an inhibitory effect on the antioxidant activity of BHT and that the level of inhibition appeared to be dependent on the source of the Cu.

Figure 1 illustrates the effects of the various Cu sources on the antioxidant activity of BHT relative to a positive control. All five Cu sources demonstrated an inhibitory effect on the antioxidant activity of BHT. However, three of the organic copper sources; Proteinate 1 (15.6%), proteinate 2 (16.3%) and the amino acid complex (21.9%) exhibited significantly less ($p < 0.05$) BHT inhibition than the samples containing the glycinate (32.1%) and Cu sulphate (28.9%) sources.

3.2 The effect of Cu sources on the stability of α-tocopherol acetate

The effect of the various Cu sources on the stability of α-tocopherol acetate was assessed through UPLC analysis of simulated premixes containing α-tocopherol acetate and one of five different Cu sources. The results noted that after the nine-week storage period the amount of α-tocopherol acetate present within all six premixes had decreased, however, the rate at which it had decreased was dependent on the source of the Cu present within each premix.

Figure 2 illustrates the effects of the various Cu sources on the stability of α-tocopherol acetate after a

Fig. 1 Impact of Cu source on the antioxidant activity of BHT relative to a positive control. Data are a means of triplicate experiments. Average values lacking a common superscript differ in statistical significance according to one-way ANOVA and separation by Tukey's test ($p \leq 0.05$)
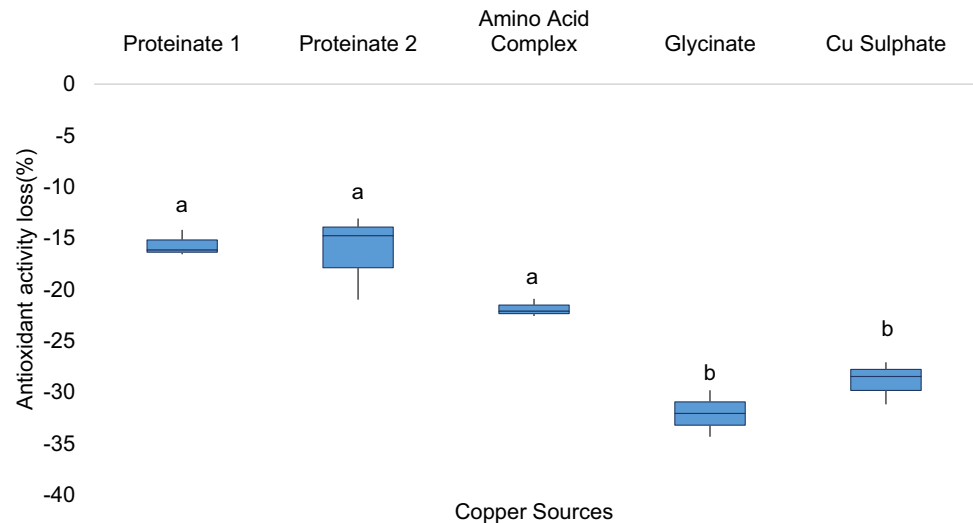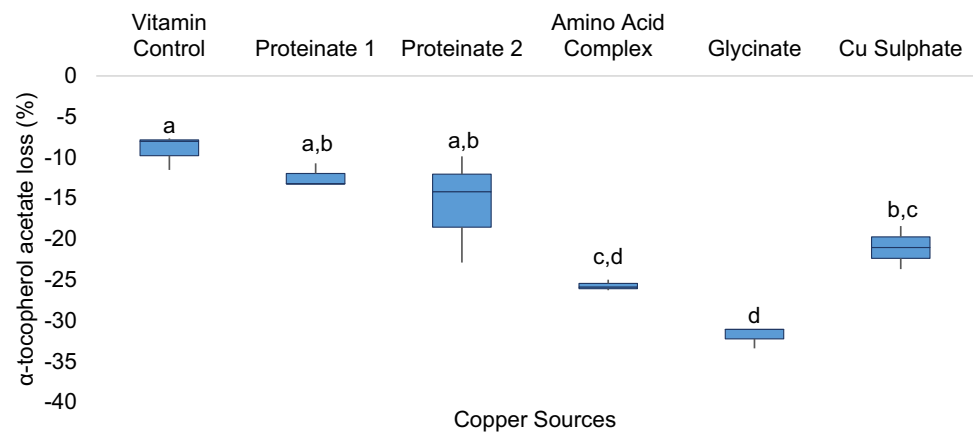

Fig. 2 The effect of the various Cu sources on the stability of α-tocopherol acetate over a nine-week period. Data are a means of triplicate experiments. Average values lacking a common superscript differ in statistical significance according to one-way ANOVA and separation by Tukey's test ($p \leq 0.05$)

nine-week storage period. The different Cu sources had varying effects on the stability of α-tocopherol acetate.

As expected, the vitamin control was found to have the lowest decrease in α-tocopherol acetate stability over the nine-week period. It exhibited a loss of 9.1% when compared to the initial 'Day 0' results. This is followed by proteinate 1 (12.4%) and proteinate 2 (15.7%), both of which were not significantly different when compared to the vitamin control. Surprisingly, Cu sulphate (21.1%), while exhibiting a significantly ($p < 0.05$) higher loss than the vitamin control, did not significantly differ from the two previously mentioned organic copper sources. The two samples that had the highest α-tocopherol acetate loss were the amino acid complex (25.7%) and the glycinate (31.9%). Both samples produced significantly ($p < 0.05$) higher losses than were found within the vitamin control and the two proteinate sources.

3.3 The effect of Cu sources on phytase activity

The effect of the various Cu sources on phytase activity was assessed by incubating the individual sources of Cu with three different phytase preparations (Phy 1–3). The results demonstrated that Cu had an inhibitory effect on phytase activity and that the rate at which the phytase activity was affected was dependent on the Cu source and the phytase in question.

Figure 3(a–c) illustrates the effects of various Cu sources on the phytase activity of Phy 1, 2 and 3. In general, proteinate 1 and proteinate 2 elicited the lowest rate of phytase activity loss. In all three cases it was noted that proteinate 1 and proteinate 2 exhibited significantly lower ($p \leq 0.05$) phytase activity loss than the phytase preparations incubated with Cu sulphate. The main difference amongst the three phytases was the effect of the amino acid complex when compared to the effect of the other three Cu sources.

Fig. 3 Phytase activity loss of **a** Phy 1, **b** Phy 2 and **c** Phy 3 after exposure to different Cu sources. Data are a means of triplicate experiments. Average values lacking a common superscript differ in statistical significance according to one-way ANOVA and separation by Tukey's test ($p \leq 0.05$). Phy 1, 6-phytase; Phy 2, 6-phytase; Phy 3, 3-phytase
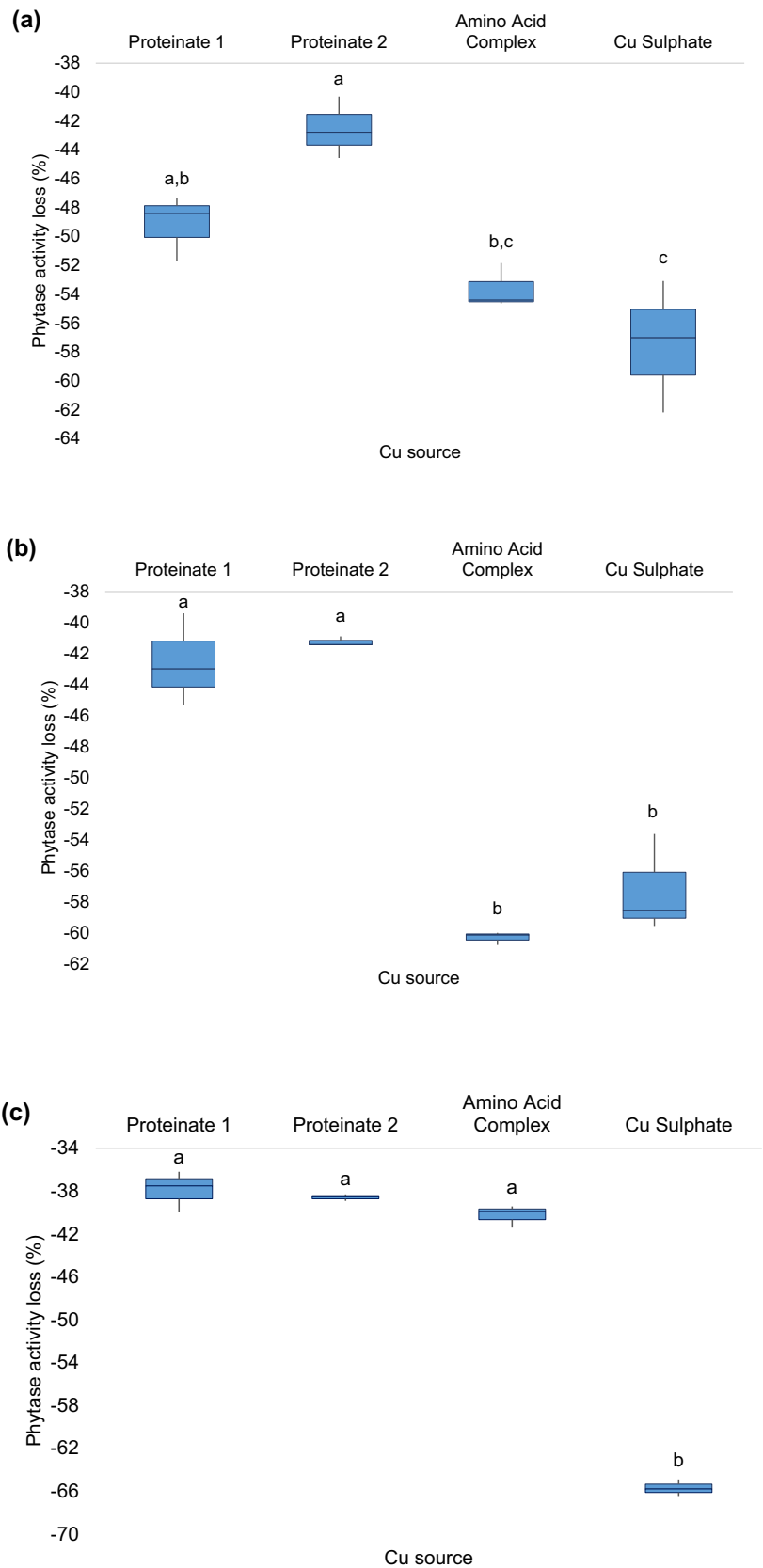
In the case of Phy 1, it was found that, while there were slight differences between them, there were no significant differences ($p \leq 0.05$) between the amino acid complex (53.6%) and both proteinate 1 (49.1%) and Cu sulphate (57.4%) in relation to the loss of phytase activity. This differed when examining Phy 2 as the amino acid complex (60.3%) was found to have significantly increased ($p \leq 0.05$) activity loss when compared to both proteinate 1 (42.6%) and proteinate 2 (41.2%). In the case of Phy 3, all three of the organic Cu sources were found to have significantly lower ($p \leq 0.05$) phytase activity loss than their inorganic counterpart, Cu sulphate (65.7%). The main difference between the three sources of phytase was that the amino acid complex was seen to be more inhibitory to the 6-phytases than it was to the 3-phytase.

4 Discussion

The redox activity of transition metals can lead to the formation of reactive oxygen species (ROS) such as hydroxyl (OH^-) and superoxide (O_2^-) radicals through Fenton type reactions [31]. These ROS can oxidise a wide range of organic substrates with high activity and are one of the main stress factors involved in the stability of a number of premix ingredients, including vitamins and synthetic antioxidants [9]. The binding of organic ligands, such as proteinates, to the trace metals can limit their ability to initiate formation of free radicals [9]. The relative strength of the interaction between the metal and the ligand can be assigned a value, which is known as the stability constant [18]. A high stability constant indicates a relatively stable complex or chelate in which the ligands are not displaced to any significant extent [20]. The bond strength between the trace elements and the ligand will vary depending on the choice of ligand and whether the trace element is either complexed or chelated to the ligand [18]. It would be expected that the strength of the bond between the trace metal and the ligand would impact the level at which the trace metals can interact with other components present within the environment. This would impact the rate at which the trace minerals are able to initiate the formation of free radicals and subsequently impact the level of oxidation of the other premix ingredients. The results from this experiment indicated that the in vitro relationship between Cu and α-tocopherol acetate, the antioxidant activity of BHT and phytase activity can be significantly influenced ($p \leq 0.05$) by the source of the Cu.

4.1 The effect of Cu source on the antioxidant activity of BHT

The results generated from this study demonstrated that the in vitro relationship between the antioxidant activity of BHT and Cu can be significantly impacted ($p \leq 0.05$) by the Cu source.

It would have been expected due to some previous studies and reviews performed in the area of mineral interaction and antioxidant activity [9, 23, 32, 33] that the organic Cu sources would inhibit the antioxidant activity of BHT at a lower rate than that of their sulphate counterparts. This was expected as the Cu present within the organic sources are bound to organic ligands which, as previously mentioned, would be expected to limit their ability to initiate the formation of free radicals and subsequent oxidation of the hydroxyl group of BHT [32, 33].

The antioxidant activity of BHT in the presence of Cu was analysed and it was found that both proteinate Cu sources and the amino acid complex exhibited significantly lower ($p \leq 0.05$) antioxidant activity loss when compared to Cu sulphate. Unexpectedly, it was also noted, that despite being complexed to glycine, the antioxidant activity loss of BHT in the presence of Cu glycinate was similar to that noted with the Cu sulphate. This would indicate that the free radicals formed by both proteinates and the amino acid complex were significantly less ($p \leq 0.05$) than that of Cu sulphate, which produced similar amounts of free radicals to Cu glycinate.

When comparing the stability of various amino acids, it can be noted that more complex ligands generally produce more stable complexes [18, 20]. This would explain why the proteinates exhibited significantly reduced antioxidant activity loss than Cu glycinate. The two proteinates contain multiple short chain peptides which produce a more stable complex with Cu [20], thus limiting the potentially negative interactions with other components.

As previously mentioned, Cu glycinate elicited similar levels of antioxidant activity loss when compared to that of Cu sulphate. There are numerous reasons that this may be the case including solubility and stability in solution. In order to limit the effect of mineral solubility on this experiment, all the samples were made up so that they would contain the same amount of Cu in solution. This, however, would not have been an issue for both the Cu glycinate complex and Cu sulphate as both are completely soluble in water at the concentrations used in this experiment [49].

Another reason behind this could be that the stability of the Cu glycinate complex is altered when added to water which, in turn, could cause it to become highly unstable or, even separate entirely. This is not the case however, as it has been shown that the Cu glycinate complex stays intact when solubilised in an aqueous solution [49].

The most plausible reasoning behind this result is that that the bond between Cu and glycine is relatively weak and, as such, can be readily dissociated in the presence of other components which would allow for the Cu ion to

initiate the formation of free radicals at a rate similar to that of Cu sulphate.

Overall, it can be determined that, in vitro, the Cu source significantly impacts ($p \leq 0.05$) the level at which the antioxidant activity of BHT is inhibited.

4.2 The effect of Cu sources on the stability of α-tocopherol acetate

The results from this study demonstrated that the in vitro relationship between α-tocopherol acetate stability and Cu can be significantly impacted ($p \leq 0.05$) by the Cu source.

The effects of trace mineral sources on vitamin stability within simulated animal premixes has been documented over the years [9, 10]. Coelho [9] compared the effect that a chelated mineral source and various inorganic mineral sources had on the stability of various vitamins within a simulated premix and noted that the chelated minerals showed less α-tocopherol acetate loss than oxide, carbonate, and sulphate sources. Shurson et al. [10] compared the effect that specific metal amino acid complexes and sulphate sources had on the stability of various vitamins within a simulated premix and found that there was no significant difference ($p \geq 0.05$) in relation to α-tocopherol acetate loss between the vitamin control, amino acid complexes and sulphate sources.

The results generated from this study suggests that the source of the Cu significantly affects ($p \leq 0.05$) the stability of α-tocopherol acetate and that, similar to the results obtained by Shurson [10], not all of the organic sources increased α-tocopherol acetate preservation when compared to the inorganic sulphate source. When compared to the vitamin control sample it was found that only two of the Cu sources, proteinate 1 and proteinate 2, did not significantly increase α-tocopherol acetate loss. As previously mentioned, the stability of proteinates is generally higher than that of amino acid complexes due to the strength of the bond between the copper ion and the peptide-based ligands [20]. This increased stability leads to the formation of more stable complexes with Cu. In this case it could be expected that the stable bonds between the proteinates and the Cu reduce the dissociation of the Cu and the subsequent formation of oxidising free radicals, which are known to oxidise and break down α-tocopherol [9]. The Cu amino acid complex, Cu sulphate and Cu glycinate samples were all found to significantly ($p \leq 0.05$) increase the level of α-tocopherol acetate loss when compared to the vitamin control. This would partially correlate with the results obtained by Shurson et al. [10] who found that there was no significant difference between their organic mineral source, which was an amino acid complex, and the sulphate mineral sources. Furthermore, the Cu glycinate sample was found to have significantly increased

($p \leq 0.05$) vitamin loss when compared to the Cu sulphate sample. This could indicate that the bond between the Cu ion and glycine is relatively weak and, as such, the Cu can easily dissociate, generating free radicals which can subsequently oxidise the vitamin.

The α-tocopherol acetate loss noted in this experiment was slightly higher than would have been noted in the previous experiments performed in relation to mineral and vitamin interactions [9, 10]. As previously mentioned, α-tocopherol would be rapidly oxidised if added directly to feeds and, as such, is added as α-tocopherol acetate which is highly resistant to oxidation and provides much higher stability within premixes [9]. However, in this case, the α-tocopherol acetate is added to a simulated premix that contains a high proportion of limestone. The presence of limestone within the premix will shift the pH of the premix to the alkaline region which can cause cleavage of the protective acetate group and allow for the rapid oxidation of the newly formed free tocopherol [9].

So, while the overall level of α-tocopherol acetate loss varies between this study and previous studies performed in this area [9, 10] it can be determined that, in vitro, the Cu source significantly impacts ($p \leq 0.05$) the level at which the α-tocopherol acetate is lost and that not all organic Cu sources will decrease α-tocopherol acetate loss when compared to inorganic Cu sources.

4.3 The effect of Cu sources on phytase activity

The results obtained from this study demonstrated that the in vitro relationship between phytase activity and Cu can be significantly impacted ($p \leq 0.05$) by the source of both the phytase and the Cu.

The effect of Cu source on phytase activity has been well documented over the years [34–36]. The present research verified the highly inhibitory effects that Cu can have on phytase activity, and how different phytases react differently upon exposure to Cu.

Each of the three phytases exhibited a dramatic reduction in their ability to dephosphorylate phytic acid when exposed to each of the four Cu sources. However, each phytase responded differently when comparing the effect of each of the Cu sources. Similar trends were noted between Phy 1 and Phy 2 in relation to the loss of phytase activity. The remaining phytase activity of Phy 1 after exposure to proteinate 2 was significantly ($p \leq 0.05$) higher than that of the phytase exposed to the amino acid complex and Cu sulphate.

Phy 2 was shown to have a similar trend to Phy 1 in that the remaining phytase activity after exposure to proteinate 2 was significantly ($p \leq 0.05$) higher than that of the phytase exposed to the amino acid complex and Cu sulphate.

The results noted for Phy 3 differed from that of the previous two phytases, in that, the three organic Cu sources were significantly better than the Cu sulphate at retaining phytase activity after exposure and that there were no significant differences in relation to the effect on phytase activity between the Cu amino acid complex and proteinate 2. The difference in the response of Phy 3 in comparison to Phy 1 and Phy 2 may have been related to the class of phytase in question, i.e.: Phy 3 was a 3-phytase whereas Phy 1 and Phy 2 were both 6-phytases. This result would not have been unexpected as phytases can respond differently to metal ions depending on their biochemical and biophysical traits [37].

Unlike BHT and α-tocopherol acetate, Cu does not affect phytase activity through oxidation. Cu can affect phytase activity through direct interaction with the enzyme itself or indirectly via phytic acid [39], however, it is difficult to ascertain which is responsible for the decline in phytase activity in this case and further experimentation is required to ascertain the specific method of inhibition.

Trace minerals can influence the enzyme function of various enzymes included in animal feed, including phytase, through interactions with amino acid residues at active catalytic sites or through changes to enzyme structure [34, 40]. These interactions can result in both agonistic and antagonistic modulation of enzyme activity [41]. Trace minerals can also affect phytase activity indirectly through interactions with phytic acid. Phytic acid is strongly negatively charged over a wide pH range [42] and as such can easily chelate divalent and trivalent metal ions, including Cu^{2+}, and form both soluble and insoluble complexes [43–45]. While the majority of these complexes are soluble at low pHs (< 3), phytate tends to chelate with Cu and/or other cations and form insoluble complexes at higher pHs (> 5) [46, 47]. These insoluble complexes are less available to phytase and as such will lower the amount of phosphorous released [48].

This reasoning may explain the differences in the loss of phytase activity after exposure to the four Cu sources. In general, proteinate 1 and proteinate 2 show a higher retention of phytase activity after exposure than the amino acid complex which, in turn, shows a higher retention of phytase activity than Cu sulphate. These results would be as expected due to the stability of the various ligands. The proteinates, which would contain the strongest bonds, would limit the interactions between the Cu and the phytases and/or the phytic acid. This would limit any agonistic or, in this case, antagonist effects on the phytases as well as limiting the formation of insoluble Cu phytate complexes, both which can affect phytase activity.

While further work will be needed to conclusively determine the specific method by which Cu inhibits the phytase activity of these three phytases, it can be determined that the in vitro relationship between phytase activity and Cu can be significantly impacted ($p \leq 0.05$) by the source of both the phytases and the Cu.

5 Conclusion

Overall, it can be determined from the results noted in this experiment that the source of Cu formulated into a premix will significantly ($p \leq 0.05$) impact the level at which the Cu is available to interact with and affect the stability and activity of the other premix components present and, in particular, vitamins, enzymes and synthetic antioxidants.

Declarations

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