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Evaluation of different indicators for mixing homogeneity of phytase in broiler feed

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Table of contents

Preface

This thesis describes some of the challenges and possibilities that lie in the field of mixing processes in animal feed. This thesis was written as an assignment for the Animal nutrition group of Wageningen University and DSM.

I would like to thank Thomas van der Poel as my supervisor for his critical review and guidance during this project. His knowledge extents to many fields in animal nutrition and feed technology which helped me a lot during the project. Other noteworthy people I would like to thank is Tamme Zandstra from the Carus facility at Wageningen University. He gave me the instructions to use the machines used in this trial and discussed with me the underlying theories.

Other people I would like to thank is the people from the animal nutrition lab and the operators of ABZ Diervoeding at the feed mill in Leusden. Without these people's contribution this project was not possible.

Abstract

An experiment was conducted to investigate the use of different indicators for the mixing homogeneity of phytase. The feed consisted of a corn-soy based diet meant as a starter diet for broiler chicks. Different indicators were investigated in several treatments, which differed in mixing time. Indicators used were phytase, measured in FTU/kg, Ti02, measure in mg/kg Ti, NaCl, measured in mg/L Cl^- and Microtracer Red, measured in particle count. Mixing times were 0, 120, 240 and 480 seconds in a vertical conical mixer. For one batch indicators and phytase were just added on top of the mixture. In the other batches phytase and indicators were added by using a premix. Mixing homogeneity was evaluated by analysing the concentration of 9 consecutive samples taken from the mixer and pelletizer. From the analysis of indicators the coefficient of variance (CV) was calculated. CV was used to indicate the degree of mixing homogeneity. Ti02 showed the best mixing homogeneity with a CV lower than 1.8% at 480 seconds mixing time. NaCl showed to have the worst mixing homogeneity, with the highest CV of 17.1%.

From this research it was concluded that phytase activity and NaCl are not very suitable indicators for mixing homogeneity of phytase. Ti02 is not very suitable as an indicator for mixing homogeneity of phytase, but can be used as an indicator for other ingredients or nutrients. Based on this research microtracer is the best indicator for mixing homogeneity of phytase. Used indicator should have the same physical traits as the investigated feed ingredient or nutrient.

Introduction

Mixing is recognized as being one of the most important processes in feed production (Behnke, 1996). The feed industry has to deal with different ingredients in their feed, which require different mixing times and ingredient addition order. Furthermore, feed has to be produced quickly, safely, with high quality and with the lowest cost possible to provide a competitive product on the market (Wornick, 1965).

In essence mixing can be described as the rearrangement of two or more ingredients in to a homogenic mixture. Rearrangement can be also seen as kinetic energy that has to put into the ingredients to mix them (Froetschner, 2005), so setting motion to particles is an essential part of mixing (Brothman, 1954). The main objective of mixing is to create a product mixture that is homogenic. This means that the predetermined concentration of ingredients and nutrients are dispersed equally over the feed mixture (Creger, 1957). This homogenic mixture ensures that all nutrients are equally distributed over the mixture, providing the animal with a diet that matches the daily requirement for optimal performance and health (McCoy, 1994). Providing a homogenic dispersion of nutrients in a diet is considered as one of the important quality aspects of feed production. Though some indicate that there is no obvious reason to believe that diet uniformity relates to animal performance (Behnke, 1996), as papers are not in abundance considering this subject. Yet, conclusion made by other authors is that especially diet uniformity plays a critical role in young animals, as more mature animals have larger feed intake and therefore can overcome the variation in diet homogeneity (Clark et al., 2007).

Mixing homogeneity is usually indicated by using Pearson's coefficient of variance (CV). By analysing the quantity of an indicator or nutrient in a set of collected samples throughout the batch of feed, a CV can be determined. A CV of 10% or lower is considered as an acceptable degree of variation (McCoy et al., 1994; Pfost et al., 1979), which indicates that the mixture is almost fully homogenic. The coefficient of variance method considers the mixture as a binary mixture; two ingredients are mixed together. A multi-ingredient mixture is considered as an indicator and the other components of the mixture.

Because of the variable characteristics of every feedstuff, mixing becomes a very complex operation. In literature particle size, particle shape and bulk density are mentioned as the most important characteristics of feedstuffs that affect mixing quality (Axe, 1995). In addition, the segregation characteristics of ingredients, cohesiveness and stickiness of the mixture are of influence. Furthermore, design of the mixer is considered as an important factor in mixing homogeneity (Barbosa-Cánovas et al., 2005). Above mentioned factors and the different use of ingredients in feed mills make particulate feed mixing a complex operation that can be influenced by many factors, especially for microingredients. These different factors and how to study the effects of mixing will be discussed in this report.

Research objective and aim

This research is part of a bigger research project which investigates the effects of different mixing homogeneities on the performance of broilers. For the animal trial multiple feeds need to be produced with different mixing homogeneities, which will be a feed below 10% CV and a feed with a CV above 25%. Therefore, the aim of is study is to produce a non-homogenous broiler feed with phytase for an in vivo trial.

The objective of this study is to evaluate different indicators for mixing homogeneity of feed. In addition, the use of these indicators should give an impression on how to achieve different mixing homogeneities for phytase in broiler feed. The final objective of this study is to compare the mixing homogeneity of phytase after the mixing process.

Research questions

Is it possible to achieve different mixing homogeneities for phytase in broiler feed?

What are suitable indicators for mixing homogeneity of phytase?

What factors are of influence on the mixing homogeneity of phytase in broiler feed?

Is there a difference in mixing homogeneity between post-mixing and post-pelleting?

Literature review

Mixing in feed mills

Mechanism of mixing

As mentioned earlier mixing involves kinetic energy in various ways, as designs of mixers differ. There are three mechanisms described that are of influence on the mixing operation in a physical way. These physical processes are convection, diffusion and shear (Williams, 1968). Although segregation is also mentioned as a possible mechanism that can influence the course of operation, this is caused by a natural physical factor that influences the mixing process instead of the three intentional physical processes instigated by kinetic force.

Firstly, convective mixing is when particles are flown in to a circulating pattern, usually cause by the rotation of mixer drum or a mixing arm (e.g. paddle or ribbon). Convective mixing is very suitable for batch mixing, due to its mainly macroscopic mixing of feed ingredients. Secondly, diffusive mixing is described as the random motion of feed particles that create a mixture, usually caused by an auger in the mixer. The rate of mixing is low in comparative to convective mixing, but the effectiveness of mixing is much higher in diffusive mixing in comparison to convective mixing. Often, these two mechanisms are applied in mixers to achieve a uniform mixture, a clear example is the Nauta mixer (see figure 1).
The last mechanism of mixing is shear, which is induced by the motion of a spinning rotor, creating a vortex as particles in the centre of the mixture move faster that the outside of the mixture. This creates slipping planes in the feed mixture and creating quick and homogenous mixture. This type of mixing is suitable for small batches of feed mixtures, it is not often applied in feed milling as the batches of feed that are produced are much larger.

Though, as described as individual mixing processes, the three cannot be seen apart from each other. As mentioned, it is impossible to have convective mixing without setting up some slipping planes due to shear mixing (Williams, 1968).

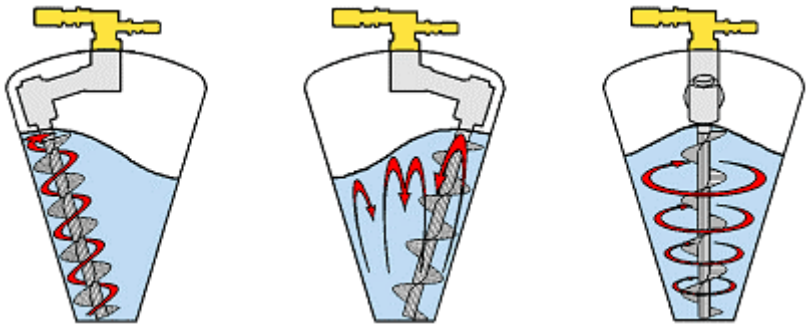

Figure 1. Different mixing processes in a vertical conical mixer

As mixing progresses the homogeneity of the mixture will increase. As mixing time progresses, the mixture will come into a steady state, in which the homogeneity will vary in a certain range (see figure 2). The mixing curve is for every ingredient (or product) unique.

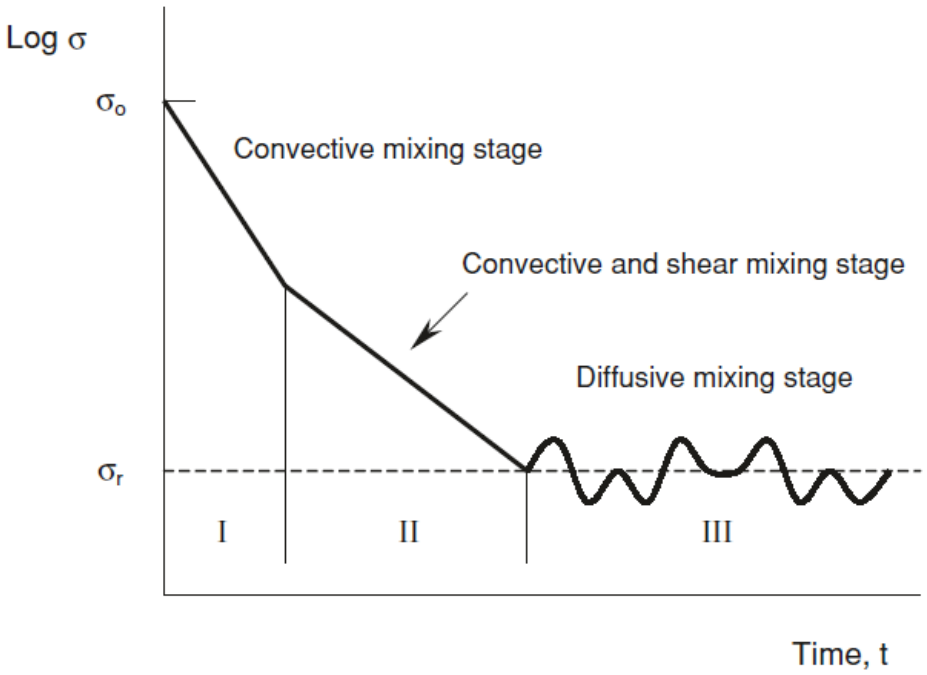

Figure 2. Characteristic curve of mixing process (adapted from Miyanami, 1997).

Mixer types in feed manufacturing

Based on the different mixing mechanisms, several different types of mixer have been designed. A well-functioning mixer employs all of the three mixing mechanisms, to ensure proper mixing. However, every type of mixer differs in the extend of mechanisms for mixing. The design of the mixer will affect the performance, and hence the efficiency of the machine and the quality of the mixed product (Ren et al., 2004).

Roughly, various types of mixers can be distinguished in to two groups; namely the mixers that rely on convective mixing and the other group of mixers that rely on diffusive and shear mixing.

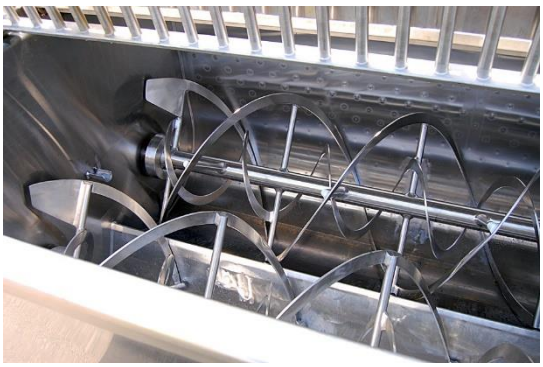

Figure 3. Double ribbon mixer

Convective mixers usually consist of a drum and are equipped with augers or mixing arms in the drum that rotate. If a ribbon or paddles are not added to the drum, segregation forces will influence the mixing homogeneity too much in a negative way. Ribbon mixers make use of a, as it already states, a ribbon that runs around to wall of the drum (see figure 3). Similar mixers are paddle mixers, which have multiple paddles as agitators to mix the product. Horizontal or rotary drum mixers are able to handle a wide range of bulk densities (Armstrong & Behnke, 1996). Furthermore, the ribbon mixer is considered as a more effective mixer, as it has fewer dead spots in comparison to a paddle mixer (Froetscher, 2005). Also, paddles are more sensitive to wear in comparison to ribbon mixers. This wear can affect the effectiveness of mixing. On the other hand, paddle mixers are more suitable for high liquid or fatty feeds in comparison to ribbon mixers, as in ribbon mixers caking of material easily occurs.

A vertical conical mixer is also a known mixer in feed manufacturing. It makes use of an auger which extends along the side of a conical shape container, where the auger rotates around its axis and circles the conical shaped container (see figure 4). The auger transports material from the bottom of the mixer to the top. These types of mixers are known for their good mixing properties, as they are widely used in feed premix manufacturing. The main disadvantage of this mixer is that it usually needs a longer mixing time (in terms of minutes) in comparison to other mixers to achieve a homogeneous mixture.

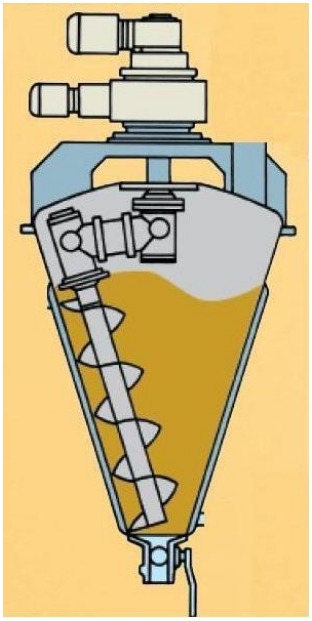

Figure 4. Vertical conical mixer

Besides the difference in mixing mechanism and mixing time, other aspects of factors are important in the overall feed manufacturing. Some mentioned are the ease of loading, suitability for applying liquids, ease of discharge and cleaning (Armstrong & Behnke, 1996). Also, manufacturers of these machines make their own small adjustments to distinguish their mixer from other competitors.

Mixing to perfect homogeneity

As a feed producer feed needs to be produced with the highest safety and quality standards. Achieving a homogenic product is the highest priority in the mixing process. However, many factors influence this process, which will be discussed in this chapter.

Ingredient factors influencing homogenic mixing

Feed mixtures, which have multiple ingredients, has multiple characteristics in terms of rheological aspects. This leads to the possibility that mixtures can un-mix, usually caused by segregation. Segregation is seen as the primary cause of reduced feed ingredient homogeneity (Axe, 1995). Segregation has been studied usually in simple ways, e.g. by using binary mixtures (Clark, 2009). Segregation can be prevented by using ingredients that have uniform particle characteristics, such as size, shape and density (Buslik, 1950; Pfost et al., 1967). Also using other techniques, such as increasing moisture content, agglomeration (e.g. pelleting) and using sticky ingredients can help to prevent segregation in feed mixtures.

Segregation also takes place during the mixture process, this explains that a fully homogenous mixture is not achievable in feed mixtures. As mixing time increases, the processes of mixing and de-mixing will reach an equilibrium, which will be a range of homogeneity also shown in figure 2. At this point increasing the mixing time will not have any effect anymore.

Main factor for de-mixing, in particular segregation, is particle size. If a non-uniform in size mixture is set into motion, for instance due to vibration, the fine particles tent to move downwards, which is called percolation. Percolation is the movement of smaller particles into the gaps of larger particles. Percolation can result in the movement of smaller particles through different layers, and the risk of ending up with a mixture that is segregated. Moreover, due to vibration larger particles move actively upwards (Williams, 1968). Due to the vibration, the larger particles bounce upwards giving the smaller particles an opportunity to move under the larger particles, ensuring a rise of the larger particle to the surface regardless of its density. When mixing homogeneity between uniform and non-uniform mixtures was

compared, the feed with the uniform particle size had a lower coefficient of variance (Djuragic et al., 2009).

As a consequence of particle size, the particle count will also differ between ingredients. Corn will have much less particles per kg of material in comparison to calcium carbonate, for instance. This difference in particle count is very critical in mixing, since different particle count ensure a difference in distribution in the mixture (Froetschner, 2005). Especially in microingredients, large particle counts are necessary to ensure a homogenic distribution through the mixture, so that every animal gets its daily requirements of this microingredient.

Another ingredient factor is the weight of each particle. Particles that are heavier are thrown further away in case of fast movement within the mixer or at a conveyor belt. Heavier and larger particles tend to fall further away than lighter particles, consequently leading to segregation (Yen et al., 1997). This effect is clearly shown in figure 5.

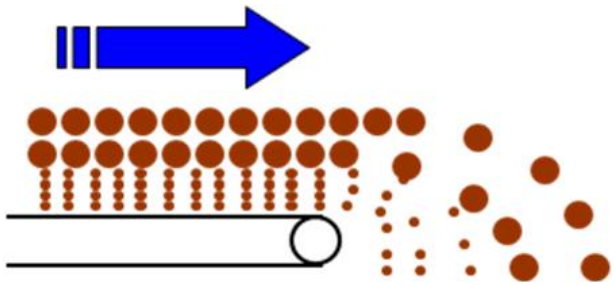

Figure 5. Heavier particles tent to fall further due to conveying, as they contain more kinetic energy

Besides this difference in trajectory of different particles there is also another effect that can cause the segregation of feed, which is caused by piling of the feed mixture for instance in a silo. This characteristic is called the angle of repose, which is the maximum angle in degrees that an ingredient maintains its slope. So, ingredients have different angle of repose, which will influence the mixture when being piled as larger particles tend to run down to the edge of the heap (Barbosa-Cánovas, 2005). Piling can also cause elutriation, which is the segregation of particles due to a counter flow of air. This counter flow stream of air concurs in a bin or silo, due to piling. These types of bulk behaviours can also occur in bunkers in the feed mill, possibly leading to a less homogenic product.

Density is also mentioned as one of the main factors that make feed particles tend to segregate during mixing (Axe 1995; Cuq et al., 2013). In essence, particles with a larger density move downwards in a mixture and low-density particles tend to move upwards. This process of segregation is called condensation (Chen et al., 2015). A clear example of condensation is the movement of wheat or barley hulls to the top of layer mash feed. In premixes it is known that carriers are selected on their density, as the density of micronutrients can be very low. It has been shown that lighter particles have less tendency to segregate than heavier materials (Creger, 1957).

Adhesive factors influencing homogenic mixing

Moreover, adhesive factors influence the mixing homogeneity of feed, as adhesive forces that take place between particles. First there is the electrostatic charge. Due to friction of particles colliding into each other or parts of the mixer, particles become charged. Eventually particles will have a different electro potential, consequently leading to the attraction of opposite charged particles. Electrostatic charge is influenced by humidity, as in lower relative humidity the particle become more conductive. This electro-adhesive force can prevent that the feed flowability is being influenced and its mixability. It is known that products such as riboflavin and several coccidiostats are vulnerable for electro charges (Wornick, 1965). Yet in mixing electrostatic forces can aid the mixing process (Karner, 2010), as particles of unlike materials can attract each other. On the other hand, particles of the same ingredient repel each other,

which can certainly be the case in micro ingredients due to their small particle size. Particles smaller in size usually become more affected by the electrostatic charge, as there is more surface area. The metal of the mixer causes the charged particles to stick to the wall or elements of the mixer, which can have negative effects on the mixing homogeneity.

Secondly there are liquid bridges. Liquid bridges originate from the water binding capacity between two particles. Some feed manufacturers make use of this principle, by adding a little bit of water to the mixture, to ensure that dry and dusty feeds bind better and the mixing homogeneity is improved. Though it is not common that water is added to the mixture in the mixing process, yet in the conditioner water is added and therefore has a big influence on the feed mixture as clogging of particles usually occurs. Liquid bridges can also be formed when the relative humidity of the atmosphere is higher than 65%. Especially hygroscopic ingredients will show clogging or caking, which will have a dramatic effect on the mixability (Clark, 2009).

The third adhesive force is the van der Waals force, as it can that clings particles together. However, this is seen as the weakest form of adhesive forces and is expected not to influence the mixability of the feed mixture. But for the sake of completeness this factor is mentioned.

These three adhesive forces mainly have effect on fine powders with a smaller size than 30µm. It is these three forces which determine the cohesiveness of powders, which is the ability of powders to stick together (Barbosa-Cánovas et al., 2005). More cohesive powders will have more flowability problems than non-cohesive powders. On the other hand, cohesive powders tent to segregate less. So, it is likely that in feed mostly micronutrients with a small particle size can have some mixing difficulties, that is why these are put on a carrier.

Managerial factors influencing mixing homogeneity

Mixing time is considered as the major component of affecting mixing homogeneity in mixing. As can be seen in figure 1, there is an optimal mixing time for a mixture. However, for every ingredient there is an optimal mixing time, dependable the physical factors of the feed ingredients, such as particle size, quantity of material and density (Young and Snaddon, 1951). Furthermore, it is indicated that too long mixing times can result in de-mixing of the mixture (Work, 1954). The explanation for this unmixing process has to do with the competition between the kinetic force and natural force on the feed mixture. Natural physical forces (i.e. segregation forces) have more time to influence the homogeneity of the mixture. Consequently, a certain configuration of particles arises, as it is more likely to occur due to the natural physical processes of segregation.

Also, rotation speed of the mixer influences the mixing homogeneity of a mixture. As rotation speed of the mixer increases, the particles become more displaced from their position and can also come to a fluidized state. In this fluidized state the particles float through the air, consequently leading to very homogenic dispersion of the mixture. Too high speeds give a centrifuge effect and as a consequence, particles are dispersed to the outside of the mixer. Also, low speeds must be avoided, as sloping effects can take effect which leads to inadequate mixing.

Furthermore, it has been shown that paddle speed is of influence on the mixing homogeneity of poultry feed (Ren et al., 2004; McCoy et al., 1994). Ren et al. (2004) also showed that there is an interaction between the rotary speed of the paddle shaft and the mixing time. Paddle speed influences the mixing

time, where increasing the RPM will result in a shorter mixing time. The optimum RPM is influenced by many factors, such as mixer design, the materials that has to be mixed, wear and ingredient build up (Fei, 2006).

Another important factor to recognize is the ingredient order. It is important to use the right order of ingredients added to the mixer, to prevent a non-optimal mixed product. For instance, if micro ingredients are added first and then the fluid products, it will ensure a clogged and caked product of micro ingredients and oils. This caked product will not mix well with the other ingredients. Also, micro ingredients, which are usually a small particle size powder, tend to stay at the bottom of the mixer as it is not scooped up easily by the paddle, screw or ribbon in the mixer. Indeed, micro ingredients should be added later in the mixing process by using a premix (Creger, 1957). This was also shown in an experiment, were big particles were added first and later the smaller, which led to a better mixing homogeneity (see figure 6) (Williams, 1968).
Interestingly, to enable a proper mixing homogeneity, both managerial factors of mixing time and ingredient order should be taken to account (Creger, 1957). It was mentioned that ingredients reach a maximum point of being mixed at different time intervals. Thus, it seems that the moment of adding every specific ingredient to the mixer has benefits for the mixing homogeneity. Remarkably, there hasn't been a lot of research taken place in this field.

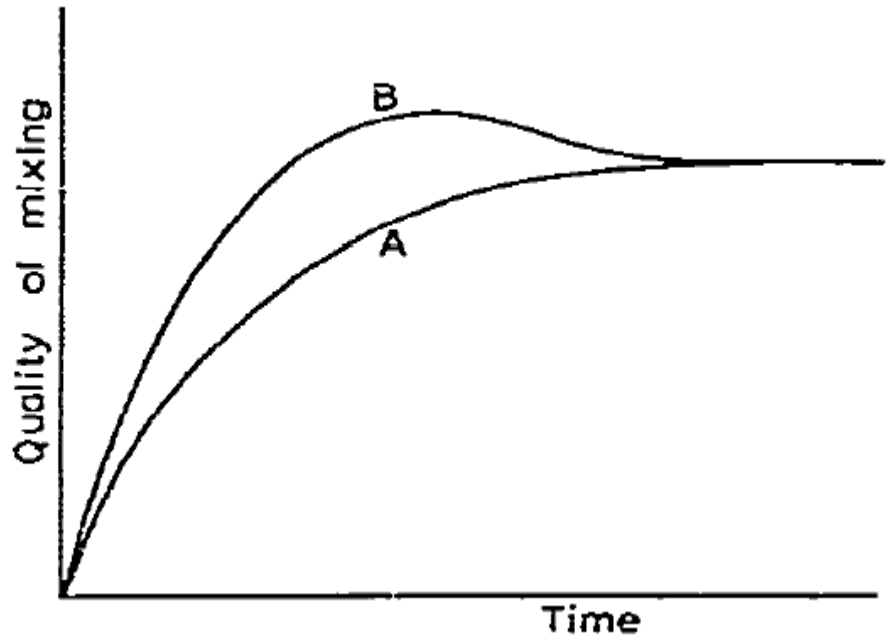

Figure 6. Progress of mixing, with A starting big particles on top and B start with big particles at the bottom (adapted from Williams, 1968).

Microingredients in animal feed

In feed products ingredients are added in different quantities, according to the formulation of the nutritionist. Most formulations have therefore so called major (>10%), minor (1-10%) and micro ingredients (<1%) (Clark, 2009). Common microingredients used in animal feed are vitamins, trace minerals, drugs, amino acids, antioxidants, enzymes, probiotics and prebiotics. In particular micro ingredients need to be distributed in a homogenic way, due to the minimal quantities mixed in to the

feed (Froetschner, 2005). A characteristic of microingredients is that they are of a small particle size, which makes the microingredients vulnerable for segregation and to be retained in death spots in the mixer. To ensure a homogenic mixture, microingredients are usually put in to a premix. When microingredients are added pure into a mixture their physical properties relative to mixing may change (Pfost et al., 1979). This is usually done by a diluent or a carrier. A diluent is an ingredient with no specific activity mixed with the pure microingredient to dilute the concentration of the active microingredient. A carrier is also an ingredient with no specific activity, but to change the physical properties of the microingredient (Pfost et al., 1979). The carrier is usually of a larger particle size than the microingredient and is used to ensure that the micro ingredient is homogenic dispersed through the final mixture (see figure 7). Common carriers that are used in the feed industry are rice hulls, calcium carbonate, corn cob fractions, wheat middlings and DDGS (Armstrong & Behnke, 1996). In some cases, it is beneficial to make a premix in two steps instead of one, as it improves the mixing homogeneity of the mixture (Ivanov et al., 2009).

Figure 7. A mixture of small particles on larger (carrier) particles, adapted from Barbosa-Cánovas et al. (2005).

Fats and oils in animal feed

In many feed formulation fats and oils are a part of the diet for broilers and are commonly referred as binding agents. In many cases, fats are added to the diet, which can be a raw ingredient, such as palm oil or salmon oil, or refined fat liquids, such as lecithin. Fats are a source of energy for the animal, but they also come in handy for the mixing of the granular material of the feed mash. It is interesting to see what positive effect adding oils has on the prevention of segregation of micro ingredients. As macro ingredients, having a large particle size, become coated in a layer of oil they seem to be a better carrier for micro ingredients. As the micro ingredients stick to the macro ingredients due to the oil, no segregation can take place.

Certainly, liquids are an essential product that affect the mixing process, as it can help dust control and improve uniformity of mixtures by the agglomeration of finer particles to larger particles (Armstrong & Behnke, 1996; Clark, 2009). Furthermore, liquids aid in reducing electrostatic charges.

Though addition of liquids can complicate the production process and agglomerates can be expected, which negatively influence the mixing homogeneity (Wornick, 1965). So, the use of liquids can be beneficial, when used properly and agglomerates are prevented. These binding agents are preferably

sprayed in the mixer, so the dispersion of the liquid is equally over the mixture and no large agglomerates of the same material appear (Armstrong & Behnke, 1996). However, the amount of liquids, types of oils and fats and the moment of addition to the mixture has not been studied very well and possible improvements can be made.

Transport and (temporarily) storage in feed mills

In feed mills feed ingredients and end products are subjected to different transport methods to get the material from one location to another for many purposes, such as storage, scaling, mixing and pelleting. Transport can be of influence on the mixing quality of the mixture. As due to handling particle movement will take place, which can lead to segregation of the mixture. Consequently, micro-ingredients become settled out, become concentrated, are collected in the dust-collection system or are attracted by dielectric material.

To complex the transportation and mixing process even further, in many factories the production line is equipped with a bin, which can hold production material temporarily. In these bins additional de-mixing can take place. This is clearly shown in figure 8 and this same effect can take place in bins. Due to the deposit of material in a bin, the particles will move differently into a heap. This is caused by the angle of repose, which is the angle of a poured heap of particles with a horizontal plane. The angle of repose is a parameter that is usually used for measuring the flowability of a granular material. So, it is dependent on the physical characteristics of the ingredient particles how they behave in depositing and flow of the feed mixture.

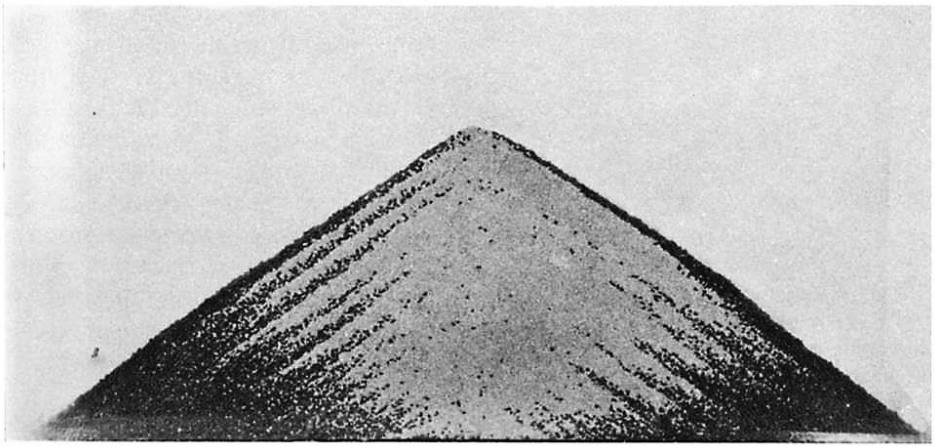

Figure 8. Segregation shown in cross-section through a heap formed from a binary mixture of small black and large white particles. Adapted from Williams 1968.

Moreover, usually feed does not flow uniformly through bins, hoppers, feeders or chutes. Common flow problems that have influence on the homogeneity of feed are ratholes and flooding. Ratholing happens when only the section of material above the opening of the silo or hopper is flowing out, while the rest of the material maintains in the silo. In combination with the segregation process mentioned before, this can be very risky. Flooding is the fluidization of particles, usually involved in ratholing as fine particles tend to pack in the middle. Preferably the mass in the silo is moved equally and constantly out of the discharge chute of the silo, this ensures that de-mixing is prevented (Barbosa-Cánovas et al., 2005).

In a perfect situation feed production lines are completely vertically installed, so ingredients can be dropped from the top and then moves down through all of the various production stages by gravity.

However, this is never the case, as it requires a very high building which is usually not feasible. Therefore, many transport systems in the feed mill are present, to move the material vertically and horizontally through their respective destinations. To accomplish this transport, multiple conveyors system is used, such as belt conveyors, chain conveyors, screw conveyors and pneumatic conveyors. These transport systems have effect on the distribution of ingredients through the mixture, as some of these transport mechanisms promote segregation in the mixture.

Assessing mixing quality

As has been shown in the previous paragraphs, many factors are of influence on the mixability of a feed mixture. Therefore, it is necessary to known to what extend the feed mixture has been properly mixed, in terms of homogeneity. It is possible to do a chemical measure for mixing homogeneity, for instance on vitamin levels or trace elements. However, these methods are time consuming and expensive.

To overcome this problem several indicator methods have been developed and tested (Eisenberg, 2004; Rocha et al., 2015; Çiftci & Ercan, 2002; Clark, 2007). Making use of indicators is a much more economically and time efficient method of determining the mixing homogeneity in comparison to analysing every nutrient. Preferably, used indicators have the same physical properties as the selected ingredients that needs to be investigated.

By using the indicator, various statistical methods are available to calculate the degree of homogeneity. To assess the mixing homogeneity of a key component, let x_i (i=1,2,…,N) be the concentration of the component in the ith sample. N are the number samples needed for analysis, usually taken from random locations in the mixer or with a regular interval at the discharge outlet. Yet, if the analytical method for a particular ingredient has a higher variation than the variation of the mixer, it will not make it as a suitable indicator for assessing mixability (Pfost et al., 1979). From these x_N samples the coefficient of variance can be calculated, which is an indication of mixing homogeneity.

Different indicators can be used in assessing the degree of homogeneity of mixtures. Using multiple coloured particles to assess the mixability of a product can be used when e.g. mixing patterns are investigated to see were ingredients are dispersed from certain locations (Chen et al., 2015). The producer of similar colour indicators provides their product mainly for testing the mixing homogeneity of microingredients (Eisenberg, 2004). This last product consists of charger iron particles with a dye, that can be used to assess the degree of homogeneity. These iron particles are isolated from the samples by using a rotary magnetic separator. The producer indicates that the method is suitable for both scientific as well as for practical purposes, such as finetuning the mixer and determining contamination levels.
Another common method for assessing mixing homogeneity is the chloride method (Çiftci & Ercan, 1994). Salt is a common ingredient in animal feed and has similar properties as microingredients in terms of particle size and density. This method analyses the concentration of chloride ions in the mixture. It is a very easy method, that does not require any lab training. In addition, it is cheap, compared to the other methods.
Many other indicators have been investigated, mainly by two papers (Clark et al., 2007; Rocha et al., 2015). From these papers it was concluded that indicators such as crude protein, phosphorous and chloride are not desirable indicators (Clark et al., 2007), as well as MnS, CuCl, ZnS and NaCl (Rocha et al., 2015). As these indicators can be present in many feed ingredients and are therefore not suitable

for accurate analyse of mixing homogeneity. This should be taken to consideration in finding a suitable indicator.

Table 1. Different investigated indicators in several compound feeds

Marker	Clark, 2007	McCoy et al, 1994	Cifti & Ercan, 2002	Ren et al, 2004	Froetschner, 2005	Rocha et al, 2015	Djuragic et al, 2
DL-Met (99%)	✓					✓	
L-Lys-HCL	✓					✓	
CP	✓						
Chloride	✓	✓	✓			✓	
Phosphourus	✓						
Manganese	✓					✓	
Microtracer Red #40 (count)	✓	✓					
Microtracer RF-Blue lake	✓	✓					✓
Roxarsone (3-nitro)	✓						
Semduramicin	✓						
Chromium		✓					
Methylene violet				✓			
Natrium ion					✓		
Vitamin B2						✓	
Copper						✓	
Zinc						✓	
L-Threonine						✓	

Materials and methods

Feed composition

A broiler mash diet was formulated without the addition of phytase enzymes. The composition can be found in Table 1. The broiler mash diet is a starter feed, meant for this preliminary technological trial and the in vivo broiler trial. In the basal feed phytase content was formulated to be as low as possible, where the target dose of added phytase will be 500 FYT/kg.

Vitamin/mineral premix was produced at Twilmij B.V. company, located in Stroe, the Netherlands.

Table 2. Feed composition of the basal feed

Ingredients (g/kg)	Basal starter diet
Corn	541.50
Brazilian HP soybean meal (480 g/kg CP)	296.60
Rye	39.10
Oat hulls	20.00
Potato protein	10.90
Corn gluten meal (prairy gold)	9.20
Soy oil	23.00
Poultry fat	7.50
Salmon oil	5.00
Limestone	17.60
Monocalcium phosphate	6.10
Sodium bicarbonate	3.80
Salt	0.80
Vitamin/mineral mix[1][2]	5.00
Lysine-HCl (L, 79%)	2.60
Methionine (DL,99%)	2.60
Threonine (L, 98%)	0.90
Valine (L, 99%)	1.90
Salinomycine (coccidiostat)	5.80
Total	1000.00
Calculated Analysis	
Metabolizable energy (kCal/kg)	2959.94
Crude protein (g/kg)	210.03
Calcium (g/kg)	9.00
Phosphorous (g/kg)	5.00
Lysine (g/kg)	13.00
Methionine (g/kg)	5.90

[1] Vitamin/mineral mix supplied per kg of diet: 100.00mg Ca, 10.15mg P, 6.75mg Mg, 14.75mg K, 0.60mg Na, 111.25mg Cl, 76.55mg S, 50.00mg Fe, 2.00mg I, 12.50mg Cu, 75.00mg Mn, 70.00mg Zn, 0.25mg Se, 10,000.00IU Vit A, 3,333.33IU Vit D3

[2] Vitamin/mineral mix carrier is baby corn (=corn starch)

Materials and mixing protocol

Production of the mash/pelleted broiler diets takes place at ABZ Diervoeding, located in Leusden, the Netherlands. Several mixing times were used to achieve different levels of mixing homogeneity of phytase in the broiler feed. A vertical, conical Nauta mixer, series 242, was used. The mixer has a capacity of approximately 700 litres. The phytase product is rhonozyme hiPhos from DSM, this is the product that also will be analysed for phytase activity. In addition, three other indicators are used to study the effect of mixing time on the mixing homogeneity of phytase. These three indicators are Chloride, Titanium dioxide and Microtracers.

Five different mixing times will be used in the mixing trial trial. Indicators are used to measure mixing homogeneity. Indicators that are used are NaCl, Microtracers and titanium dioxide.

Phytase product – phytase premix

For one of the batches (batch no. 1) phytase product will be added on top. This means that the product is directly added to the complete broiler feed. For the other batches, a premix is made of the phytase product and put onto a corn meal carrier or we will use part of the broiler feed. The mixer that is used to prepare the premix, is an Imperator 40 dough mixer, mixing time for premix is set at 6 minutes. To use a premix of phytase product, broiler feed is mixed to ensure that a 1% inclusion of phytase premix results in a 500IU/kg concentration in the broiler diet. Mixing time is 4 minutes to produce the phytase premixture. Microtracers, with a particle size of 150µm, are added in a concentration of 0,016 g/kg. Titanium dioxide (TiO2) is added in a concentration of 2,00 g/kg.

Mixing protocol

Table 3 shows the mixing times to be used to achieve a <10% or >25% coefficient of variance (CV). The mixer was cleaned and checked for any abnormalities or malfunction before the experiment started. Batches of 500 kg of broiler feed mash were put in to the mixer. The phytase and indicator premix were added on top of the broiler feed in the centre, according to Table 3.

Table 3. Mixing times used and method of adding phytase & indicator premix to get the target coefficient of variance of phytase

Batch	Phytase dosage	Target CV	Mixing time	No. of samples
0	Diet before addition of phytase	N.A.[2]	Negative control	3
1	No premix = adding phytase (on top)	> 35%	no mixing (<2sec)	9
2	1% Premix[1]	> 25%	no mixing (<2sec)	9
3	1% Premix[1]	< 20%	120 sec	9
4	1% Premix[1]		240 sec	9
5	1% Premix[1]	< 10%	480 sec = above recommended mixing time[3]	9
	Total			48

[1] 1% premix = Mix phytase and indicators with ground corn from the mix or blank diet;

[2] N.A. = not applicable

[3] Recommended mixing time by feed manufacturer is 240 seconds.

Basal diet was fully mixed, phytase was added as final ingredient to the mixture. After adding the phytase mixing started, at the same moment a timer was started to control the mixing time. When the required mixing time was reached, the machine was stopped and the mixer outlet was opened.

Pelleting

After mixing, the feed mash was discharged into a bin at a lower floor and then unloaded in a pit with a bucket elevator conveyor belt. The bucket elevator transported the feed to the top floor and then a chain conveyor transported the feed into the hopper above the pelletizer. The feed mash was put through a PTN BOA compacter. Also, the pelletizer was from PTN using three rollers to press the feed outside the mash. Broiler feed was pelleted with a 3mm die and the knife was set at 10mm. Conditioning temperature was set at 70 degrees Celsius. Temperature and moisture of conditioned mash and pellets was being measured using an infrared temperature measurer.

Sampling

Sampling mash from mixer before pelleting

The mixing time was started when the last ingredient is added to the mixer and ends when the mixer begins to discharge, therefore it does not include discharging time. When the required mixing time was completed, the discharge outlet was opened.

Multiple samples were taken from the outlet of the mixer by putting a bucket under the opening of the mixer. These samples were taken with regular time intervals, of approximately 20 seconds. When the mixer was fully discharged there were approximately 20 samples to choose from. For batch 0, three samples were taken. For batch 1-5 batches feed (see also Table 3) nine feed samples for each were taken. The samples were approximately 500 grams in weight.

All of the procedures given above were repeated for each mixing time, in total 6 batches. The three samples from batch 0 and the 45 samples from batch 1-5, resulted in a complete total of 48 mash samples for analysis. The 500-gram sample was split into four identical samples of 125 gram, by using a sample splitter, one for phytase analysis, one for Microtracer analysis, one for titanium oxide analysis and one for storage (4°C; spare samples).

Sampling pelleted diet from pelletizer

Pellet samples were taken at the outlet of the pelletizer. Pellet samples were taken with a time interval proportionally to the total pelletizing time. Samples were spread over the ground to cool down, before being put into a sample bag.

Head and tail of the production was not sampled, to prevent cross contamination between treatments. All of these procedures are repeated for each mixing time, in total 6 batches. Three negative control samples are taken from batch 0. For batch 1-5 nine samples were taken for each batch. This results in a complete total of 48 pellet samples for analysis. The 500-gram sample is split in four samples of 125 gram similar to the method described for the mash samples; one for phytase analysis, one for Microtracer analysis, one for titanium oxide analysis and one for storage (4°C; spare samples).

Assay and spare samples

From every sample taken from the technological trial, a spare sample of ~100grams was stored at Carus facilities of Wageningen University, may something go wrong with analysis of the samples.

Evaluation of mixing homogeneity

To evaluate mixing homogeneity four evaluation tests will be used, a Quantab chloride test, Microtracer test, titanium dioxide and Phytase analysis. For the chloride test, the salt in the feed is used to determine the mixing homogeneity. The chloride colorimetric test consists of test strips that react to the chloride in the feed. These colour samples can be compared to standard solutions to determine the salt concentration in each sample. The chloride analysis will be performed using a Hach Quantab Chloride colorimetric test strips (See annex 1). The chloride test will be executed for both mash and pelleted samples.

Secondly, Microtracers (Jadis Agri, Schiedam, the Netherlands) will be used to evaluate mixing homogeneity. Microtracer particles are separated from the feed sample with rotary magnet where iron particles were fixed on the magnetic surface of rotary detector. The iron particles are demagnetized and then sprinkled onto a large filter paper. The filter paper is then moistened with 70 % ethanol. When spots begin to develop, the paper is transferred to a preheated hot plate and dried (Arlet, 2003). All particles are counted. Number of spots represents the concentration of added tracer in the sample (Djuragic et al., 2009).

Thirdly titanium dioxide will be used as an evaluation maker. Titanium dioxide is usually used as an indicator for digestibility trials. Analysis will take place at Nutricontrol laboratory, located in Veghel, the Netherlands. Analysis method consisted of a microwave destruction of the material, after which the titanium was analysed using an inductively coupled plasma mass spectrometry (van Bussel et al., 2009).

Finally, samples will be analysed on its phytase activity per kg (FTU/kg) at Biopract Gmbh laboratory, located in Berlin.

Data analysis

Coefficient of variance (CV) was calculated as followed:

$$CV\ (\%) = \frac{\sigma}{\bar{x}} * 100$$

With

CV = coefficient of variance in percentage

σ = standard deviation

$\bar{x}$ = mean of sample

From the nine samples taken from the mixer or pelletizer, the sample mean and standard deviation is calculated. Using these two numbers the CV was calculated and comparisons were made to evaluate mixing homogeneity.

Pellet quality

Also pellet quality parameters will be analysed. Pellet durability was analysed by using a Holmen NHP 100 pellet tester (see Annex 2). Schleuniger pellet hardness was determined by using the sotax hardness tester (see Annex 3). And finally, water activity was determined using a Ro-tronic water activity meter (Rotronic AG, 2009).

Results

In Figure 9 the results of the mash feed are shown, where samples were taken directly after the mixer. It starts for Ti02 with a high CV, which indicates that the mixture is not properly mixed. After 120 seconds mixing it declines rapidly, and finally come to a steady state level lower than 5% CV, which is considered as very good (McCoy, 1994).

The phytase shows a similar pattern as the Ti02, as it starts with a CV of about 60% and quickly moves to CV of around 17%, after which it stays at 17.3% at 240 seconds. Until it goes higher to 21.5% at 480 seconds mixing time.

For the chloride the CV varies between 12% and 39%. It does not show a mixing curve as expected.

Microtracers also have a similar pattern as the Ti02 and the phytase, as it moves from the high CV to a lower CV. After 120 seconds the mixing homogeneity stays around the 26% until it drops to a mixing homogeneity of 14.7%.

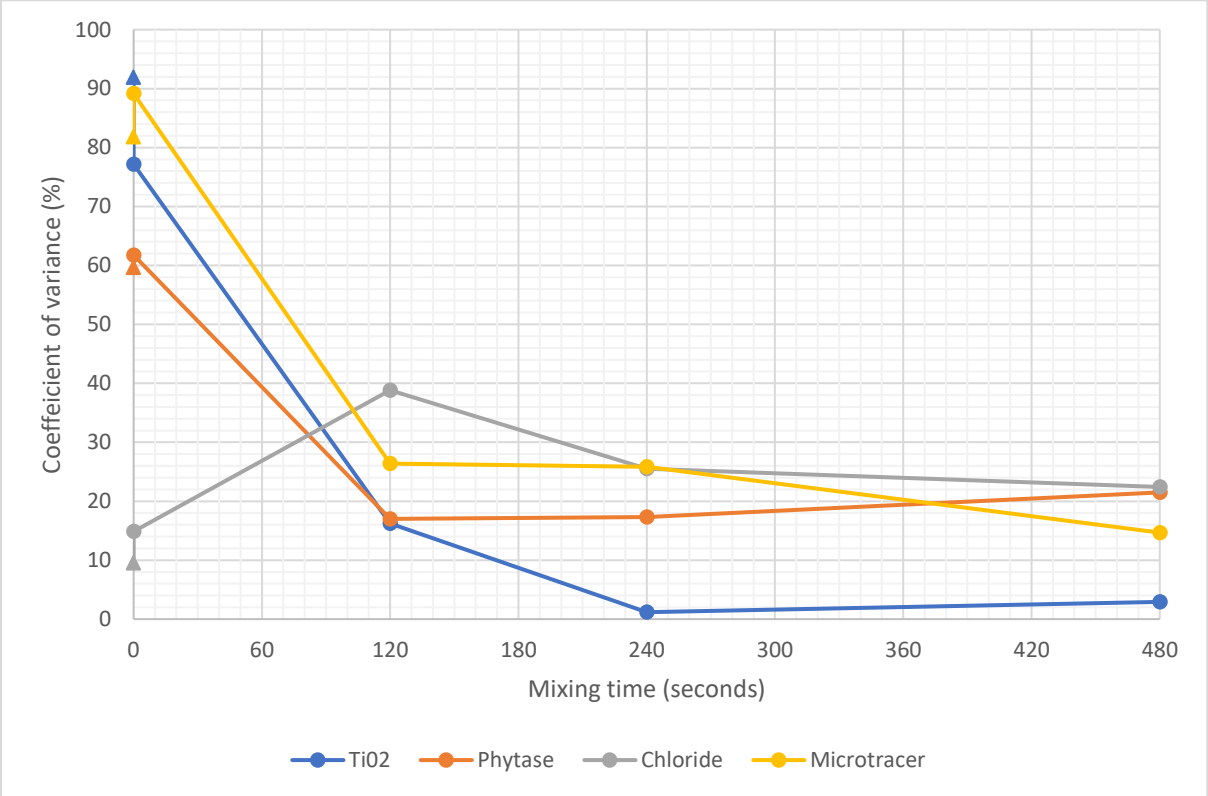

Figure 9. Coefficient of variance at different mixing times for the used indicators in the feed mash directly after the mixer. Note that the triangular points in the graph is dosing on top and no mixing (Batch 1).

Table 4 indicates the CV of Batch 1-5 for after mixing. It shows that chloride exceeded in all cases, but batch 1, the 10% CV point. Also, for the phytase and microtracer mixing homogeneity does not drops below the 10% CV.

Table 4. Comparison of the coefficients of variation (%) of the analytical results of mixing indicator comparison test of the samples taken after the mixer.

	Batch no.	1	2	3	4	5
Indicator, % CV	Mix time (s)	0	0	120	240	480
Ti02		91.9	77.2	16.2	1.2	2.9
Phytase		59.7	61.8	17.0	17.3	21.5
Chloride		9.5	14.9	38.8	25.5	22.4
Microtracer		81.8	89.1	26.4	25.8	14.7

In Figure 10 the results of the pelleted feed are shown. These samples were taken directly at the outlet of the pelletizer. For all treatments lower CV's can be seen, than expected according to the treatment plan. Furthermore, no clear mixing curve is distinguishable in this graph for all indicators. Ti02 shows the lowest CV, whereas microtracer show the highest CV. If we do not consider 0 seconds mixing time, microtracer seems to follow the same mixing curve as seen in figure 9.

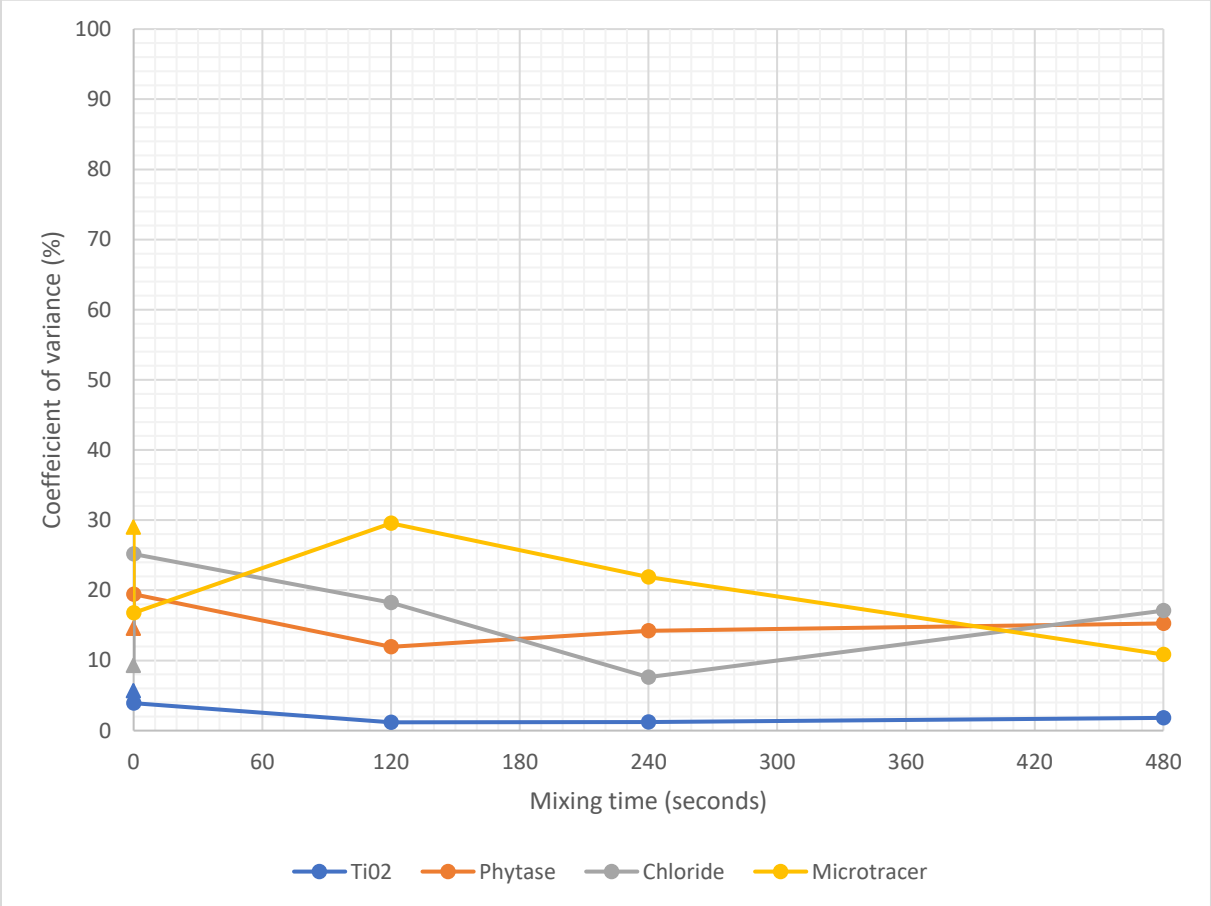

Figure 10. Coefficient of variance at different mixing times for the used indicators directly after the pelletizer. Note that the triangular points in the graph is dosing on top and no mixing (Batch 1).

In Table 5 can be seen that microtracer and phytase do not come below the 10% CV. Ti02 shows to be well mixed.

Table 5. Comparison of the coefficients of variation (%) of the analytical results of mixing indicator comparison test of the samples taken after the pelletizer.

	Batch no.	1	2	3	4	5
Indicator, % CV	Mix time (s)	0	0	120	240	480
Ti02		5.6	3.9	1.2	1.2	1.8
Phytase		14.6	19.4	11.9	14.2	15.3
Chloride		9.3	25.2	18.2	7.6	17.1
Microtracer		29.0	16.8	29.6	21.9	10.8

Discussion

Indicators for mixing homogeneity of phytase

In this trial several indicators were compared with each other to look into their suitability as an indicator to be used for phytase. In this part the suitability of every indicator will be discussed.

First of all, the phytase itself was analysed for phytase activity, to see if this is a good indicator for mixing homogeneity. From the results it became clear that there is a high variation in analysis, as samples sent to the laboratory were analysed in Duplo. These Duplo results showed high variation, indicating that the variation originates from the analytical method, rather than the variance created by the mixing treatments. This shows that phytase activity does not seems to be a very good indicator for the mixing homogeneity of phytase. Certainly, it has been advised that indicators with high variation in analysis should not be used as an indicator for mixing homogeneity (Pfost et al., 1979).
In addition, from the results it was shown that phytase activity was average 152 FTU/kg in the negative control. This is higher than expected, since the original diet was formulated to be low in native phytate. The high FTU/kg of phytase is due to the fact that the formulated oats were replaced by rye during production. This was done without prior consult with the research present during production. Rye has a very high phytase activity (Eeckhout & de Paepe, 1994), possibly contributing to the variance that is seen in the analytical results.
Also, the phytase activity could be influenced by the amount of shear in the pelletizer. Also pelleting temperature can be of negative influence on the phytase activity (Jongbloed & Kemme, 1990). In this trial the temperature was kept constant aimed at 70 degrees Celsius.

Chloride as an indicator is a very easy and practical method. The use of the Quantab strips was easy and also low in costs (approx. €1,00 per analysis). Concentration of chloride found in the feed were in comparison to what was expected. However, the salt was already in the basis feed and was properly mixed before the indicator materials were added to the feed. Salt should have been dispersed together with the Ti02, phytase and microtracer to the feed, to ensure that it would have followed a mixing pattern that would have been expected (see Figure 2). But from the results many variations were seen in the results, it seems that the accuracy of this test is not very high. Indeed, the accuracy of using chloride as an indicator for mixing homogeneity has been debated for many years (Creger, 1957). This usually is due to the high variance of results are reported from using this analysis. It is therefore possible to conclude that sodium chloride is not a very good indicator for research purposes, but could be used for practical purposes (McCoy et al., 1994). Though, even for these purposes some do not agree and mention that NaCl is not a good indicator for mixing homogeneity, even for practical purposes, as chloride can also be present in other feed ingredients (Rocha et al., 2015). This makes it a less suitable indicator, as it is known that if the analysed component is present in multiple ingredients the results can be influenced, e.g. crude protein (Pfost et al., 1966). The use of chloride as an indicator is not very suitable as an indicator for scientific purposes, as not only salt, but also other ingredients contain chloride ions. In particular choline chloride and L-Lysine-HCl contain chloride ions and could influence results (Clark et al., 2007). Based on this research and those in the past decade, it can be concluded that chloride is not a very good indicator for mixing homogeneity.

Titanium dioxide is an indicator that is usually used in digestibility trials (Short et al., 1996), but has not been used for the purposes of mixing homogeneity. As titanium is not present in other feed ingredients, it makes it already a very suitable indicator for mixing homogeneity. Also, it has similar properties to other microingredients, such as a very small particle size (approx. 25µm). However, titanium dioxide is not a material with good flowability properties and has a high angle of repose, which was seen by the researcher of this project. However, no accurate particle size analysis and angle of repose measurements were executed. Nonetheless, the dispersion in the premixture was good, as the material showed little clogging.
CV patterns found in this trial for Ti02 were similar those with Miyanami (1997) for the mash samples, which were taken directly after the mixer. Batch 2, in which materials were supplied in a premix on top, showed a better mixing homogeneity than batch 1, for which indicators were added not via a premix and on top. This possibly has to do with the size of the premix, increasing the size of the premix with ingredients shows a better mixing homogeneity (Pfost et al., 1979). Also, the rapid numerical decline in CV during the increase in mixing time was similar to what was found by Clark et al. (2007). Yet, the mixing curve was not the same as for the phytase product. This probably had to do with the difference in physical characteristics of the Ti02 and the phytase product. It is important that the
So, titanium dioxide seems to be a very good indicator for the assessment of mixing homogeneity and can also be used to study the further effects of digestibility in animal trials. This makes it a very attractive indicator for mixing homogeneity and the effect of different mixing homogeneities on the performance of animals. However, since it is not fully alike as the phytase product, in terms of physical characteristics, it is not suggested to use Ti02 as an indicator for phytase.

Microtracer is a familiar method for investigating mixing homogeneity of animal feed (Eisenberg, 2004; Clark et al.,2009; Rocha et al., 2015). The microtracer used in this research, F-Red Lake, was chosen because it had the most similar physical characteristics as the phytase product. This is important as it ensures that the particles behave the same as the phytase product would do. The microtracer company also provides microtracers in different particle sizes, giving the researcher the opportunity to select the right microtracer as marker for the investigated ingredient.
Microtracer also showed a mixing curve according to the description of Myanami (1997) at the mixer. Also, the mixing curve was most similar to the one of phytase. So, it seems that this indicator followed the same mixing curve, due to the similar physical properties. Yet, no repetitions were done in this research, due to time availability and financial reasons. It would take further research to fully confirm that microtracer F-Red lake is a good indicator for mixing homogeneity of phytase, by substantiating the results with a statistical analysis.

Based on this research Microtracer is the best indicator for the phytase. However, in other research DL-methionine was mentioned as a good indicator for mixing homogeneity (Reese et al., 2017). But this depends on the product or nutrient that has to be investigated for mixing homogeneity. Based on the feed ingredient that has to be investigated the proper marker has to be chosen, that preferably can be analysed with a high accuracy.

A big remark for this research is that more factors influence the variation in the CV. CV does not only indicate the degree of mixing, but also includes variation from sampling procedure, assay method, assay accuracy and randomness (Behnke & Beyer, 2002). So, it is not just an indication of mixing homogeneity, as it will partly consist of the variance of these factors.

The sampling procedure used in this experiment was based on the flow of material out of the mixer and pellet press. However, in other trials samples were taken from the machine in different locations (Rocha et al., 2015). Yet, due to the flow of material out of the machine, it seems more logical to take samples with regular intervals from the mixer or pelletizer. As this method comes closest to practice.
Furthermore, there has been discussion on how many samples have to be taken from the mixture to properly assess mixing homogeneity (Barbosa-Cánovas et al., 2006). Some studies advice that ten samples may not be sufficient; others mention that higher numbers are over the top. However, it is mentioned that based on the feed composition (with all of their different traits) you want to assess, and especially what degree of mixing is expected, the number of samples should be established (Creger, 1957). If the mass of mix is unmixed, it requires more samples to assess the mixing homogeneity in comparison to a well-mixed mass. Thus, maybe in case of batch no. 1 and 2, where a high CV was expected, more samples should have been taken and analysed to determine the high CV.
Moreover, analysis accuracy of the indicators might also play their part in the CV. As it is known that phytase activity analysis is not a very accurate method in comparison to a chemical analysis (De Jonge, 2018).

Optimal mixing for mixing homogeneity

Post-mixing processes seem to be highly influential on the mixing homogeneity of the mixing of the indicators in this research. Pelleted feed had a mixing homogeneity with a CV lower than 30% for all indicators in comparison to the mash feed. Mash feed was directly sampled at the outlet of the mixer. For the pelleted feed it had to be put into a bunker, after which the feed was transported by a transport screw to the bunker above the conditioner/pelletizer. This could possibly influence the mixing homogeneity to result in a lower CV. This in contrast to what is stated in literature, as it is mentioned that transport can negatively influence the mixing homogeneity of animal feed (Barbosa-Cánovas et al., 2005). In this research it did not become clear if these transport processes were of negative of positive influence on the mixing homogeneity of the feed, but it can be stated that they have an effect.

From an optimal mixing perspective, feed manufacturers should focus on particle uniformity to achieve a high mixing homogeneity, to ensure a high-quality product (Axe, 1995). High particle uniformity consists of similar particle shape, similar density and similar particle size. Also, high particle numbers and a high flowability are of importance in creating a low CV, thus resulting in a perfect mixing homogeneity.
Though many researches have used the mixing time on the CV of certain products, these results cannot be used as a guideline for other feeds. This is caused by the unique diet formulations, particle size of ingredients, revolutions per minute of the augers in the mixer, mixer cleanliness and wear, mixing time and the specific indicator used for analysing mixing homogeneity.

The addition of fat or other adhesive products can contribute to a better mixing homogeneity of the feed (Armstrong & Behnke, 1996). It is likely that the very good mixing homogeneity of the feed could be aided by the fat in the feed. However how much the quantity, quality and stickiness of fat were of influence is not known in the feed production sector. It could be interesting to investigate this field, as it can be of aid during the mixing process and therefore improve mixing homogeneity. However, when high quantities of fat are added, caking may take place (Wornick, 1965). In addition, when fat is added at the incorrect moment this can lead to an improper dispersion of fat through the mixture and influence mixing homogeneity (Reese et al., 2017).

Conclusions

It was not possible to produce a pelleted broiler feed with different mixing homogeneities of phytase. As it was planned to have a diet >25% CV and a diet <10% CV. It is therefore suggested that a mash diet is fed in the animal trial. Another suggestion is to simulate different mixing homogeneities in the animal trial, by making different diets with different level of phytase and provide this according to a time schedule, in which phytase content varies.

Titanium dioxide is a good indicator for the assessment of mixing homogeneity in mash diets. It can be questioned to what extent it is suitable as an indicator of phytase mixing homogeneity in both mash and pelleted diets. Based upon this research microtracer is the suitable indicator for mixing homogeneity of phytase.
Chloride and phytase activity are no good indicators for mixing homogeneity of phytase, due to their high variation in analysis and presence in multiple ingredients. It is recommended that an indicator is chosen based on the lowest variation in analysis as possible, to prevent any misinterpretations of mixing homogeneity by using CV.

It is recommended that a particle size analysis is executed for all markers and the feed. Also the flowability should be measured to get a good indication on the flow pattern of the feed and indicators.

There was an effect of transport seen if mixing homogeneities of post-mixing and post-pelleting were compared. The extend of this effect and if it had a positive effect or negative effect, did not became clear in this research.

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Annex

Table 6. Comparison of averages of indicators for mixing homogeneity and their standard deviation for the samples directly taken after the mixer.

	Treatment	Negative control	On top	On top premix	1% premix	1% premix	1% premix
	Batch no.	0	1	2	3	4	5
Indicators	**Mixing time (s)**	240	0	0	120	240	480
Ti (mg/kg)		13.0 ± 0.0	1183.6 ± 1087.7	947.4 ± 731.0	1094.9 ± 177.8	1130.0 ± 13.3	1120.0 ± 33.0
Phytase (FTU/kg)		0.0 ± 0.0	1069.6 ± 638.2	669.4 ± 413.4	595.1 ± 101.2	538.7 ± 93.2	860.2 ± 184.9
Chloride (mg/L)		42.2 ± 5.3	41.0 ± 3.9	46.3 ± 6.9	56.3 ± 21.9	54.6 ± 13.9	49.3 ± 11.1
Microtracer (Count)		0.0 ± 0.0	26.1 ± 21.3	22.3 ± 19.9	35.5 ± 9.4	32.1 ± 8.3	30.3 ± 4.5

Table 7. Comparison of averages of indicators for mixing homogeneity and their standard deviation for the samples directly taken after the pelletizer.

	Treatment	Negative control	On top	On top premix	1% premix	1% premix	1% premix
	Batch no.	0	1	2	3	4	5
Indicators	**Mixing time (s)**	240	0	0	120	240	480
Ti (mg/kg)		17.0 ± 0.0	1074.1 ± 60.7	1122.2 ± 43.9	1112.2 ± 13.1	1118.9 ± 13.7	1104.4 ± 20.1
Phytase (FTU/kg)		152.0 ± 54.8	628.7 ± 91.7	629.6 ± 122.4	681.3 ± 81.4	606.6 ± 86.2	889.4 ± 135.9
Chloride (mg/L)		51.3 ± 12.3	63.2 ± 5.8	45.9 ± 11.6	38.8 ± 7.1	37.5 ± 2.9	44.8 ± 7.7
Microtracer (Count)		8.1 ± 13.2	61.7 ± 17.9	74.0 ± 12.4	66.1 ± 19.5	65.0 ± 14.2	60.8 ± 6.6

Table 8. Production conditions of the six batches

Batch	Mixing time (s)	Temp cold mash (°C)	Temperature BOAmash (°C)	Temp pelletizer (°C)	Steam%
0	240	28	50-55	60-66	20
1	0	28	50-55	56-65	15 - 20
2	0	29	50-55	60-67	20
3	120	30	50-55	64-68	17
4	240	30	50-55	63-68	15
5	480	30	50-55	62-68	15

Table 9. Comparison of pellet quality parameters between different batches.

Parameter	1	2	3	4	5	6
Pellet hardness	10.40	7.60	8.20	8.80	7.50	7.80
Pellet durability	0.27	0.31	0.30	0.30	0.21	0.19
Aw	0.63	0.63	0.62	0.62	0.61	0.61

Annex 1. Procedure used for Hach titrators test for chloride

Used product: Hach titrators for chloride, low range 30-600ppm Cl^- (0.005-0.1% as NaCl), 40 tests, Cat. 27449-40

Procedure:

1. Dissolve 10 gram feed in 100 ml of demineralised water
2. Stir feed mixture actively, to ensure good dissolvement of the salt
3. Remove a titrator from bottle and replace cap immediately.
4. Insert lower end of titrator into solution. Do not allow solution to reach the yellow completion band at top of titrator.
5. Allow solution to completely satureate wick of titrator. Reaction is complete when yellow band turns dark.
6. Note were the tip of the white chloride peak falls on the numbered Quantab® scale. This represents the Quantab® unit value.
7. Refer to the table on the bottle to convert Quantab® units into salt concentration.

Hach Company, P.O. Box 389, Loveland, CO 80539 U.S.A.

Annex 2. Procedure used for Holmen Ligno NHP 100 pellet tester

1. Weigh 50 grams of material
2. Sieve any fines from the sample
3. Open Holmen machine by swinging the lid and filter frame open. Open the filter support plate.
4. Insert pellets into the pellet chamber
5. Close filter frame and the lid
6. Set time on the timer selection switch, it was set at 30 seconds.
7. Press start button, the blower will start to run and circulate the pellets for the selected duration and then automatically stop.
8. Swing the lid and the filter frame open.
9. Open the flap on the filter support plate and the move the plate toward the left.
10. Collect the remainder of the sample
11. Weigh back the sample
12. Pellet durability is calculated by dividing the weight of the remainder of material by the weight at the start of the sample.

TekPro Limited, Willow Park, North Walsham, Norfolk NR 28 0BD, United Kingdom

Annex 3. Procedure used for Sotax Schleuniger pellet hardness tester

1. Grab 10 pellets of every sample
2. Test every pellet for pellet hardness by placing the material between the press and the wall
3. Press the start button
4. Wait for the hardness to show on the screen
5. Write down result
6. Take the average of the ten samples to get an representative indication of the pellet hardness

Pharmatron AG, Untigenstrasse 28, CH-3600 Thun, Switzerland